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Neural regulation of secretion from tracheal submucosal glands

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NEURAL REGULATION OF SECRETION

FROM TRACHEAL SUBMUCOSAL GLANDS

by

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ABSTRACT

To determine which neural mechanisms mediate mucus secretion from tracheal glands of ferrets, we developed methods for studying secretion of fluid and macromolecules separately *in vitro*. To activate neural mechanisms *in vitro*, we used electrical field stimulation and drugs. We monitored fluid secretion by measuring the diameters of tantalum-covered droplets of fluid secreted by glands, and we monitored glycoprotein secretion by measuring the release of $^{35}SO_4$-labeled macromolecules. Using these methods, we found that adrenergic and cholinergic nerves mediate secretion of fluid. In addition to adrenergic and cholinergic nerves, nonadrenergic noncholinergic nerves also mediate secretion of macromolecules. The role of this unidentified mechanism may be to regulate secretion of macromolecules separately from fluid, whereas the cholinergic and adrenergic systems mediate secretion of both macromolecules and fluid. We also found evidence of interaction between peripheral nerves in regulating secretion. By measuring the efflux of ^{3}H from nerves after preloading them with radio-labeled norepinephrine, we found that stimulation of cholinergic nerves in the airway inhibits adrenergic neurotransmission to glands. Thus, nerves regulate secretion directly via stimulating secretion, and indirectly, by modulating transmission by other nerve types.

ACKNOWLEDGEMENTS

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CONTENTS

INTRODUCTION

The lung is the most vulnerable organ in the body to environmental materials because in order to promote efficient exchange of respiratory gases, the exposed surfaces are very large ($\simeq$ 70 m^2) and are very thin (only 1 cell thick). Thus, there is great a potential hazzard from inhaled particles including bacteria, viruses, and chemical aerosols. However, a complex system of branching airways and a mucociliary clearance mechanism protect the alveolar membranes from damage caused by inhaled particles. During breathing, particles larger than 1 μm are deposited on the airway wall both by gravity and the relative inertia of air and particles. The path that the air follows to reach the alveoli changes direction at each of approximately 24 branch points [246]. Since air molecules have much less inertia than suspended particles, the air changes direction relatively easily. However, due to their greater inertia, particles are less able to follow the path that the air takes, and consequently hit the airway walls, and stick in a layer of mucus. The mucus layer is composed of an aqueous sol layer in which cilia beat, and a more viscous and sticky gel layer that is moved by the tips of the cilia [218, 258]. This moving layer of mucus carries trapped particles to the mouth (FIG. 1.1), where they are either expectorated or swallowed. If the sol layer is too thin, the cilia can get stuck in the viscous gel layer, thereby decreasing their effective strokes. Conversely, if the sol layer is too thick, the tips of the cilia may not touch the gel layer, and clearance may likewise decrease. Additionally, if the

viscoelastic properties of the gel layer are not appropriate, then the gel layer will not be moved efficiently by the cilia [129]. For example, in chronic bronchitis or asthma in man, the secreted mucus may be more viscous and sticky than normal mucus [36], and thus may be cleared less efficiently [129]. Conversely, if the concentration of glycoprotein molecules in the secretions is too low, the mucus will not form a weak gel, and will not be cleared efficiently [145].

Mucus is a complex mixture of water, ions, glycoproteins, lysozyme, IgA, and other solutes. Of these, the water and glycoproteins are the most important in determining the physical properties of mucus [129, 256]. Since the visco-elastic properties of mucus are due in part to the relative amounts of water and glycoproteins present, effective clearance of particles requires the proper quantity and quality of each of these primary components.

Mucus is secreted from at least two sources, epithelial goblet cells and submucosal glands [41, 66, 119, 146]. Furthermore, water [253] and sodium chloride [164] are also secreted by the epithelium. Submucosal glands are found in many but not all species [91]. When found, they are tubuloacinar glands with acini composed of both serous and mucous cells, joined by tight junctions, surrounding acinar lumina [147, 148]. Myoepithelial cells surround acini [9] and may aid in the expulsion of secreted mucus out of the tubules. The lumina of different tubules join together to form a series of collecting tubules that join to form an excretory duct leading to the surface of the tracheal lumen (CHAPTER 2) [148]. In the species that have submucosal glands, the major source of airway mucus is the glands [187]. In

humans, the submucosal glands occupy 25 - 35 % of the radial dimension of the cartilagenous airways, and thus represent approximately 40 times the volume of secretory tissue compared to the goblet cells [187].

NEURAL REGULATION

The lungs and airways derive embryologically from the primative foregut. It is therefore reasonable to expect that autonomic mechanisms control secretion from airway glands as they regulate salivation (see CHAPTER 6) and gastrointestinal function. It is not surprising then, that there are early reports of neural control of mucus secretion. Stimulating the vagus nerves of dogs caused droplets of fluid to appear on the surface of the exposed trachea [114]. Since then, numerous anatomical and physiological studies have confirmed the presence of cholinergic autonomic innervation, and have shown that cholinergic nerves regulate secretion from the trachea. Additionally, anatomic studies have demonstrated the presence of adrenergic innervation to these glands, but few studies of the role(s) of adrenergic or other nerve types have been reported.

A. ANATOMIC STUDIES

1. EVIDENCE FOR CHOLINERGIC INNERVATION

The tracheas of dogs, humans, and cats receive innervation from the parasympathetic nervous system via the vagus nerve. Fibers from the medulla leave the skull near the auditory bulla, and form the vagal trunk. Fibers leave the vagal trunk to form the superior

laryngeal nerve that innervates the larynx and the upper trachea. Caudally, below the inferior cervical ganlgion, the recurrent laryngeal nerve (RLN) leaves the vagus, curves cranially and ascends the trachea. From the RLN, small bundles of nerves run to the submucosal surface of the trachea, and presumably innervate airway ganglia. Early anatomic studies using methylene blue or silver staining techniques showed ganglia within the airway that send post-ganglionic fibers to glands of human beings [122]. Because cutting the vagus nerves of rabbits results in degeneration of nerves innervating airway ganglia [123], whereas cutting the sympathetic nerves of dogs does not [57], airway ganglia were presumed to be primarily parasympathetic. More recent studies relying on acetylcholinesterase staining techniques indicated the presence of cholinergic innervation to submucosal glands of monkeys [56], dogs [243], and sheep [135]. Electron microscopic studies showed axon varicosities that contained small unfilled vesicles, suggesting cholinergic nerves, to serous and mucous cells of glands in cats [151], ferrets [9], opossums [212], sheep [135], and human beings [12, 147].

2. EVIDENCE FOR ADRENERGIC INNERVATION

Dogs, cats, and humans have sympathetic innervation to the airways. In dogs, fibers from the superior cervical ganglion enter the vagus immediately caudal to the nodose ganglion. Some of these fibers may innervate the upper trachea via the superior laryngeal nerve. Additionally, fibers from the stellate ganglion enter the

vagus both directly and via the inferior cervical ganglion. Some of these fibers may enter the recurrent laryngeal nerve and innervate airway ganglia. Early studies were inconclusive, but did not rule out the possibility of sympathetic innervation of glands of man [122] or dogs [57]. Catecholamine fluoresence was found in the bronchial ganglia of calves [104, 135], dogs [104], pigs, rabbits, sheep [135], and cats [151]. Additionally, catecholamine fluoresence was found in the extrachondral nerve bundles in sheep [135]. Catecholamine fluoresence suggested the presence of adrenergic innervation to glands of cats [151], but not to those of dogs [243], calves, rabbits, or pigs [135]. Electron microscopic studies have shown the presence of nerve profiles containing small dense cored vesicles near the submucosal glands of cats [151], ferrets [9], and man [12, 147]. This type of vesicle is characteristic of adrenergic nerves. Additionally, this type of nerve takes up 5-hydroxydopamine [151], indicating the presence of a catecholamine uptake mechanism. These findings suggest that there is adrenergic innervation to glands of these species.

3. EVIDENCE FOR NONADRENERGIC-NONCHOLINERGIC INNERVATION

In addition to adrenergic and cholinergic innervation, there is recent evidence that peptides may be neurotransmitters in the airway. Immunocytochemical studies have demonstrated the presence of VIP-containing nerves in ganglia and in the submucosa of cats [231] and ferrets [8]. Electron microscopic studies have shown the presence, in submucosal nerves, of axon varicosities containing large dense-cored vesicles. These "P"-type varicosities may be the nerves in which VIP

immunoreactivity is contained [8]. Additionally, substance P is found in tracheal epithelial nerves [162]. Substance P may also play a role in neural regulation of secretion. If substance P is a sensory neurotransmitter in the airway as it is in peripheral afferent nerves [100], substance P may regulate secretion by acting on airway ganglia, other nerve(s), or directly on the glands. However, it is not known whether any of these substances are neurotransmitters that regulate airway secretions.

B. REGULATION OF FLUID SECRETION

1. EFFECTS OF CHOLINERGIC NERVES

Cholinergic agonists stimulate fluid secretion. Pilocarpine increases the secretion of fluid from the tracheas of cats [66, 173] and rabbits [173], and methacholine increases the secretion of tracheobronchial fluid from dogs [24, 186]. Although the presence of cholinergic nerves and the effects of cholinergic drugs strongly imply functional innervation, cholinergic neural regulation of secretion can only be shown by nerve stimulation. Kokin first demonstrated that stimulating the vagi of dogs caused fluid to be secreted from the ducts of tracheal submucosal glands [114]. Since then, several studies have shown cholinergic neural regulation of fluid secretion from tracheas of cats [66, 173] and dogs [117, 153].

However, since in few of the earlier studies was the site of secretion identified, it was possible that in some cases the secretions were not from glands. Stimulating the cholinergic nerves

causes fluid secretion from airways of rabbits [173], a species without submucosal glands [141]. The source of fluid may be the epithelium of the posterior membrane. The posterior membrane of the canine trachea secretes fluid [253] and sodium chloride [164] at rest, and the rate of chloride secretion increases in response to acetylcholine [136]. Because the hypothesis that water movement follows the movement of osmotically active ions appears to be correct under these conditions, cholinergic stimulation could result in increased water secretion from the epithelium. This finding complicated the interpretation of studies of fluid secretion from intact segments of the airways that have both submucosal glands and epithelium such as the anterior portion of the tracheas of cats [66].

To surmount this problem, two methods were developed to isolate functionally the glands from the epithelium as a whole. The first was the "hillocks" method [45, 153, 154]. The airway is opened, the surface wiped free of mucus, and a thin layer of tantalum powder (mean mass diameter, 0.5 μm) is applied to the lumen. Stimulation of secretion from glands causes elevations or "hillocks" to appear directly over submucosal gland duct openings [154]. Using this method, Davis et al found that local arterial injection of acetylcholine or electrical stimulation of the vagus or superior laryngeal nerve increased the rate of fluid secretion via muscarinic mechanisms [46].

The second method was a micropipette collection technique [233]. A fine glass micropipette is placed over a gland duct opening, and the junction between the pipette and duct is sealed with paraffin oil to

insure both that the fluid secreted by the gland does not leak out onto the tracheal surface and that fluid secreted by the epithelium does not flow into the pipette. Using this method, Ueki et al showed that stimulating the cervical vagi of cats increased the flow rate of fluid from glands via a muscarinic mechanism [233].

The cholinergic system is at least partially under reflex control. Hypoxia [44] or sulfur dioxide [95] increased secretion of fluid from glands of dogs, as did mechanical irritation of the larynx [82] or stomach [81] of cats. Since each of these responses was inhibited by either cooling the vagi or infusing atropine, the responses must have been mediated by vagal muscarinic mechanisms.

2. EFFECTS OF ADRENERGIC NERVES

Adrenergic agonists cause secretion from both airway epithelium and glands. Terbutaline [48] and phenylephrine [176] each stimulate the secretion of chloride ions towards the tracheal lumen of cats *in vitro*. Similarly, isoproterenol, epinephrine, norepinephrine, and phenylephrine each stimulate chloride ion transport towards the tracheal lumen of dogs [2]. Since some of the effect of epinephrine was blocked by propranolol, modulation of ion and presumably water secretion is at least partially under β-adrenergic control [2]. Epinephrine increases the secretion of tracheobronchial fluid from unidentified sites of airways in guinea pigs but not rabbits, whereas amphetamine increases the secretion of fluid from rabbits [24]. The failure of sympathetic nerve stimulation to cause secretion from airways of rabbits [24] may be due to the paucity of glands in this species [91, 141].

Phenylephrine increased the rate of fluid secretion from glands of cats [181, 233] and dogs [47]. Since the effect was prevented by prior treatment with the alpha-adrenergic antagonist phentolamine [233], fluid secretion is mediated via alpha-adrenergic receptors. Beta-adrenergic mechanisms are much less potent in causing fluid secretion than are alpha-adrenergic mechanisms [20, 181, 233]. In dogs however, atropine prevented all of the response evoked by stimulation of the vago-sympathetic trunk [47, 117], suggesting that adrenergic nerves do not mediate secretion of fluid in dogs.

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C. REGULATION OF GLYCOPROTEIN SECRETION

1. EFFECTS OF CHOLINERGIC NERVES

Cholinergic nerves mediate secretion of glycoproteins. This conclusion is based primarily on studies with radioactive precursors of mucous glycoproteins. Glucosamine and SO_4 are constituents of airway glycoproteins [58, 215]. The glucosamine is found at branch points in the carbohydrate side chains, and SO_4 is often the terminal group on the side chains. After incubating segments of the airways of cats [74], dogs [32, 58], humans [16], or ferrets [83] with $^{35}SO_4$, acetylcholine stimulated the release of radio-labelled molecules whose composition and very high molecular weights suggested that they were glycoproteins [33]. Because the secretory effect of acetylcholine was prevented by atropine, muscarinic receptors mediated the response. Additionally, stimulating the vagus nerves of cats causes the release of $^{35}SO_4$-labelled glycoproteins from tracheal segments *in vivo* [74]. Because nearly all of the effect of stimulating the vagus was prevented by atropine, glycoprotein secretion is under vagal muscarinic control.

Because both epithelial goblet cells and submucosal glands produce mucus [32, 41, 66, 119, 146], it became important to differentiate between them. After separating the epithelium with goblet cells from the submucosa with glands, Ellis found that methacholine caused secretion from glands in the absence of the epithelium [58]. Using autoradiographic methods, Sturgess found that acetylcholine, pilocarpine, and carbamylcholine each depleted human gland cells of ^{3}H-labeled materials [216]. Because atropine prevented

the depletion, muscarinic mechanisms were responsible. Furthermore, physostigmine increased the resting rate of secretion in the same preparation, suggesting that acetylcholine was spontaneously released in vitro, and that this released acetylcholine stimulates secretion at rest [40]. In other studies in vivo, pilocarpine depleted glands of cats of $^{35}SO_4$-labeled materials without depleting the goblet cells [75]. In contrast, inhalation of ammonia vapor depletes both the glands and the goblet cells [75]. The effects of ammonia vapor are partially prevented by atropine [66, 189], suggesting that there may be muscarinic regulation, but Florey was unable to show a clear effect of vagus nerve stimulation on goblet cells in the cat [66]. Recent studies of goblet cells in the gut reveal that few stimuli cause release of goblet cell contents [159]. However, both cholinergic nerve stimulation and acetylcholine stimulated the release of $^{35}SO_4$-labeled macromolecules from tracheas of geese, a species without glands [178]. Thus, neural regulation of goblet cells may occur only in species with no submucosal glands. However, in species with many glands, the problem may be less significant, as in humans, whose submucosal gland mass is approximately 40 times greater than the goblet cell mass [187]. Thus, even if goblet cells are under neural control, the effect of their output on the overall secretion from the airway may be small.

2. EFFECTS OF ADRENERGIC NERVES

There are conflicting data concerning the effects of adrenergic agents on tracheal glycoprotein secretion. Numerous studies have failed to show adrenergic influences on glycoprotein secretion. Norepinephrine failed to stimulate secretion from airways of dogs *in vitro* [32], geese [178], and man [16]. Similarly, guanethidine [216] or isoproterenol [16, 216] failed to deplete granules or cause secretion of glycoconjugates [208] from human glands *in vitro*. However, in another study of human airways, the alpha-adrenergic agonist phenylephrine, the β_1-adrenergic agonist terbutaline, and the β_2-adrenergic agonist salbutamol each increased the rate of both $^{35}SO_4$ and ^{3}H-labeled glycoprotein secretion [175]. Both alpha [176] and beta [74, 176] adrenergic agonists stimulate secretion of $^{35}SO_4$-labelled glycoproteins from cats *in vivo*. Additionally, isoproterenol depleted granules from mucous cells of ferrets [79]. The alpha-adrenergic antagonist, phentolamine, prevented the effect of phenylephrine, and the beta-adrenergic antagonist, propranolol, prevented the secretory effects of terbutaline [176].

A probable reason for the differences in results between the physiological and the less quantitative morphological studies is that measurable secretion may occur in the absence of morphologic change [216]. A likely reason for the differences between physiological studies is that in several of the studies [16, 32, 208], tissues were incubated *in vitro* for up to several days prior to the addition of secretagogues. The human or canine airways used in these studies were of full thickness (≈2 - 3 mm). Because diffusion of O_2, CO_2, and

nutrients into the interior of the tissue may be insufficient [31], the tissues may have not been responsive.

There are very little data regarding the roles of adrenergic nerves in secretion. Recent studies in cats showed that stimulating the stellate ganglion increased the release of $^{35}SO_4$ [74, 170] or ^{3}H labelled glycoproteins [170] via beta-, but not alpha-adrenergic mechanisms. However, because alpha-adrenergic mechanisms mediate the response to phenylephrine [176], alpha-adrenergic receptors are present on the glands. Since some of the reflex effects of stimulating the upper airways may be mediated by sympathetic neural pathways [177], sympathetic as well as parasympathetic nerves may be important in mediating secretory responses of cats. In dogs, the effects of adrenergic nerves are less well known. Phenylephrine and acetylcholine each increase the release of fluid from glands [47], but since atropine entirely prevents the response to stimulation of the vagus nerve, it appears that although there are adrenergic receptors on glands, there may be little adrenergic neural regulation of secretion in dogs.

In sum, although there is anatomic evidence demonstrating the presence of adrenergic nerves, there are conflicting data about the roles of adrenergic nerves in the regulation of either fluid or glycoprotein secretion from the tracheobronchial tree.

AIMS OF THE STUDIES

The primary purpose of these studies was to determine what neural mechanisms regulate mucus secretion from tracheal glands. One major difficulty with previous studies *in vivo*, was the presence of reflexes and circulating mediators that caused secretion [44, 189, 191], and thus confused the interpretation of studies intended to demonstrate specific neural regulation. One way to overcome this problem is to stimulate the cut end of the efferent parasympathetic or sympathetic nerves to the airways. However, the superior and recurrent laryngeal nerves of cats and dogs contain both parasympathetic and sympathetic nerves. Thus, it is difficult to stimulate the efferent sympathetic nerves to the airways without also stimulating the efferent parasympathetic nerves. Additionally, *in vivo* studies have the disadvantage that it is possible to monitor only a limited number of tissues from each animal [74, 153]. To overcome these problems, I wanted to do studies *in vitro*, in the absence of reflexes or circulating mediators. Another complication is the presence of goblet cells in several of the species used for studies (e.g., cats, humans, dogs). Thus, I chose to do these studies with tracheas of ferrets because they have large numbers of glands distributed throughout the trachea (CHAPTER 2) and have goblet cells located not in the trachea but primarily in the bronchi [103] (Basbaum, personal communication). To study neural mechanisms *in vitro*, We used electrical field stimulation [166] to activate nerves. We measured secretion of both fluid (CHAPTER 3) and radio-labeled macromolecules (CHAPTER 4) from

tracheal glands.

These studies demonstrated that adrenergic nerves mediate secretion of fluid via alpha-adrenergic mechanisms (CHAPTER 3), and glycoproteins via both alpha-adrenergic and beta-adrenergic neural mechanisms (CHAPTER 4). These studies provide the first evidence of nonadrenergic-noncholinergic neural regulation of secretion. Additionally, we found that cholinergic nerves inhibit adrenergic neurotransmission to glands of ferrets (CHAPTER 5), indicating that nerves regulate secretion both directly, by stimulating glands, and indirectly, by modulating other neural pathways to glands.

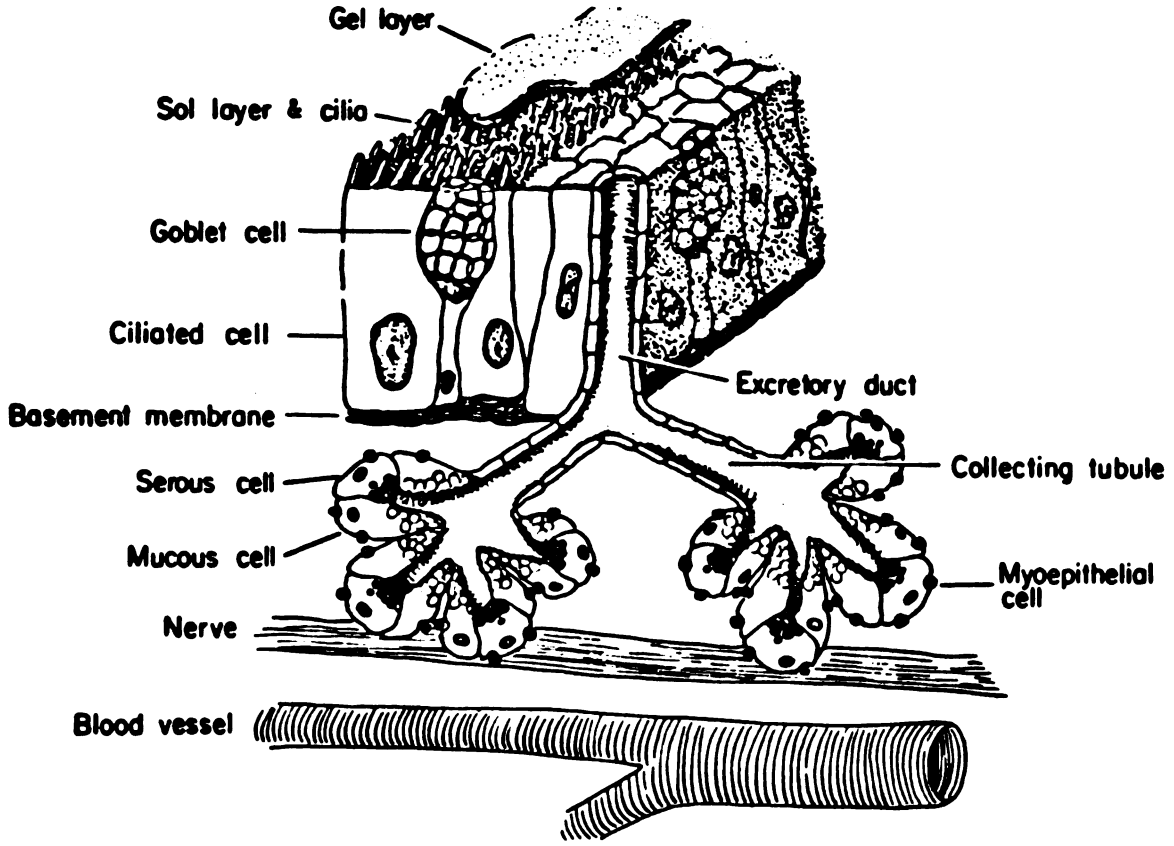

FIG. 1.1. Diagram of airway epithelium, showing epithelial ciliated and goblet cells, tubulo-acinar submucosal glands composed of serous cells, mucous cells, and myoepithelial cells. Acini are connected to collecting tubules which merge with each other to form an excretory duct that opens onto the airway lumen. The cilia beat in an aqueous sol layer, and their tips move a gel layer of mucus secreted by the glands and goblet cells. Ciliary beating moves the layers of mucus towards the mouth, where they are swallowed or expectorated. Regulation of secretion may be via nerves or blood vessels in the submucosa.

ANATOMY

INTRODUCTION

To study the functional innervation, we had to develop methods for studying nerves and secretion in vitro in the absence of central nervous system reflexes or circulating mediators that can cause secretion. The presence of either of these mechanisms may be responsible for the variability between animals [74], that might make interpretation of studies difficult. Furthermore, in whole animals, a supply of radio-labeled precursor cannot remain constant after intravenous injection. Thus, the rate of baseline release of $^{35}SO_4$-labeled macromolecules declines with time after injection of $^{35}SO_4$ [74]. Additionally, the concentration at the glands of injected agonists or antagonists cannot be accurately known. These problems may further increase the variability of the measured secretory responses in vivo. To overcome these problems, we wanted to design experiments that used within animal and within tissue controls. To do this, we needed to use a species that has sufficient submucosal glands distributed throughout the trachea, and thus enable us to use several pieces of tissue from each animal. Also, the tissue (trachea) has to be thin enough to allow rapid diffusion of nutrients to the cells to occur (≈ .5 - 1 mm) [31].

Neither chickens [241] nor geese [178] have submucosal glands. Guinea pigs and rabbits have few glands [141], and rats have only a few submucosal glands, which are located only near the larynx [91,

116]. In these species however, epithelial goblet cells are responsible for supplying mucus to the ciliated epithelium. Pigs have submucosal glands distributed throughout the trachea and bronchi [91, 106], as do opossums [212], sheep and goats [135], cats [74], dogs [114], monkeys [56], and human beings [226, 230]. Of these species, only cats and opossums have tracheal walls thin enough for sufficient diffusion of nutrients to occur in vitro. However, the random source cats obtained at the University of California, San Francisco often had upper airway disease. Since upper airway infections cause changes in the responsiveness of airway smooth muscle to inhaled aerosols [62], we felt that secretion might also be altered and that we should not use unhealthy animals. Thus, we could not be assured of sufficient numbers of healthy animals for the studies. Additionally, since there is no commercial source of opossums, we needed to find a species that was available in sufficient numbers for our studies.

In a survey of several species, Ueki found that ferrets (Mustella putorius) had ducts that opened onto the tracheal epithelium (Ueki, personal communication), suggesting that unlike other small animals, ferrets might have submucosal glands. Therefore, the purposes of these anatomic studies were to see whether ferrets have sufficient numbers of submucosal glands to be suitable animals for studies of the neural regulation of secretion, and whether there is anatomic evidence of adrenergic and cholinergic innervation of tracheal glands.

METHODS

We anesthetized 8 adult male ferrets by injecting them with sodium pentobarbital (45-60 mg/kg, ip). After making a ventral midline incision, we removed the entire trachea, removed most of the loose fat and some of the loose connective tissue, and rinsed the trachea in 0.9 % NaCl. To study submucosal glands, we cut through the posterior membrane in the midline, opened the trachea flat, and pinned the edges of the tissue onto a Silgard Polymer plate (Dow Corning Inc.). To study the ganglia and nerve supply to the surface of the trachea, we cut through the anterior portion of the trachea, and pinned the trachea with the submucosal side up. To stain the airway, we added neutral red (0.1 %, Sigma Chemical Co., St. Louis, MO), and left it in contact with the tissue for 45 min. We then washed off the trachea with fresh saline and wiped the surface to remove any dye crystals that settled onto the tissue. We observed the tissue via transmitted light using a dissecting microscope (Wild model M5), and made photographs using a 35 mm camera (Pentax). To determine the relative distribution of glands, we made 12.5 x prints of photographs of the top, middle, and lower portions of the tracheas from 3 ferrets. Each segment photographed contained approximately 15 cartilagenous rings. We measured the area enclosed by glands using a rolling disk planimeter (LaSICO model 1250 S1; Los Angeles, CA). Results are expressed as gland area/ total area of each segment of trachea. Additional tissues were processed for conventional light microscopy by fixing them in Bouin's solution, embedding them in glycol methacrylate, cutting 5 µm sections and staining them by the periodic

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acid Schiff method. For electron microscopy, tissues were fixed *in situ* by perfusing anesthetized animals with formaldehyde-glutaraldehyde fixative.

To study gross innervation, we studied 2 ferrets by making careful dissections of the nerves around the trachea.

RESULTS

Tracheas from adult ferrets are approximately 10 cm long, and contain about 60 cartilagenous rings. The rings are discontinuous, with a posterior membrane connecting the ends. The trachaelis muscle inserts near each end of each ring, and when freshly removed from the animal, the muscle is contracted enough to cause the ends of the rings to overlap. Every trachea stained with neutral red dye. The most densely stained areas were between the cartilagenous rings (FIG. 2.1) where submucosal glands lie [187]. There are more glands near the central part of the anterior trachea (FIG. 2.2). The glands are evenly distributed longitudinally. In the upper, middle, and lower trachea, they occupy 31.5 ± 4.3, 35.9 ± 6.7, and 32.1 ± 2.8 (SEM) %, respectively, of the total area (n=3 ferrets).

At higher magnification (FIG. 2.3), these densely staining areas have the typical appearance of submucosal glands, with acini surrounding collecting tubules that merge with each other and finally connect with a single excretory duct that leads to the surface of the trachea. These glands have similar structure when observed after conventional histological preparation (FIG. 2.4). The acinar cells stain with the periodic acid Schiff reagent indicating that carbohydrates are present in these cells. Electron micrographs indicate clearly the cell types present in the glands (FIG. 2.5). The gland acini are composed of two main secretory cell types, serous cells and mucous cells, that surround a central lumen. The serous cells are filled with electron-dense granules that are mostly supranuclear in the cell. The mucous cells contain granules of mixed

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morphology. Some of the granules are electron-lucent, and some granules have dense cores or areas of heterogeneous staining. Myoepithelial cell processes are commonly found in close proximity to the acini, often with one process near each individual cell (e.g., FIG. 2.5). Bundles of nerves are found between acini, as are fibroblasts (FIG. 2.5).

Parasympathetic and sympathetic nerves innervate the trachea. Dissections show that roots of the vagus nerve leave the skull, and they join to form the vagus nerve cranially from the jugular and nodose ganglia (FIG. 2.6). The jugular ganglion lies immediately medial to the auditory bulla within 3-4 mm of the exit of the nerve roots from the skull. The superior laryngeal nerve leaves the main branch of the vagus approximately 2 mm below the nodose ganglion to innervate the larynx and possibly the upper trachea. Below the inferior cervical ganglion, the recurrent laryngeal nerve leaves the vagosympathetic trunk to innervate the lower trachea. Sympathetic fibers may enter the trachea via short branches from the superior cervical ganglion. Additionally, a bundle of fibers approximately 5 mm long runs from the stellate ganglion into the recurrent nerve near the most caudal protion of the loop.

Ganglia in the submucosa of the airway lie in rows, arranged along the posterior border of the anterior portion of the trachea (FIG. 2.7). Nerve bundles in the submucosa contain at least two types of varicosities (FIG. 2.8). The most common type contains small electron-lucent granules characteristic of cholinergic nerves. The other common type contains small vesicles with electron-dense cores

characteristic of adrenergic nerves. Additionally, processes of supporting cells are seen between varicosities (e.g., FIG. 2.8). In some cases however, the cholinergic varicosity is close to the adrenergic nerve without any supporting cell in between (FIG. 2.9).

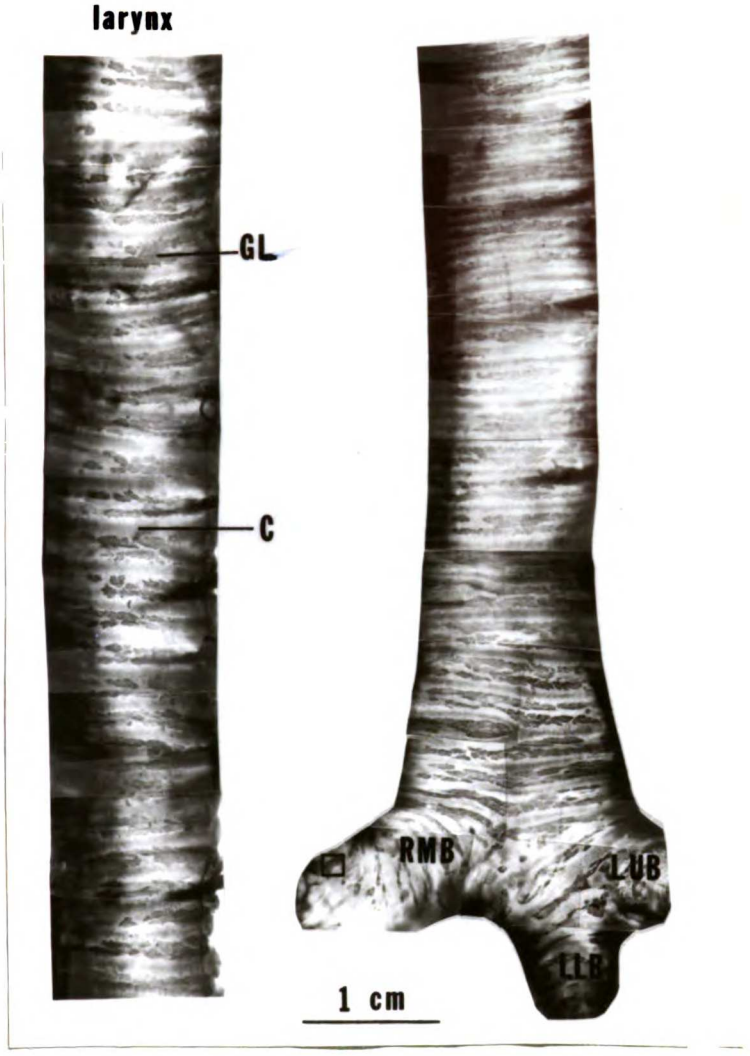

FIG. 2.1. Photomicrograph of an entire trachea in vitro from a ferret. The posterior membrane was incised, the trachea was opened flat, stained with neutral red (0.1 %; 45 min), and photographed via transmitted light. The tracheal submucosal glands (GL) appear as darkly staining bands throughout the trachea between cartilage rings (C). One gland (in square) in the right mainstem bronchus (RMB) is seen in FIG. 2.3. Other glands are in the left lower lobe (LLB) and left upper lobe (LUB) bronchi.

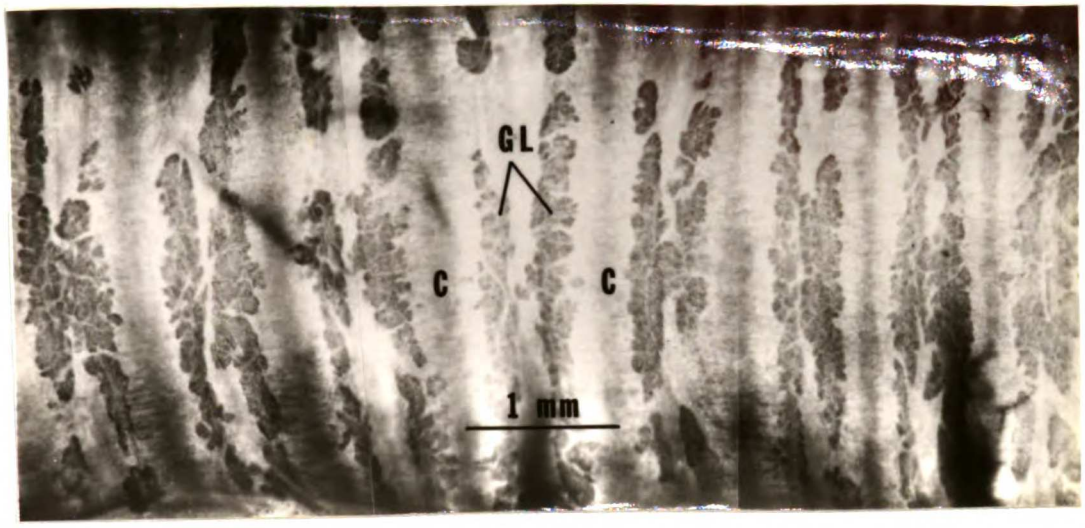

FIG. 2.2. Photomicrograph of a segment of the anterior portion of a ferret trachea stained with neutral red. Glands (GL) appear in double rows between cartilage rings (C).

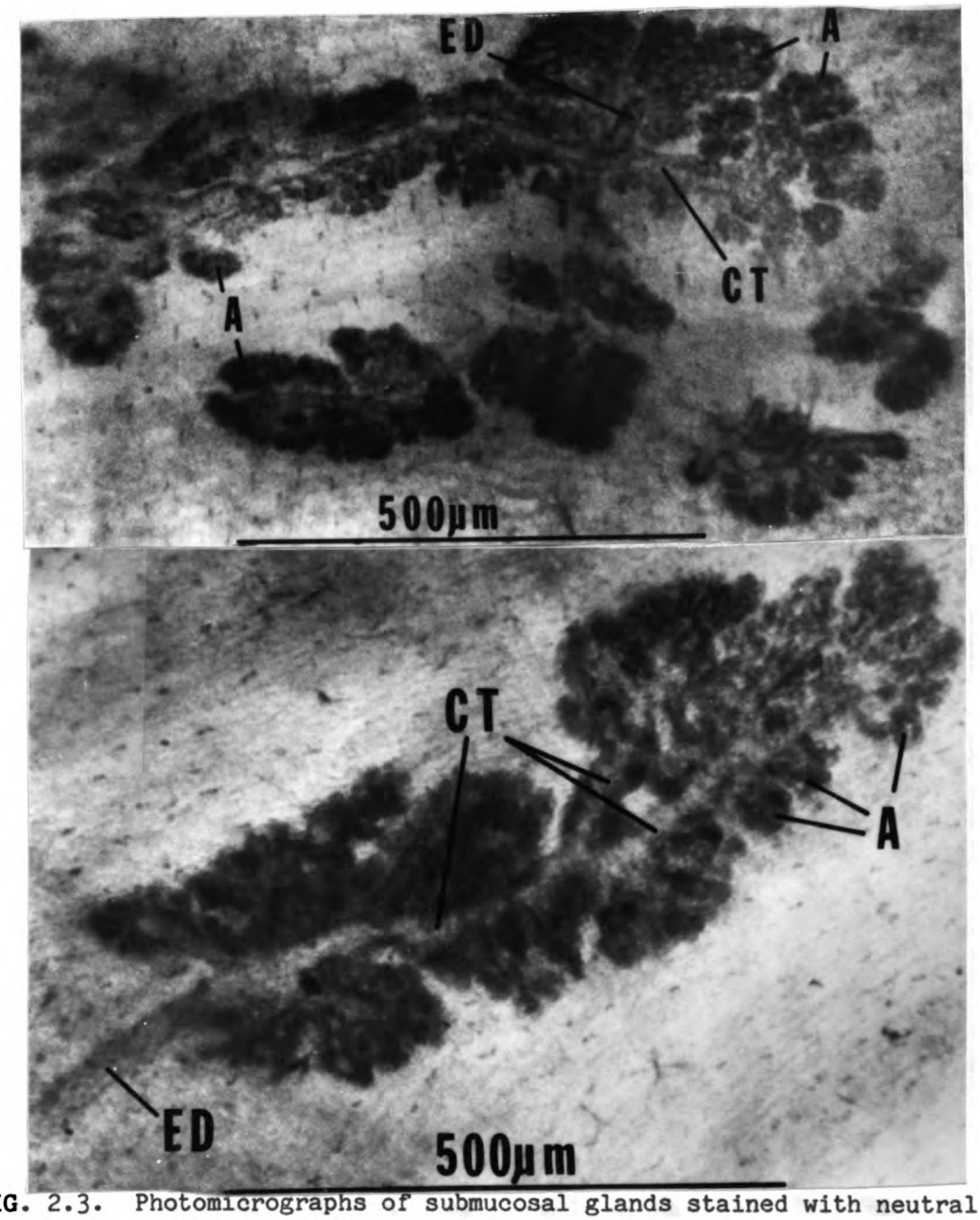

FIG. 2.3. Photomicrographs of submucosal glands stained with neutral red. Top: A gland from the midtrachea near the posterior membrane. Bottom: The gland in the Right Lower Lobe Bronchus shown in the square in FIG. 2.1. Acini (A) connect to collecting tubules (CT) which in turn, connect with a single excretory duct (ED) that opens onto the surface of the trachea.

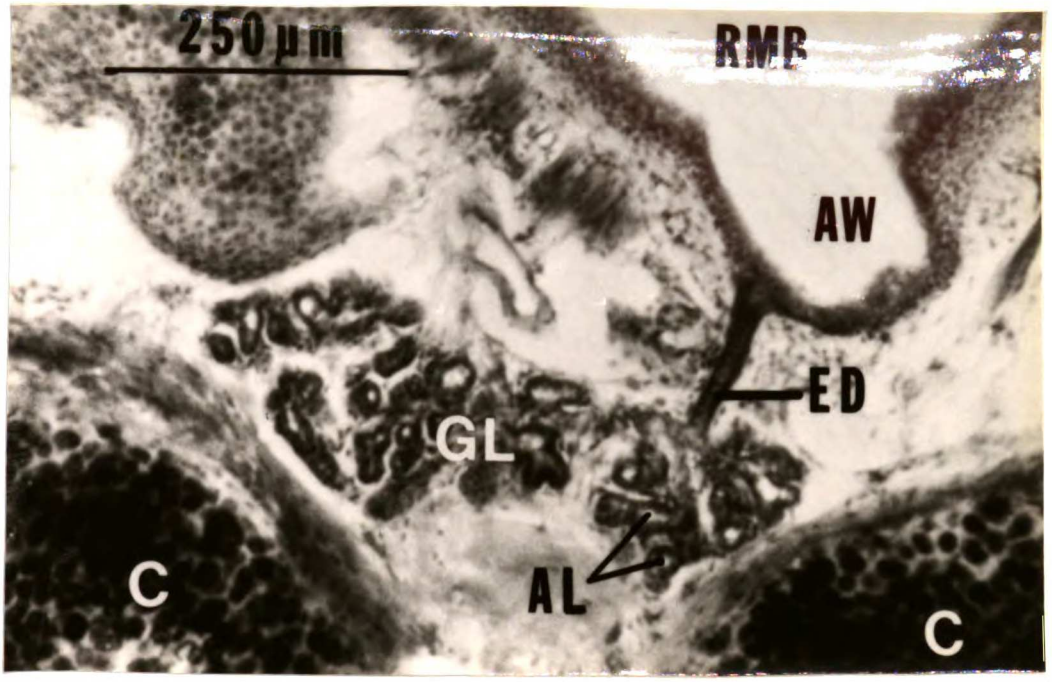

FIG. 2.4. Light micrograph of a 5 µm thick section of a tracheal gland in a small airway near the right mainstem bronchus. Tissue was fixed in Bouin's solution and stained by the periodic acid Schiff method. Acinar lumina (AL) of the gland (GL) connect, via an excretory duct (ED), to the surface of a small airway (AW) in the right mainstem bronchus (RMB). Cartilage rings (C) are on each side of the gland.

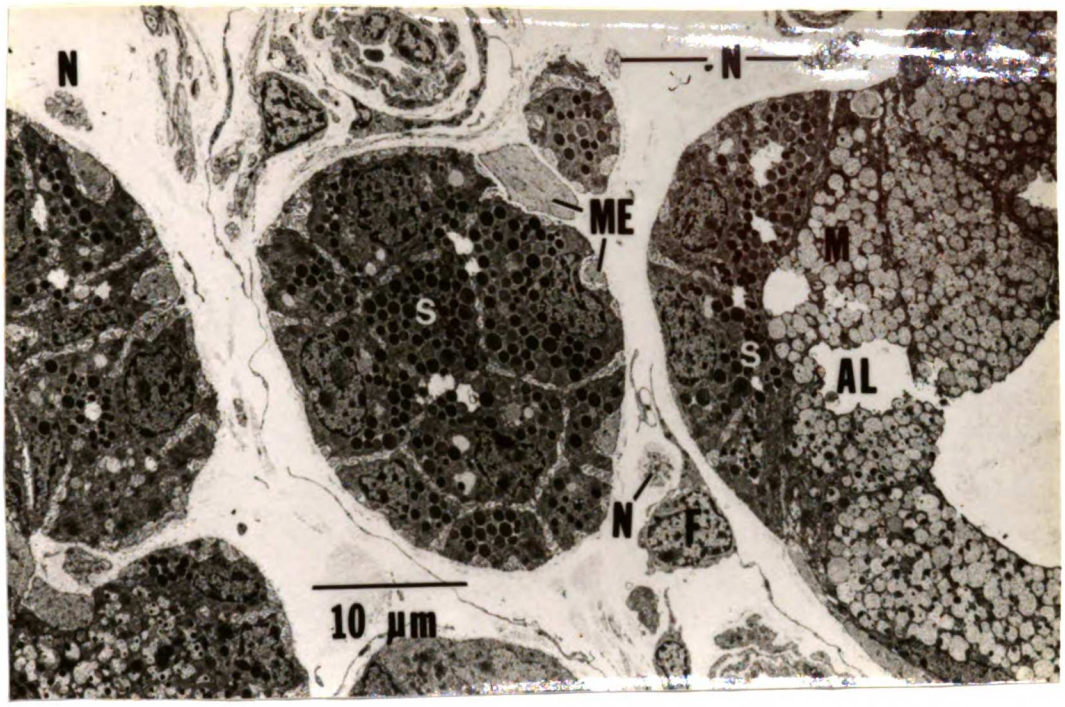

FIG. 2.5. Electron micrograph of a submucosal gland from a ferret showing serous (S) and mucous (M) cells surrounding an acinar lumen (AL) (on right). The plane of section missed the lumina of other acini. Processes of myoepithelial cells (ME) surround acini. Nerve bundles (N) are found close to acini, as are fibroblasts (F). Tissue was fixed with glutaraldehyde and stained with uranyl-acetate. Photomicrograph courtesy of C. Basbaum.

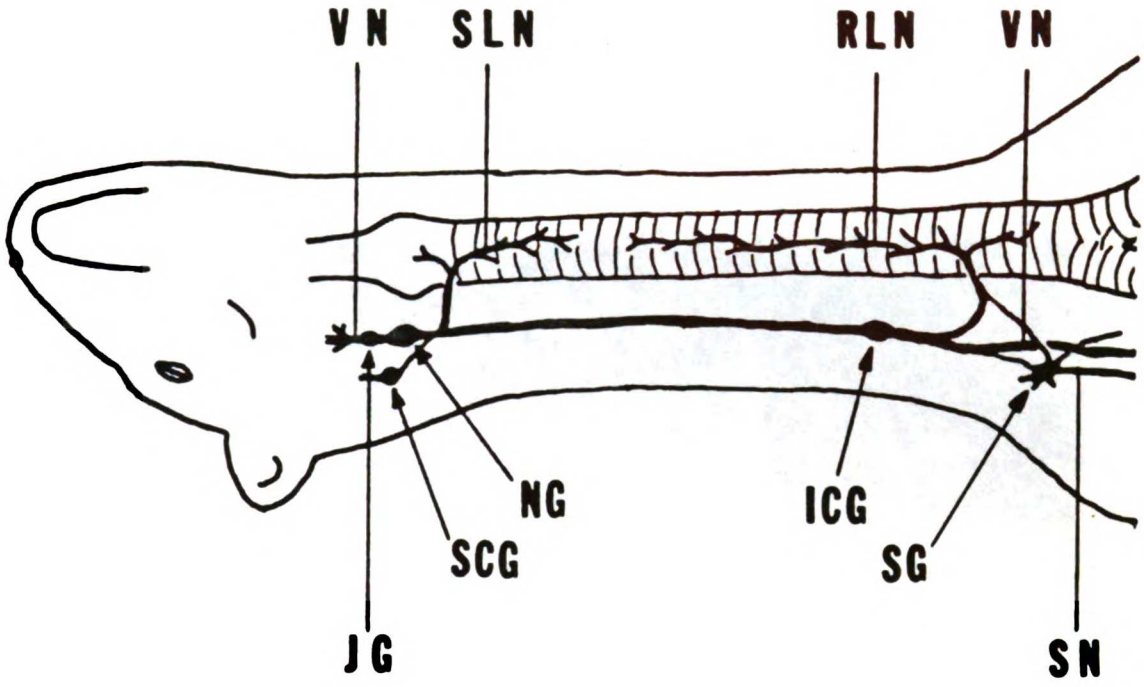

FIG. 2.6. Diagram of parasympathetic and sympathetic innervation of the ferret trachea. From the skull, roots of the X nerve join together to form the vagus nerve (VN). Caudally from the jugular (JG) and nodose (NG) ganglia, the superior laryngeal nerve (SLN) innervates the larynx and upper trachea. Below the inferior cervical ganglion (ICG), the recurrent laryngeal nerve (RLN) leaves the vagus to innervate the lower and middle trachea. Nerves from the superior cervical ganglion (SCG), stellate ganglion (SG), or the sympathetic trunk (SN) may provide sympathetic innervation to the trachea via the SLN and RLN, respectively.

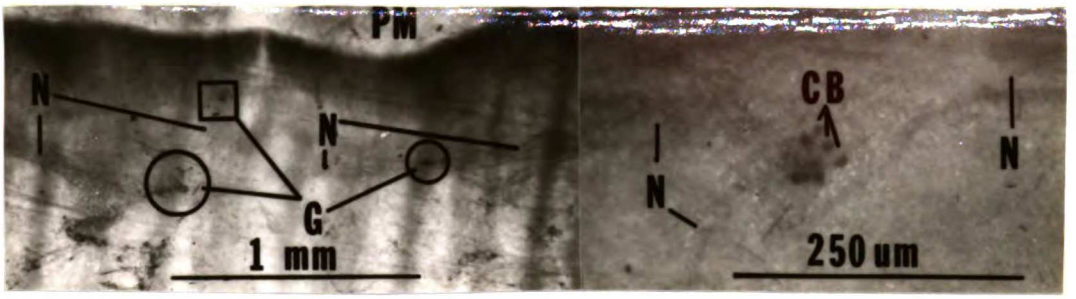

FIG. 2.7. Posterior portion of the mid-trachea of a ferret stained with neutral red. Left: Near the posterior membrane (PM) and near the ends of the cartilage rings, a longitudinal nerve (N) connects several ganglia (G)(circled). Right: Higher magnification photograph of the ganglion on left photograph in the square, showing ganglion cell bodies (CB). Nerve bundles (N) enter the ganglion from several directions.

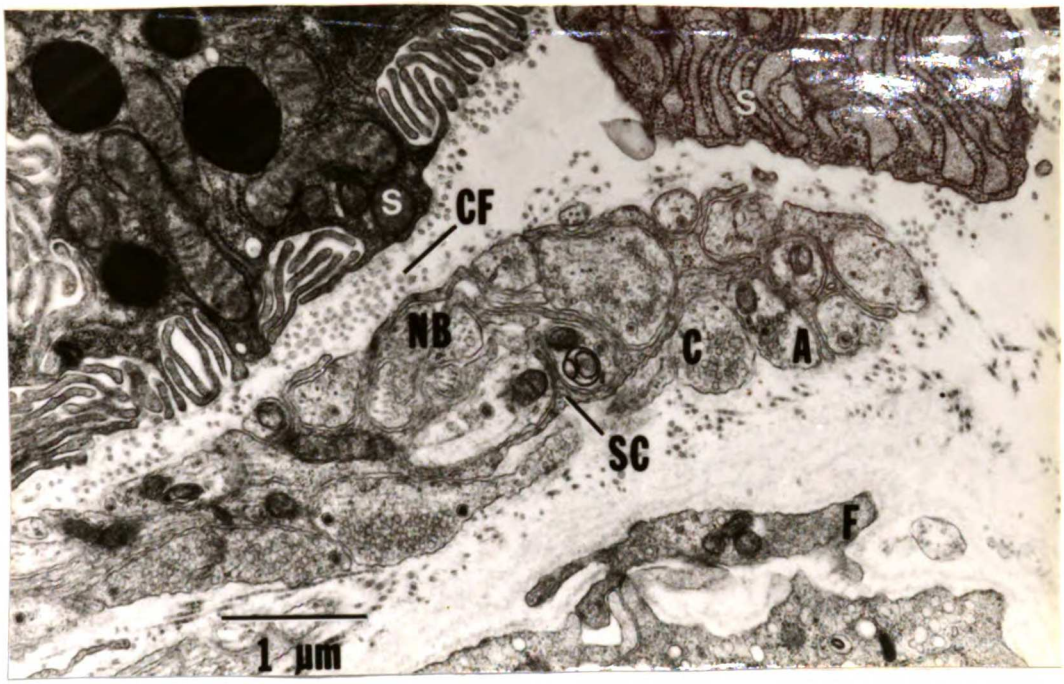

FIG. 2.8. Electron micrograph of a nerve bundle (NB) near a tracheal submucosal gland. Nerve varicosities containing synaptic vesicles characteristic of cholinergic (C) and adrenergic (A) nerves are close to 2 serous cells (S). Processes of supporting cells (SC) partially surround axons in the bundle. Collagen fibers (CF) and fibroblasts (F) are nearby. Tissue was fixed with glutaraldehyde and stained with uranyl-acetate.

Photomicrograph courtesy of C. Basbaum.

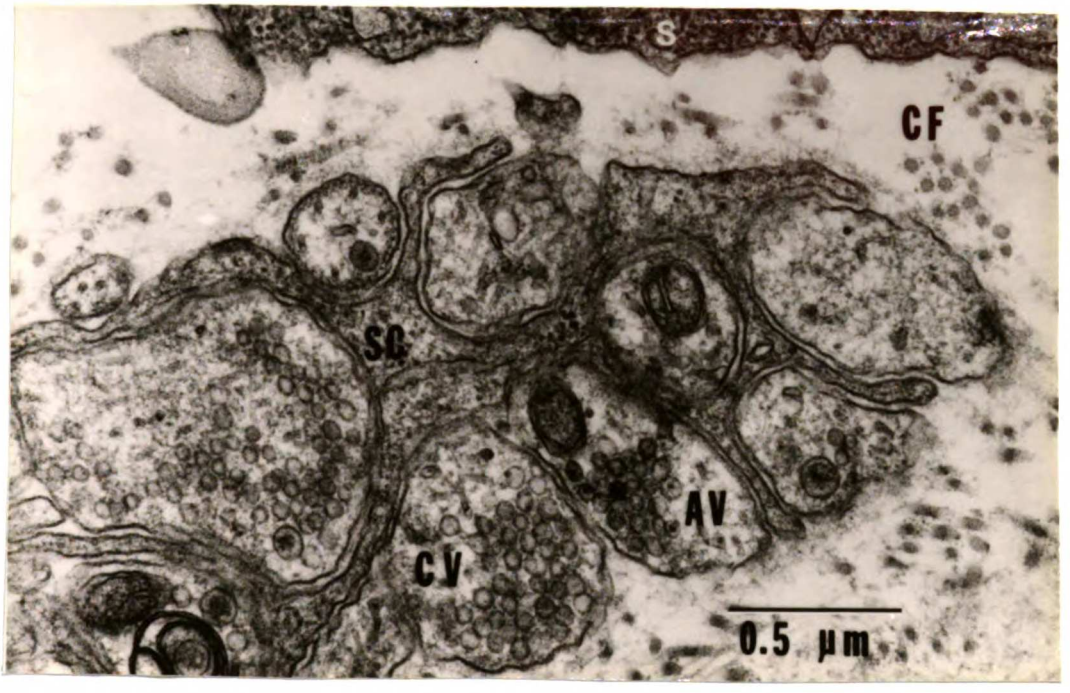

FIG. 2.9. Higher magnification photograph of a portion of the nerve bundle in FIG. 2.8. The varicosities of adrenergic (AV) and cholinergic (CV) nerves are close to each other without being separated by a supporting cell (SC). Collagen fibers (CF) may help maintain the relative positions of the bundle and the secretory cells. Photomicrograph courtesy of C. Basbaum.

DISCUSSION

Our aim was to determine whether ferrets have sufficient numbers of glands to be useful for studies in the regulation of tracheal secretions. Additionally, we wanted to determine whether parasympathetic and sympathetic nerves innervate the glands. Our results show that ferrets have many submucosal glands (FIGS. 2.1 - 2.5), and that they are distributed evenly throughout the trachea. This last result was based on the neutral red studies. Neutral red stains lysosomes and vacuoles [98], and the secretory granules of basophils and mast cells [192], and neuron cell bodies [93]. Neutral red is avidly taken up by macrophages, neutrophils, and eosinophils, whereas tumor cells and fibroblasts take up neutral red to a lesser degree [98]. Mature red blood cells and small lymphocytes do not take up the stain [98], possibly because these cell types do not contain large numbers of lysosomes or granules. Additionally, because the color of a solution of neutral red is dependent of pH [179], neutral red has been used to monitor the intracellular pH of plant cells [179]. Thus, neutral red stains a wide variety of cell types, and therefore may be useful for the identification of submucosal gland cells. The secretory materials in tracheal gland cells may stain because the sialic acid or sulfate [214] in acidic glycoproteins [49, 106, 213] may provide the proper pH for neutral red to be appear red, and thus stain many parts of submucosal gland cells (FIG. 2.3), including acinar cells, collecting tubules, and excretory ducts. However, the neutral red method is not sufficiently sensitive to distinguish between serous and mucous cells because the unfixed

trachea cannot be adequately cut for frozen sections. Furthermore, freezing the tissue disrupts the cells and the dye leaks out. Thus, we needed to fix the tissues and use conventional histological methods to distinguish the cell types. Using these methods, we found that ferrets have both serous and mucous cells in their submucosal glands. The structure of the glands of ferrets is therefore similar to the structure of glands of human beings [147, 148], cats [74, 151], pigs [106], and sheep [135].

We also found that branches of the vagal parasympathetic and sympathetic nerves appear to innervate the trachea (FIG. 2.6). However, the superior laryngeal nerve and the sympathetic nerves from the superior cervical and stellate ganglia are all quite small and would be very difficult to isolate in vivo. Branches from the laryngeal nerves may connect with metamerically arranged ganglia in the airway wall. These ganglia may give rise to the nerve varicosities characteristic of cholinergic nerves seen in electron micrographs (FIGS. 2.8 - 2.9)[9]. The sympathetic nerves may give rise directly to the profiles characteristic of adrenergic varicosities. The finding that cholinergic and adrenergic varicosities are occasionally seen without any supporting cell processes in between suggests the possibility that one nerve type may interact pre-synaptically with the other(s). This close proximity of nerves in airways has been demonstrated previously only in cats [151].

Thus, ferrets have some similarities to other species. As with ferrets, goats and horses [91], sheep [135], pigs [106, 141], cats [74], opposums [212], and human beings [216, 230] each have many

tubulo-acinar submucosal glands. This is not true for birds [178], or mice, rats, guinea-pigs, hamsters, or rabbits [91, 141]. As in humans [226, 230], the glands of ferrets are present throughout the trachea. Therefore, we could perform experiments on several tissues from each animal. Unlike cats, dogs, and humans, ferrets do not have many tracheal goblet cells [103](Basbaum, personal communication). This finding simplifies the interpretation of studies with $^{35}SO_4$-labeling techniques, since goblet cells as well as glands incorporate sulfate into macromolecules [105]. Additionally, as with cats [151] and humans [122], ferrets have adrenergic, cholinergic [9], and possibly peptidergic [8] innervation.

These results suggest that ferrets may be suitable for studies of the neural regulation of secretion from tracheal submucosal glands. However, ferrets are probably not suitable for experiments involving the stimulation of parasympathetic or sympathetic nerves separately. These results also suggest that in addition to classical, direct efferent innervation of tracheal glands, there may be interaction between cholinergic and adrenergic nerves in the airway glands as has been suggested for tracheal smooth muscle [240]. Although there is good anatomic evidence for innervation of airway glands, we do not know what the functions of the nerves are. Therefore, we did a series of studies to determine whether cholinergic, adrenergic, and possibly other nerves mediate secretion of fluid or macromolecules from tracheal glands. Additionally, we wanted to see if nerves to glands interact with each other.

HILLOCKS

INTRODUCTION

The purpose of these studies was to determine which nerves mediate the secretion of fluid from tracheal glands. Only a few studies have measured effects on submucosal glands without the complications of other secretory cell types (e.g., tracheal epithelial and goblet cells). Therefore, we performed a series of experiments in vitro and measured the secretions from only the submucosal glands using the "hillocks" technique of Nadel [153]. Fluid secreted from tracheal submucosal glands comes to the surface of the luminal surface of the trachea via an excretory duct. As the fluid reaches the surface, the surface tension of the mucus prevents the fluid from immediately spreading over the epithelium. Therefore, a droplet of mucus is visible on the trachea for a time after secretion has occurred [114]. To increase the contrast of the secretion droplets against the background of the tracheal epithelium, we apply a thin layer of tantalum powder to the surface of the trachea before secretion has occurred. During secretion, the mucus elevates the tantalum layer over the duct openings to form "hillocks". Hillocks occur only directly over the duct openings [155] Thus, the secretions from the glands can be easily seen and measured.

EXPERIMENTAL DESIGN

Our approach to the problem was to stimulate nerves to tracheal glands in vitro using a modification of the electrical field stimulation technique developed in 1955 by Paton [166], and to inhibit neural effects with pharmacological antagonists of neural mechanisms. To interpret results, we need to know the specificity of each antagonist.

ELECTRICAL FIELD STIMULATION

Application of electrical fields stimulates cholinergic nerves. Paton found that electrical pulses of less than 0.5 ms duration activate cholinergic nerves to ileal smooth muscle of the guinea pig, and that the chronaxie of the intensity/duration curve was 0.2 ms [107, 166]. However, after denervation, the response to electrical field stimulation disappeared [168]. Additionally, the muscle contraction was prevented by low concentrations (10^{-8} M) of atropine and augmented by the acetylcholinesterase inhibitor, physostigmine. Because acetylcholine was released by electrical stimulation of intact preparations but not of denervated tissues, the muscular response was due to the stimulation of the intramural nerves, and not due to direct stimulation of the smooth muscle [167]. In the rabbit ileum, electrical field stimulation results in increased transepithelial transport of ions [102]. Since this effect was prevented by atropine, electrical stimulation also activates cholinergic nerves that mediate secretion.

Application of electric fields also can also activate adrenergic nerves that cause either relaxation or contraction. Paton [167] found that field stimulation caused progressive relaxation in gastrointestinal tract smooth muscle/nerve preparations treated with progressively larger doses of atropine. Additionally, electrical field stimulation of isolated artery smooth muscle/nerve preparations causes contraction via the release of endogenous norepinephrine [51]. Furthermore, reserpine, a drug that depletes catecholamines from adrenergic nerve terminals, and choline-2:6-xylyl ether (TM 10), a drug, similar to guanethidine, that inhibits release of catecholamines from spleen and adrenergic nerves [3, 23], also prevented relaxation of gastric smooth muscle due to electrical stimulation. Cocaine potentiated this relaxation [167], presumably by preventing re-uptake of norepinephrine thereby increasing the local concentration of norepinephrine at adrenergic effector sites [121]. Finally, the alpha-adrenergic receptor antagonist, phentolamine, reduced the relaxation response to electrical stimulation. Thus, adrenergic and cholinergic nerves can be stimulated simultaneously. Using electrical field stimulation pulses of 0.1 ms, Foster [69] stimulated both the adrenergic and cholinergic nerves to isolated guinea pig trachea. Electrical stimulation caused contraction followed by relaxation. Cocaine potentiated the relaxation in this tissue as it did in the ileum. The contraction was prevented by atropine, and the relaxation was prevented by either the beta-adrenergic antagonist, dichloroisoproterenol, or the sympatholytic drug, guanethidine.

Further evidence that electrical stimulation activates nerves derives from experiments in which either cholinergic denervation of guinea pig ileum [168], or sympathetic denervation of vascular smooth muscle [13] abolished contractions in response to short electrical pulses. Thus, there is ample evidence that both adrenergic and cholinergic nerves can be stimulated in vitro by short electrical pulses.

In addition to adrenergic and cholinergic effects, some responses of smooth muscles of gastrointestinal tract or airway to electrical field stimulation are not prevented by adrenergic or cholinergic antagonists, but are prevented by blocking nerve conduction [28, 38, 190]. There are many possible neurotransmitter substances for these nonadrenergic noncholinergic neural effects, including vasoactive intestinal peptide (VIP) [139] or the adenine nucleotides [28]. VIP is released from nerves in the ileum and mediates relaxation of smooth muscle [139]. Since antibodies to VIP prevented some of the relaxation of the ileum, the probable neurotransmitter for this effect is VIP [139]. Adenine nucleotides have also been suggested as transmitters in intestinal nerves that mediate nonadrenergic noncholinergic relaxation [28, 132]. Although the identities of all of the possible neurotransmitters are not known, I conclude from these studies of smooth muscle of the vascular beds and gastrointestinal tract, and of ion transport across the ileum, that electrical fields can be used to stimulate a variety of different nerve types in vitro. Furthermore, pharmacological antagonists can be used to study the effects of simultaneously stimulating several types of nerves.

TETRODOTOXIN

To verify that the secretory responses to electrical field stimulation are mediated by nerves, we studied the electrically-evoked responses before and after incubating the tissues with the puffer fish toxin, tetrodotoxin. Tetrodotoxin blocks the rapid entry of sodium ions through depolarized nerve membranes without affecting permeability to either K^+ or Cl^- [157], and thereby abolishes action potentials in both giant fibers of lobsters [157] and skeletal muscle [85]. However, tetrodotoxin does not prevent either spontaneous miniature end plate potentials [59] or those evoked by electrotonic depolarization of pre-synaptic nerve terminals [107, 108]. Tetrodotoxin also does not inhibit spontaneous contractions of smooth muscle or those induced by either acetylcholine or norepinephrine [85, 118]. Similarly, tetrodotoxin inhibited the secretion of ions from the rabbit ileum evoked by nerve stimulation but not by either cholinergic or adrenergic agonists [102]. Furthermore, we showed that tetrodotoxin does not inhibit the release of ^{3}H-norepinephrine evoked by K^+ (CHAPTER 5). Thus, tetrodotoxin can be used acutely to block nerve conduction, and thereby to cause functional denervation.

AUTONOMIC ANTAGONISTS

To determine which neural mechanisms mediate secretion, we used the competitive antagonists, atropine, phentolamine, and propranolol. We chose competitive antagonists for two reasons. First, because the inhibition produced is likely to be more selective, because the antagonist must have close structural similarity to the agonist in order to be effective [160]. Second, the inhibition can be overcome

by using high concentrations of the corresponding agonists or by stimulating nerves at higher frequencies. We wanted to use within-tissue controls wherever possible because in previous studies of secretion from airways in vivo there was wide variability [74]. Ideally, the first response would be under control conditions. Subsequent responses would be under specific experimental conditions, usually inhibitory, and the last response would be under conditions expected to overcome the inhibition. The last response would provide a positive control for the tissue. Non-competitive antagonists act by forming irreversible covalent bonds with receptors and other sites as well [160]. This means that the inhibitory effects persist long after the drug has been washed out of the tissue as a whole [161]. This may reduce the likelihood of significant non-specific (e.g., local anesthetic) effects, but since inhibition cannot be overcome, we would not be able to use a positive control at the end of the experiment. The positive control would allow us to conclude that the inhibition in the presence of the antagonists was not due to either depletion of mucus from the glands or to nerve or gland death.

Because the antagonists are the major tools we used to study mechanisms of secretion, we have to define the specificity of each of the antagonists. To do this, we need to show that each of the antagonists used inhibits only the effects of the corresponding agonists (e.g., phentolamine inhibits only the effects of phenylephrine), and fails to inhibit effects of other non-corresponding agonists (e.g., phentolamine does not inhibit the effects of either acetylcholine or terbutaline).

METHODS

To demonstrate a role for specific nerves in the secretion of fluid, we first have to show that stimulation of nerves causes secretion. Next, we have to show that specific receptor mechanisms mediate secretion, and finally, that receptor antagonists prevent at least some of the response to nerve stimulation.

A. PREPARATION

We anesthetized 27 adult male ferrets weighing 1 - 1.5 kg each by injecting sodium pentobarbital (45-60 mg/kg ip). After making a ventral midline incision in the neck, we excised the entire trachea and placed it in Krebs-Henseleit solution maintained at 37° C and bubbled with 95% O_2 - 5% CO_2. We removed individual segments of the trachea 1.5 cm long, cut through the segment in the posterior midline, and opened the segment flat. Then, we mounted the segment, luminal side up, on top of a plastic half-chamber 1 cm in diameter. We impaled the edges of the tissue with pins and secured the tissue in place with a ring clamp (FIG. 3.1). Then we superfused the submucosal surface of the tissue with Krebs-Henseleit solution.

To prepare a field for viewing, we removed the mucus from the luminal surface of the epithelium by carefully wiping it with tissue paper pledgets, and then we coated the surface with powdered tantalum. Tantalum increases the contrast between the fluid droplets and the surrounding epithelial surface (FIG. 3.2)[153]. Thus, the secretions are more easily seen. I viewed the tantalum surface through a binocular dissecting microscope (Zeiss Epitechniscope) and recorded

the image on tape using a Sony Videotape System (Camera model AVC-1400; Tape Recorder model VO-2600) (FIGS. 3.1,3.2).

B. ELECTRICAL STIMULATION

To study secretion, we first needed to know whether electrical field stimulation resulted in secretion, and if it did, what the optimum stimulus parameters were. We chose a pulse duration of 0.5 ms on the basis of the work of Paton [166], and therefore studied only the voltage and frequency dependence of the response to electrical stimulation. First, we stimulated 2 tissues from each of 3 ferrets at voltages from 1 - 20 volts via the 3 pins on each side of the chamber (FIG. 3.1) (Pulse duration, 0.5 ms; frequency, 6 monophasic cycles/s for 1 min) using a Grass Stimulator (model S88) and counted the number of hillocks on the field. Since the circuit was shorted through the tissue and the Krebs-Henseleit solution underneath, the nominal voltage output from the stimulator was nearly 10 times that applied to the tissue, and the current was 500 mA. These results showed that at 10 V applied to the tissue (500 mA), the number of glands stimulated had reached a maximum (FIG. 3.3). We therefore stimulated all other tissues at at least 10 V. Next, to determine the frequency dependence of the response to electrical stimulation, we stimulated 2 other tissues from the same 3 other ferrets at frequencies of 0.5 - 20 cycles/s for 1 min (intensity, 10 V; pulse duration, 0.5 ms), and counted the number of glands stimulated to secrete fluid.

C. AUTONOMIC AGONISTS

To see whether cholinergic or adrenergic agonists stimulate secretion, we measured the diameters of 6 round hillocks in one tissue from each of 4 ferrets, 3 and 4 min after adding either acetylcholine (10^{-7} - 10^{-4} M; Sigma Chemical Co., St. Louis, MO) or phenylephrine (10^{-7}- 10^{-3}M; Neo-Synephrine; Winthrop Laboratories, New York, NY) to the submucosal bathing solution.

To study the mechanisms of secretion, we began by measuring the responses of 1 tissue from each of 5 ferrets to electrical stimulation, acetylcholine, and phenylephrine in the absence of inhibitors. To determine which glands responded to electrical stimulation, 1 min after preparing a field for viewing, we photographed it, stimulated the tissue electrically for 1 min (intensity, 10 V; pulse duration, 0.5 ms; frequency, 6 pulses/s; train duration, 0.6 s; train rate, 1/s), and then measured diameters of the selected hillocks. Next, to determine which glands responded to cholinergic agonists, we prepared a new field in each tissue and photographed it after 3 min without stimulation, and then again 3 min after adding acetylcholine (5×10^{-5} M) to the submucosal bathing solution via the bubbling chamber (FIG. 3.1). Then, to determine which glands responded to adrenergic agonists, we prepared a new field in each tissue and photographed it after 4 min without stimulation, and again 4 min after adding phenylephrine (5×10^{-5} M) to the solution bathing the submucosal side. After each stimulus, we measured the diameters of the selected hillocks. Preliminary experiments showed that the stimulation time used for each stimulus

was long enough so that the hillocks were easily measurable. Furthermore, the stimulation times were short enough so that the hillocks were small enough to remain round and not merge with each other. Not all of the glands studied responded to each stimulus: 58% of 325 glands responded to all three stimuli, electrical stimulation, acetylcholine, and phenylephrine. The probable reason for this difference was due to the presence of bubbles in the chamber that prevented the agonists from reaching all parts of the tissue. Therefore, we selected only those glands that responded to electrical stimulation, acetylcholine, and phenylephrine under control conditions. To determine whether the responses to stimulation decreased with time, we repeated this sequence of stimulation procedures at 45 min, 90 min, and 135 min after the first sequence.

D. AUTONOMIC NERVES

In the next series of experiments we used the electrical stimulation-acetylcholine-phenylephrine sequence to determine whether the secretory response to electrical stimulation was mediated by nerves. We determined whether stimulation still elicited secretion from each of 5 ferrets after adding tetrodotoxin (10^{-7} M; 20 min; citrate-free; Calbiochem-Behring, La Jolla, CA) to the bathing solution.

In the next 3 series of experiments, we determined which autonomic nerves mediated the response to electrical stimultion. Tyramine releases norepinephrine from adrenergic nerves (CHAPTER 5) [127, 194, 239]. Therefore, if adrenergic nerves mediate secretion of fluid from tracheal glands, then tyramine should release

norepinephrine onto the glands and thereby stimulate the production of hillocks. We tested this hypothesis by photographing 1 tissue from each of 3 ferrets after 4 min without stimulation, and again, after 4 min incubation with tyramine (10^{-4} M; Sigma Chemical Co., St. Louis, MO), and measured the diameters of selected hillocks. To determine whether the effect of tyramine is mediated by alpha-adrenergic receptors, we repeated the stimulus after incubation in phentolamine (10^{-6} M; 20 min).

Then, to determine whether alpha-adrenergic and cholinergic nervous mechanisms mediate the response to electrical stimulation, we studied the responses of 1 tissue from each of 5 other ferrets to electrical stimulation, acetylcholine, and phenylephrine before and after incubating the tissues with muscarinic and alpha-adrenergic antagonists. To inhibit muscarinic effects, we incubated each tissue for 20 min with atropine sulfate (10^{-6} M; 20 min; J.T. Baker Chemical Co, Phillipsburg, NJ). Our criterion for muscarinic inhibition was the prevention of the secretory response to acetylcholine. To wash out the atropine and to inhibit alpha-adrenergic effects, we then incubated each tissue for 20 min with phentolamine methanesulfonate (10^{-6} M; Regitine Mesylate; CIBA Pharmaceutical Co, Summit, NJ). Our criterion for alpha-adrenergic inhibition was the prevention of the secretory response to phenylephrine. To inhibit both muscarinic and alpha-adrenergic effects, we then incubated each tissue with both atropine and phentolamine (each drug, 10^{-6} M; 20 min). Then, in a separate experiment to determine whether beta-adrenergic mechanisms mediate fluid secretion, first we studied 1 tissue from each of 5

additional ferrets by photographing a field after 4 min without stimulation and again 4 and 10 min after adding terbutaline sulfate (10^{-4} M; Bricanyl; Astra Pharmaceutical Co, Worcester, Mass) to the bathing solution. Then, to determine whether beta-adrenergic nervous mechanisms mediated secretion, we inhibited muscarinic and possible beta-adrenergic effects by incubating these tissues for 20 min with both atropine and DL-propranolol (10^{-6} M; Sigma Chemical Co, St. Louis, MO). After determining the responses to electrical stimulation, acetylcholine, and phenylephrine, we incubated each tissue with atropine, propranolol, and phentolamine together, and repeated the stimulation sequence. At the end of each experiment, we showed that the nerves and glands still functioned by electrically stimulating the tissue at 20 pulses/s for 1 min.

E. DATA ANALYSIS

Stimulation of glandular secretion either electrically or pharmacologically produced elevations, which we call "hillocks" [153] on the surface of the trachea. In the experiments designed to determine the parameters of electrical stimulation, we counted the numbers of hillocks. We considered an elevation to be a hillock only if it was not present immediately after applying tantalum, and if it formed in response to stimulation. The minimum diameter of recognizable hillocks was approximately 50 μm.

After electrical stimulation, there is a lag period of a few seconds, after which time fluid first appears over the duct opening. The size of the hillock increases rapidly at first, but within 1 min, the diameter of the hillocks does not increase as rapidly. This is

because, as the hillock gets larger, the ratio of the volume to diameter gets larger. Volume increases as the cube of the radius. Thus, a volume added to a large hillock produces less change in diameter than the same volume added to a smaller hillock. To calculate the volume of a round hillock, we assumed that the hillocks were hemispherical. Then the volume of a hillock is:

$$\text{VOLUME} = \frac{1}{12} \pi d^3,$$

where d is the diameter of the hillock. After stimulation with either acetylcholine or phenylephrine, there were longer lag periods, of between 1.5 and 2.5 min, respectively. 4 min after adding either acetylcholine or phenylephrine, the diameters of the hillocks were not increasing very rapidly. To calculate secretory rate, we measured hillock diamters after 3 and 4 min of incubation with acetylcholine or phenylephrine. We calculated the volume of each hillock at each time and expressed the difference in volume over the 1 min interval as the secretory rate.

For each experiment, we measured the diameters of only round hillocks. For the experiments to determine the mechanisms of the response to electrical stimulation, we selected 6 glands from each tissue that responded to electrical stimulation, acetylcholine, and phenylephrine in the absence of inhibitors, and still secreted fluid in response to electrical or pharmacological stimulation at the end of the experiment. To determine the diameters of selected hillocks, after each experiment we identified the locations of the glands by placing a piece of plastic over the video image of the last field of each experiment and marking the pattern of hillocks with a pen.

Because the relative positions of the duct openings remained constant in each tissue, we identified the positions of the same glands throughout each experiment by placing the plastic over the images of previous fields. We measured the diameters of the hillocks by using a video image shearing monitor (IPM model 907, IPM, San Diego, CA) to bisect images of individual hillocks horizontally and displace the upper half from the lower half by the diameter of the hillock. The output of the shearing monitor was proportional to the the displaced diameter. We calibrated the output by bisecting the image of the chamber and displacing the upper portion from the lower portion by the diameter of the chamber (1 cm).

We used Student's paired t test to compare hillock diameters within tissues and Student's unpaired t test to compare diameters between tissues.

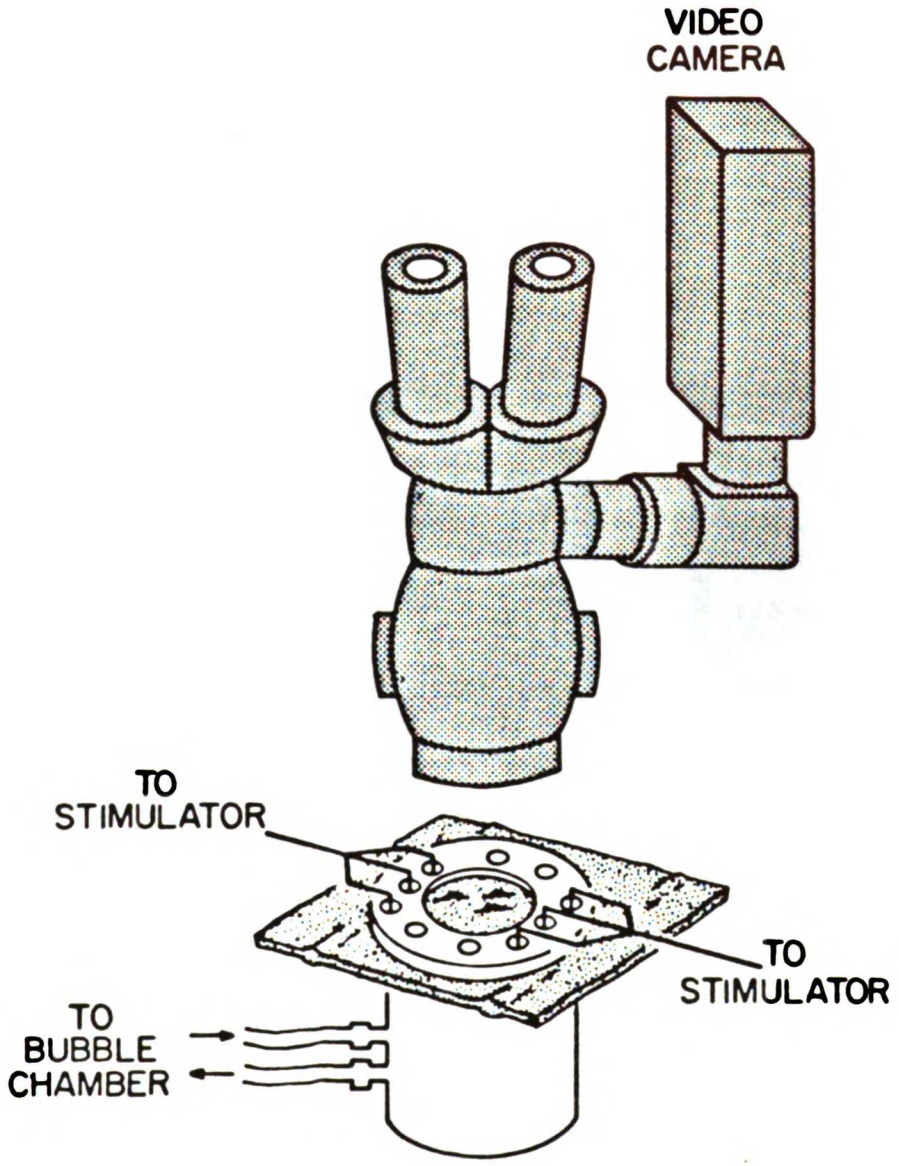

FIG. 3.1. Apparatus used to study fluid secretion in vitro. Tissue was impaled by pins and held on top of a half-chamber by a ring clamp. The luminal surface of the trachea was coated with tantalum powder and viewed through a microscope. For electrical stimulation of the tissue, three pins on either side of the chamber were connected to a Grass stimulator. For pharmacological stimulation and inhibition, drugs were added to the solution bathing the submucosal surface via a bubbling chamber (not shown).

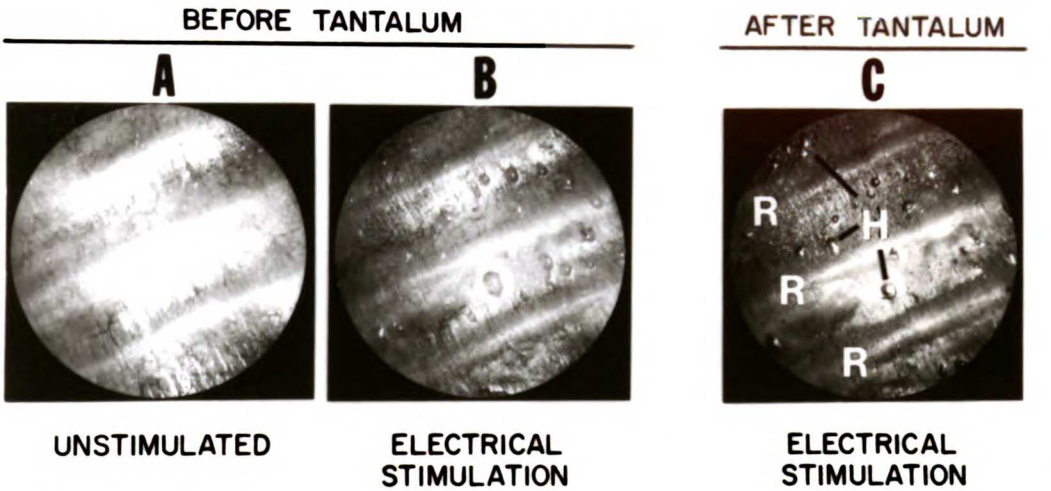

FIG. 3.2. Use of powdered tantalum to increase the visibility of secretions from tracheal submucosal glands of one tissue from a ferret. A: Unstimulated segment of trachea before application of powdered tantalum. B: Same segment after electrical stimulation (intensity, 10 V; pulse duration, 0.5 ms; frequency, 6 cycles/s for 1 min). C: Same segment after removal of the fluid, application of tantalum powder, and electrical stimulation as in B. The hillocks (H) are aligned in rows between cartilage rings (R).

RESULTS

In each of the glands studied, there was little spontaneous secretion during the first minute after tantalum was applied for the first time: the mean hillock diameter was 50 ± 10 μm (SEM). During the next minute, however, electrical stimulation (pulse duration, 0.5 ms; frequency, 6 monophasic pulses/s, train duration, 0.6 s; train rate, 1/s for 60 s)(e.g., FIG. 3.2) produced hillocks in a voltage-dependent fashion. The threshold of the response was approximately 1 V (FIG. 3.3), and the number of glands stimulated to secrete fluid increased to a maximum at 10V (FIG. 3.3). At a stimulus intensity of 10V, electrical stimulation produced hillocks in a frequency-dependent fashion. The threshold was approximately 0.5 cycles/s, and the number of glands stimulated reached a maximum at approximately 6 cycles/s (FIG. 3.4). Acetylcholine stimulated the production of hillocks in a dose-dependent fashion with a threshold of approximately 10^{-7} M, and a maximum response at 10^{-5} M (FIG. 3.5). Similarly, phenylephrine produced hillocks in a dose-dependent fashion, with a threshold of 10^{-6} M and a maximum response at 10^{-4} M (FIG. 3.5). Additionally, tyramine (10^{-4} M; 4 min) caused the formation of hillocks (TABLE 3.1). However, terbutaline failed to stimulate the secretion of visible fluid at either 4 or 10 min from any gland.

Each antagonist decreased the response only to its corresponding agonist (TABLE 3.1). Atropine prevented 88 ± 10% of the response to acetylcholine (5×10^{-5} M) ($p<0.001$; n=30), and phentolamine prevented 100% of the responses to phenylephrine ($p<0.001$; n=30) or tyramine ($p<0.05$; n=18). However, each antagonist or combination of

antagonists failed to decrease the responses to the non-corresponding agonists. Thus, atropine did not prevent the response to phenylephrine, phentolamine did not decrease the response to acetylcholine, and atropine plus propranolol did not diminish the response to phenylephrine (each comparison, $p>0.5$; n=30 glands)(TABLE 3.1).

Electrical stimulation (10 V; 6 cycles/s for 1 min) reproducibly produced hillocks whose mean diameter was 482 ± 24 μm (SEM; n=30). Subsequent periods without stimulation resulted in no hillock formation. The secretory response to repeated electrical stimulation decreased with time: the mean response at 135 min (after 9 previous electrical and pharmacological stimulations) in one tissue from each of 5 ferrets was 68 % of the first response (FIG. 3.8).

Tetrodotoxin prevented the response to electrical stimulation but did not decrease the responses to acetylcholine or phenylephrine ($p>0.5$; n=30) (e.g., FIGS. 3.6,3.7), suggesting that the response to electrical stimulation was mediated by nerves. Atropine alone decreased the response to electrical stimulation to 73% of the control response at the equivalent time (45 min) ($p<0.05$; n=30) (FIG. 3.8). Phentolamine alone decreased the response to electrical stimulation to 68% of the control response at the equivalent time (90 min) ($p<0.05$; n=30) (FIG. 3.8). Because the volume of a hemisphere decreases as the cube of the decrease in diameter, atropine and phentolamine each inhibit more than 50 % of the fluid secretion. However, we did not measure volume of the hillocks, and thus, the changes in diameter are not necessarily proportional to changes in volume. Therefore, we

cannot precisely determine the relative effects of muscarinic and alpha-adrenergic mechanisms. Atropine and phentolamine together almost completely prevented the response to electrical stimulation (FIG. 3.8). The response remaining (8 ± 5% of the control response at the equivalent time of 135 min) was not significantly different from zero ($p>0.3$; $n=30$). Atropine plus propranolol decreased the response to electrical stimulation. Mean hillock diameter was 263 ± 47 (SEM) μm. However, this decrease was no greater than that caused by atropine alone ($p>0.5$; $n=30$), but the further addition of phentolamine reduced mean hillock diameter in the same tissues to 10 ± 8 (SEM) μm ($p<0.001$; $n=30$).

The results are similar for intrathoracic segments of trachea. The response to electrical stimulation (460 ± 28 μm) in the absence of drugs was not different from the response of extrathoracic segments (482 ± 24 μm; $p>0.5$; $n=15$). Atropine decreased the response to electrical stimulation to 178 ± 39 μm (54 % of the control response at the equivalent time). The addition of phentolamine further reduced hillock diameter to 47 ± 24 μm (16 ± 8 % of the control response at the equivalent time).

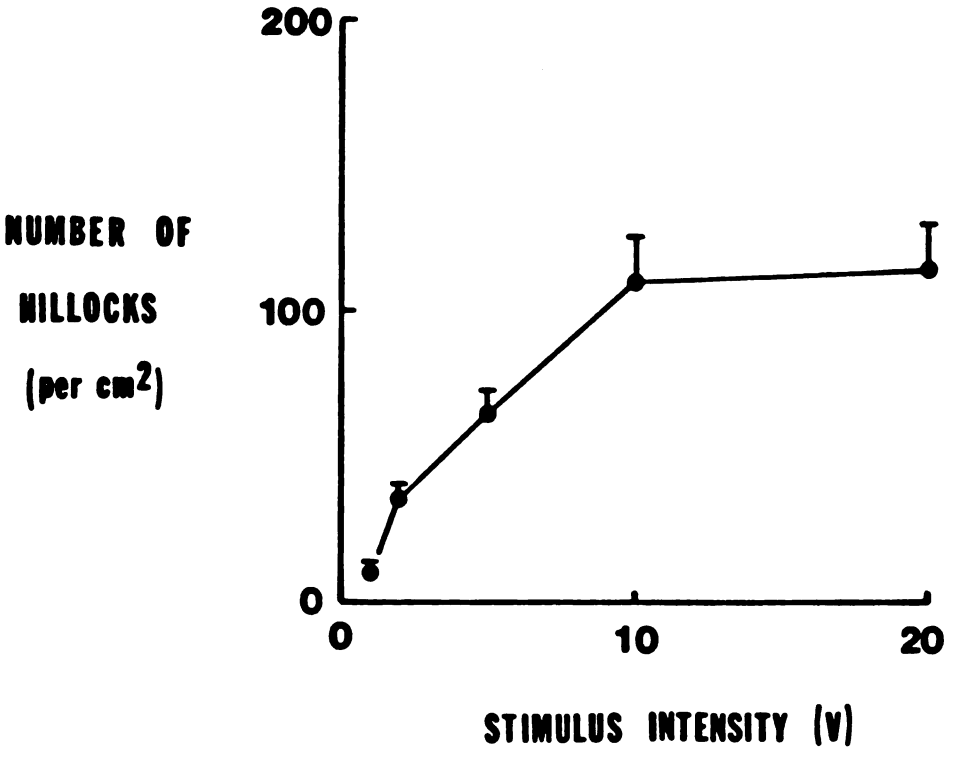

FIG. 3.3. Effect of stimulus voltage on the number of glands stimulated to secrete fluid. Two tissues from each of 3 ferrets were electrically stimulated (frequency, 6 cycles/s for 1 min) at the voltages indicated. Data are expressed as mean ± SEM.

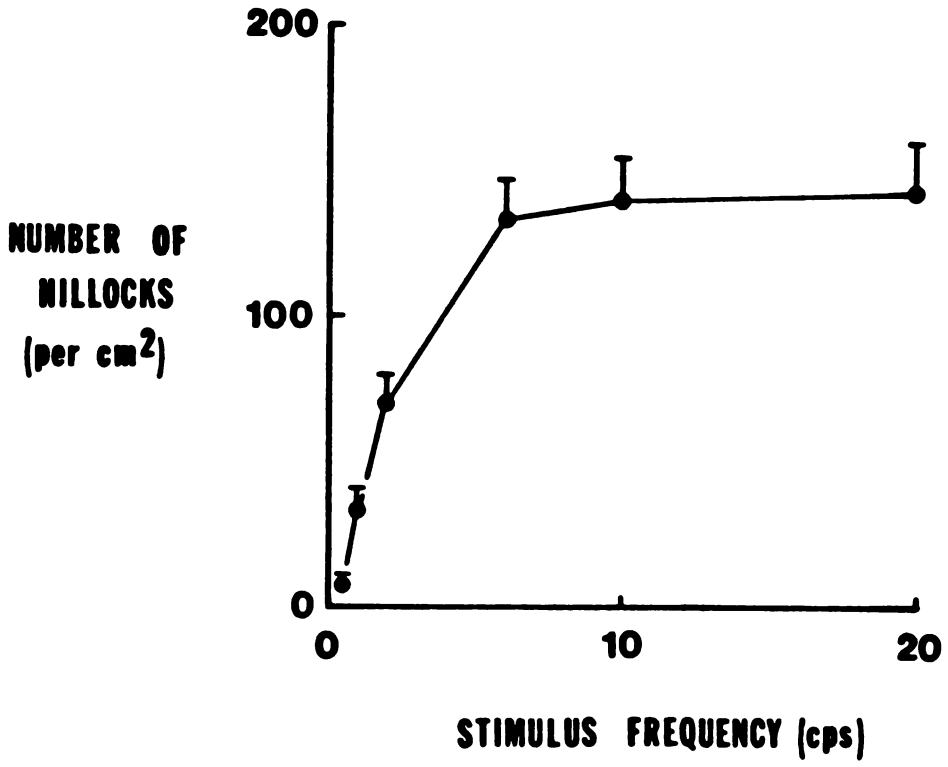

FIG. 3.4. Effect of stimulus frequency on the number of glands stimulated to secrete fluid from 2 tissues from each of 3 ferrets. Segments were electrically stimulated (intensity 10 V) for 1 min at the frequencies indicated. Data are expressed as mean ± SEM.

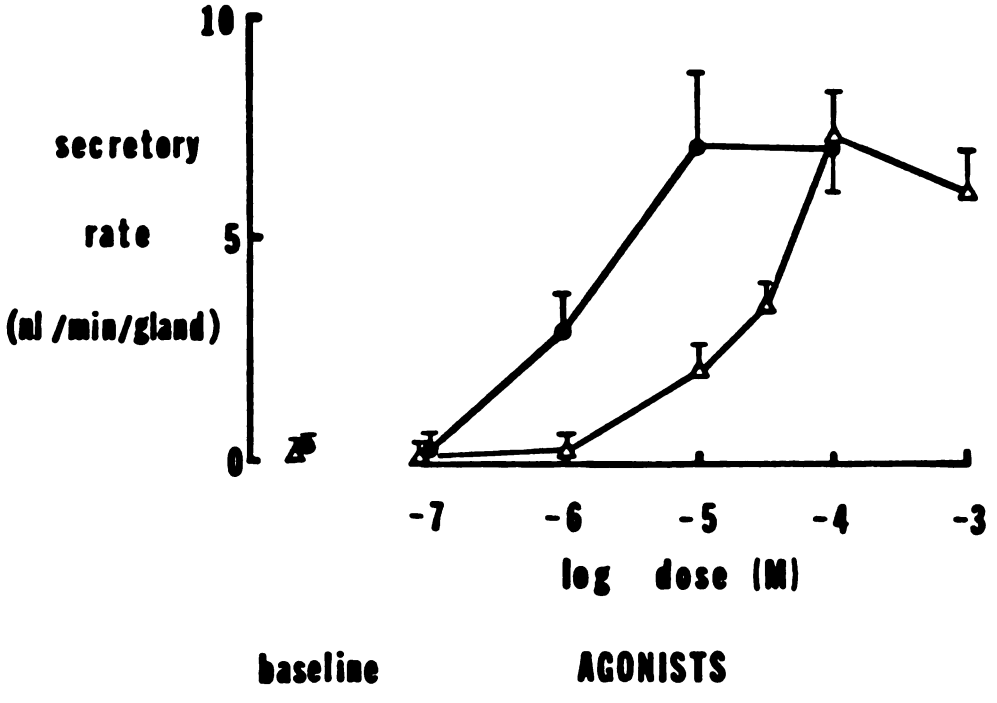

FIG. 3.5. Effects of acetylcholine (●) and phenylephrine (Δ) on secretion of fluid from 6 glands from one tissue from each of 4 ferrets. Change in volume was calculated from hillock diameters measured after 3 and 4 min of exposure to acetylcholine or phenylephrine. Data are expressed as mean ± SEM.

TABLE 3.1

EFFECTS OF AUTONOMIC AGONISTS AND ANTAGONISTS

ON HILLOCK DIAMETER

	ACETYLCHOLINE	PHENYLEPHRINE	TYRAMINE	TERBUTALINE
CONTROL	355 (μm)	318	206	0
(±SEM)	33	24	73	-
ATROPINE	44	327	-	-
(±SEM)	16	32	-	-
PHENTOLAMINE	326	0	0	-
(±SEM)	44	0	6	-
ATROPINE plus PHENTOLAMINE	0	0	-	-
ATROPINE plus PROPRANOLOL	0	297	-	-
(±SEM)	0	29		

TABLE 3.1. Effects of autonomic agonists and antagonists on the formation of hillocks. Tissues were stimulated with acetylcholine, phehylephrine (each drug, 5 x 10^{-5} M, tyramine (10^{-4} M), or terbutaline (10^{-4} M), before and after incubation with atropine, phentolamine, and propranolol as indicated (each antagonist, 10^{-5} M; 20 min) (n=5 ea).

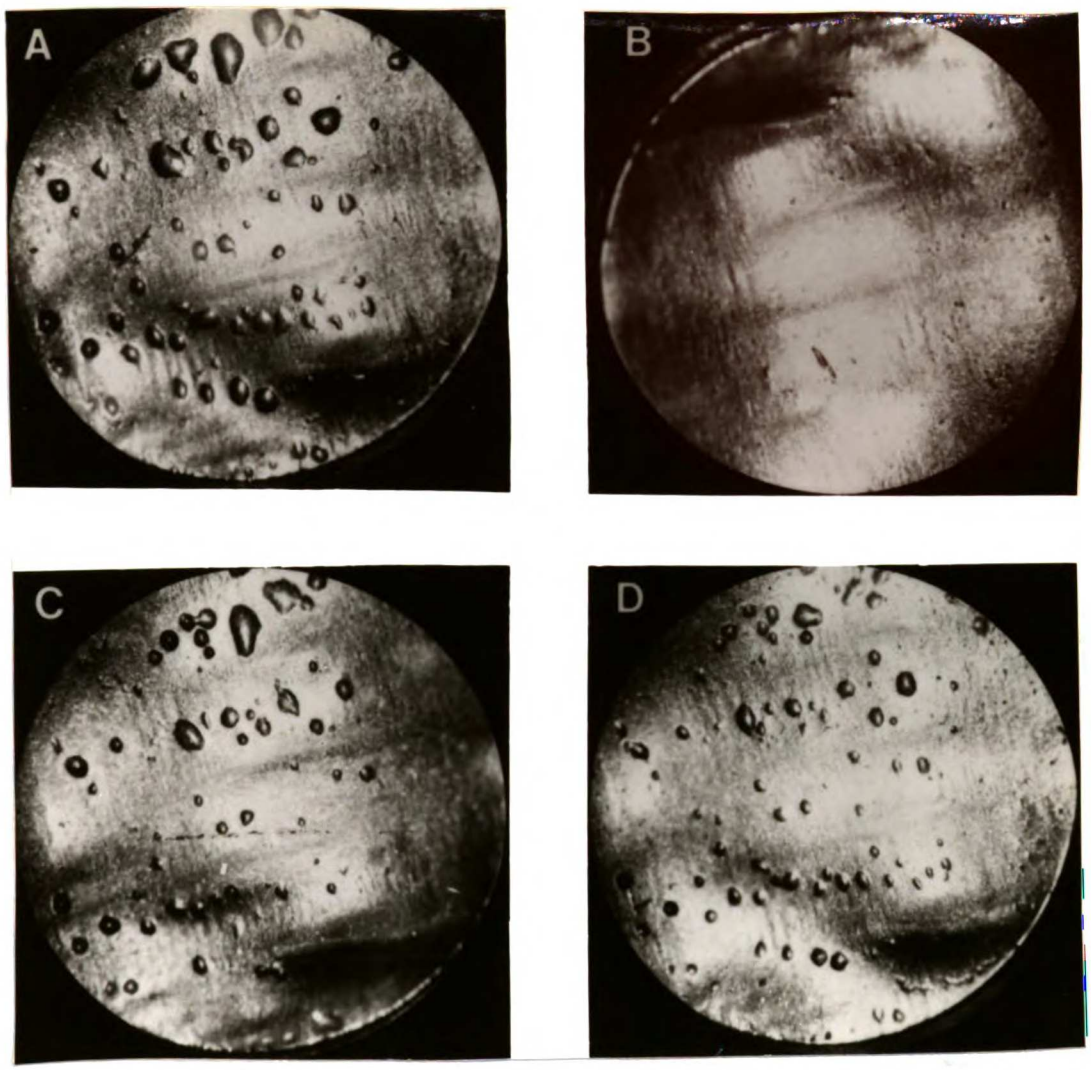

FIG. 3.6. Effect of tetrodotoxin on secretion of fluid from glands of one tissue from a ferret. A: Electrical stimulation (intensity, 10 V; frequency, 6 cycles/s for 1 min) before tetrodotoxin. B: Electrical stimulation after tetrodotoxin (10^{-7} M; 20 min). C: Acetylcholine (5×10^{-5} M; 3 min) plus tetrodotoxin. D: Phenylephrine (5×10^{-5} M; 4 min) plus tetrodotoxin.

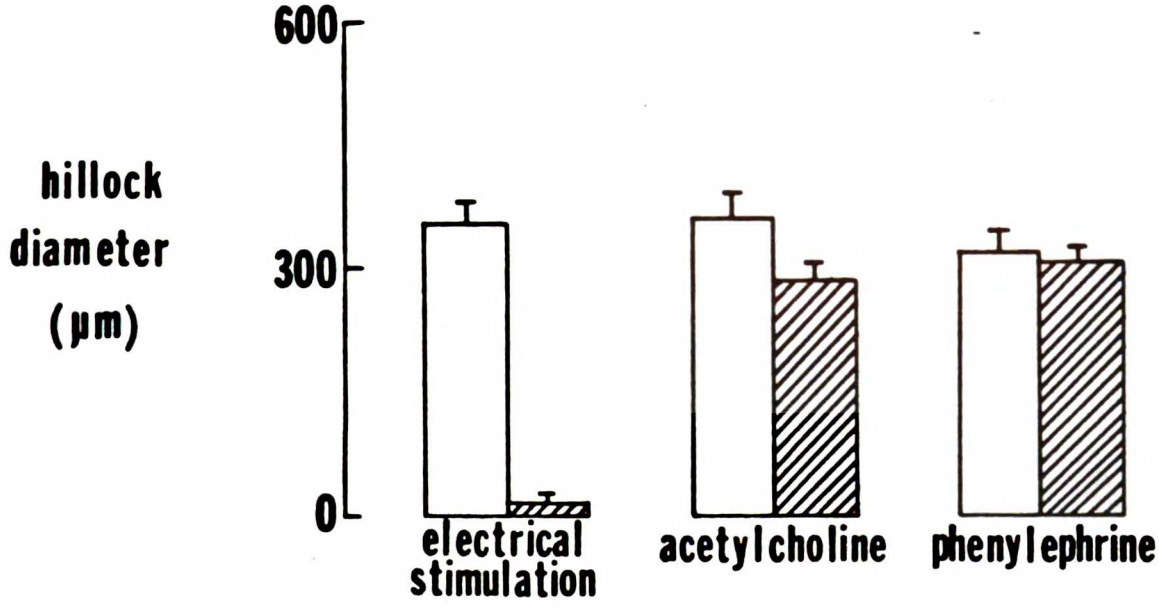

FIG. 3.7. Effect of tetrodotoxin on secretion of fluid from glands in one tissue from each of 5 ferrets. Each tissue was stimulated first electrically (10 V; 6 cycles/s for 1 min), then by acetylcholine (5×10^{-5} M; 3 min), then by phenylephrine (5×10^{-5} M; 4 min) before (□) and after (▨) incubation with tetrodotoxin (10^{-7} M; 20 min). Data are expressed as mean ± SEM.

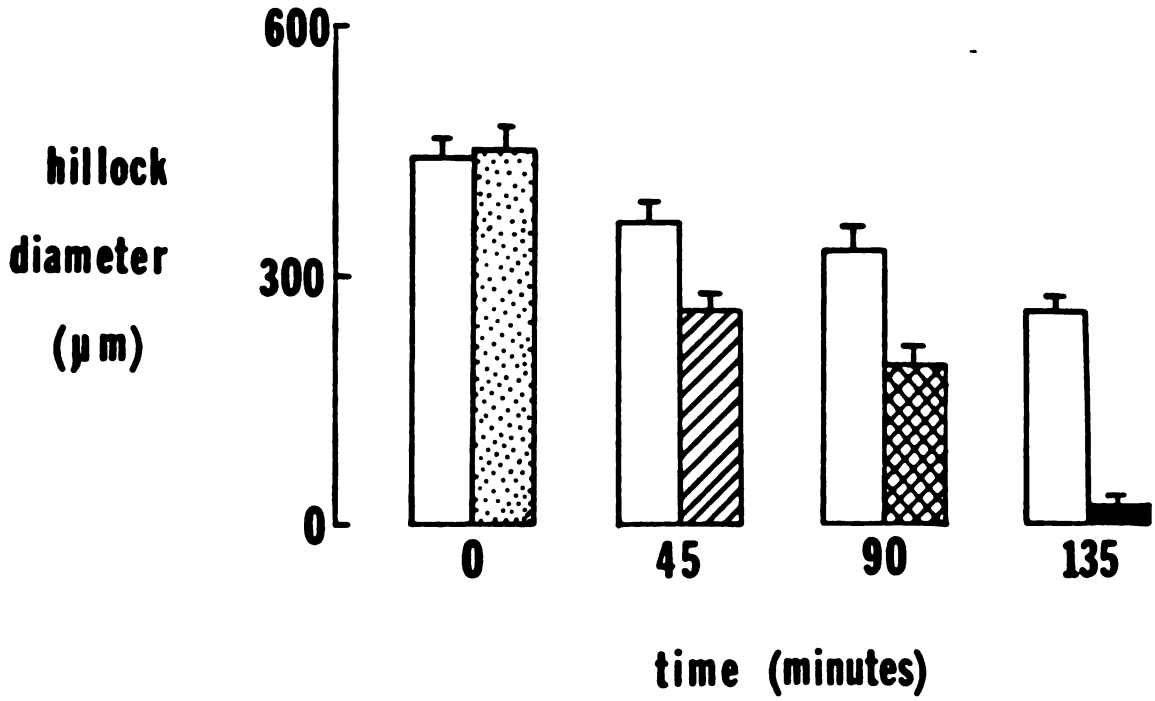

FIG. 3.8. Effects of autonomic antagonists on hillock diameter. Repeated electrical stimulation of one tissue from each of 5 ferrets at the times indicated (□) resulted in progressively smaller diameters with time. Electrical stimulation of one tissue from each of 5 other ferrets before antagonists (▩), after atropine alone (10^{-6} M; 20 min)(▨), phentolamine alone (10^{-6} M; 20 min)(▩), or atropine plus phentolamine (each drug, 10^{-5} M; 20 min)(■). Data are expressed as mean ± SEM.

DISCUSSION

Our goal was to determine whether adrenergic and cholinergic nerves mediate secretion from submucosal glands. As in dogs [155] and cats [74, 233], cholinergic nerves mediate secretion from tracheal glands of ferrets (FIG. 3.8). In addition, we conclude that adrenergic nerves mediate fluid secretion. The adrenergic response is mediated via alpha-adrenergic and not by beta-adrenergic receptors.

These conclusions are based on the following findings. First, the secretory response to electrical stimulation is probably mediated by nerves since this response was prevented by the nerve conduction blocker, tetrodotoxin, in doses that did not inhibit the responses to acetylcholine or phenylephrine (FIGS. 3.6,3.7). Next, alpha-adrenergic receptors probably mediate secretion because the secretory response to phenylephrine was specifically inhibited by the alpha-adrenergic antagonist, phentolamine (TABLE 3.1). Additionally, muscarinic receptors probably mediate secretion because the secretory response to acetylcholine was specifically inhibited by the muscarinic antagonist, atropine. Finally, the response to electrical stimulation was prevented only by the combination of alpha-adrenergic and muscarinic antagonists (FIG. 3.8). Thus, when sufficient atropine was present to prevent the secretory response to a high dose of acetylcholine, a response to electrical stimulation nonetheless appeared. Since atropine blocked the muscarinic mechanism, a non-muscarinic nervous mechanism must have mediated this response. Conversely, when sufficient phentolamine was present to prevent the response to a high dose of phenylephrine, a response to electrical

stimulation appeared. Therefore, a non-alpha-adrenergic nervous mechanism must have mediated this response. Furthermore, since the combination of phentolamine and atropine was necessary and sufficient to prevent the response to electrical stimulation, no other nervous mechanisms (e.g., nicotinic-cholinergic or beta-adrenergic) mediated the response. This last conclusion was corroborated by our results showing a lack of stimulation by terbutaline and a lack of antagonism by propranolol, on the adrenergic response to electrical stimulation. Additionally, these results suggest that there are no effects of stimulating nonadrenergic noncholinergic nerves on the secretion of fluid from the glands.

The decreasing magnitude of the control responses to electrical stimulation (FIG. 3.8) does not jeopardize these conclusions. The average decrease was only 32% over the time of the experiments. The probable reason for the decreasing response was a progressive depletion of cellular stores of mucus. This decline was corrected for by comparing the diameters of hillocks from tissues incubated with antagonists to hillock diameters from tissues used for control studies at the equivalent time in the experiment.

Our results agree with those of previous work showing that muscarinic-cholinergic nerves regulate fluid secretion from submucosal glands of cats [66] and dogs [155]. However, our results do not agree with other studies on adrenergic neural regulation of fluid secretion. Only a few studies on the effects of stimulating adrenergic nerves exist. In rabbits, sympathetic nerve stimulation did not cause secretion [24]. This may have been due to the paucity of submucosal

glands in this species [91]. In cats, the secretion of $^{35}SO_4$-labeled macromolecules *in vivo* in response to sympathetic nerve stimulation was mediated by beta-adrenergic receptors [74]. Since mucus is composed of fluid as well as glycoproteins, it is possible that beta-adrenergic mechanisms also mediate fluid secretion from glands. Using micropipettes, it was subsequently found that injecting adrenergic agonists into cats causes fluid secretion by both alpha-adrenergic and beta-adrenergic mechanisms [233]. However, we found that beta-adrenergic mechanisms do not mediate the formation of hillocks from submucosal glands of ferrets.

Although it is possible that the neural regulation of fluid secretion is different in ferrets, cats, and man, a more likely explanation for the differences is that the beta-adrenergic effect on fluid secretion is small [181, 233], and sufficient fluid may not have been secreted to form hillocks. A likely reason why our results differ from those of Gallagher [74] is that we measured hillock diameter, a measurement related to fluid volume, and Gallagher et al measured sulfate bound to high-molecular weight molecules, a measurement related to secretory glycoprotein concentration and not to fluid volume. Another likely reason for the difference is that because the studies of Gallagher et al were performed *in vivo*, stimulating the stellate ganglion may have activated many sympathetic nerves other than those to airways. Therefore, the secretory response of the trachea may have been due to stimulation of either reflexes or the release of circulating mediators. In contrast, our studies were performed *in vitro* in the absence of intact reflexes and the

circulation, and therefore reflect only local innervation of the trachea.

The findings that glycoprotein secretion is mediated by alpha-adrenergic [176], beta-adrenergic [74, 176], and muscarinic [74] receptors whereas fluid secretion is mediated by muscarinic and alpha-adrenergic receptors, but only weakly by beta-adrenergic receptors [181, 233], suggest the possibility that fluid secretion and protein secretion may be under separate neural regulation in tracheal glands as they appear to be in salivary glands [78, 202].

To further test this hypothesis, we performed studies to determine whether autonomic nervous mechanisms mediate the secretion of $^{35}SO_4$-labeled macromolecules.

$^{35}SO_4$-LABELED MACROMOLECULES

INTRODUCTION

The purpose of these experiments was to determine whether autonomic nerves also mediate the secretion of [$^{35}SO_4$]-labeled macromolecules from submucosal glands, and if they do, by which mechanisms.

We used an *in vitro* preparation consisting of tracheal segments mounted between half-chambers. To measure secretion of macromolecules, we used a modification of the method of Phipps [176] to label the SO_4 component of glycoproteins. Although $^{35}SO_4$ is taken up into cartilage and into cell surface glycoproteins, it is taken up mostly by submucosal glands [74, 79, 105], where it is incorporated predominately into secretory glycoproteins in dogs [58], cats [74], and ferrets [83], and therefore can be used to monitor glycoprotein secretion.

To show neural control of secretion of macromolecules, we had to show, as for hillocks (CHAPTER 3) that the stimulation of nerves evokes secretion, that exogenous drugs that mimic neurotransmitters evoke secretion, and that antagonists of these exogenous effects at least partially inhibit secretion evoked by nerve stimulation. To stimulate nerves *in vitro*, we used electrical field stimulation [20, 22](CHAPTER 3). To show that electrical field stimulation activated nerves, we used the nerve conduction blocker tetrodotoxin. Tetrodotoxin blocks the rapid entry of sodium through depolarized

nerve membranes and abolishes action potentials in nerves [157] and thereby prevents the secretion of fluid from tracheal glands in response to electrical stimulation, but does not prevent secretion induced by either acetylcholine or norepinephrine [20](CHAPTER 3). Tetrodotoxin, therefore, abolishes nerve conduction only, and thereby creates functional denervation.

Because goblet cells secrete [$^{35}SO_4$]-labeled glycoproteins [178], it was fortunate that ferrets have less than 5% goblet cells in their tracheal epithelium [103](Basbaum, personal communication), and have a large number of glands distributed throughout the trachea (CHAPTER 2)[9].

METHODS

A. PREPARATION

We anesthetized 52 adult male ferrets weighing 1-1.5 kg each by injecting pentobarbital sodium (45-60 mg/kg ip). After making a ventral midline incision in the neck, we excised the entire trachea, removed most of the loose fat and connective tissue, and placed the trachea in Medium 199/Earles HCO_3 solution maintained at 38° C and bubbled with 95% O_2 - 5% CO_2, with 20 units of Pen-Strep added per ml. We removed individual segments of the trachea 1.5 cm long, cut through the posterior membrane of each segment in the midline, and opened the segment flat. Then we mounted each segment between two half-chambers (0.8 cm inside diameter)(FIG. 4.1). We impaled the edges of each tissue with pins, clamped the two halves of the chamber together, and bathed each side of the tissue with 7 ml of Medium 199. To the submucosal side of each tissue we added 0.17 mCi of $^{35}SO_4$, and left it in contact with the tissue for the duration of the experiment. Every 15 min, we drained the solution from the luminal side of each tissue and replaced it with fresh medium. We placed each sample in cellulose dialysis tubing (Spectrapor No. D1615-1), and exhaustively dialysed it along with the rest of the samples from each experiment (≈ 100-150) against 6 changes of distilled water (4 l; 3 h ea) containing excess unlabeled SO_4 to displace non-covalently bound $^{35}SO_4$, and 10 mg sodium azide per liter to prevent biological degradation of the macromolecules. The pore size of the dialysis tubing allows molecules of less than 12,000-14,000 daltons to pass through. After dialysis, we determined the radioactivity of each sample to an accuracy of 0.5%

using a Beckman Instruments beta counter (model LS 7500).

Preliminary experiments showed that unlabeled macromolecules collected and handled the same way did not bind exogenous $^{35}SO_4$. Before adding radiolabel, the average radioactivity of the collected materials was 74 ± 23 cpm, and after incubation for 2 h with 0.17 mCi $^{35}SO_4$ and then dialysis, the average radioactivity in the samples was 81 ± 16 cpm. Neither of these values is significantly different from the background radioactivity of the water used for dialysis ($p > 0.5$; $n=6$).

B. BASELINE

To study secretion, we began by determining the baseline release of bound $^{35}SO_4$ from one tissue from each of 9 ferrets over a period of 5 h in the absence of stimulation. Preliminary experiments showed that after 2 h of incubation with $^{35}SO_4$, secretion had nearly reached a steady state (FIG. 4.2). Therefore, we stimulated other tissues only after 2 h.

C. ELECTRICAL STIMULATION

To determine the reproducibility of the response to electrical stimulation, we studied 1 other tissue each from 6 of the same ferrets. After 2 h, 3 h, and again, after 4 h of incubation with $^{35}SO_4$, we stimulated each tissue electrically via 4 pins that impaled the tissue (2 on each side of the chamber)(FIG. 4.1), using a Grass model S88 stimulator and 2 Grass model SIU 5 stimulus isolation units (stimulus intensity, 10 V biphasic; pulse duration, 0.5 ms; frequency, 5 cycles/s for 5 min). Then, to determine the frequency dependence of

the response to electrical stimulation, we studied the responses of 3 other tissues from each of 8 of the same ferrets. We stimulated each tissue twice, at either 1, 2,3, or 7 cycles/s for 5 min, once after 2 h of incubation with $^{35}SO_4$, and again after 3 h of incubation.

Then, in the next series of experiments, we determined whether the response to electrical stimulation was mediated by nerves by seeing whether, in one tissue from each of the 9 ferrets, electrical stimulation (3, 5, or 7 cps) still evoked the release of $^{35}SO_4$ after incubation with the nerve conduction blocker tetrodotoxin (10^{-7} M; 30 min; citrate-free, Calbiochem-Behring, La Jolla, CA). To show that these tissues were not depleted of $^{35}SO_4$, and that the gland cells were capable of responding to agonists, we then stimulated secretion from each tissue using either acetylcholine, phenylephrine, or terbutaline (each drug, 10^{-5} M; 10 min).

D. AUTONOMIC AGONISTS AND ANTAGONISTS

To determine whether muscarinic, alpha-adrenergic, and beta-adrenergic mechanisms mediate secretion, we studied 3 pairs of tissues from each of 6 other ferrets. To study muscarinic effects, we added acetylcholine chloride (10^{-5}M, 10 min) to the the solution bathing the submucosal side of 1 control tissue and 1 tissue pretreated with atropine sulfate (10^{-5} M, 45 min). Similarly, to study alpha-adrenergic effects we added phenylephrine (10^{-5}M, 10 min) to the solution bathing the submucosal side of 1 control tissue and 1 tissue pretreated with phentolamine methanesulfonate (10^{-5} M, 45 min). Then, to study beta-adrenergic effects, we added terbutaline sulfate (10^{-5} M, 10 min) to the solution bathing the submucosal side of 1 control

tissue and 1 tissue pretreated with DL-propranolol (10^{-5} M, 10 min, Sigma Chemical, St. Louis, MO). Because terbutaline is a partial agonist, we also studied the response of 2 tissues from 2 other ferrets to the complete beta-adrenergic agonist, isoproterenol (10^{-5} M; 10 min; Sigma Chemical Co., St. Louis, MO).

Next, to determine whether the effects of atropine, phentolamine, and propranolol are specific to muscarinic, alpha-adrenergic, and beta-adrenergic receptors respectively, we stimulated release of $^{35}SO_4$ from 3 tissues from each of 4 other ferrets. For each ferret, we added acetylcholine to 1 tissue pre-incubated with phentolamine plus propranolol, phenylephrine to 1 tissue pre-incubated with atropine plus propranolol, and terbutaline to 1 tissue pre-incubated with atropine plus phentolamine (each agonist, 10^{-5} M, 10 min; each antagonist, 10^{-5} M, 45 min).

Because adrenergic nerves secrete neither phenylephrine nor terbutaline, but do secrete norepinephrine, we determined whether norepinephrine causes secretion via alpha- or beta-adrenergic mechanisms in 4 tissues from each of 8 other ferrets. We added DL-norepinephrine (10^{-5} M, 10 min, Sigma Chemical, St. Louis, MO) to the solutions bathing the submucosal sides of 1 control tissue and 3 tissues pretreated with either phentolamine alone, propranolol alone, or phentolamine plus propranolol (each drug, 10^{-5} M; 45 min).

E. AUTONOMIC NERVES

In the next set of experiments, to determine whether autonomic nervous mechanisms mediate the response to electrical stimulation, we

studied the responses of 3 tissues from each of 8 other ferrets to stimulation at 5 cycles/s in the presence of first two, and then three of the antagonists, atropine, phentolamine, and propranolol. To determine whether muscarinic nervous mechanisms mediate secretion, we stimulated tissues in the presence of phentolamine plus propranolol (each drug, 10^{-5} M; 45 min), and again after adding atropine (10^{-5} M; 45 min). Similarly, to determine whether beta-adrenergic nervous mechanisms mediate secretion, we stimulated another tissue from each of the same ferrets in the presence of atropine plus phentolamine (each drug, 10^{-5} M; 45 min), and again after adding propranolol (10^{-5} M; 45 min). Then, to determine whether alpha-adrenergic nervous mechanisms mediate secretion, we stimulated another tissue from each of the same ferrets in the presence of atropine plus propranolol (each drug, 10^{-5} M; 45 min), and again after adding phentolamine (10^{-5} M; 45 min).

Preliminary experiments showed that there was a significant response to electrical stimulation in the presence of atropine, phentolamine, and propranolol together. To determine whether the response remaining was mediated by non-cholinergic non-adrenergic nerves, we had to determine whether the response was neurally mediated. Therefore, we repeated electrical stimulation at 4 h, after adding tetrodotoxin (10^{-7} M; 45 min) to each tissue. Because the response was abolished by tetrodotoxin (see RESULTS), the response was mediated by nerves, and it was crucial therefore, to determine whether the response remaining after atropine, phentolamine, and propranolol was due to incomplete inhibition of muscarinic, alpha-, and beta-

adrenergic mechanisms by the competitive antagonists.

Because competitive antagonism can be increased by either increasing the concentration of the antagonists or by decreasing the stimulus intensity (e.g., frequency), we repeated these experiments and stimulated 3 tissues from each of 5 other ferrets at a decreased frequency of 2 cycles/s in the presence of combinations of 2, then 3 antagonists at 10^{-4} M.

In another series of experiments, we compared the responses to electrical stimulation at different frequencies in the presence of different concentrations of all 3 antagonists together. At 3 and 5 cycles/s, we studied 3 tissues from each of 3 additional ferrets. At 3 cycles/s, we stimulated each tissue first in the presence of 10^{-6} M antagonists, then 10^{-5} M, and finally, 10^{-4} M (each combination of drugs, 45 min). At 5 cycles/s, we stimulated each tissue first in the absence of antagonists, then in the presence of 10^{-6} M, and finally, 10^{-5} M (each combination of drugs, 45 min). We stimulated 2 tissues from each of 2 other ferrets at 7 cycles/s, first in the presence of 10^{-6} M, and finally, 10^{-5} M antagonists (each combination of drugs, 45 min).

Because there is a collagen layer on the submucosal side of the tissue, it was possible that the antagonists may not have diffused well enough into the tissues, and therefore may not have had access to all of the receptors that mediate secretion. Therefore, we stimulated 7 tissues from 2 additional ferrets at 3 cycles/s, first in the absence of antagonists, then in the presence of atropine, phentolamine, and propranolol together on both the submucosal and

luminal sides of each tissue (each drug, 10^{-5} M; 45 min).

Although atropine abolished the response to exogenous acetylcholine, indicating that muscarinic mechanisms mediate the response, nicotinic mechanisms might possibly mediate some of the response to nerve stimulation. To test this hypothesis, we stimulated 1 tissue from each of 5 ferrets at 5 cycles/s, first in the absence of antagonists, then in the presence of atropine, phentolamine, propranolol, and hexamethonium (each drug, 10^{-5} M; 45 min).

F. DATA ANALYSIS

To calculate the evoked response to each stimulus, we subtracted the radioactivity of the pre-stimulus sample from the radioactivity of the sample collected immediately after stimulation. Unless otherwise stated, all data is expressed as mean ± SEM. To compare results within tissues, we used Student's paired t test, and to compare results between tissues, we used Student's unpaired t test.

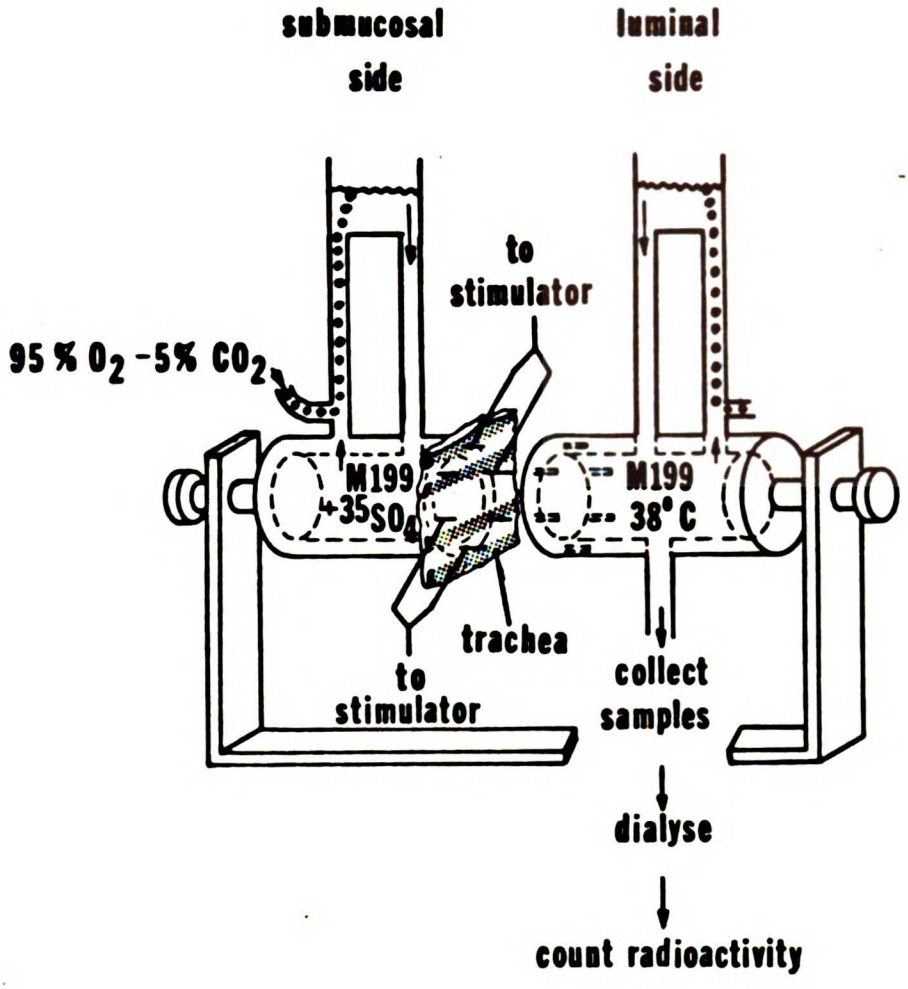

FIG. 4.1. Apparatus used for in vitro studies of secretion of macromolecules. Tissue was impaled by pins and held between two plastic half chambers by a clamp. $^{35}SO_4$ was added to the submucosal side, and samples were collected from the luminal side. For electrical stimulation of tissue, two pins on either side of chamber were connected to a Grass stimulator. For pharmacological stimulation and inhibition, drugs were added to the submucosal surface via a bubbling chamber.

RESULTS

A. BASELINE RELEASE

During the 5 h of exposure to $^{35}SO_4$, each tissue incorporated $^{35}SO_4$ into high molecular weight materials and released them into the tracheal lumen (FIG. 4.2, top). After a lag of about 1 h, the amount of non-dialysable $^{35}SO_4$ released from each tissue increased with time from 253 ± 275 to 3216 ± 650 cpm/h at 5 h of incubation. By 2 h, the amount of bound $^{35}SO_4$ released from each tissue reached 75 ± 5 % of the amount released at 5 h (FIG. 4.2, bottom), indicating that a steady state had nearly been reached. Although secretion was not constant after 2 h, the baseline secretory rate was linear and increased only by 20% from 2 to 4 h, the duration of all other experiments. Therefore, the release of $^{35}SO_4$ evoked by electrical stimulation or drugs could be calculated.

B. ELECTRICAL STIMULATION

Repeated electrical stimulation reproducibly increased the amount of bound $^{35}SO_4$ released into the lumen at all frequencies studied. (FIG. 4.3). The second and third responses to 5 cycles/s were 108 ± 13% and 100 ± 13% of the first response, respectively (FIG. 4.3, bottom). The second responses to electrical stimulation at 2,3, and 7 cps were 102 ± 12 %, 107 ± 14 %, and 99 ± 17 %, respectively. Furthermore, increasing stimulus frequency increased the evoked secretion (FIG. 4.4). The highest frequency at which we obtained reproducible responses was 7 cps. The evoked response at this frequency was 4632 ± 517 (SEM) cpm/h, or approximately 2 times the

baseline secretory rate at 2 h. At 5 Hz, the largest evoked response was from segments from the upper trachea. However, the response (mean, 3812 ± 752 cpm/h) was not significantly greater than the responses from segments from the middle 3388 ± 836 cpm/h, or lower trachea 3320 ± 512 cpm/h.

Tetrodotoxin prevented the response to electrical stimulation at all frequencies (e.g., FIG. 4.5). The mean response for all frequencies was 27 ± 20 cpm/hr, a value not significantly different from zero ($p>0.5$; $n=24$). However, tetrodotoxin did not prevent the responses to either phenylephrine, acetylcholine, or terbutaline: the mean response after tetrodotoxin for all the agonists was 72 ± 23 % of the response to each agonist before tetrodotoxin ($p>0.1$; $n=9$).

C. AUTONOMIC AGONISTS AND ANTAGONISTS

Acetylcholine, phenylephrine, terbutaline ($n=6$ each), and norepinephrine ($n=8$) each increased the amount of bound $^{35}SO_4$ released into the tracheal lumen ($p<0.05$) (TABLE 4.1), with the order of potency, norepinephrine > acetylcholine > phenylephrine > terbutaline. Each antagonist prevented the response to its corresponding agonist: atropine prevented 98 ± 2 % of the response to acetylcholine, phentolamine prevented 96 ± 2 % of the response to phenylephrine, and propranolol prevented 94 ± 3 % of the response to terbutaline ($p<0.05$; $n=6$ ea). However, each combination of two antagonists failed to decrease the response to the non-corresponding agonists significantly: phentolamine plus propranolol did not decrease the response to acetylcholine, atropine plus propranolol did not decrease the response to phenylephrine, and atropine plus phentolamine did not significantly

decrease the response to terbutaline (n=4 ea). Propranolol reduced the response to norepinephrine by 43 %, phentolamine reduced the evoked response by 89 %, and phentolamine plus propranolol together prevented 98 % of the response to norepinephrine ($p<0.05$; n=8) (TABLE 4.1).

D. AUTONOMIC NERVES

After incubation with each combination of two of the antagonists atropine, phentolamine, or propranolol, electrical stimulation resulted in secretion, and the addition of the third antagonist reduced the response by an average of 44 ± 12% for all tissues (FIGS. 4.6,4.7) ($p<0.05$; n=30). Atropine, phentolamine, and propranolol each reduced the responses by 33 %, 40 %, and 49 %, respectively, from the responses in the presence of the other two antagonists.

However, the response to electrical stimulation in the presence of 3 antagonists together was not abolished at any frequency, whereas the response was abolished after the addition of tetrodotoxin (FIGS. 4.6, 4.7). Furthermore, the degree of inhibition was not increased by increasing the concentration of atropine, phentolamine, and propranolol above 10^{-5} M ($p>0.5$; n=33) (FIG. 4.8). The proportion of the response remaining was the same (24 ± 7%; n=70), for all frequencies and concentrations of antagonists above 10^{-6} M. Furthermore, we were unable to increase the inhibition significantly by adding the antagonists to both sides of the tissues (21 ± 7 % of control; $p>0.5$; n=6), or by further adding hexamethonium (23 ± 8 % of control; n=5).

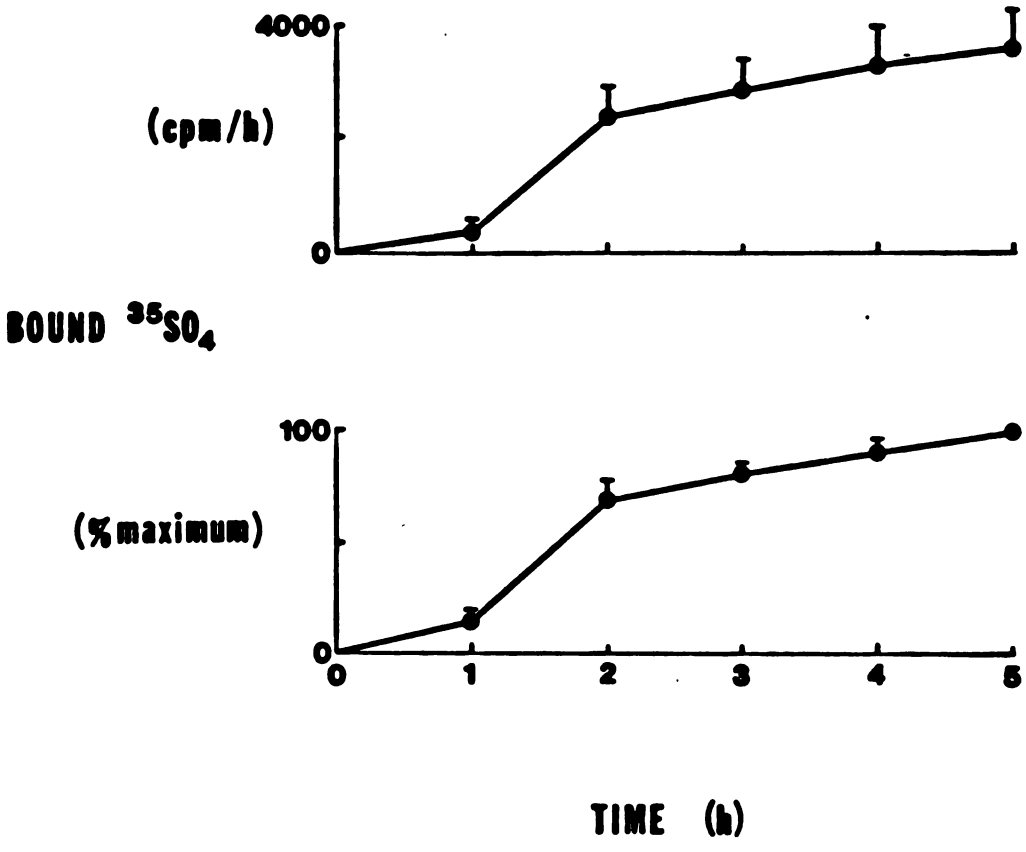

FIG. 4.2. Baseline $^{35}SO_4$ secretion from one tissue from each of 9 ferrets. $^{35}SO_4$ (0.17 mCi) was added to the submucosal side at 0 h. Every 15 min, the luminal bathing solution was collected and replaced with fresh solution. After dialysis, the bound radioactivity was determined for each sample. Top: Data at each hour are expressed as mean ± SEM. Bottom: Data at each hour are expressed as percent maximum for that tissue (mean ± SEM).

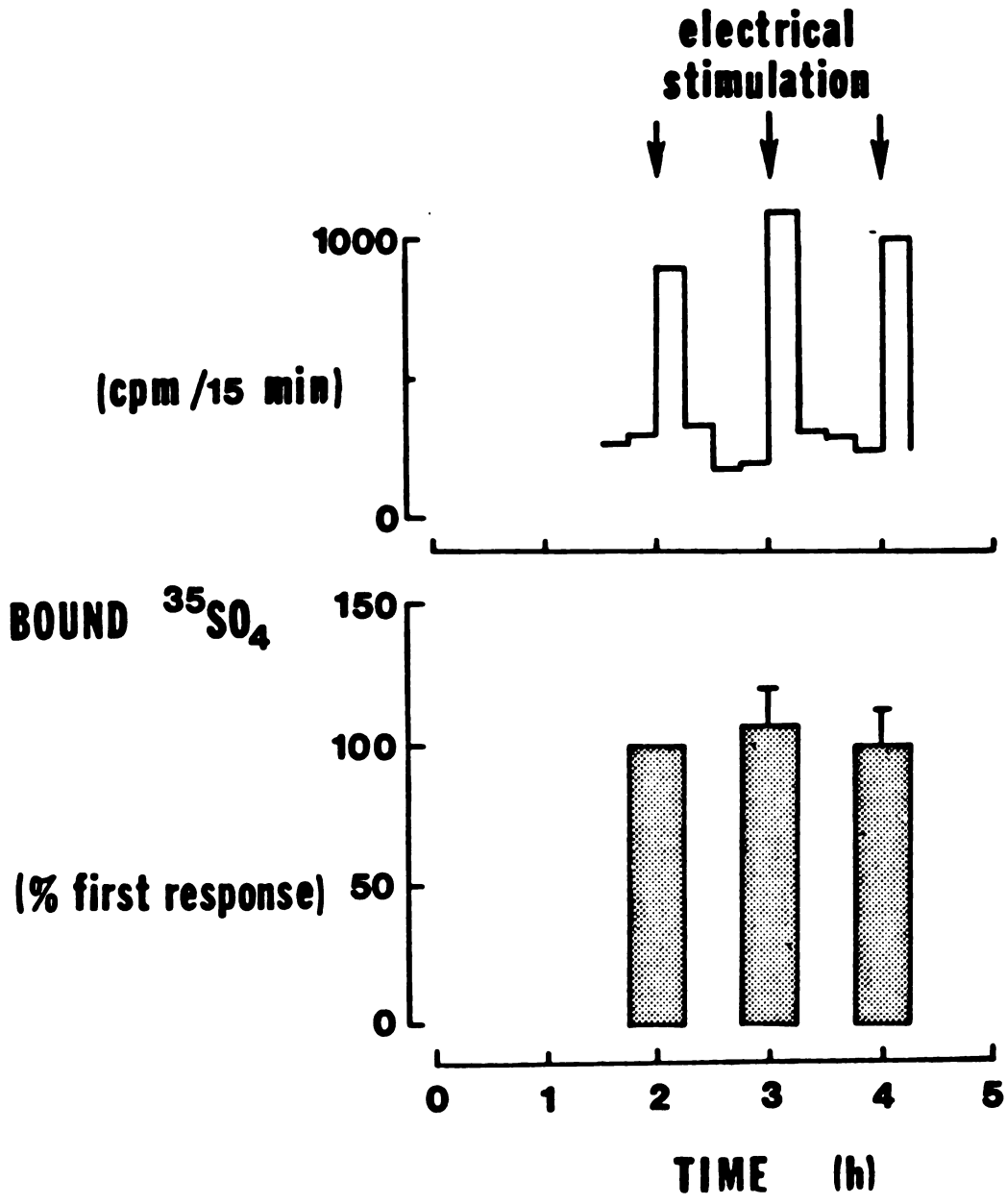

FIG. 4.3. Reproducibility of the secretory response to electrical stimulation at the times indicated (10 V; 5 cycles/s for 5 min) (arrows). Top: One tissue from a ferret. Bottom: One tissue from each of 6 ferrets. Data are expressed as mean ± SEM.

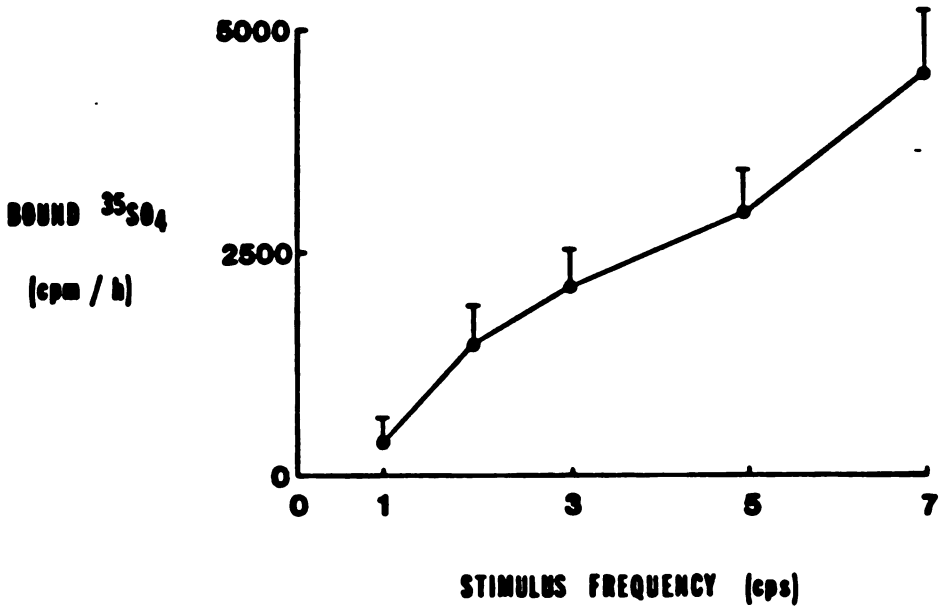

FIG. 4.4. Effect of frequency of electrical stimulation on secretion of $^{35}SO_4$. At each frequency, 1 tissue from each of 8 ferrets was stimulated twice (at 2 and 3 h; 10 V; 0.5 ms; frequency, as indicated for 5 min). Evoked responses are expressed as mean ± SEM.

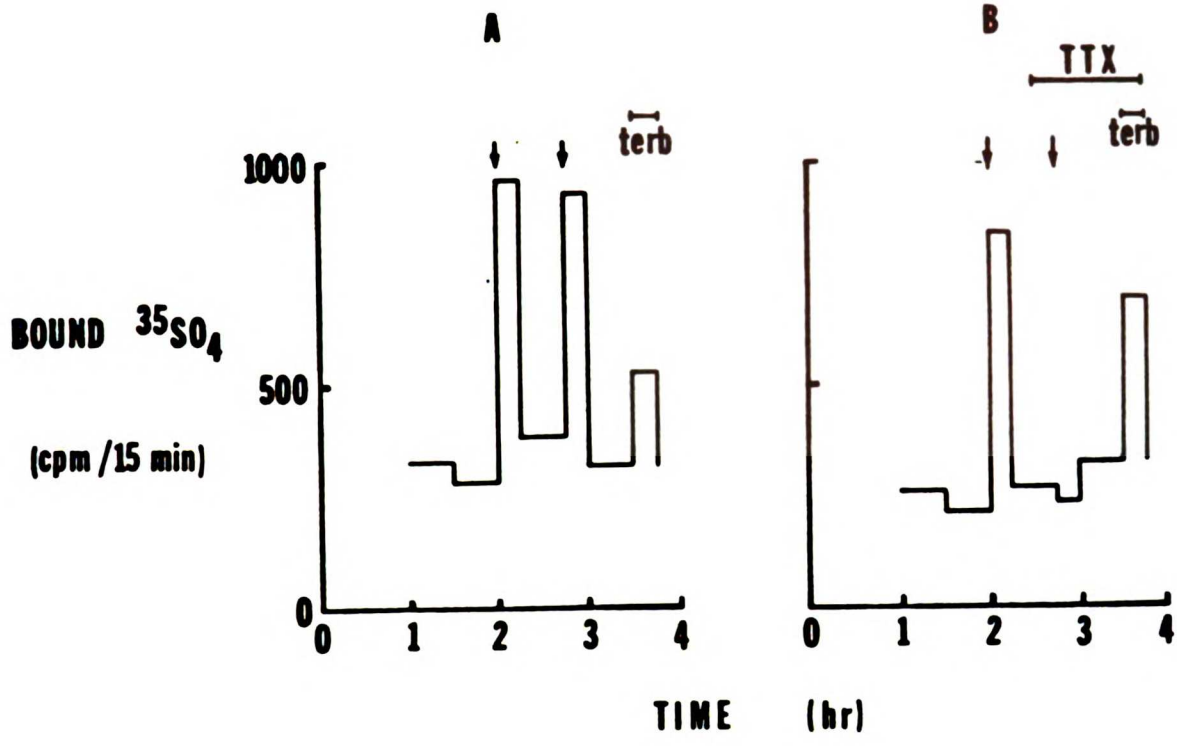

FIG. 4.5. Effect of tetrodotoxin on secretion of $^{35}SO_4$ from two tissues from a ferret. A: Control electrical stimulation (intensity, 10 V; pulse duration, 0.5 ms; frequency, 5 cycles/s for 5 min) (arrows) or terbutaline (terb; 10^{-5} M; 10 min) causes secretion of $^{35}SO_4$. B: After a control electrical stimulation, tetrodotoxin (TTX, 10^{-7} M; 30 min) prevented the response to electrical stimulation but not to terbutaline.

TABLE 4.1

EFFECTS OF AUTONOMIC AGONISTS AND ANTAGONISTS ON THE EVOKED SECRETION OF $^{35}SO_4$

	ACH	NOREPI	PHENYL	TERBUT	ISOPROT
CONTROL (cpm/h)	8790	16280	3657	1959	4576
(±SEM)	2050	3212	625	310	1465
ATROPINE	53±70	---	---	---	---
PHENTOLAMINE	---	1740±552	145±60	---	---
PROPRANOLOL	---	9220±2132	---	114±63	---
PHENTOLAMINE plus PROPRANOLOL	8180±1975	364±144	---	---	---
ATROPINE plus PROPRANOLOL	---	8120±3523	4660±855	---	---
ATROPINE plus PHENTOLAMINE	---	---	---	1230±695	---

TABLE 4.1. Effects of autonomic agonists and antagonists on secretion of $^{35}SO_4$. Tissues were stimulated with acetylcholine (ACH), norepinephrine (NOREPI), phenylephrine (PHENYL), terbutaline (TERBUT), or isoproterenol (ISOPROT) (each agonist; 10^{-4} M, 10 min) in the absence and presence of combinations of the antagonists atropine, phentolamine, and propranolol (each antagonist; 10^{-5} M, 45 min). Each evoked response was calculated by subtracting the cpm in the sample collected immediately before adding the agonists from the cpm in the sample collected 10 min after adding the agonists.

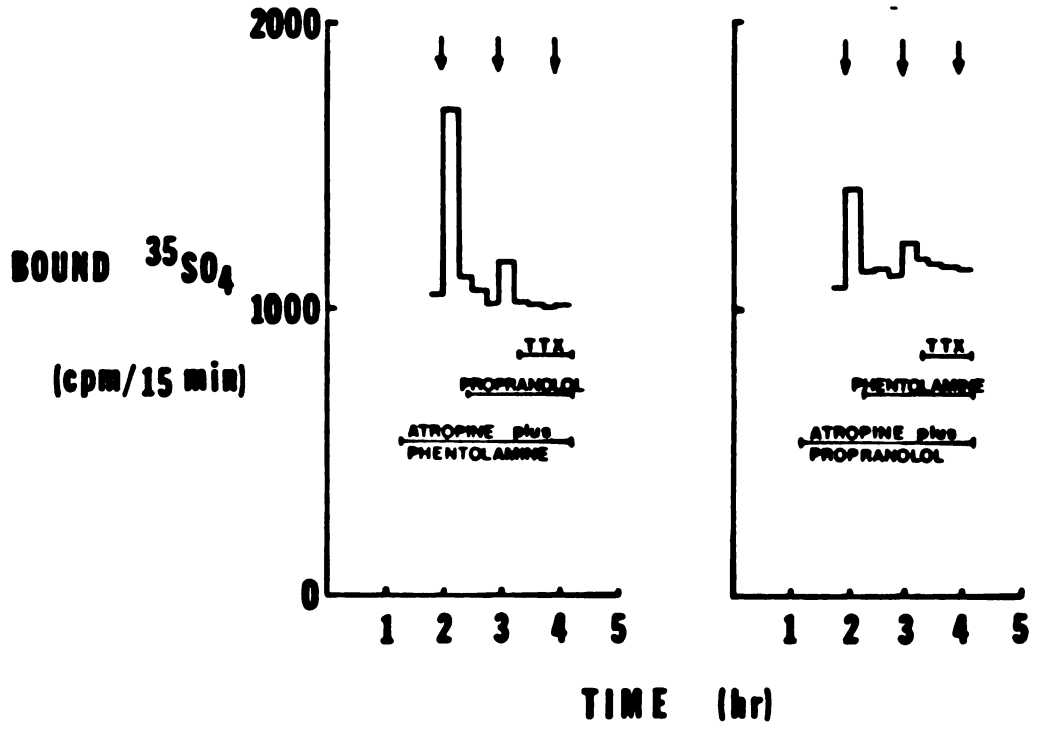

FIG. 4.6. Effect of autonomic antagonists and tetrodotoxin on the secretion of $^{35}SO_4$ evoked by electrical stimulation (10 V; 5 cycles/s for 5 min) of 2 tissues from a ferret. Left: Tissue electrically stimulated (arrows) first in the presence of atropine plus phentolamine (each drug, 10^{-5} M; 45 min), again after the addition of propranolol (10^{-5} M; 45 min), and again after the addition of tetrodotoxin (TTX, 10^{-7} M; 45 min). Right: Tissue stimulated (arrows) first in the presence of atropine plus propranolol (each drug, 10^{-5} M; 45 min), again after the addition of phentolamine (10^{-5} M; 45 min), and again after the addition of tetrodotoxin (10^{-7} M; 45 min).

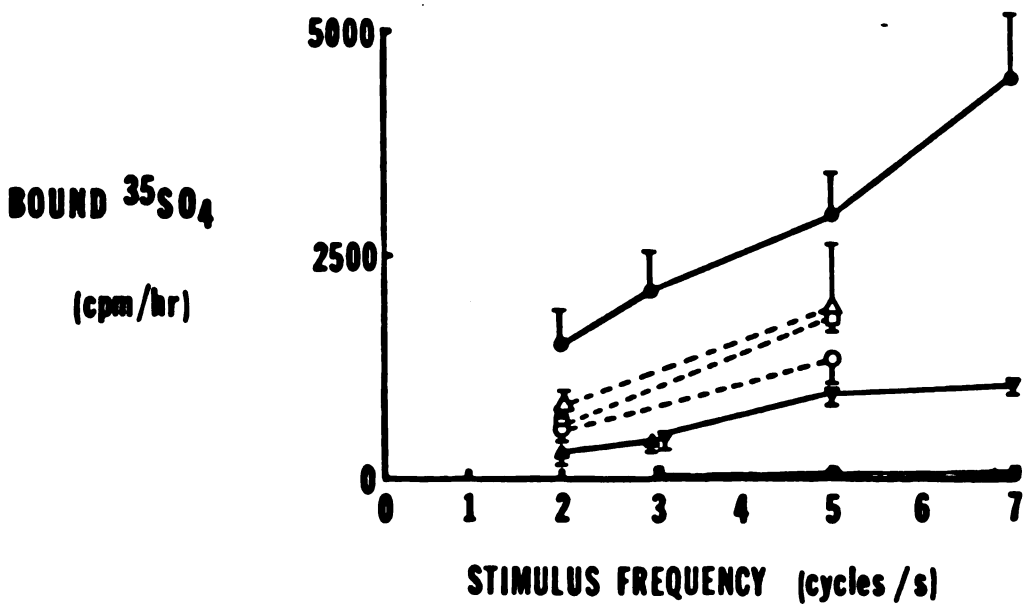

FIG. 4.7. Effects of autonomic antagonists and tetrodotoxin on the secretion of $^{35}SO_4$ evoked by electrical stimulation. Tissues were stimulated in the absence of autonomic antagonists (●), in the presence of two antagonists (--): either atropine plus phentolamine (△), phentolamine plus propranolol (□), or atropine plus propranolol (○) (2 cycles/s: 10^{-4} M, 45 min; 5 cycles/s: 10^{-5} M; 45 min), and in the presence of all three antagonists (10^{-4} M), (▲); (10^{-5} M, 45 min) (▼). Stimulation after tetrodotoxin, (■). Data are expressed as mean ± SEM.

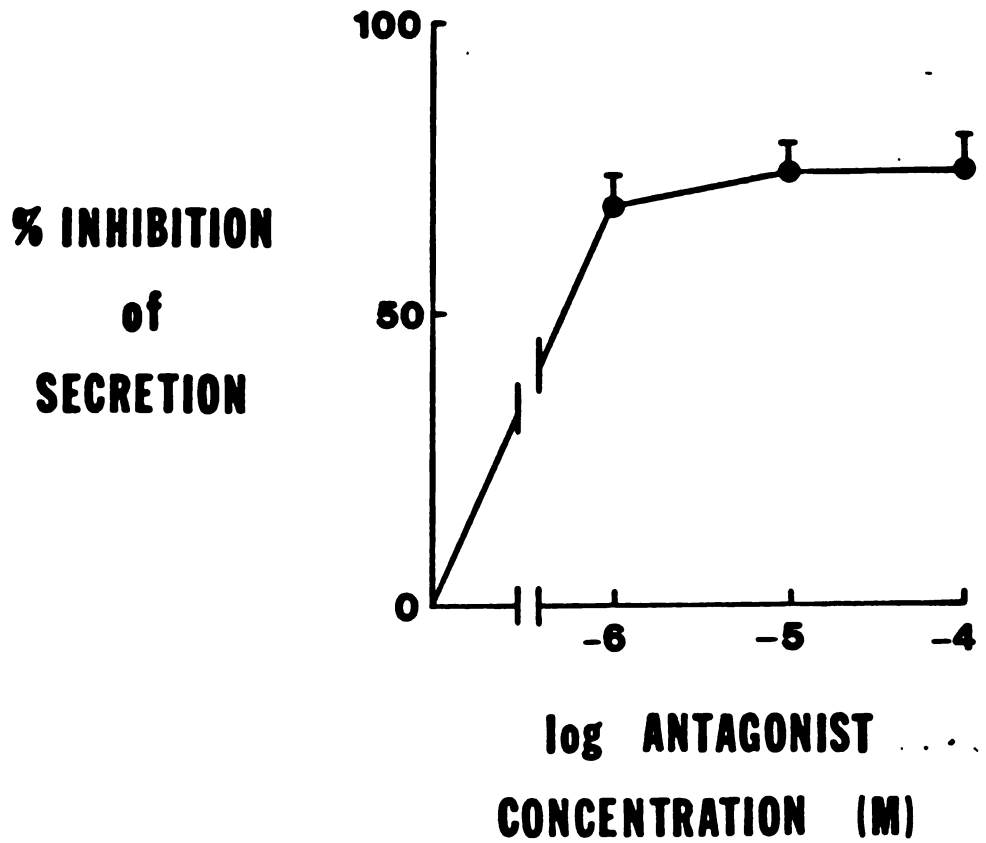

FIG. 4.8. Effect of antagonist concentration on the inhibition of $^{35}SO_4$ secretion evoked by electrical stimulation. 2 tissues from each of 5 ferrets were stimulated before and after incubation with atropine, phentolamine, and propranolol together at the doses indicated for 45 min prior to stimulation. Data are expressed as mean ± SEM.

DISCUSSION

Our aim was to determine whether autonomic nerves mediate the secretion of [$^{35}SO_4$]-labeled macromolecules from tracheal glands of ferrets. Our results indicate that, as for fluid secretion in ferrets (CHAPTER 3) [20, 22], and in cats [233], both cholinergic and adrenergic nerves mediate secretion of [$^{35}SO_4$]-labeled macromolecules (FIG. 4.7). Additionally, we conclude that another, unidentified nervous mechanism also causes secretion.

These conclusions are based on the following findings. First, the secretory response to electrical stimulation must be mediated by nerves because this response was frequency-dependent (FIG. 4.4) and was completely prevented by tetrodotoxin in doses that did not inhibit the secretory responses to acetylcholine, phenylephrine, or terbutaline (FIG. 4.5).

Next, specific autonomic receptor mechanisms cause secretion. Muscarinic and not nicotinic cholinergic mechanisms must mediate secretion of $^{35}SO_4$, since atropine prevented all of the response to acetylcholine (TABLE 4.1), and hexamethonium did not inhibit any of the response to electrical stimulation. Similarly, alpha-adrenergic receptors must mediate secretion because phentolamine prevented all of the response to phenylephrine and most of the response to norepinephrine. Beta-adrenergic receptors also must mediate secretion because both terbutaline and isoproterenol cause secretion, and propranolol prevented the response to terbutaline and some of the response to norepinephrine (TABLE 4.1). Additionally, atropine, phentolamine, and propranolol were specific to muscarinic, alpha-

adrenergic, and beta-adrenergic receptor mechanisms respectively (TABLE 4.1).

Finally, specific autonomic neural mechanisms mediate secretion. Muscarinic, alpha-adrenergic, and beta-adrenergic neural mechanisms each mediate secretion because atropine, phentolamine, and propranolol each inhibit a significant portion of the response to electrical stimulation. Furthermore, the inhibition of each neural mechanism was specific at all the concentrations of antagonists used, because if there had been any non-specific local anesthetic effects, there would have been progressively greater inhibition of the response to electrical stimulation with increasing concentration of the antagonists until the response was completely inhibited. Thus, the antagonists used were specific to their expected mechanisms of action. However, we found that the proportion of the response inhibited was no greater at 10^{-4} M than at 10^{-6} M (FIG. 4.8). This finding also indicates that the inhibition of each known neural mechanism was complete at 10^{-5} M.

Thus, when atropine and phentolamine were present, the response to electrical stimulation must have been mediated by non-cholinergic non-alpha-adrenergic nervous mechanisms. Similarly, when atropine and propranolol were present, the response to electrical stimulation must have been mediated by non-cholinergic non-beta-adrenergic nervous mechanisms, and when phentolamine and propranolol were present, the response to electrical stimulation must have been mediated by non-adrenergic mechanisms. Finally, in the presence of all three antagonists together, the response to electrical stimulation must have

been mediated by nerves, because the response increased with increasing frequency (FIG. 4.7), and tetrodotoxin prevented the response (e.g., FIG. 4.5). Therefore, the response to electrical stimulation in the presence of atropine, phentolamine, and propranolol together must have been mediated by non-cholinergic non-adrenergic nervous mechanisms.

Although the neurotransmitter that mediates this response has not been identified, a number of substances do mediate secretion. Prostaglandins E_1 and $F_{2\text{-alpha}}$, and histamine each mediate secretion of $^{35}SO_4$ from tracheal segments of cats in vivo [189], as does leukotriene C_4 [171]. Additionally, $PGF_{2\text{-alpha}}$, and PGE_2 each cause secretion from segments of ferret trachea in vitro (work in progress). However, preliminary experiments suggest that leukotrienes C_4 and D_4 are not secretagogues. Additionally, none of these substances have been specifically localized to peripheral nerves, and they are not likely to be the neurotransmitters responsible for this non-cholinergic non-adrenergic effect.

Other candidates are peptides and purines. Vasoactive Intestinal Peptide (VIP), is found in ganglia and in nerves to airway glands and smooth muscle [231, 232], may be present in "P" type varicosities in nerves to glands of ferrets [8]. Moreover, it is thought to mediate non-adrenergic non-cholinergic relaxation of airway smooth muscle [139]. VIP may mediate salivation [131], and secretion of thyroid hormone [1]. Additionally, exogenous VIP causes both an increase in C-AMP fluoresence in serous and mucous cells, and the release of $^{35}SO_4$ from tracheas of ferrets via non-neural mechanisms (work in progress).

Other transmitter candidates are adenyl nucleotides: adenosine triphosphate (ATP) may mediate some effects of stimulating non-adrenergic non-cholinergic nerves to the smooth muscle of the intestine of guinea pigs [132].

However, most of the studies of the non-adrenergic non-cholinergic system have relied on electrical field stimulation in vitro (CHAPTERS 3-5) [132, 139], which may activate both efferent and afferent nerves. It is possible that there is a local neural reflex mechanism in the airways, mediated by stimulation of afferent nerves in the airways, which in turn causes the release of a neurotransmitter or neuromodulator. A possible candidate for such a material is substance P, which is found in tracheal epithelial nerves [162], peripheral afferent nerves [100], and causes salivation [126]. If substance P is released from peripheral afferent nerves in the trachea, and if it causes secretion of mucus from airways, it may be the mediator of such a local reflex.

Our results agree with some of the previous work on the adrenergic control of secretion. Beta-adrenergic nervous mechanisms mediate the secretion of [$^{35}SO_4$]-labeled macromolecules from tracheas of cats in vivo [74]. In addition, we found that alpha-adrenergic nervous mechanisms mediate secretion of macromolecules (FIG. 4.7), as well as of fluid (CHAPTER 3)[20]. Norepinephrine is the probable neurotransmitter for these adrenergic effects, because norepinephrine stimulates the secretion of $^{35}SO_4$ via both alpha- and beta-adrenergic mechanisms (TABLE 4.1), is found in the trachea of ferrets, is taken up into nerves to tracheal glands, and is released by electrical

stimulation of nerves (CHAPTER 5) [21]. Thus, the secretion of macromolecules and fluid from the trachea appear to be regulated separately. Glycoprotein secretion is mediated by muscarinic-cholinergic, alpha- and beta-adrenergic, and non-cholinergic non-adrenergic neural mechanisms, whereas fluid secretion is mediated by muscarinic and alpha-adrenergic, but only weakly by beta-adrenergic mechanisms (CHAPTER 3) [19, 20, 181].

Morphologic studies have also found differences in the effects of the different agonists. Only muscarinic and alpha-adrenergic stimulation degranulate serous cells of ferrets and cause the formation of lucent vacuoles, "watery vacuolation" [9, 224], whereas beta-adrenergic agonists do not [9]. In contrast, beta-adrenergic stimulation causes degranulation of mucous cells of ferret glands [79]. The significance of watery vacuolation is not understood, but it may reflect the secretion of fluid from secretory cells [223]. Regardless of the mechanisms or role of this vacuolation, these studies do suggest the presence of different regulatory mechanisms on the different cell types. This differential regulation is not due to differences in the proportion of adrenergic to cholinergic innervation of serous and mucous cells. Adrenergic nerves account for 10 % of the varicosities seen near submucosal glands, and that proportion is constant regardless of the relative numbers of serous and mucous cells nearby [151]. It is probable therefore, that differential regulation of fluid and glycoprotein secretion is due to the presence of different receptor mechanisms on the different cell types.

The implications of the different mechanisms are poorly understood. Although beta-adrenergic stimulation causes only a slight increase in the volume of fluid secreted, the bound $^{35}SO_4$ in the fluid is high (FIG. 4.6, left, table 4.1). In contrast, since both muscarinic and alpha-adrenergic stimulation cause substantially more fluid secretion, the glycoprotein concentration may be lower than for beta-adrenergic stimulation [234]. Additionally, alpha-adrenergic stimulation releases more lysozyme from serous cells [228] and a 50,000 dalton $^{35}SO_4$-labeled component [84] than does beta-adrenergic stimulation. The functions of lysozyme and the 50,000 dalton protein in airway secretions are unknown, but it may be possible that they change the physical properties of the mucus. These differences between alpha- and beta-adrenergic stimulation may account for the higher viscosity of mucus secreted in response to beta-adrenergic stimulation (Leikauf, personal communication). The quality of the mucus secreted in response to stimulating non-cholinergic non-adrenergic nerves is unknown. But because muscarinic and alpha-adrenergic neural mechanisms are the dominant mechanisms for fluid secretion, we expect that the glycoprotein concentration might be higher than that of the mucus secreted in response to either muscarinic or alpha-adrenergic stimulation.

The balance between the different neural mechanisms is subject not only to central control, but also to local neural interaction. The sum of the responses to electrical stimulation at 5 Hz in the presence of combinations of 2 antagonists was 145 % of the control response (FIG. 4.7)($p>0.1$). This suggests that in the absence of

drugs, there possibly was inhibition of at least one neural mechanism by others. This may be so because the addition of the antagonists may have prevented inhibitory effects by antagonizing pre-synaptic mechanisms. Other evidence for neural interaction in the neural regulation of secretions is that the mechanisms by which exogenous and endogenous norepinephrine act are not identical. The response to exogenous norepinephrine is mediated mostly via alpha-adrenergic mechanisms (TABLE 4.1), whereas the response to endogenous norepinephrine released by electrical stimulation is mediated more by beta- than by alpha-adrenergic mechanisms (FIG. 4.7). This may be because phentolamine may inhibit a presynaptic neural auto-inhibition of norepinephrine release [239]. In the presence of auto-inhibition of norepinephrine release, stimulation of adrenergic nerves releases less norepinephrine than in the absence of the inhibitory mechanism. After phentolamine however, the amount of norepinephrine released may increase, and the amount of transmitter may be greater at the glands causing a greater secretion via the uninhibited beta-adrenergic mechanism.

Adrenergic nerves may also inhibit cholinergic neurotransmission to glands as they appear to in airway smooth muscle [240]. Conversely, cholinergic nerves might inhibit adrenergic neurotransmission to glands. There is evidence for this hypothesis. Acetylcholine inhibits the evoked release of both endogenous and ^{3}[H]-norepinephrine from airways [67, 240], and a variety of vascular smooth muscle [209, 219, 239]. Because atropine reversed all of these effects, the inhibition was via muscarinic receptors. In the presence

of atropine, this inhibitory mechanism is inhibited, and thus, the amount of norepinephrine released increases, thus stimulating adrenergic mechanisms of secretion.

Thus, the autonomic nervous system may regulate the quantity and composition of mucus by acting at two different levels. First, central adrenergic or cholinergic neural mechanisms stimulate the secretion of characteristic mucus, and second, nerves may alter the activity of the other secretomotor nerves. To test the second possibility, we performed a series of experiments in which we measured changes in the neurally evoked release of radio-labeled adrenergic neurotransmitter in response to stimulating cholinergic nerves.

^{3}[H]-NOREPINEPHRINE

INTRODUCTION

The adrenergic and cholinergic nerves that probably mediate secretion are found in the same nerve bundles close to each other often without any supporting cell processes between them (FIG. 2.9)[9, 151]. This proximity of the two nerve types suggests that, in addition to their direct effects on the gland cells, the nerves may interact with each other. There is some evidence for neural interaction in secretion from airways. The sum of the secretory responses to stimulating nerves in the presence of combinations of two of three antagonists was greater than the response to electrical stimulation in the absence of drugs (CHAPTER 4). This may mean that in the absence of drugs, there is inhibition by nerves on the neurotransmission of others.

There is precedent for neural interaction to smooth muscle. Endogenous catecholamines inhibit cholinergic neurotransmission to bronchial muscle of dogs [240]. Conversely, acetylcholine inhibits the release of ^{3}[H]-norepinephrine from nerves in tracheal smooth muscle [67]. Since atropine inhibited the effects of acetylcholine, muscarinic receptors inhibit adrenergic neurotransmission to this muscle. It is less clear however, whether cholinergic nerves inhibit adrenergic neurotransmission. Therefore, our aim was to determine whether the stimulation of cholinergic nerves inhibits adrenergic neurotransmission to the submucosal glands of ferrets, and if so, by

which mechanisms.

If cholinergic nerves inhibit adrenergic nerves, then exogenous acetylcholine should inhibit release of norepinephrine, and if muscarinic mechanisms are responsible, then atropine should reverse the inhibition. Additionally, atropine by itself might increase the release of norepinephrine, and finally, potentiating the effects of stimulating cholinergic nerves should inhibit the release of norepinephrine.

To test this hypothesis, we measured the release of ^{3}H after pre-incubating segments of trachea with ^{3}[H]-norepinephrine [219]. The release of total ^{3}H after pre-incubation parallels the release of ^{3}[H]-norepinephrine [67, 239] suggesting that measurement of total ^{3}H released reflects adrenergic neurotransmission. Norepinephrine is the most likely candidate for the adrenergic neurotransmitter, since it is present in the lung [138], and catecholamine fluorescence is found near tracheal glands of cats [151]. Additionally, ^{3}[H]-norepinephrine is taken up into nerves that innervate the trachealis muscle [194, 240] and submucosal glands [21]. Furthermore, norepinephrine causes secretion of glycoproteins from tracheal glands of ferrets (TABLE 4.1)[18]. To show that the ^{3}[H]-norepinephrine was taken up into nerves, we used desmethylimipramine, a drug that inhibits the uptake of catecholamines into adrenergic nerves [65, 70, 227]. To stimulate nerves <u>in vitro</u>, we used electrical field stimulation [21]. To show that electrical stimulation releases ^{3}H via neural mechanisms, we used tetrodotoxin, a drug that prevents the rapid influx of sodium into depolarized nerve membranes [157], prevents action potentials, and

thereby prevents adrenergic neurotransmission to tracheal glands [18, 20].

METHODS

A. PREPARATION

We anesthetized 31 adult male ferrets weighing 1-1.5 kg each by injecting sodium pentobarbital (45-60 mg/kg, ip). After making a ventral midline incision, we excised the entire trachea, removed most of the loose fat and connective tissue, and rinsed the trachea in Krebs-Henseleit solution maintained at 38° C and bubbled with 94% O_2-6% CO_2. To eliminate effects of adrenergic innervation of airway smooth muscle, we removed the entire posterior membrane, including the trachealis muscle and its insertions into the cartilage rings. Since the airway ganglia are located in rows along the posterior membrane (FIG. 2.7)[93], this procedure also removed the ganglia with their adrenergic innervation [93, 151]. Then we cut the remaining anterior portion into segments approximately 10 cartilage rings long.

B. NOREPINEPHRINE MEASUREMENT

To determine whether norepinephrine is present in the anterior trachea, whether tissues can take up norepinephrine, and whether the uptake is mediated by a specific catecholamine mechanism, we incubated one tissue from each of 4 ferrets in Krebs-Henseleit solution either without drugs, with DL-norepinephrine (5×10^{-7} M; 90 min; Sigma Chemical Co, St. Louis, MO), or with norepinephrine plus desmethylimipramine (DMI; 10^{-5} M; Merck). We then made duplicate determinations of the content of norepinephrine in each tissue, using high-performance liquid chromatography [113]. Preliminary experiments

showed that although there were substantial amounts of norepinephrine in the tissue, there was not enough endogenous norepinephrine released into the medium to be measured chromatographically. Therefore, to study the release of neurotransmitter, we had to label the norepinephrine pools in the nerves with ^{3}[H]-norepinephrine.

C. ^{3}[H]-NOREPINEPHRINE

We first determined whether the tissues take up ^{3}[H]-norepinephrine and release ^{3}H in the absence of stimulation. After incubating 1 tissue from each of 9 ferrets in ^{3}[H]-norepinephrine (5 x 10^{-7} M; 90 min; ICN, Irvine, CA), we placed each segment in a chamber (FIG. 5.1) containing 5 ml of Krebs-Henseleit solution without ^{3}[H]-norepinephrine. Every 5 min for 6 h, we drained the chamber and refilled it with fresh Krebs-Henseleit solution. Preliminary experiments showed that the release of ^{3}H followed a predictable exponential pattern, and that after 2 h of washout, release followed a single exponential pattern (e.g., FIG. 5.2). We believe that after this time, most of the ^{3}H released comes from extraneuronal sites (see Discussion), and that the release from nerves had reached a low value.

D. ELECTRICAL STIMULATION

We electrically stimulated tissues after at least 2 h of washing them. First, we determined the voltage-dependence of the response to electrical stimulation. We stimulated 2 tissues each from an additional 3 ferrets electrically, at an intensity of either 5, 10, or 20 volts (pulse duration, 0.5 ms; frequency, 10 monophasic cycles/s

for 4.5 min). Preliminary experiments showed that electrical stimulation caused the release of ^{3}H, and that at 10 V, the response had reached a maximum. Therefore, we stimulated all other tissues at 10 V. Next, to determine the frequency dependence of the response, we stimulated 2 other tissues from the same ferrets at 2, 6, or 10 cycles/s.

To determine whether neural mechanisms are responsible for the uptake and release of ^{3}H, we did another series of experiments. First, 1 h after electrically stimulating 2 tissues from each of 3 additional ferrets (10 cps for 4.5 min), we added tyramine (10^{-4} M; 4.5 min, Sigma Pharmaceutical Co., St. Louis, MO). Then, to determine whether the ^{3}H recovered was from ^{3}[H]-norepinephrine taken up into nerves, we incubated 2 tissues each from 4 additional ferrets with ^{3}[H]-norepinephrine in the absence of inhibitors or in the presence of desmethylimipramine, DMI, (10^{-6}-10^{-5} M; 90 min). After washing the tissues for 120 min, we stimulated each tissue electrically at 10 cycles/s, and collected samples for an additional 70 min. Finally, to determine whether the release of ^{3}H evoked by electrical stimulation was mediated by nerves, we stimulated the release of ^{3}H from 1 tissue from each of 6 additional ferrets, first by an electrical field and then by K^{+} (100 mM; 4.5 min), before and after incubating the tissues with tetrodotoxin (10^{-9} - 10^{-6} M; 70 min; Calbiochem-Behring, La Jolla, CA).

E. AUTONOMIC NERVES

In the last series of experiments, we determined whether cholinergic neural mechanisms inhibit adrenergic neurotransmission. To determine the within-tissue reproducibility of the response to electrical stimulation, we stimulated 1 tissue from each of 13 additional ferrets (10 V; 10 cycles/s for 4.5 min) at 120, 195, and 270 min after removing the tissues from the solution containing ^{3}[H]-norepinephrine. After the final stimulation, we collected samples for an additional 70 min. Then, to see if exogenous acetylcholine inhibited the release of ^{3}H evoked by electrical stimulation, we stimulated 1 other tissue from 8 of the same ferrets first in the absence of drugs, and again after adding acetylcholine (10^{-5} M; 70 min; Sigma). We then repeated the stimulation after adding atropine sulfate (10^{-6} M; 70 min; Sigma). Then, to determine whether endogenous acetylcholine inhibits the release of ^{3}H evoked by electrical stimulation, we stimulated 1 other tissue from 6 of the same ferrets, first in the absence of drugs, and again, after adding atropine sulfate (10^{-6} M; 70 min). Finally, we potentiated cholinergic neural mechanisms in 1 other tissues from 8 of the same ferrets by inhibiting acetylcholinesterase. We stimulated each tissue first in the absence of drugs, and then again after adding physostigmine (10-6 M; 70 min; Sigma). To see if the effects of physostigmine were mediated by muscarinic mechanisms, we then added atropine (10^{-6} M; 70 min) and repeated the stimulation.

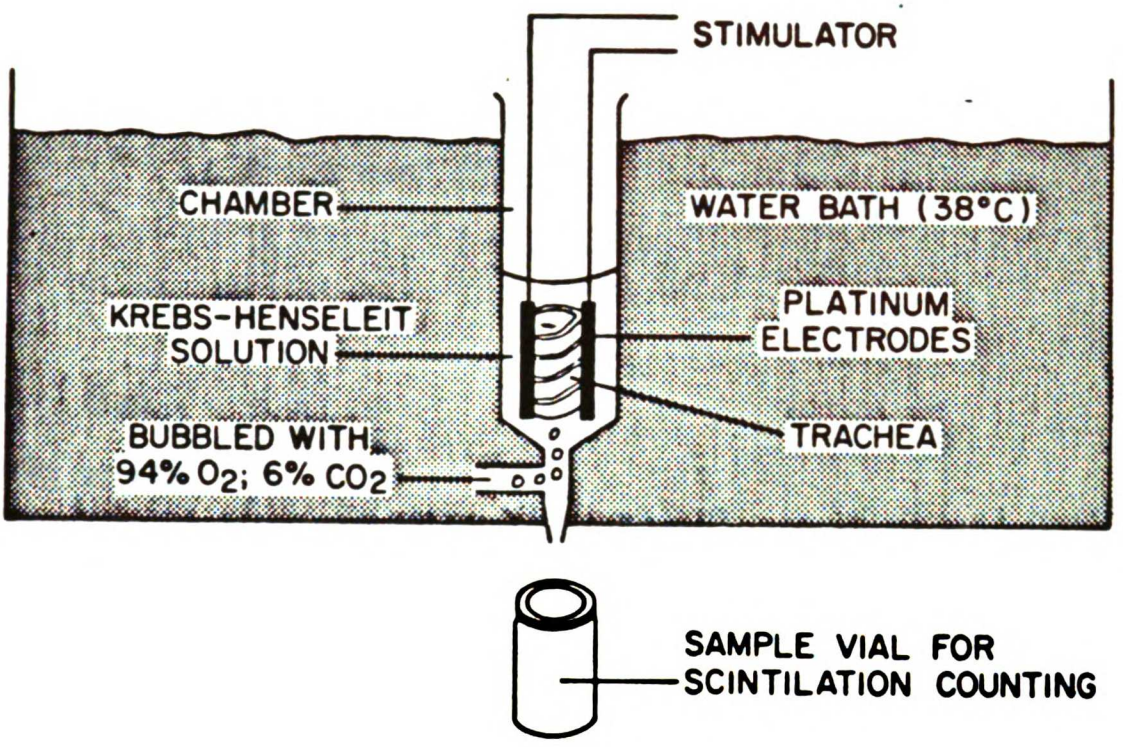

FIG. 5.1. Apparatus used to study release of ^{3}H. After incubation with ^{3}[H]-norepinephrine ($5x10^{-7}$ M; 45 min), muscle-free segments of anterior trachea were held between the two platinum plate electrodes used for electrical stimulation. Every 5 min, the Krebs-Henseleit solution was drained from the chamber, and fresh solution was added. Drugs were added to the solution prior to refilling the chamber.

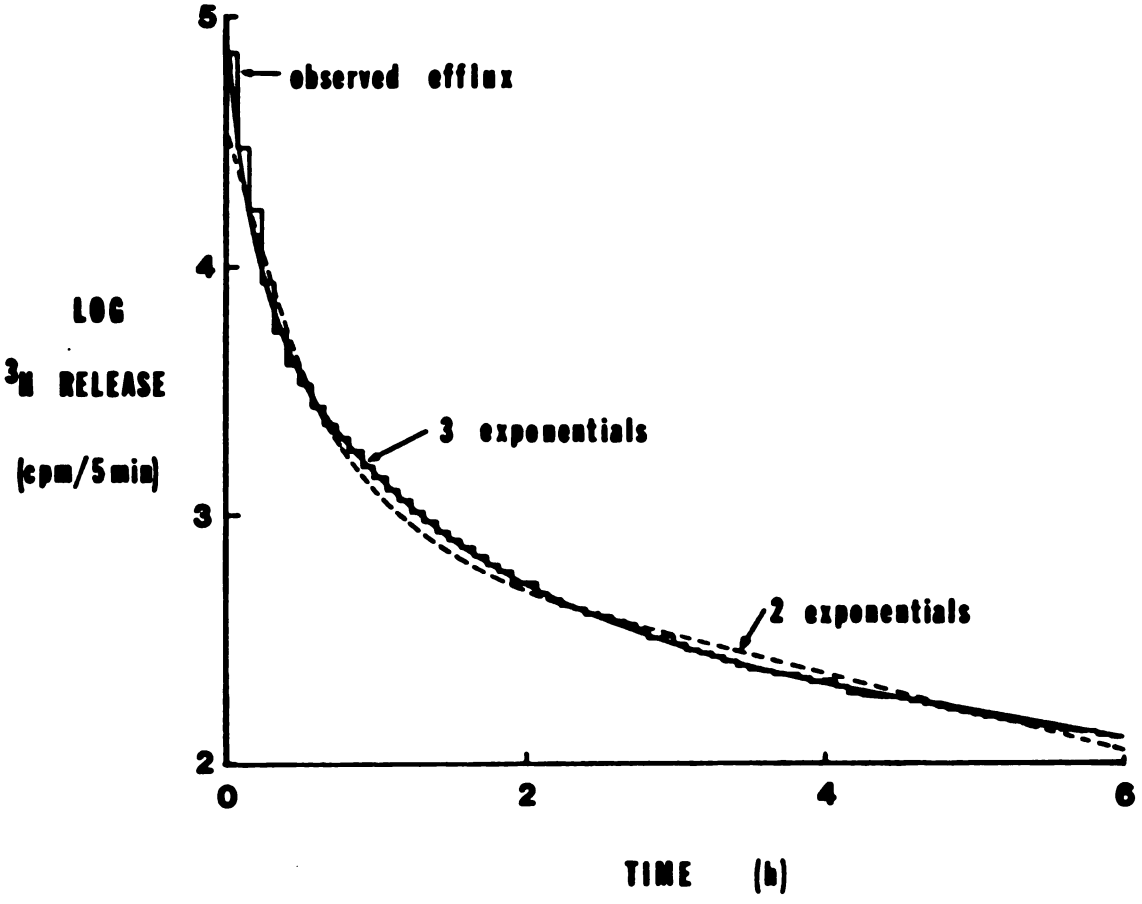

FIG. 5.2. Semilogarithmic plot of baseline ^3H release from one tissue. Data points were fitted to the sum of 2 (--) or 3 (__) exponential terms. From 30 min on, the line of best fit was achieved by assuming 2 exponential terms (not shown). The line of best fit for data after 2h was achieved by assuming a single exponential term (not shown). Similar patterns of secretion were obtained from one tissue from each of 9 ferrets.

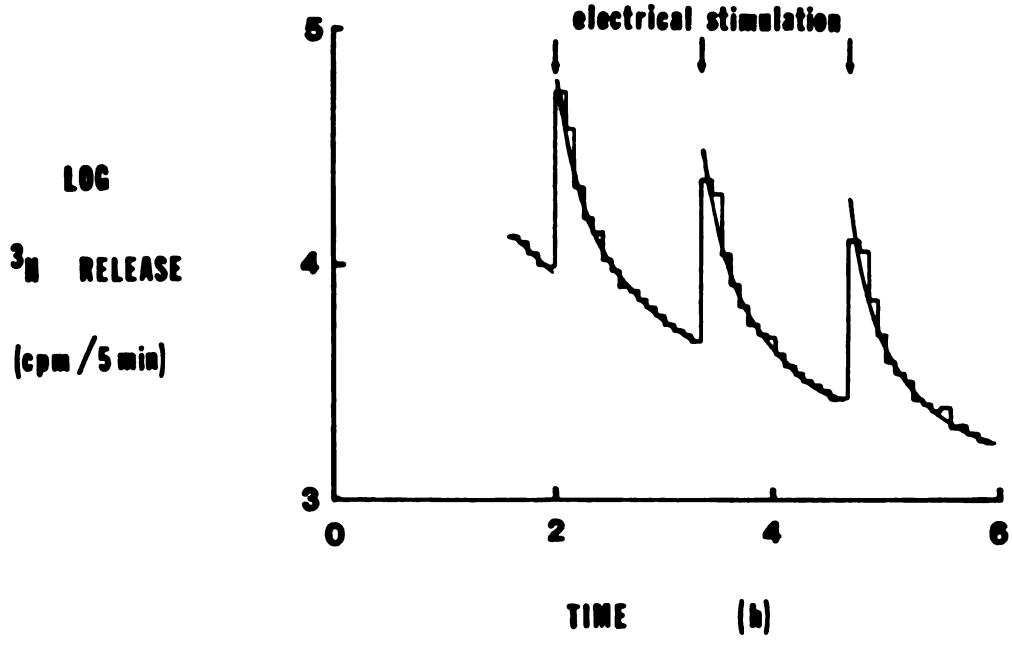

FIG. 5.3. Release of ^{3}H from one tissue in response to repeated electrical stimulation (intensity, 10 V; pulse duration, 0.5 ms, frequency, 10 cycles/s for 4.5 min)(arrows).

F. DATA ANALYSIS

1. Exponential Model

After transferring tissues from Krebs-Henseleit solution containing ^{3}[H]-norepinephrine to solution without the label, or after electrical or pharmacological stimulation, the washout of ^{3}H from tissues followed an exponential pattern (FIGS. 5.2, 5.3). The lines of best fit were achieved by assuming at most, 3 exponential terms (FIG. 5.2, TABLE 5.1). The secretory rate at any time, $S_{(t)}$ is therefore given by the sum of 3 exponentials:

$$S_{(t)} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t}$$

Equation 1,

where each term represents a first order exponential process. A_1, A_2, and A_3 are the parameters that represent the sizes of the ^{3}H pools at t=0, of each of the exponential processes, and k_1, k_2, and k_3 are the respective rate constants. Electrical stimulation releases ^{3}H from nerves and thereby increases the content of ^{3}H in the pools that are washed out after stimulation. By integrating the washout curve from the onset of electrical stimulation to an arbitrary time t, we obtain the total quantity of ^{3}H released, $C_{(total)}$:

$$C_{(total)} = \int_0^t A_1 e^{-k_1 t} + \int_0^t A_2 e^{-k_2 t} + \int_0^t A_3 e^{-k_3 t}$$

Equation 2.

By subtracting the quantity of ^{3}H released during the same time due to baseline processes, we obtain the quantity of ^{3}H released by

electrical stimulation, $C_{(total)}-C_{(baseline)}$. However, we cannot determine the component of total secretion that represents baseline secretion, since electrical stimulation depletes pools of ^{3}H available for baseline secretion (APPENDIX I). Thus, after electrical stimulation, baseline secretion is probably reduced during the time that total ^{3}H release is still high. Thus, we cannot accurately predict baseline release during and after electrical stimulation. To bypass this problem, we relied on a property of exponential processes, namely that the solution to equation 2 for $C_{(total)}$ is proportional to the sum of $A_1 + A_2 + A_3$. (i.e., at t=0, $C_{(0)} = A_1+A_2+A_3$). Therefore, the relative difference between the total label released $C_{(tot)}$, and the baseline secretion $C_{(bl)}$, $[C_{(tot)}-C_{(bl)}]\ /C_{(bl)}$, is equal to the relative differences between the initial pool sizes (at t=0), of the two states.

$$\frac{[C_{(tot)}-C_{(bl)}]}{C_{(bl)}} = \frac{[A_1+ A_2+ A_3]_{(tot)} - [B_1+ B_2+ B_3]_{(bl)}}{[B_1+ B_2+ B_3]_{(bl)}},$$

where B_1, B_2, and B_3 represent the intercepts of the pre-stimulation baseline efflux curves extrapolated forward in time to the onset of stimulation (FIG. 5.4). Thus, we do not have to predict baseline secretion because the pool sizes of each are determined for the same point in time (the onset of stimulation).

2. Data Reduction

Given the cpm of ^3H released during each 5 min interval for each experiment, we solved the differential equation (Equation 1) using a non-linear least squares regression program run on a Hewlett Packard computer (model 9825A). To calculate the evoked response, we determined the cpm released immediately before and after stimulation (FIG. 5.4). We found the pre-stimulus baseline cpm (B) by extrapolating the pre-stimulus efflux curve from 30 min prior to stimulation forward to the time of stimulation. The line of best fit was achieved by assuming a single exponential term. Then, we found the peak height of the evoked response ($A_1 + A_2$) by extrapolating the post-stimulus efflux curve from 75 min after stimulation backward to the time of the onset of stimulation. We did not use the first point after stimulation because during the time of stimulation, an additional rapid kinetic process was occurring (release of ^3H evoked by action potentials), that we could not evaluate using the relatively long sampling interval (5 min). The line through the remaining 15 points that gave the most precise estimates of the evoked response was achieved by assuming 2 exponential terms (TABLE 5.2). Although the fit is not as good with 2 exponential terms as with 3, the estimated standard error of calculating the peak height (APPENDIX II) using the 2 exponential model was only 18 %, whereas the standard error using the 3 exponential model was 39 %. Therefore, the 2 exponential model resulted in more precise calculations.

The absolute evoked response, $ER_{(abs)}$, is the extrapolated maximum secretory rate minus the pre-stimulus baseline secretory rate:

$$ER_{(abs)} = A_1 + A_2 - B.$$

Since we washed the label out of the tissue during the experiment, both the baseline secretion and the absolute evoked response decreased progressively (FIG. 5.3). We corrected for these decreases by expressing the evoked response as the relative evoked response, $ER_{(rel)}$, by dividing the absolute evoked response by the pre-stimulus baseline secretory rate:

$$ER_{(rel)} = \frac{[A_1 + A_2 - B]}{B}$$

(see APPENDIX I).

3. Statistical Analysis

For within-tissue comparisons, we expressed the relative evoked responses as percent of the first (control) response. Since there was significant uncertainty of estimating the evoked responses (APPENDIX II), we could not compare results using either standard "t" tests or analysis of variance since both of these methods of comparison require that the values of the data points be precisely known. Therefore, we used maximum likelihood estimation [15] to estimate the mean, X, and the biological variance, σ^2, of $ER_{(rel)}$, and to determine whether the means of two groups of responses are different from one another (see APPENDIX III). Unless otherwise specified, data is expressed as the mean, X, and the biological variance σ^2.

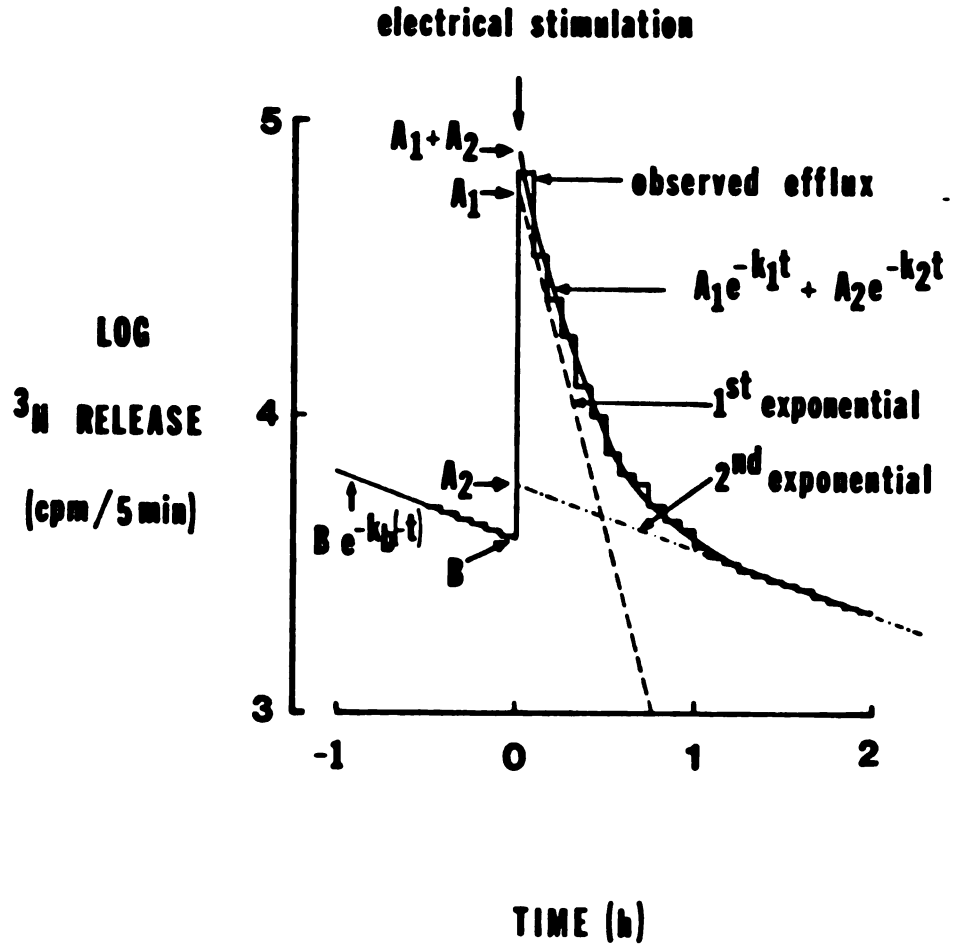

FIG. 5.4. Exponential analysis of ^{3}H release. For 75 min after stimulation (arrow), ^{3}H efflux, $S_{(t)}$, is described as the sum of two exponential terms.

$$S_{(t)} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}.$$

The extrapolated secretory rate at the onset of stimulation, (A_1 + A_2), minus the secretory rate just before stimulation (B) is the absolute evoked response $ER_{(abs)}$. (A_1 + A_2 - B)/B is the relative evoked response $ER_{(rel)}$. Since $-k_1$ is steep, small errors in estimating sampling will cause large errors in estimating A_1.

RESULTS

A. NOREPINEPHRINE MEASUREMENT

Segments of trachea with neither trachealis muscle nor ganglia contained norepinephrine. Control tissues contained 131 ± 6 (SEM) ng/mg norepinephrine (n=4). Incubation with unlabeled norepinephrine ($5x10^{-7}$ M) increased the content slightly to 142 ± 10.5 (SEM) ng/mg (n=4). Addition of DMI (10^{-5} M) decreased the content of norepinephrine by 38% to 90 ± 13 (SEM) ng/mg (n=4).

B. ^{3}[H]-NOREPINEPHRINE BASELINE

^{3}[H]-norepinephrine was taken up into, and ^{3}H was released from each tissue. The efflux of ^{3}H followed a predictable pattern under baseline conditions (e.g., FIG. 5.2, TABLE 5.1). The line of best fit was acheived by assuming 3 exponential terms. After 2h, the line of best fit was acheived by assuming a single exponential term, because by assuming more terms, the computer program would not calculate the parameters, presumably because the errors of estimating them were be too high. Similar results were obtained for each of the other 8 tissues studied under baseline conditions.

C. ELECTRICAL STIMULATION

Electrical stimulation released ^{3}H (e.g., FIG. 5.3). For the 70 min of efflux following electrical stimulation, the line of best fit was achieved by assuming 3 exponential terms (TABLE 5.2). However, the accuracy of the estimates of the exponential parameters was better

using 2 exponential terms (TABLE 5.2). The intercept and slope of the slowest exponential in each case was nearly the same, suggesting that after stimulation, release had become nearly log-linear. Increasing the voltage applied to the tissues increased ^{3}H release, with a plateau in the response at 10 V (FIG. 5.5); increasing the frequency of stimulation also increased the release of ^{3}H (FIG. 5.6). The relative evoked response, $ER_{(rel)}$, increased from 248 ± 33 % at 2 cycles/s, to 797 ± 70 % at 10 cycles/s (FIG. 5.6). The absolute evoked response to a single period of electrical stimulation (frequency, 10 cps, 4.5 min) at 2 h was 328 ± 72 cpm/mg tissue ($p<0.005$; n=13). Repeated electrical stimulation caused reproducible secretion of ^{3}H (e.g., FIG. 5.3). The mean responses for the second and third responses were 109 ± 17 % and 111 ± 8 %, respectively, of the first response ($p>0.4$; n=13). When corrected for by tissue weight, neither baseline release, $ER_{(abs)}$, nor $ER_{(rel)}$ were different between segments taken from the lower trachea versus segments taken from the upper trachea (FIG. 5.7).

In addition to electrical stimulation, tyramine and K^{+} released ^{3}H. The mean responses, $ER_{(rel)}$, were 644 ± 86 % and 713 ± 91 %, respectively ($p<0.05$; n=6 tissues each)(FIGS. 5.8, 5.9, 5.10). DMI inhibited the uptake of ^{3}[H]-norepinephrine in a dose-dependent fashion, and thereby decreased the release of ^{3}H evoked by electrical stimulation (FIGS. 5.8, 5.9). The maximum inhibition was 71 ± 6 % at 10^{-5} M. DMI did not diminish baseline secretion. At 2 h after incubation with ^{3}[H]-norepinephrine, tissues incubated in the absence of DMI released 2361 ± 354 (SEM) cpm/5 min, and tissues incubated with

DMI released 2667 ± 311 (SEM) cpm/5 min. Corrected for tissue wet weight, untreated tissues released 13.3 ± 1.6 (SEM) cpm/5 min/mg, and treated tissues released 12.0 ±0.7 (SEM) cpm/5 min/mg. Neither of these differences in baseline release between DMI treated and untreated tissues are significant. Tetrodotoxin caused a concentration-dependent decrease in the secretion of ^{3}H evoked by electrical stimulation; the maximal inhibition was 89 ± 3 % at 10^{-6} M. However, tetrodotoxin did not inhibit the response to K^{+} (FIGS. 5.10, 5.11).

D. AUTONOMIC NERVES

Acetylcholine decreased the release of ^{3}H evoked by electrical stimulation by a mean of 17 ± 7 % of the first response ($p<0.05$; n=8) (e.g., FIG. 5.12). After acetylcholine, atropine increased release by 50 ± 12 % to 122 ± 14 % of the first response in the absence of drugs ($p<0.05$; n=8). Atropine alone increased evoked release to 121 ± 9 % of the first response (P=0.05; n=6) (e.g., FIG. 5.13). Physostigmine decreased evoked release by a mean of 37 ± 6 % ($p<0.05$; n=6) (FIGS. 5.14, 5.15). After physostigmine, atropine increased release by a mean of 68 ± 21 % to 109 ± 8 % of the control response in the absence of drugs (n=8).

TABLE 5.1

EXPONENTIAL PARAMETERS OF BASELINE ^{3}H EFFLUX

MODEL	PARAMETER						
	A_1 (cpm/5 min)	k_1 (min^{-1})	A_2	k_2	A_3	K_3	FIT (±cpm/5 min)
3 EXPONENTIALS	814,189	-1.51	50486	-.240	13,325	-.035	101
(±m²)	78,991	.100	6139	.035	1824	.005	8 (SEM)
(% error)	9	7	12	13	9	15	8
2 EXPONENTIALS	43,821	-.665	7713	-.050	-	-	134
(±m²)	7596	.170	1338	.008	-	-	15 (SEM)
(% error)	19	23	21	16	-	-	11

TABLE 5.1. Exponential parameters for baseline ^{3}H efflux (see FIG. 5.2). One tissue from each of 9 ferrets was incubated with ^{3}[H]-norepinephrine for 90 min, and then rinsed every 5 min with unlabeled Krebs-Henseleit solution. The data points were fit to the sum of either 3 or 2 exponential terms by a computer with a nonlinear least squares regression program. For each exponential term there is one intercept parameter A, and one slope parameter, k. The goodness of fit is expressed as Standard Error Around the Curve (FIT) ± SEM. Other data are expressed as mean ±m².

TABLE 5.2

EXPONENTIAL PARAMETERS OF ^{3}H EFFLUX

EVOKED BY ELECTRICAL STIMULATION

MODEL	PARAMETER						
	A_1 (cpm/5 min)	k_1 (min^{-1})	A_2	k_2	A_3	k_3	FIT (± cpm/5 min)
3 EXPONENTIALS	108,840	-.355	25,904	-.035	7185	-.020	97
(±m^2)	8630	.031	8604	.024	1380	.012	32 (SEM)
(% error)	8	8	33	64	19	70	33
2 EXPONENTIALS	58,649	-.198	6681	-.015	-	-	126
(±m^2)	6367	.011	907	.0002	-	-	15 (SEM).
(% error)	11	6	14	11	-	-	12

TABLE 5.2. Exponential parameters of ^{3}H efflux evoked by electrical stimulation. One tissue from each of 13 ferrets was stimulated once, after 120 min of washing out each tissue. The following 15 points were fit to the sum of either 3 or 2 exponential terms. For each exponential term, there is one intercept parameter A, and one slope parameter k. The goodness of fit is expressed as the Standard Error Around the Curve (FIT). Other data are expressed as mean ±m^2. The values of the parameters A and k calculated for the slowest exponential process, are nearly the same regardless of whether we assume 2 or 3 exponential terms.

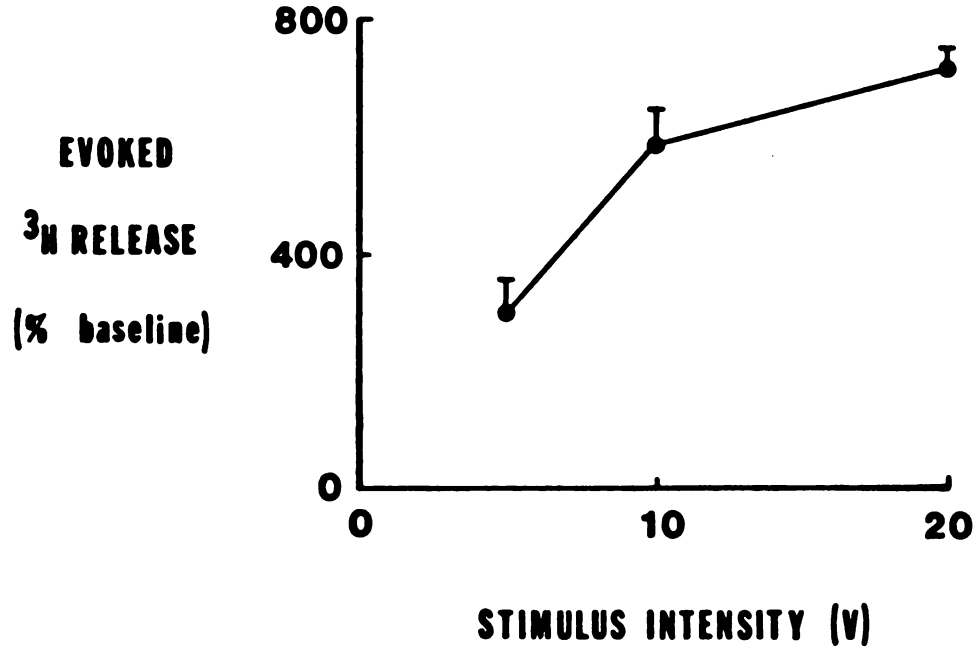

FIG. 5.5. Effect of stimulus voltage on release of ^{3}H evoked by electrical stimulation. Each point represents the mean ± SE of responses from one tissue from each of 5 ferrets.

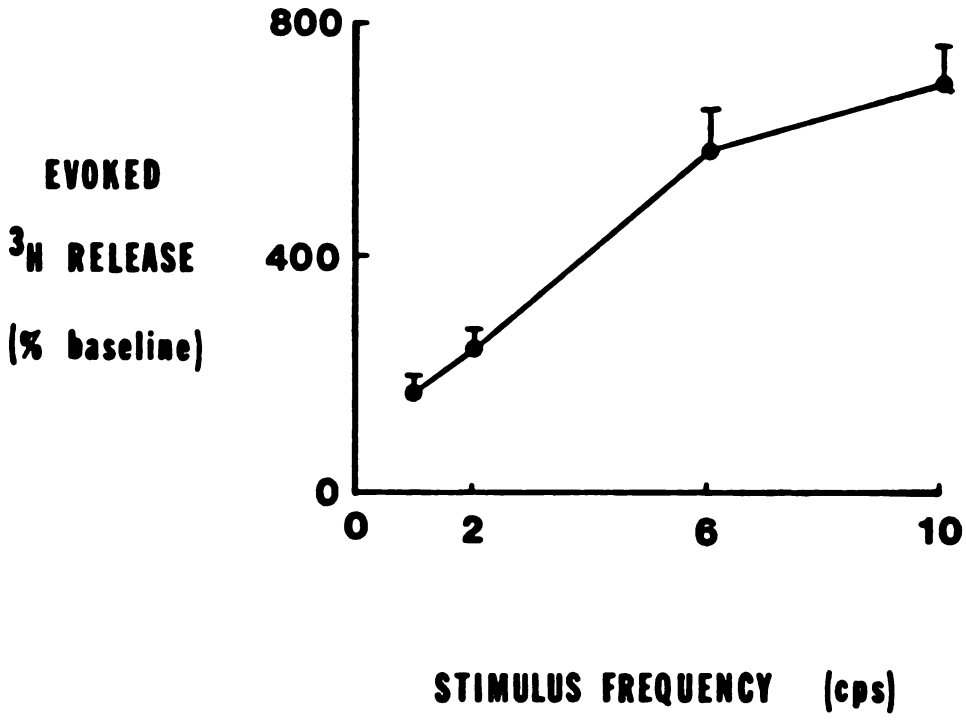

FIG. 5.6. Effect of stimulus frequency on release of ^{3}H evoked by electrical stimulation. Each point represents the mean ±m^{2} of responses from one tissue from each of 5 ferrets.

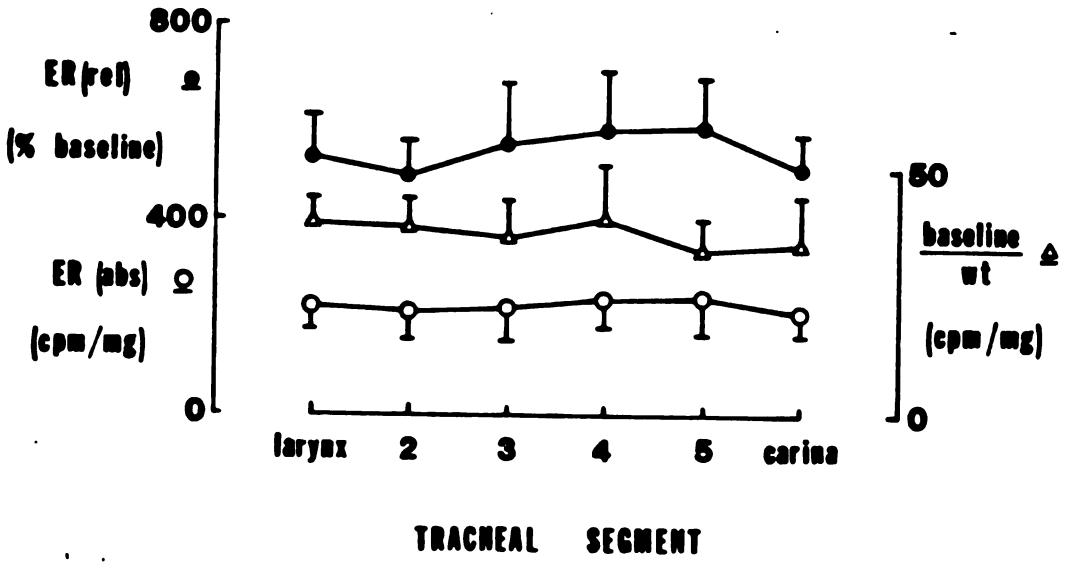

FIG. 5.7. ^{3}H release from segments taken from different parts of the trachea. Baseline (Δ), peak height/wt, $ER_{(abs)}$ (O), and relative peak height, $ER_{(rel)}$ (●) are expressed as mean ± m^2 of 6 tissues from each of 6 ferrets.

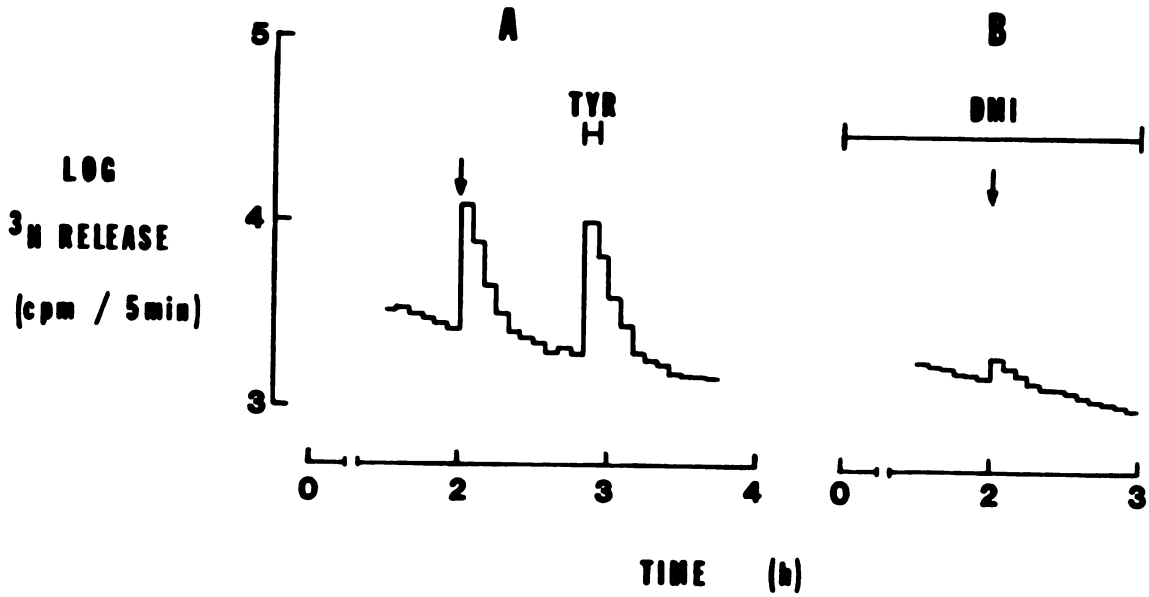

FIG. 5.8. Effects of tyramine and desmethylimipramine of ^{3}H release from 2 tissues from a ferret. A. Tissue incubated with ^{3}[H]-norepinephrine alone (5 x 10^{-7} M; 90 min), washed out starting at 0 h, and then stimulated by an electrical field (10 cps; 4.5 min) (arrow), or by tyramine (10^{-4} M; 4.5 min; TYR). B. Another tissue incubated with ^{3}[H]-norepinephrine plus desmethylimipramine (10^{-5} M; DMI), washed out starting at 0 h, and then stimulated electrically (10 cps; 4.5 min) (arrow).

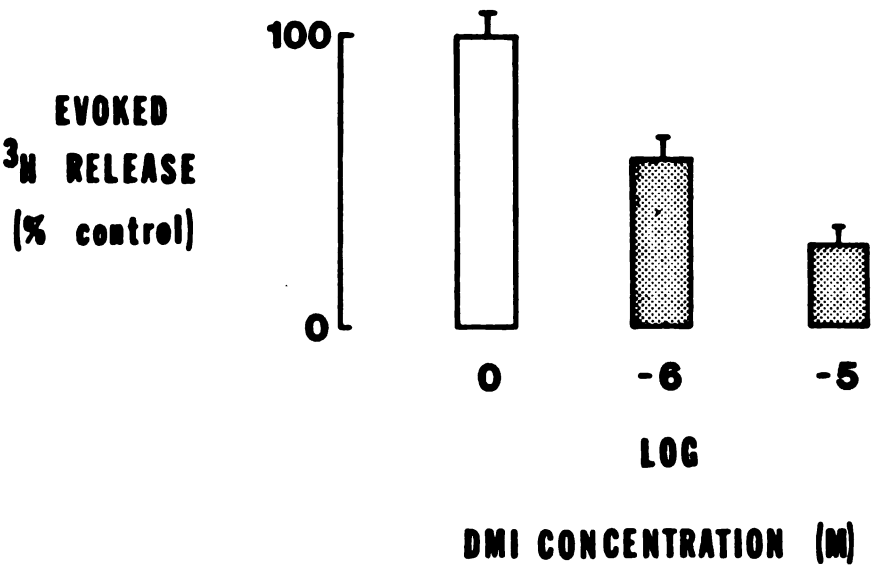

FIG. 5.9. Effect of incubating segments with desmethylimipramine during pre-incubation on secretion of ^{3}H evoked by electrical stimulation. Data from 2 tissues from each of 3 ferrets at each concentration of DMI are expressed as mean ± SE.

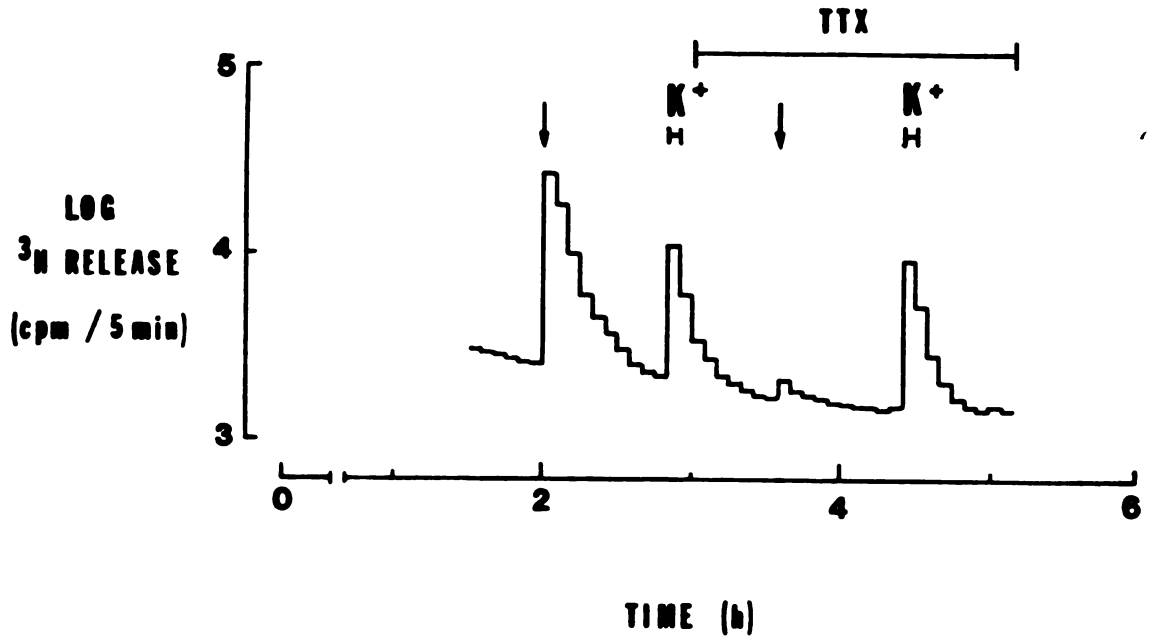

FIG. 5.10. Effect of tetrodotoxin (TTX) on ^{3}H release from a tissue from a ferret. Tissue was incubated with ^{3}[H]-norepinephrine alone (5 x 10^{-7} M; 90 min), washed out starting at 0 h, and then stimulated either electrically (10 cps; arrows) or with K^{+} (100 mM; 4.5 min) before and after incubation with TTX (10^{-7} M).

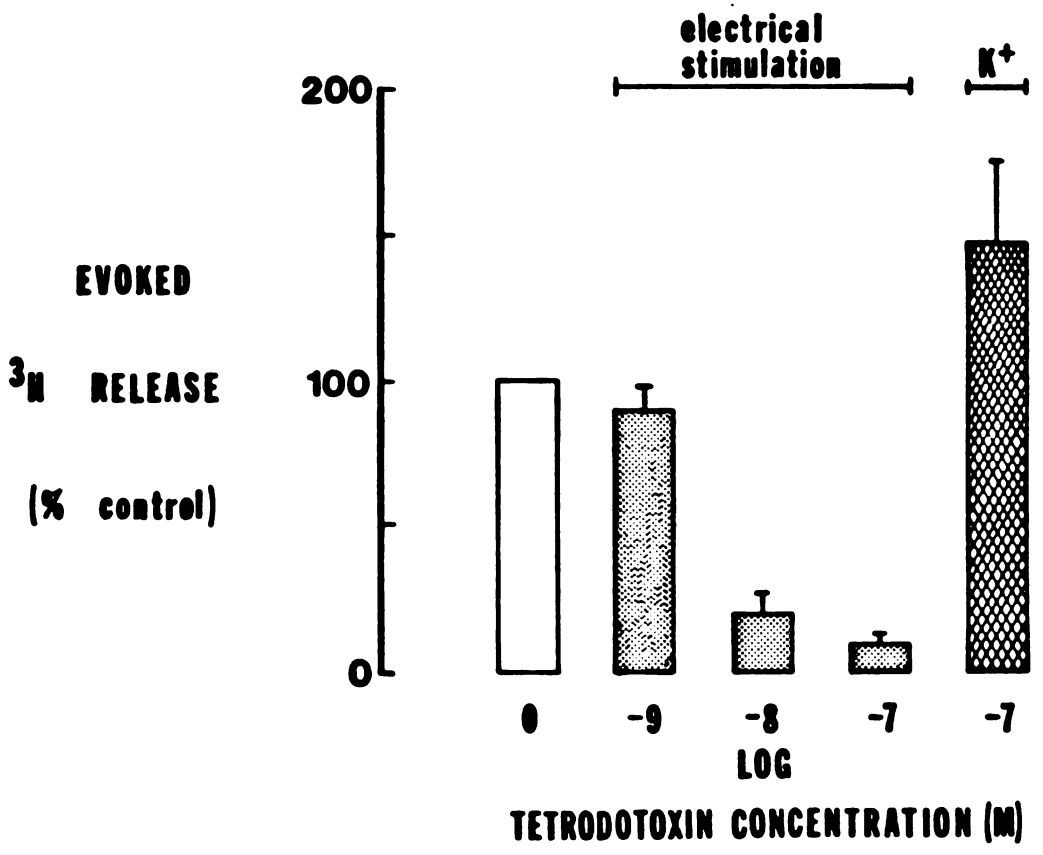

FIG. 5.11. Effect of tetrodotoxin on the release of ^{3}H evoked by either electrical stimulation or potassium. One tissue from each of 6 ferrets was stimulated in the absence of tetrodotoxin (□) first by an electrical field (10 cycles/s for 4.5 min) and then by potassium (100 mM; 4.5 min). After incubation with tetrodotoxin at the concentrations indicated, each tissue was again stimulated by an electric field (▩) and then by potassium (K^+, ▩). Data are expressed as mean ± SE.

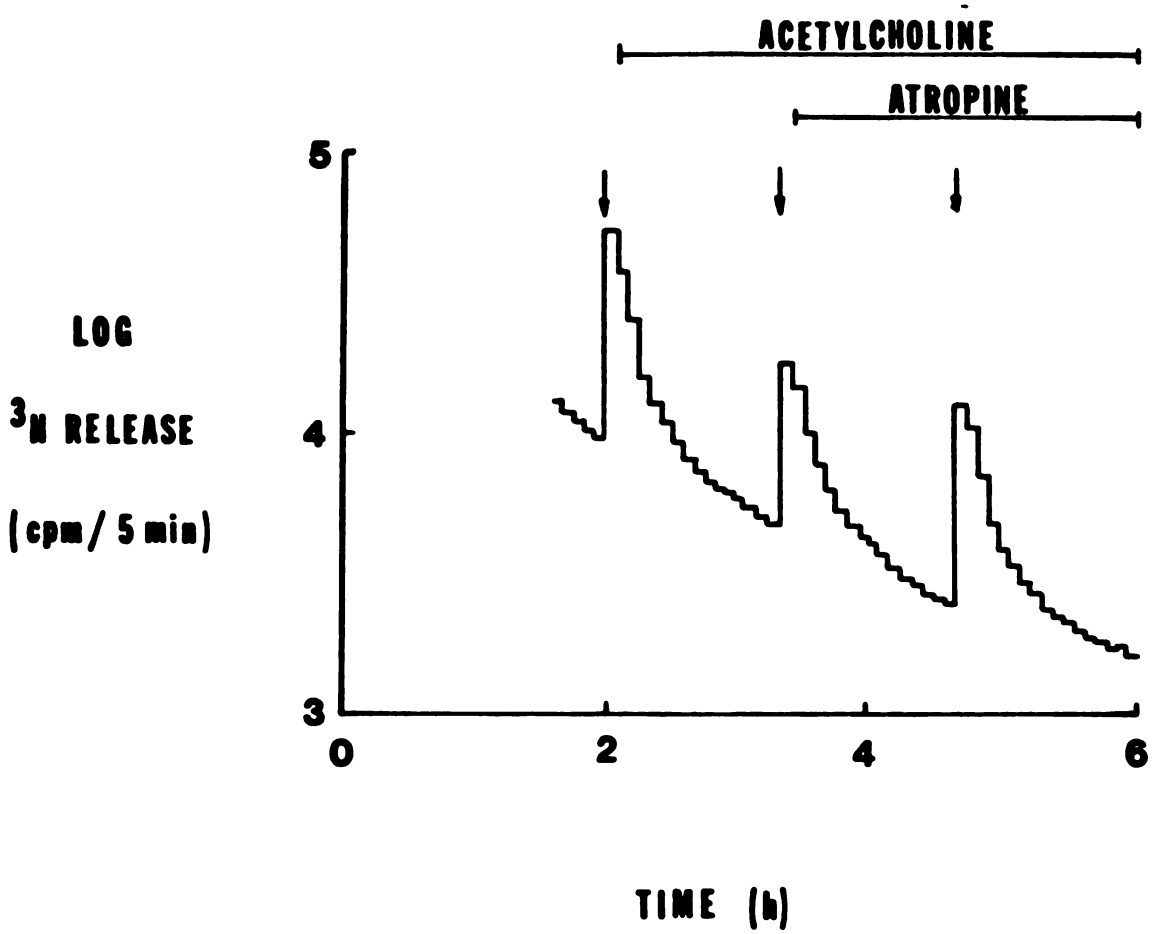

FIG. 5.12. Effects of acetylcholine and atropine (each drug, 10^{-5} M) on the release of ^{3}H evoked by electrical stimulation (arrows) of one tissue.

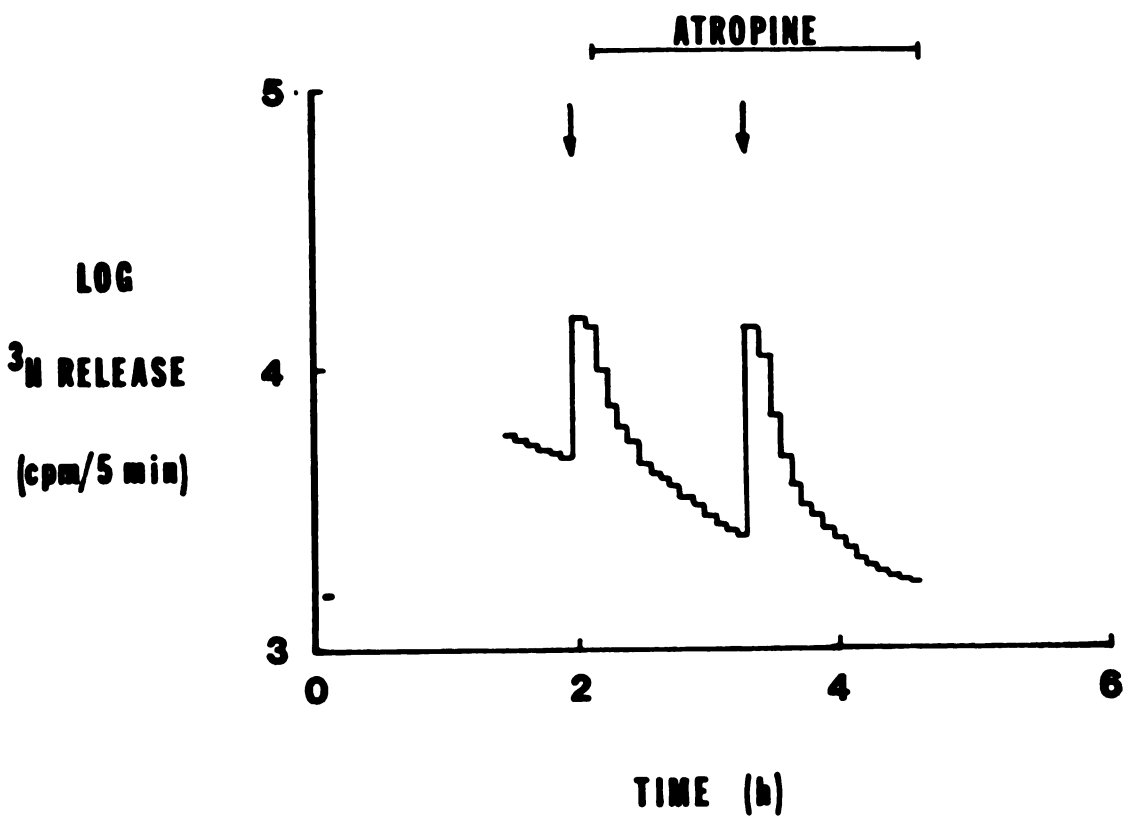

FIG. 5.13. Effect of atropine (10^{-5} M) on release of ^{3}H evoked by electrical stimulation (arrows) of one tissue.

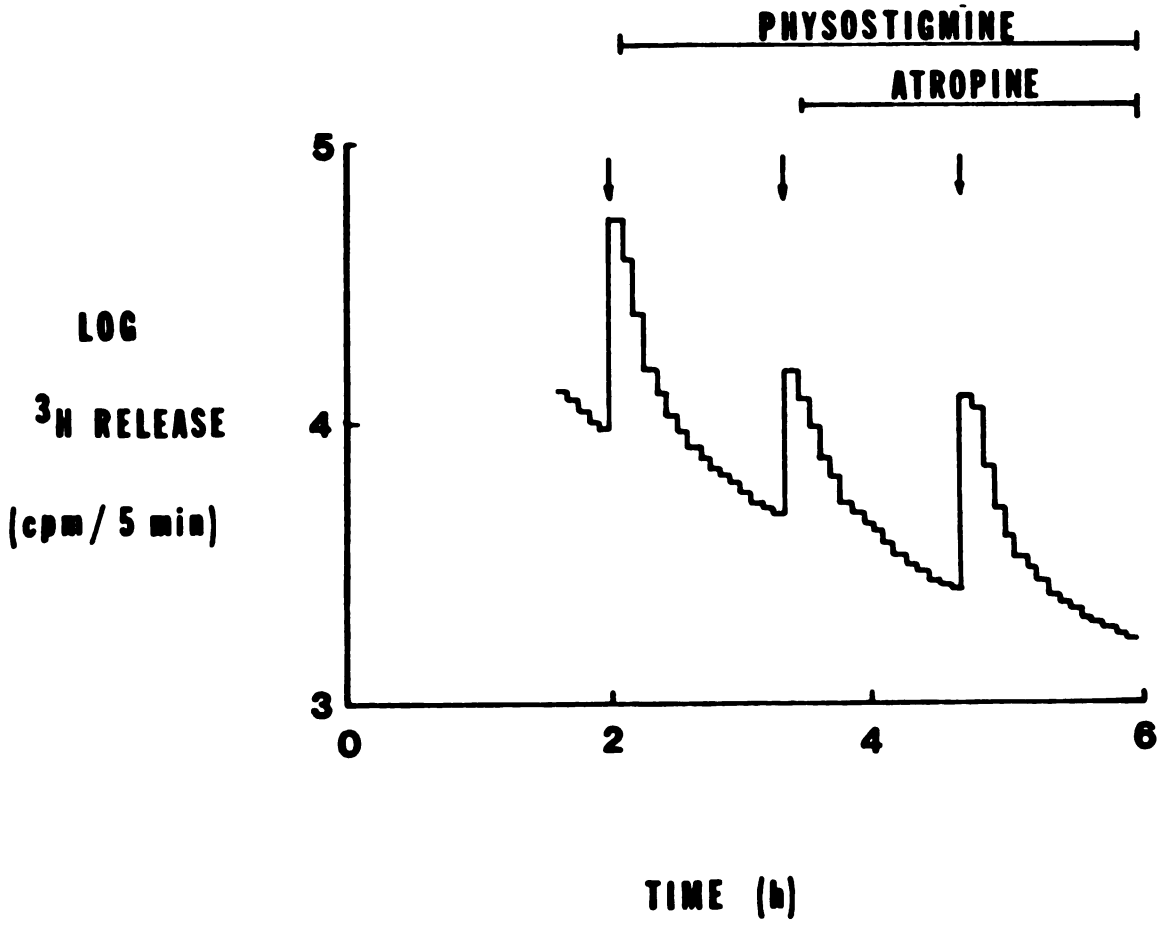

FIG. 5.14. Effects of physostigmine and atropine (each drug, 10^{-5} M) on the release of ^{3}H evoked by electrical stimulation (arrows) of one tissue.

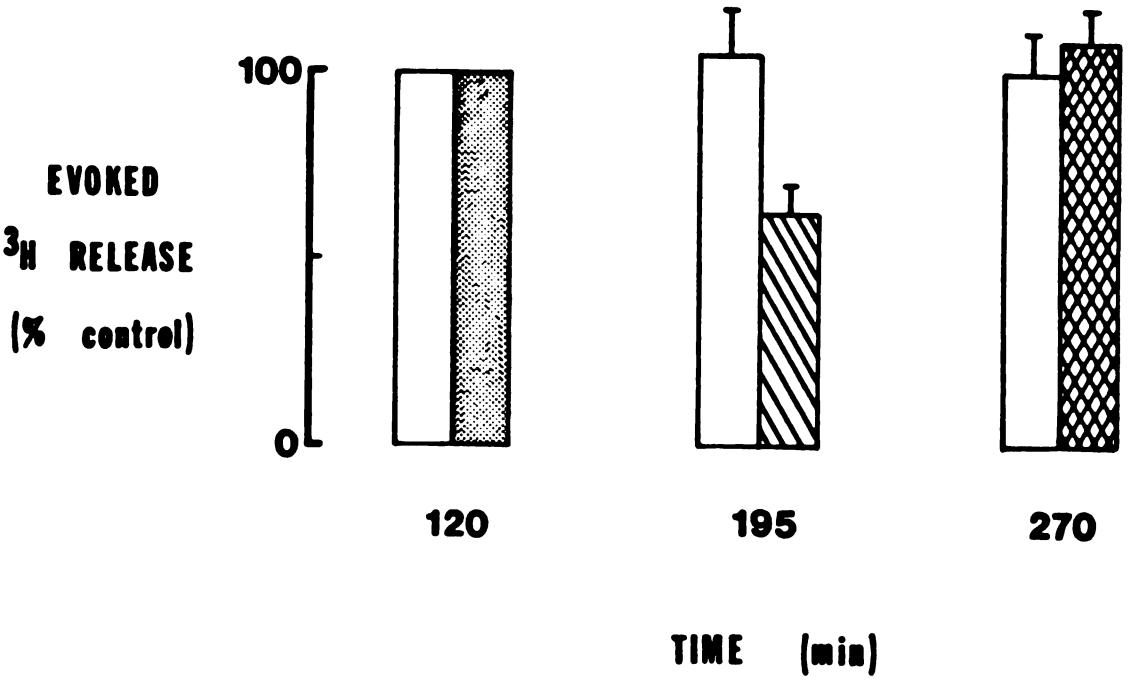

FIG. 5.15. Effects of physostigmine and atropine on the release of ^{3}H evoked by electrical stimulation. One control tissue from each of 13 ferrets was stimulated at the times indicated in the absence of drugs (□). One other tissue from each of 8 of the same ferrets was first stimulated in the absence of drugs (▩), again after incubation with physostigmine (10^{-5} M; 70 min)(▨), and again after incubation with physostigmine plus atropine (10^{-5} M; 70 min)(▩). Data are expressed as mean ± m^2.

DISCUSSION

The purpose of these studies was to determine whether cholinergic nerves interact with adrenergic nerves to tracheal submucosal glands. We found that cholinergic nerves inhibit adrenergic neurotransmission to tracheal glands. We reached this conclusion based on the use of radio-labeled neurotransmitter for measuring changes in adrenergic neurotransmission. We first determined that the anterior portion of the tracheas of ferrets have measurable quantities of norepinephrine. Thus, norepinephrine could be the adrenergic transmitter that mediates secretion of fluid (CHAPTER 3)[20] and glycoproteins (CHAPTER 4), [18, 74]. However, the amounts of norepinephrine in the medium were below the threshold for measurement by high performance liquid chromatography (50 ng/ul) [113]. We therefore used ^{3}H efflux to measure changes in adrenergic neurotransmission. To verify the method, we did a number of experiments to study the characteristics of ^{3}H release evoked by electrical stimulation or neurally active drugs. The findings that tyramine causes ^{3}H release (FIG. 5.8) and that DMI inhibits the uptake of both unlabeled norepinephrine and ^{3}[H]-norepinephrine releasable by electrical stimulation (FIG. 5.9) suggests that ^{3}[H]-norepinephrine labels neural pools [65]. Furthermore, electrical stimulation causes release of ^{3}H via neural mechanisms since the release of ^{3}H is voltage and frequency dependent and tetrodotoxin inhibits most of the release of ^{3}H evoked by electrical stimulation (FIG. 5.8).

Using this method, we obtained evidence that cholinergic nerves inhibit release of ^{3}H. First, exogenous acetylcholine inhibited the

release of ^{3}H evoked by electrical stimulation. Atropine reversed this inhibition, indicating that muscarinic receptors inhibited ^{3}H release (FIG. 5.12). Next, atropine alone increased the evoked ^{3}H release (FIG. 5.13), suggesting that cholinergic nerves may inhibit adrenergic neurotransmission. Finally, potentiating the effects of cholinergic nerve stimulation caused significant inhibition of the evoked ^{3}H release (FIG. 5.15). Since physostigmine does not bind to muscarinic receptors [152], these results strongly suggest that acetylcholine released from nerves inhibits ^{3}H release by activating inhibitory muscarinic mechanisms.

Thus, with progressively greater effects of stimulating cholinergic nerves, there was a progressive decrease in ^{3}H released by electrical stimulation. With the smallest degree of cholinergic effect (after atropine), evoked ^{3}H release was the greatest (121 % of control). Increasing the effect of stimulating cholinergic nerves, by stimulating nerves in the absence of atropine, decreased the release of ^{3}H (100 % of control). By preventing the enzymatic destruction of acetylcholine by incubating tissues with physostigmine, the concentration of acetylcholine locally near the muscarinic receptor sites may have increased. This may have increased the effects of stimulating cholinergic nerves, and thereby further reduced the evoked release of ^{3}H (to 63 % of control). Thus, the cholinergic system is capable of inhibiting about 50 % of adrenergic neurotransmission.

Although we did not directly measure norepinephrine overflow, the release of total ^{3}H reflects adrenergic neurotransmission. First, electrical stimulation causes the rapid efflux of ^{3}H. The first

sample after stimulation was always the highest in ^{3}H, and after stimulation, the release of ^{3}H decreased rapidly. This means that the transmitter washes out or is taken back into the nerves rapidly. The half-time for this process is approximately 2 min, which probably represents a diffusion-limited process by which small molecules wash out of tissues of this thickness (1 mm) [31]. Additionally, the intercept parameter of the rapid exponential process is 3.28 times higher than the sum of the other intercept parameters (TABLE 5.2). Thus the pool size for the rapid process is the highest, and accounts for ≈ 70 % of the evoked response under control conditions. The rapid time course of release and removal of the neurotransmitter is consistent with nerve mediated effects. Because tetrodotoxin and desmethylimipramine affect primarily the most rapid exponential process, and the evoked responses are very small (FIGS. 5.8, 5.10), most of the ^{3}H released by electrical stimulation comes from nerves.

However, some of the ^{3}H released by electrical stimulation may come from extraneuronal sites. Tetrodotoxin at concentrations that prevent smooth muscle contraction [207] and secretion of both fluid (CHAPTER 3)[21] and $^{35}SO_4$-labeled macromolecules (CHAPTER 4)[18], does not completely prevent the release of ^{3}H evoked by electrical stimulation (FIG. 5.11) [207]. ^{3}H may be released by electrotonic depolarization of the nerve terminal by the electric field even though action potentials are abolished at this concentration of tetrodotoxin [157]. However, if this ^{3}H represents norepinephrine, then the quantity of transmitter released must be insufficient to cause secretory effects in the glands.

Twenty minutes after stimulation, the rapid process contributes little to total ^{3}H release because the transmitter either washed out of the tissue, was taken back into nerve terminals by the DMI sensitive catecholamine uptake mechanism, or was bound to extraneuronal sites (FIG. 5.16). Some of this ^{3}H may then be released from the tissue more slowly, with a half-time of approximately 20 min, and accounts for about approximately 22 % of the total evoked response. The slowest exponential process contributes approximately 8 % to total ^{3}H release, most to ^{3}H release after 2 h, and because it is insensitive to DMI, it probably represents non-neuronal baseline release [65]. Alternatively, some ^{3}H may not reflect norepinephrine, but instead, the release of a metabolite from the nerve, and thus may not reflect neurotransmision [67, 240].

Thus, during both baseline and electrical stimulation, some non-neuronal ^{3}H may be recovered, but because baseline release accounts for only a small proportion of the total ^{3}H released after stimulation ($\simeq$ 8 %), changes in the non-neuronal pools could not account for the magnitude of the changes in adrenergic transmission caused by cholinergic nerves. In spite of the uncertainties, we conclude that exponential analysis of ^{3}H release after electrical stimulation is useful in seeing whether neural interaction can regulate adrenergic nerve function.

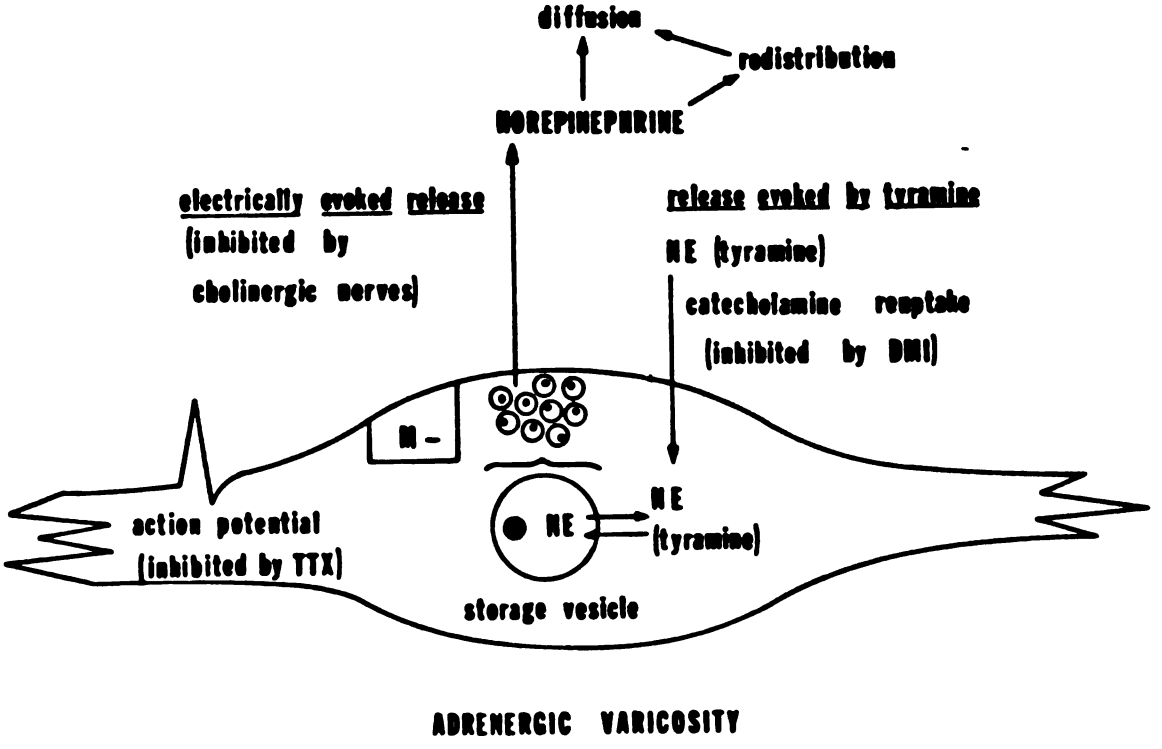

FIG. 5.16. Diagram of ^{3}H release evoked by electrical stimulation or tyramine. Action potentials stimulate release of ^{3}H from adrenergic nerve. Most of the released ^{3}H diffuses out into the bathing medium, and is lost to the tissue. Some of the ^{3}H is taken back up into the nerve via the catecholamine pump. Tyramine also is taken into nerves, where it displaces ^{3}[H]-norepinephrine from sites in the nerve. After release, some ^{3}H may be redistributed to extraneuronal sites (e.g., tissue binding) and is then released slowly. Muscarinic receptors (M^{-}) inhibit the release of ^{3}H evoked by electrical stimulation.

Some of our findings are similar to those of others. The amounts of norepinephrine in human and canine bronchi are 752 and 350 ng/mg, respectively (Vermiere, personal communication). These values are somewhat larger than for ferrets (131 ng/mg). However, using radioenzymatic assay techniques, Duckles and Vermiere each found that electrical field stimulation in vitro increased the amount of norepinephrine released into the bathing medium in a frequency-dependent fashion [51] (Vermiere, personal communication). These amounts released were below the threshold for measurement by high performance liquid chromatography (Nelson, personal communication).

Studies measuring ^{3}H efflux from a variety of other adrenergically innervated tissues also found that cholinergic nerves inhibit adrenergic neurotransmission. Many of these studies also showed parallelism between adrenergic nerve function and ^{3}H release. After incubating tissues with ^{3}[H]-norepinephrine, the overflow of total ^{3}H parallels overflow of ^{3}[H]-norepinephrine and muscle contraction [194, 209, 219, 239]. Also, acetylcholine decreased the release of both ^{3}H and ^{3}[H]-norepinephrine and inhibited muscle contraction evoked by electrical stimulation, and atropine reversed the inhibition of each in parallel [194, 209, 237, 239]. Furthermore, tyramine causes secretion of fluid (CHAPTER 3) and the release of ^{3}H suggesting that the ^{3}H was released from adrenergic nerves, as it is from nerves to the heart [127] and saphenous veins [209, 239]. Similar results were subsequently found in airway smooth muscle [194]. The finding that muscarinic receptors inhibit adrenergic neurotransmission were also shown for adrenergic nerves to airway

smooth muscle [194] and pulmonary artery [238]. In addition, we found that stimulation of cholinergic nerves inhibits the release of ^{3}H from adrenergic nerves to tracheal glands (FIGS. 5.12 - 5.15). Similarly, stimulating the vagus nerve to the heart decreases the overflow of endogenous norepinephrine evoked by stimulating sympathetic nerves [128, 130, 138]. The finding that atropine by itself increased the evoked release of ^{3}H (FIG. 5.13) suggests that in the absence of drugs, cholinergic nerves inhibit adrenergic neurotransmission. This does not mean that in vivo, there is tonic inhibition. Parasympathetic and sympathetic mechanisms are regulated separately in vivo, whereas in vitro, all nerve types are stimulated at the same frequency. Although the range of the inhibition is ≈ 50 % at the matched frequencies, the inhibition may be most effective when cholinergic frequency is high and adrenergic frequency is low.

The roles of the inhibition are not known, but there are differences in the secretions produced by adrenergic and cholinergic mechanisms (see CHAPTER 6). Additionally, adrenergic mechanisms may regulate blood flow to glands. If this is correct in vivo, then stimulating cholinergic mechanisms may help prevent vasoconstriction caused by sympathetic mechanisms (see CHAPTER 6).

SUMMARY

The aims of these studies were to determine whether autonomic nerves regulate the secretion of mucus from airway submucosal glands. These studies have demonstrated that several different types of nerves regulate secretions from airways (FIG. 6.1). Cholinergic nerves, acting via muscarinic mechanisms, mediate secretion of both fluid and macromolecules from the trachea. Adrenergic nerves cause the release of fluid via alpha-adrenergic mechanisms and act via both alpha- and beta-adrenergic mechanisms to release $^{35}SO_4$-labeled macromolecules. Additionally, there is evidence that nonadrenergic noncholinergic nerves mediate the release of macromolecules but not of fluid. The balance between these different mechanisms may be important in modulating the physical properties of mucus. In addition to neural effects that directly cause secretion, neural interaction may play a significant role in the regulation of secretion. During electrical stimulation of both nerve types, cholinergic nerves inhibit, via muscarinic mechanisms, the release of norepinephrine from adrenergic nerves. This mechanism may be responsible for maintaining blood flow to glands or increasing the specificity of the cholinergic response.

CHOLINERGIC NERVES

1. AIRWAY GLANDS

Cholinergic nerves are potent mediators of secretion of both fluid and macromolecules from the airways. The quality of the mucus secreted in response to stimulating cholinergic nerves is similar to that of mucus secreted at rest. Although the volume of fluid is high after stimulation, the protein concentration [234] and the viscosity and elasticity (Leikauf, personal communication) are similar to those of resting secretions. Cholinergic vagal mechanisms are important in mediating reflex responses to mechanical stimulation of the larynx [82], inhaled SO_2 [95], or hypoxia [44]. Because cooling the vagi of cats decreased the resting flow rate [233], muscarinic neural mechanisms might be important in regulating both resting and evoked secretion. However, cholinergic mechanisms are not the only ones responsible for maintaining secretion at rest because atropine does not abolish resting secretion (CHAPTER 4) [74, 82, 233].

2. OTHER EXOCRINE GLANDS

Cholinergic nerves are important regulators of many other exocrine glands. Mechanical stimulation of the teeth or stomach causes salivation via cholinergic reflex mechanisms [97, 101, 204]. Cholinergic agonists cause release of amylase and K^+ from isolated parotid gland cells of rats [29, 133]. The increases in K^+ permeability and efflux are responsible for hyperpolarization of the secretory cells [72] after cholinergic stimulation. The role of the

hyperpolarization is unknown, but it is possible that the electrochemical gradient for Ca^{++} is increased. Cholinergic stimulation also increases the intracellular concentration of C-GMP and not C-AMP in rat parotid glands [29].

Cholinergic mechanisms also mediate secretion from submaxillary [27] and submandibular glands of rats [183, 184] and cats [124]. Cholinergic stimulation causes only a small amount of glycoprotein secretion from submandibular glands of rats, whereas beta-adrenergic mechanisms are potent secretagogues [184].

Sweat glands are also under cholinergic control [35, 43, 185]. Increased hypothalamic temperature activates sympathetic nerves to cause vasodilation in the skin and sweating. Sweating is also stimulated by injected acetylcholine [185]. Because the reflex sweating and the response to acetylcholine are inhibited by atropine, the reflex is mediated by cholinergic nerves via muscarinic mechanisms [35, 68]. Denervation results in supersensitivity to injected methacholine [156, 196], suggesting that cholinergic motor activitgy to the glands normally exists. The response to cholinergic agonists requires the presence of extracellular Ca^{++} [200].

Stimulating the vagus nerve to the pancreas of cats causes secretion of fluid and HCO_3^- [64]. Acetylcholine causes pancreatic acinar cells to depolarize and release amylase [39, 125, 140, 174]. Because radiolabeled atropine specifically binds to acinar cells [76], and the secretory effects are inhibited by atropine [39], muscarinic mechanisms mediate these secretion. Spontaneous minature end plate potentials occur, and are associated with decreases in membrane

resistance, effects mimicked by acetylcholine. These findings suggest strongly that acetylcholine released from cholinergic nerves mediates pancreatic secretion [163].

ADRENERGIC NERVES

1. AIRWAY GLANDS

The significance of adrenergic innervation to airway glands is not well understood. Adrenergic mechanisms cause secretion of mucus, and thus, are not diametrically opposed to cholinergic effects as is the case in the smooth muscle of the airway (e.g., beta-adrenergic bronchodilation vs. muscarinic bronchoconstriction) [240]. However, there are differences in the quantity and quality of mucus secreted in response to stimulation of different autonomic mechanisms. The quantity of mucus reflects the fluid components because solutes account for only 5 % of the total mucus. Although beta-adrenergic stimulation does not cause much secretion of fluid, the mucus recovered has more protein [234] and higher viscosity (Leikauf, personal communication) than mucus recovered during baseline, alpha-adrenergic, or muscarinic stimulation. Conversely, alpha-adrenergic stimulation causes secretion of mucus with a great deal more fluid and less protein than mucus recovered during baseline secretion or during either cholinergic or beta-adrenergic stimulation. Also, alpha-adrenergic mechanisms cause the release of more lysozyme [228] and a 50,000 dalton protein [84] than do beta-adrenergic mechanisms.

How these differences in the composition of the secretions influence clearance are not well understood. For optimum clearance,

the mucus layer should be a weak gel [145]. The viscoelasticity of a weak gel is due in part to the formation of intermolecular disulfide bonds between cysteine residues in the exposed protein cores, and in part to electrostatic attraction between charged groups in the carbohydrate side chains [145]. Increased cross-linking increases the viscosity of the layer, which opposes deformation of the gel by ciliary beating. Deformation of the gel is somewhat similar to stretching a spring. When deformed, the gel tends to return to its original configuration. However, the viscosity of the layer tends to resist flow back to its original configuration [145]. Thus, energy can be stored by the gel and released slowly, effectively transducing the energy from periodic strokes of the cilia into a smooth continuous flow. Glycoproteins are therefore important determinants of mucus viscosity [129]. Because the weak gel state occurs within a narrow band of glycoprotein concentration [145], viscosity and elasticity can be significantly altered by changing the glycoprotein concentration. The most viscous mucus has the highest protein concentration (Ueki, personal communication), and is secreted in response to beta-adrenergic stimulation, whereas the least viscous mucus may be secreted in response to alpha-adrenergic stimulation (Leikauf, personal communication). The finding that the two adrenergic mechanisms produce mucus with the most different viscoelastic and chemical properties is of uncertain significance because the adrenergic neurotransmitter is probably norepinephrine, which has mixed alpha- and beta-adrenergic effects (CHAPTER 4). Unless there is another adrenergic neurotransmitter with different specificity for alpha- and beta-adrenergic receptors, neural mechanisms may not

regulate the alpha- and beta-adrenergic mechanisms separately. Therefore, the net effect of adrenergic stimulation must depend on the distribution of alpha- and beta-adrenergic receptors on the secretory cells and the function of the individual cells.

There is some difference between alpha- and beta-adrenergic receptor mechanisms on different cell types. Stimulating alpha-adrenergic mechanisms causes degranulation, the formation of watery vacuoles [9], and loss of immunocytochemical staining for lysozyme [228] of serous but not mucous cells. Conversely, stimulating beta-adrenergic mechanisms causes degranulation of mucus cells but not serous cells [79]. However, beta-adrenergic receptors appear to be on serous cells, because isoproterenol increases the intracellular content of C-AMP in serous cells as well as mucous cells (Lazarus, personal communication). Beta-adrenergic functions may be regulated via circulating epinephrine released from the adrenal medulla, which has potent beta-adrenergic effects. In contrast, norepinephrine is a potent alpha-adrenergic agonist (TABLE 4.1). Therefore, the balance between the release of local norepinephrine versus systemic epinephrine may determine the degree to which the alpha- and beta-adrenergic secretory mechanisms are activated.

In addition to its effects fluid and macromolecule secretion, adrenergic nerves may be important in the regulation of blood flow to the glands. Angiography showed that in addition to its secretagogue effects, phenylephrine constricts the arteries to canine submucosal glands [46]. Because systemic blood pressure did not change during the experiment, blood flow to the glands must have decreased. Thus,

under control conditions, the entire dose of injected phenylephrine may not have reached the glands, and therefore, the response was smaller under control conditions. This conclusion is corroborated by the finding that if papavarine, a direct acting vasodilator, is infused prior to phenylephrine, the arteries do not constrict, and the secretory response to phenylephrine is significantly increased [47]. Alternatively, the decreased blood flow to the glands may not have allowed sufficient fluid for maximal secretion to occur. Because phenylephrine decreased the diameter of the arteries to glands, alpha-adrenergic receptors are probably involved. Because the adrenergic nerves in the submucosa are not specifically associated with the gland cells [151], it is possible that norepinephrine released by these nerves may also act on the blood vessels nearby. Vasoconstrictor effects of stimulating nerves may be partially overcome by increased levels of circulating epienphrine released by reflex stimulation of sympathetic nerves.

The significance of cholinergic inhibition of adrenergic neurotransmission in the airway is not well understood. The composition of the mucus secreted in response to stimulating each of the different neural mechanisms is probably different. If this is true, then presynaptic inhibition may increase the specificity of the secretory stimulus, and thus, cause a more specific cholinergic response. Additionally, because stimulation of adrenergic nerves might decrease blood flow to glands, it is likely that cholinergic inhibition of norepinephrine release partially counteracts adrenergic vasoconstrictor effects. This inhibition would then permit sufficient

blood flow to allow the high rates of fluid secretion stimulated by cholinergic mechanisms.

2. OTHER EXOCRINE GLANDS

Adrenergic nerves also have secretory effects on other glands. Tasting sucrose or carrot juice stimulates release of amylase by activating sympathetic reflexes [90]. Direct stimulation of the sympathetic nerves to the parotid glands of cats caused salivation, but the flow rates were not as high as for stimulating the parasympathetic nerves [78, 89]. Although the flow rate is low, the concentration of amylase is high [78]. The adrenergic response is mediated via both alpha- and beta-adrenergic mechanisms. Alpha-adrenergic stimulation releases K^+ [134] and amylase, and is associated with a rise in the cellular content of 3'-5' cyclic-GMP [30]. In contrast, beta-adrenergic mechanisms are potent stimulators of protein secretion [11, 17, 134, 254], and are associated with increases in the intracellular concentration of C-AMP [11, 30]. In addition to effects directly on acinar cells, stimulation of alpha-adrenergic mechanisms increases the hydrostatic pressure in the ducts of parotid glands [61, 225]. This effect is due in part to contraction of the myoepithelial cells decreasing the size of the acini and forcing the secreted fluid into the secretory duct [77].

There are differences in the composition of the saliva produced by stimulating different secretory mechanisms. Stimulating sympathetic nerves to parotid glands of rats *in vivo* causes relatively less fluid secretion than does stimulating parasympathetic nerves

[78]. During continuous stimulation of parasympathetic nerves, superimposed sympathetic stimulation results in decreased secretion of fluid from parotid glands of dogs [61]. Stimulating sympathetic nerves of cats produces submaxillary saliva whose glycoproteins had higher neuraminic acid/fucose and neuraminic acid/hexosamines ratios than the saliva produced by parasympathetic stimulation These differences in chemical composition may be responsible for the lower sedementation rates of the glycoproteins released with sympathetic stimulation [50]. The higher relative content of neuraminic acid after sympathetic stimulation may result in the secretion of glycoproteins with more charged carbohydrate groups which may attract charged groups on other glycoprotein molecules. Thus, the secretions produced by sympathetic stimulation may form large aggregates of glycoproteins that may not sediment rapidly.

In addition to secretory effects, stimulation of sympathetic nerves decreases blood flow during parasympathetic stimulation [61]. The decreased blood flow may be responsible for the decrease in salivation with superimposed sympathetic stimulation in vivo [60]. The quantity of protein secreted is comparable during parasympathetic or sympathetic stimulation [78], but because stimulating sympathetic mechanisms produces less fluid [61, 72, 78], the protein concentration in the saliva is higher.

Adrenergic regulation of secretion is similar in submandibular glands, salivary glands with numerous mucous cells that secrete glycoproteins. Alpha-adrenergic agonists stimulate the release of K^+ from isolated acinar cells [183]. However, alpha-adrenergic

stimulation results in relatively little glycoprotein secretion [182]. In contrast, beta-adrenergic agonists are relatively weak stimulators of K^+ release, but are potent secretagogues of glycoproteins. The secretion of glycoproteins evoked by beta-adrenergic stimulation is associated with an increase in intracellular C-AMP [182, 183].

Eccrine sweat glands are regulated locally by adrenergic nerves, and systemically via the adrenal glands. The major role of adrenergic regulation is to mediate sweat responses to exercise, and to potentiate heat-induced sweating during exercise. Catecholamine fluoresence around the secretory coil suggests that adrenergic nerves are present [235], and denervation produces supersensitivity to epinephrine [35]. Epinephrine injected into the skin produces secretion at lower rates than after acetylcholine [198]. Because the adrenergic response was inhibited by the alpha-adrenergic antagonist dibenamine [96], the response is mediated by alpha-adrenergic mechanisms. However, beta-adrenergic mechanisms also mediate some responses to epinephrine [211, 244, 245]. Because denervation of the adrenal glands reduced the sweat response to exercise and the potentiation of the heat-induced response, some of the adrenergic regulation during exercise is mediated by circulating catecholamines [193]. Although there are few studies of the interaction between adrenergic and cholinergic mechanisms, reducing blood flow to the skin reduces the volume of sweat secreted during cholinergic stimulation [199].

NONADRENERGIC-NONCHOLINERGIC NERVES

1. AIRWAY GLANDS

The roles of nonadrenergic-noncholinergic nerves in airway secretion are not well known, but on the basis of these experiments using electrical stimulation, these nerves may regulate mucin secretion independently from fluid secretion (CHAPTERS 3,4). The nonadrenergic-noncholinergic response is similar to the beta-adrenergic response in that stimulation of each mechanism causes the release of $^{35}SO_4$- labeled materials without causing significant secretion of fluid. A possible transmitter for these effects is VIP, which is found in airway ganglia and in nerves to glands [8, 231], and mediates effects of stimulating nonadrenergic mechanisms is smooth muscle [139]. Like the beta-adrenergic agonists terbutaline and isoproterenol, VIP increases secretion of $^{35}SO_4$- labeled materials [172] (work in progress), and increases the intracellular content of 3'5'-cyclic AMP in both serous and mucous cells (work in progress). Additionally, VIP and beta agonists each stimulate secretion of Cl^- towards the airway lumen [158] and increases the immunocytochemical staining for C-AMP in the epithelial ciliated cells (Lazarus, personal communication) that are responsible for ion transport and fluid secretion [252].

There is suggestive evidence that VIP may also modulate neurotransmission in the airways. Because VIP is present in the same nerve bundles as are cholinergic and adrenergic nerves [8], VIP is in the right place to interact with these other nerve types. Although VIP inhibits the release of norepinephrine evoked by electrical field

stimulation of smooth muscle (Vanhoutte, personal communication), it is not known whether VIP inhibits the evoked release of norepinephrine from adrenergic nerves of ferrets. Additionally, VIP is present in nerve terminals that are close to airway ganglia [8], suggesting that VIP may stimulate ganglia, modulate cholinergic transmission, or inhibit adrenergic transmission in ganglia. If VIP mediates some effects of stimulating nerves, then VIP should be released by nerve stimulation, and VIP-antibodies or antagonists should inhibit the effects. Because VIP has long lasting effects [172], it may help regulate baseline secretion or provide sustained stimulation of synthesis. It is also possible that other gastrointestinal hormones or peptides (e.g., cholecystokinin, gastrin) may be responsible for some effects of nerve stimulation. However, there have been no studies of either the effects of any of these other peptides on airway secretions, or any anatomic studies to determine their presence.

Another possible role for nonadrenergic noncholinergic nerves may be as part of a local airway reflex that mediates secretion. Stimulation of afferent nerves in the airways may release VIP or some other peptide (e.g., substance P) near secretomotor ganglion cells, stimulating them to fire action potentials, and thus causing secretion from glands. It is also possible that peptides mediate axon reflexes in the airway as in the skin [100]. These possible mechanisms may mediate responses to local irritation of the airways without invoking the central nervous system. This local response may be an efficient way of trapping inhaled particles. By mechanical or chemical stimulation of the local area, a particle might evoke the secretion of

a highly viscous mucus nearby to trap the particle without causing widespread secretion of mucus. This might help to conserve glycoproteins and also help prevent mucus plugging of the airways. There is precedent for local reflexes in the G.I. tract. Mechanically stimulating the mucosa of the denervated small intestine causes local vasodilation [55] that is not inhibited by either adrenergic or cholinergic antagonists [14]. Because VIP is released into the venous blood during local stimulation, VIP is possibly the neurotransmitter [55, 63].

2. OTHER EXOCRINE GLANDS

Infusion of VIP into the arterial circulation perfusing isolated parotid glands does not cause salivation [131], but its effects on protein or glycoprotein secretion have not been well studied. VIP nerves may, however, regulate of blood flow. Stimulation of the parasympathetic nerves to parotid glands of cats increased the flow rate of saliva and increased blood flow in the absence of changes in systemic blood pressure. Atropine failed to inhibit all of this vasodilation, whereas the additional infusion of VIP anti-serum did, suggesting that a role of VIP nerves in salivation is to maintain blood flow during secretion [131].

VIP-containing nerves are found in the vagus nerve to the pancreas of cats [64, 220]. Stimulation of the vagus causes the release of fluid rich in HCO_3^-. Although some of the secretory response is inhibited by atropine, it is not abolished. The remaining secretion is associated with the release of VIP into the pancreatic

venous blood [64]. Both the VIP release and the secretion induced by vagal stimulation was inhibited by infusion of somatostatin, but the response to exogenous VIP was unchanged [64], suggesting that the effect of somatostatin was to decrease the release of endogenous VIP.

VIP binds to specific receptors on acinar cells to increase C-AMP and stimulate secretion of enzymes [76]. Because radio-labeled VIP and secretin compete for the same saturable binding site, they apparently share a common receptor [76]. Additionally, cholecystokinin and gastrin share a common receptor that increases C-AMP and stimulates the release of amylase [42]. Substance P also has a specific receptor that mediates increases in Ca^{++} efflux and enzyme release [76].

In one study of the mucous Brunner's glands of the small intestine of rats, VIP-containing nerves were found near the gland acini, and exogenous VIP caused the release of mucous glycoproteins, proteins, and HCO_3 into the intestinal lumen [111]. VIP nerves also stimulate the transport of Cl^- across the ileum. VIP is present in the longitudinal muscle and is released by electrical field stimulation. Both the release of VIP and the increased secretion of Cl^- were abolished by tetrodotoxin. Removing the circular and longitudinal muscle layers decreased the amount of VIP in the tissues, and inhibited the release of VIP into the serosal medium [73].

All of these results suggest strongly that VIP nerves mediate secretion from a variety of glands. The dominant effect of stimulating these nerves is the release of proteins, and not of fluid or electrolytes. Additionally, VIP stimulates the accumulation of C-

AMP in glands that respond to VIP. In most of the glands described, the effects of stimulating VIP mechanisms is strikingly similar to stimulating beta-adrenergic mechanisms. Thus, peptidergic or VIP nerves in the airway are likely to regulate glycoprotein secretion separately from fluid secretion.

3. SMOOTH MUSCLE

Noncholinergic nonadrenergic nerves also mediate relaxation of several smooth muscles. Electrical field stimulation of airway smooth muscle causes contraction followed by relaxation. Some of the relaxation is inhibited by cholinergic or beta-adrenergic antagonists, but a substantial proportion of the relaxation remains [190]. Similarly, electrical field stimulation of the lower esophageal sphincter [92] or airway [139] causes relaxation. These effects may be mediated by VIP because VIP antibodies inhibit a substantial proportion of the responses [92, 139].

NONSECRETORY EFFECTS OF NERVES

1. MUCOCILIARY CLEARANCE

Clearance is a complex process requiring coordinated ciliary activity and the presence of mucus macromolecules that crosslink to form a weak gel [144, 145, 195]. The most efficient clearance is acheived by cilia beating with high frequency [86] propelling mucus with low elasticity [88] and viscosity [53]. The viscosity of mucus is determined to a large degree by the relative contents of water [37, 52] and glycoproteins [129, 137] in it. If the concentration of

glycoprotein molecules in the fluid is too low, they do not form sufficient numbers of cross-links to create a weak gel, and therefore the layer does not have enough elasticity to be transported [145]. With concentrations of glycoproteins higher than needed to form a weak gel, the mucus becomes more viscous and elastic [129, 145]. The pH of the mucus is also important in determining the viscosity. Decreasing the pH of the mucus from the optimum of 8 units [80] to 6.0 increases cross linking of macromolecules, which may increase the viscosity [37, 129].

The roles of nerves in regulating the entire process are not well understood. Denervation of the lung either by hilar stripping [26] or by autotransplantation [54] decreased clearance. The mechanisms by which the decrease occur are not well known, but both cholinergic and adrenergic antagonists affect clearance.

Cholinergic mechanisms can regulate ciliary beating, mucus secretion, and net clearance. Acetylcholine, physostigmine [115, 242], and pilocarpine [143] each increase the frequency of ciliary beating. Methacholine in low doses increases the secretion of mucus with low viscosity, but higher doses increases viscosity and decreases ciliary transport [110]. Because atropine decreased baseline beating frequency and prevented the increase due to of acetylcholine, muscarinic mechanisms were involved in regulating ciliary activity [115]. Thus, the effects of cholinergic nerves on ciliary activity parallel those on secretion. Stimulating cholinergic mechanisms could increase clearance above baseline by increasing the beat frequency and the amount of mucus in the airway. Up to a point, increasing the

supply of mucus should be beneficial. As mucus is swept towards the mouth at higher rates, it needs to be replaced at the same rate in order for a steady-state to be maintained.

Inhibiting cholinergic effect by denervation [26, 54] or atropine decreases clearance rates in dogs [87]. Similarly in humans, atropine decreases clearance [71, 169, 257]. This may be because atropine may have decreased ciliary activity and transportability of mucus. Atropine increases the viscosity of mucus when viscosity is low [109] and thereby decreases the transportability of the mucus [110]. However, it is likely that if clearance were low because of an excess of mucus, then increasing the mucus load may actually decrease clearance even more. Therefore, depending on the circumstances, stimulation of cholinergic mechanisms may either increase or decrease clearance.

Adrenergic mechanisms can also regulate ciliary activity and clearance. Beta-adrenergic agonists increase the frequency [142, 236] and amplitude [142] of ciliary beating. These effects may increase [71, 257], or have no effect on [197, 255] clearance in normal humans. Beta-adrenergic agonists increase clearance in patients with chronic bronchitis [197] or cystic fibrosis [255].

However, the mechanisms for the changes are not known. Beta-adrenergic agonists decrease blood pressure and thus stimulate cardiovascular reflexes to increase heart rate and secretion of mucus [19]. Because the effect is mimicked by the direct-acting vasodilator nitroprusside (Borson, unpublished data), the secretion may be due to activation of cardiopulmonary reflexes and not necessarily due to

beta-adrenergic stimulation of gland cells by terbutaline. It is possible that similar cardiovascular reflexes may affect clearance. In addition to its effects on cilia, epinephrine may also increase the viscosity of secreted mucus [142], an effect that may impair clearance [53]. The beta-adrenergic effect, which increases viscosity, is consistent with the weak effects of beta-adrenergic agonists in causing fluid secretion (CHAPTER 3). Thus, stimulation of beta-adrenergic mechanisms may either increase or decrease clearance, depending on the relative effects on ciliary activity and secretion.

The effects of alpha-adrenergic agonists on clearance are not known, but if it is true that optimum clearance requires mucus of low viscosity, then stimulating the secretion of watery mucus may increase clearance if clearance is reduced by too much sticky mucus. It is difficult to interpret clearance studies *in vivo*, because of the multiple effects on cilia, secretion, and bronchomotor tone.

Stimulation of potent reflexes also causes coughing. For cough to be most effective, enough mucus must be on the airway to allow high linear velocities of airflow to shear the mucus off the airway. The quality and quantity of mucus that promotes effective cough is is unknown, but without sufficient volume, waves may not be set up in the mucus layer, and thus cannot be sheared off.

2. NEURAL REGULATION OF GLAND SIZE

In addition to effects on secretion and clearance, cholinergic and adrenergic nerves also probably modulate growth responses of airway glands. Many cholinergic reflexes that are known to cause

secretion from airways. The chronic stimulation of these reflexes may lead to hypertrophy of secretory cells. Chronic stimulation of the airways by cigaret smoke increases the number of goblet cells [120, 141, 165], and increases the size of the airway glands [99, 141, 149]. Chronic inhalation of sulfur dioxide has similar effects [4, 34, 141]. The increasing numbers of goblet cells may be due to increased mitotic index [119]. The increase in gland mass is associated with increased presence of acidic glycoproteins [99, 141]. The increase in cellular stores of mucus of the glycosyltransferase enzymes that are important in the addition of sugar groups onto glycoproteins [4]. In some cases, chronic stimulation increases the amount of mucus in the airway lumen [34, 188], but it is not known whether this reflects increased secretion or decreased clearance rates. In addition to reflex effects of inhaled irritants, both smoke and SO_2 exert direct effects on the airway epithelium that complicate the interpretation of these studies.

To overcome this problem, investigators attempted to mimic the reflex effects by chronically exposing animals to the known neurotransmitters of acute reflexes. Chronic injection of cholinergic agonists causes hypertrophy of glands [5, 112, 217] and increases the activity of the glycosyltransferases in them [5]. As with cholinergic agonists, beta-adrenergic agonists caused growth of the glands of pigs [10]. Similarly in ferrets, isoproterenol injection for 5 d increased the volume of glands by increasing the proportion of each gland occupied by mucous cells, and by increasing the total volume of the mucous granules [229]. Because beta-adrenergic agonists cause depletion of mucous cells of ferrets [79], it is apparent that after

chronic injection, either synthesis is enhanced or the secretion mechanisms are inhibited, causing the cells to remain full of secretory materials and thus enlarging. A possible mechanism for the decreased secretion is down regulation of the beta-adrenergic receptors that mediate secretion of macromolecules (CHAPTER 4).

However, the mechanisms of hypertrophy in airways is not known. Both cholinergic and adrenergic agonists stimulate cardiovascular reflexes that mediate secretion. It is therefore necessary to stimulate the airway only locally, without causing systemic effects. Chronic stimulation by any of these methods produces conditions that are similar to spontaneous chronic bronchitis in pigs [106] or humans [187, 222]. It may be that these diseases are the result of chronic stimulation of irritant or hypoxic reflexes.

Parasympathetic and sympathetic nerves regulate the growth responses of salivary glands [248]. Chronic reflex stimulation, by feeding solid pellets [251] or proteolytic enzymes [249], or by cutting the incisor teeth [210, 248] caused glands of rats to enlarge. The increased size is associated with increased DNA synthesis and mitosis. Changing to a liquid diet reverses the hypertrophy and the increased DNA and protein synthesis [201]. Because cutting the parasympathetic nerves prevented the increased mitosis and caused atrophy [205, 206], and depleted the cells of amylase [204], the regulation of both cell number and synthesis of protein is under cholinergic regulation.

The sympathetic nervous system is also important in mediating hypertrophic responses. Cutting the sympathetic post-ganglionic

nerves to the glands prevented the hypertrophy due to incisor amputation [210, 248], or ingestion of enzymes [249]. Reflex salivation due to feeding is partially mediated by alpha-adrenergic mechanisms [203], suggesting that norepinephrine is released. Norepinephrine may also be responsible for the increase in size and in mitotic index of glands within 1 hr of nerve stimulation [150]. The norepinephrine may act via beta-adrenergic mechanisms because chronic isoproterenol injection mimics the effects of stimulating sympathetic nerves [25, 150, 180, 221, 247]. Some effects of stimulating nerves may be mediated by the release of epidermal growth factor [7]. However, as with the airway studies, systemic reflexes were probably stimulated by the isoproterenol, thus confusing interpretation.

The hypertrophy may be due to increased synthesis of DNA [94] resulting in increased cell number, or to inhanced RNA and protein synthesis [6]. The beta-adrenergic effects are associated with increases in cellular C-AMP [94], and the hypertrophy due to incisor amputation is potentiated by the phosphodiesterase inhibitor, theophylline [250]. Therefore, hypertrophic and secretory responses may be mediated by similar mechanisms. This is consistent with the idea that synthesis and growth responses might be stimulated by the secretory process of the individual cells.

CONCLUSIONS

We used electrical field stimulation and autonomic drugs *in vitro* to study the neural regulation of secretion of mucus from the trachea in the absence of circulating mediators or central nervous system

reflexes. These techniques allowed us to do within-tissue experiments, and thus we could achieve accurate, reproducible responses to stimulation.

Using these techniques, we confirmed previous work that demonstrated cholinergic-muscarinic regulation of fluid and glycoprotein secretion, and beta-adrenergic regulation of glycoprotein secretion. Additionally, we were able to show, for the first time, that adrenergic nerves, acting via alpha-adrenergic mechanisms, mediate secretion of fluid from tracheal submucosal glands (CHAPTER 3). Additionally, we showed that adrenergic nerves, acting via both alpha-adrenergic and beta-adrenergic neural mechanisms, and a nonadrenergic-noncholinergic nerve type mediate secretion of glycoproteins (CHAPTER 4). We also found the first direct evidence that cholinergic nerves inhibit adrenergic neurotransmission in the airway (CHAPTER 5).

Thus, nerves may affect mucociliary clearance by many different mechanisms. They regulate the secretion of fluid and macromolecules from glands and can affect ciliary activity, and thus regulate mucociliary clearance. They also regulate the synthesis of export proteins and the capacity of glands to secrete mucus. Although the integrated function of the mucociliary clearance system is not well understood, it is apparent that neural regulation of glands involves integration at several different levels. The central nervous system regulates secretion directly or via reflexes. Most of the reflexes studied to date are mediated by cholinergic mechanisms, although adrenergic or peptidergic reflexes may be present. The

parasympathetic efferent neural activity must ultimately activate ganglion cells that also recieve input from other nerve types, including adrenergic and VIP-containing nerves. Thus, the ganglion cell may be responsible for modulating secretomotor output by peripheral neural mechanisms. Additionally, there is evidence for inhibitory interaction between post-ganglionic nerve terminals near glands. This may provide another level for neural interaction in secretion. Each of the secretory cell types has several specific receptor mechanisms. When activated, these stimulate intracellular mechanisms to produce the characteristic secretory products. The intracellular mediators responsible for the effects may interact with each other, providing another level of integration. All of these mechanisms acting together may provide rapid, flexible, specific responses to irritation of the airways.

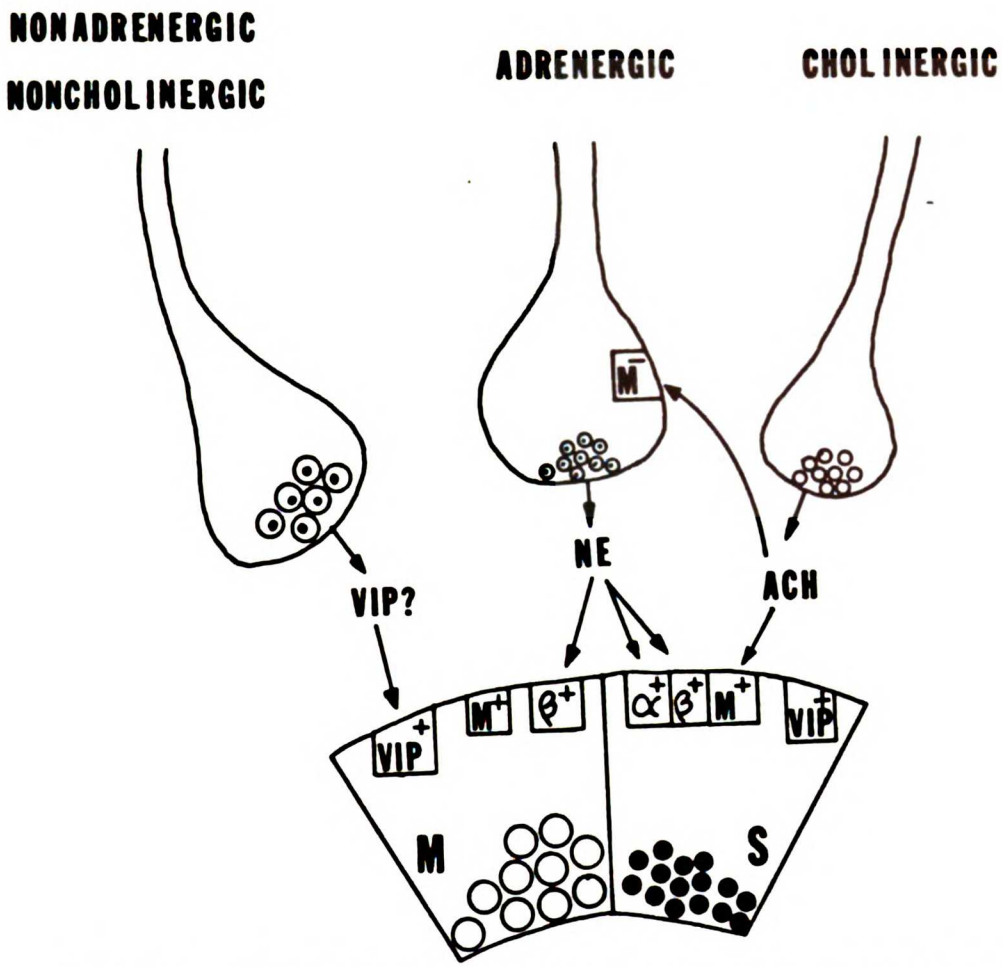

FIG. 6.1. Neural mechanisms in secretion from tracheal glands. Adrenergic nerves release norepinephrine (NE) that stimulates alpha-adrenergic receptors (α^+) on serous cells (S) to cause secretion of fluid and macromolecules. Norepinephrine stimulates beta-adrenergic receptors (β^+) on mucous cells (M) to cause release of macromolecules. Cholinergic nerves release acetylcoline (ACH) that stimulates muscarinic receptors (M^+) on glands resulting in secretion of fluid and macromolecules. ACH also stimulates muscarinic receptors (M^-) on adrenergic nerves to inhibit norepinephrine secretion. Nonadrenergic noncholinergic nerves release a transmitter (VIP?) that may stimulate serous and mucous cells, and cause the release of macromolecules.

APPENDIX I

QUANTIFICATION OF NOREPINEPHRINE RELEASE

The purpose of this appendix is to describe and evaluate several methods for analysing data on ^{3}H release. We used the first two methods, area under the curve and secretion averaging, to estimate changes in the quantity of ^{3}H released by stimulation. We used exponential analysis to estimate changes in the rates of secretion. We found that one of the latter methods, relative peak height $ER_{(rel)}$, produced the most precise and reproducible measure of the evoked response (APPENDICES I, II, III).

1. AREA UNDER THE CURVE

Baseline secretion before stimulation and > 45 min after stimulation are both approximately log-linear. This suggests that the observed secretory rate may have returned to "true" baseline. We supposed that this baseline might be the same baseline that would have been observed in that tissue if it had not been electrically stimulated. There is some evidence for this supposition. Because DMI does not decrease the amount of ^{3}H released during unstimulated conditions at 2h, some of the baseline efflux of ^{3}H may come from extraneuronal sites (DISCUSSION) [Farnebo 1971] and thus, may be independent of electrical stimulation. If this baseline is independent of electrical stimulation, then drawing a line underneath the efflux curve would provide a useful baseline to compare with the evoked response. The problem to solve was where and how to draw the baseline.

A. Area by eye

We drew a straight line by eye underneath the peak of the evoked secretion curve, connecting the pre-stimulus baseline portion of the curve to the post-stimulation portion (FIG. 5.17). To calculate evoked responses, we subtracted the estimated baseline cpm at each 5 min interval from the observed cpm in the sample at the same time and divided the result by the estimated baseline value. We then added the results for each of the 5 min intervals together to achieve the area underneath the curve, or evoked response, expressed as % baseline (FIG. 5.17). Mean data for the 13 control experiments showed that the second and third responses were 87 ± 8 (SEM) % and 90 ± 11 (SEM) %, respectively, of the first response.

The major objection to this method is that to connect the pre- and post-stimulus baselines by a log-linear baseline underestimates the responses because secretion is not linear during that time (e.g., FIG. 5.3). However, we used this method to estimate the effects of DMI and TTX because the magnitudes of the evoked responses after treatment with these drugs were small (see CHAPTER 5: RESULTS). We needed to use this method because, when they could be calculated, the standard errors of the estimates of $ER_{(rel)}$ were very high (≈ 200 %) making the estimates themselves were unreliable. This was probably due to the few points on the efflux curve that were not on the apparent baseline (FIG. 5.8, 5.10). By contrast, the standard error of the mean (SEM) of the same responses estimated by eye was much less (<8 %). Because the evoked responses were small, most of the points were close to the predicted baseline which was nearly log-linear.

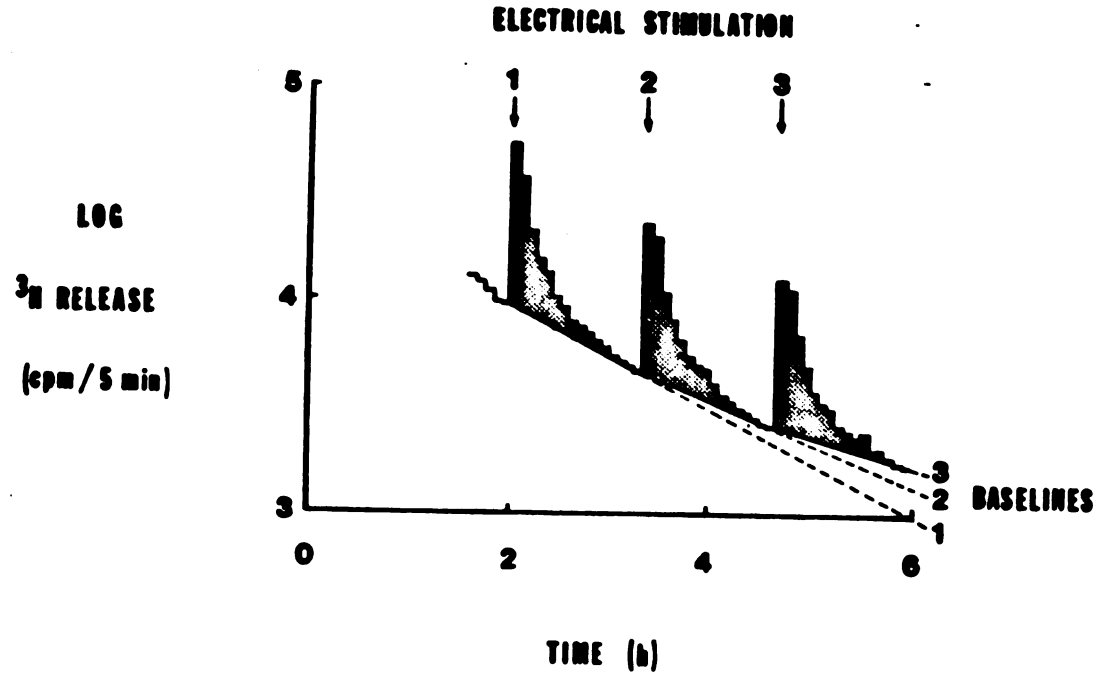

FIG. 5.17. Area by eye (▨). Log-linear baselines were drawn underneath each ^{3}H peak. For each 5 min sample, the relative difference between the observed cpm in the sample and the predicted baseline were added together.

B. Computer Derived Area

Because baseline efflux of ^{3}H is not log-linear, but is best described by the sum of at least 2 exponentials. We calculated the expected baseline from data from another series of experiments at times equivalent to the peak of ^{3}H efflux from stimulated tissues under otherwise identical conditions. To do this, we first determined the baseline efflux from 6 unstimulated tissues beginning at the end of the 90 min incubation period and ending at 165 min (the end of the stimulus efflux for the first electrical stimulation) (see CHAPTER 5: METHODS). This baseline data will be compared to the data from tissues recieving a single period of electrical stimulation (at 2 h). Then, we determined the efflux from 6 other tissues from the beginning of a single electrical stimulus to 165 min later (the end of the efflux curve for a second electrical stimulation). This data will be compared to the data from tissues receiving 2 periods of stimulation. Finally, we determined the baseline efflux from 6 other tissues from the beginning of their second electrical stimulus to 165 min later (the end of the efflux curve for a third electrical stimulation). This data will be compared to data from tissues recieving 3 stimulations.

Using the computer, we superimposed the pre-stimulus portion of each evoked peak and the corresponding baseline efflux curve at the equivalent time (FIG. 5.18). The computer then subtracted the predicted baseline secretion from the observed cpm at each time point, divided the result by the predicted baseline, and added the results for all the time points together, thus expressing the evoked response

as % baseline.

However, we found that after stimulation, the efflux approached values below the predicted baseline (FIG. 5.18). After stimulation, the rate of release decreased more rapidly than did the baseline at the equivalent time. This is reflected in the higher time constant (k_1) than the baseline process at the equivalent time (k_2) (TABLE 5.3)($p=0.05$). Thus, after each stimulation, the evoked ^3H efflux curves were always steeper than the baseline curves at the equivalent times (TABLE 5.3). However, because the intercepts (A_2) of the baseline and evoked effluxes were not different from each other ($p>0.3$), after about 45 min after stimulation, observed release was always less than the baseline release at the equivalent times.

Some of the baseline ^3H was therefore released from the same neural pools as was the evoked ^3H, i.e., electrical stimulation shifted the baseline efflux curve down. Furthermore, the calculated area under the peak progressively decreased from the first to the third stimulus. The second and third responses were 93 ± 8 % and 81 ± 13 %, respectively, of the first response. Thus, this estimate of evoked secretion is not reproducible.

Although the estimation error is relatively low (<10 %), neither of these methods is satisfactory because some baseline release is independent of nerves, and some is dependent on nerve activity. Therefore, we could not reliably predict baseline efflux and therefore we could not achieve accurate estimates of secretion.

It may be possible to draw a different baseline and compare the evoked response with the "new" baseline after stimulation. It is

possible to extrapolate the slowest exponential backwards in time to the onset of stimulation. However, the error in estimating evoked release is too large to use this method. The estimation error of A_3 is 19 %, and that of k_3 is 70 % (TABLE 5.2). The calculation of the evoked response would require propagating these errors by the number of times that we used estimated baseline release (≈ 10) in the calculations (see APPENDIX II). Considering only the first 4 points after stimulation, the estimated standard error of the calculated evoked response would be ≈ 40 %. Considering more points would progressively increase the error. Furthermore, this problem would not be solved by using the two exponential model. The standard errors of the parameter estimates for the intercept and slope parameters for the slowest exponential are 14 and 11 %, respectively (TABLE 5.2). Therefore, the estimate of the standard error of the predicted baseline from these is approximately 50 % for the entire evoked response. Thus, because we could not accurately predict the baseline release, we sought methods that did not require predicting baseline secretion.

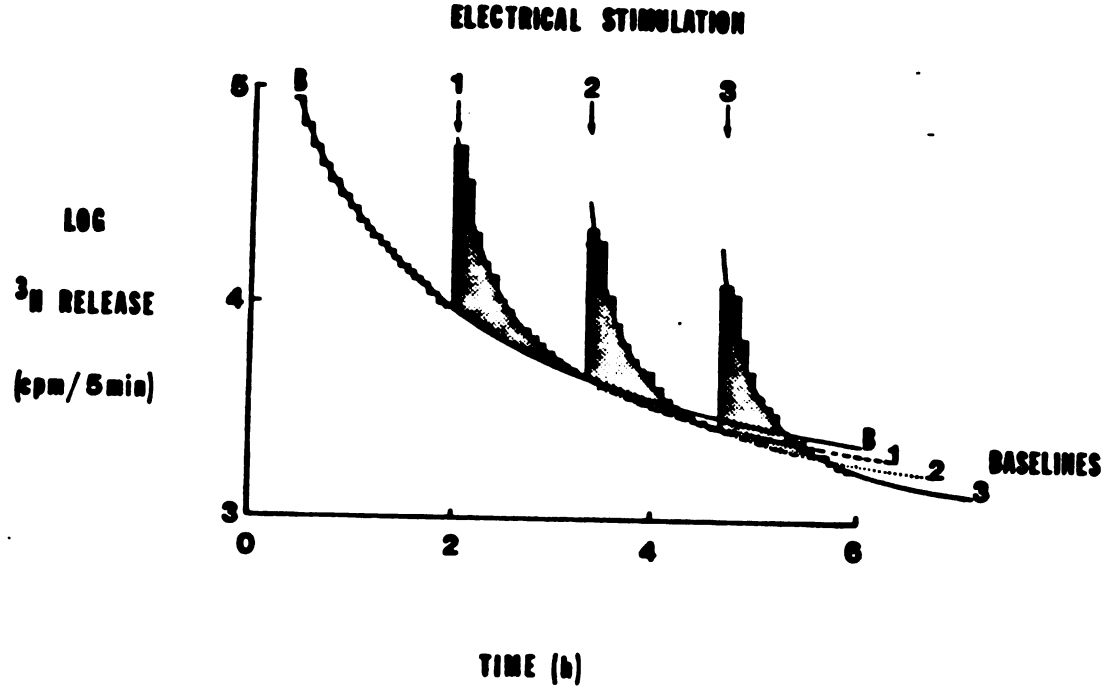

FIG. 5.18. Computer derived area (▨). After stimulation (arrows), the efflux curves intersect the baseline curve and remain below the expected baselines (B,3,2,1), suggesting that electrical stimulation depleted the pools of ^{3}H responsible for baseline secretion.

TABLE 5.3

EXPONENTIAL PARAMETERS FOR BASELINE

AND STIMULATED ^{3}H RELEASE

ELECTRICAL STIMULATION PERIOD	A_1	k_1	A_2	k_2
	(cpm/5 min)	(min^{-1})		
1				
Baseline	61615	-.070	14933	-.0104
($\pm$ m^2)	6612	.004	2086	.0004
Electrical Stimulation	41841	-.189	7959	-.0328
($\pm$ m^2)	9231	.018	1327	.0132
2				
Baseline	7276	-.052	3410	-.007
($\pm$ m^2)	1111	.006	600	.001
Electrical Stimulation	21269	-.163	5001	-.014
($\pm$ m^2)	4401	.013	916	.001
3				
Baseline	4614	-.074	2217	-.006
($\pm$ m^2)	679	.009	250	.000
Electrical Stimulation	13269	-.165	2744	-.008
($\pm$ m^2)	1452	.018	537	.001

TABLE 5.3. Exponential parameters for baseline and evoked ^{3}H release. Each curve was fit to the sum of 2 exponential terms, each described by an intercept parameter, A, and a slope parameter, k.

One way to avoid having to predict the baseline secretion is to consider that because electrically evoked release of neurotransmitter is a rapid process, the early part of the post-stimulus curve contains most of the ^{3}H evoked by stimulation. We therefore considered only the times immediately before and after stimulation, allowing us to determine baseline secretion during the pre-stimulus period without having to predict baseline release for any time after stimulation.

2. SECRETION AVERAGING

The first method was to calculate the average cpm of the few points immediately before stimulation to represent an estimate of baseline secretion. The average cpm of the points after stimulation minus the average before stimulation divided by the baseline is the evoked response as expressed as % baseline. If we used the two points each before and after stimulation from each of 13 control tissues to calculate the evoked response, the second and third responses to electrical stimulation were 112 ± 9 (SEM) % and 124 ± 13 (SEM) %, respectively, of the first response. If we used the three points each before and after stimulation, the second and third responses were 123 ± 9 (SEM) % and 129 ± 14 (SEM) %, respectively, of the first response.

The major problem with either of these procedures is that the estimates of the evoked responses in the absence of drugs are not reproducible. The third response is 24 or 29 % greater than the first response. The lack of reproducibility is probably due to the use of only a few points to estimate the responses. The cpm in any given sample is sensitive to errors in either sampling time or volume. By

increasing the number of points used, the accuracy of the method would increase, but with additional points, we would have to assume that baseline release was linear, otherwise an arethmetic average would not be meaningful. However, "true" baseline release is not predictable and is not linear, and therefore, arithmetic averaging methods are not justifiable.

3. PEAK HEIGHT BY EXPONENTIAL ANALYSIS

Although the first few points after stimulation contain most of the ^{3}H released by electrical stimulation, the efflux processes is best described as the sum of exponentials (FIGS. 5.3, 5.4). Therefore, all of the points in the efflux curve contain useful information about the evoked response. By increasing the number of points considered for each efflux curve, the precision of the computations must increase (i.e., the standard errors of their estimates must decrease). From the values of the exponential parameters obtained by nonlinear regression, we estimated the evoked response in two ways, first by considering only the most rapid exponential process, and then considering the sum of the exponential processes.

A. First Exponential

We computed the area underneath the most rapid exponential process after stimulation (FIG. 5.4). The area under the first exponential term from the onset of stimulation to infinite time is the total ^{3}H released by that exponential process. Because DMI and TTX

affect only the rapid exponential processes, the rapid efflux represents mostly neural release, and the analysis probably reflects changes in neural release. By approximately 20 min (4 samples) after stimulation, the rate of release of ^{3}H decreased to approximately 10 % of the subsequent baseline rate (e.g., FIG. 5.3). The relative standard error of the estimate of the first response was 19 %. This poor precision is probably due to the relatively large effects on calculated evoked responses of small errors in either sampling time or volume. The slope of the first exponential is steep, and therefore, a small error in estimating the slope will cause a large error in estimate of the intercept. The poor precision results in the poor reproducibility of the estimates of evoked responses. Furthermore, there are at least 2 exponential processes occurring after stimulation. By considering only the most rapid one, we use only a fraction of the total information about the efflux curve. Although the release of ^{3}H evoked by electrical stimulation is rapid, tissue redistribution of ^{3}H may occur, therefore delaying the appearance of some of the released ^{3}H in the bath and thus, adding slower exponential processes to the total efflux. Adding the slow component of release increases the amount of label recovered and increases the calculated evoked responses to stimulation.

B. Sum of Exponentials

By considering the efflux curve as a sum of exponential processes, we can accurately describe the pattern of efflux (TABLES 5.1, 5.2). The average standard error of the regression was 126 cpm/

sample. Because the average lowest cpm recovered during the experiment is usually 2000 cpm (e.g., FIG. 5.3), the relative error is 6.3 %. This relatively high accuracy means that we have a great deal of information about the efflux processes. We can use this accuracy to extrapolate the efflux curve backwards in time to the onset of stimulation, a time when baseline secretion is known. Therefore, we do not have to predict baseline release after stimulation. The extrapolated points are useful because they represent the size(s) of the pool(s) from which ^{3}H is released from the tissue, and are proportional to the amount of ^{3}H released at that time. Thus, changes in the values of these extrapolated points are equal to the changes in the release of ^{3}H (see CHAPTER 5: METHODS). There are several ways to express results as the sum of exponentials.

i. Absolute Peak Height

To calculate the absolute magnitude of the evoked response, we subtracted the cpm of the pre-stimulus baseline point from the cpm of the intercept of the post-stimulus efflux curve extrapolated to the same time (FIG. 5.4). For the same 13 control experiments, the mean response to the first stimulus was 43537 ± 7377 cpm. The relative standard error of the estimate is 17%. The peak height decreased substantially with successive stimuli (see FIG. 5.3) because both time and stimulation reduced the ^{3}H available for secretion. Thus, this estimate of the response is neither precise nor reproducible within a tissue.

ii. Absolute Peak Height / Tissue Weight

We thought that the density of adrenergic innervation might be related to gland mass. Since gland mass is evenly distributed throughout the trachea (i.e., is proportional to tissue size)(CHAPTER 2), we felt that dividing the absolute peak height by tissue size (weight) might give a more precise and reproducible measure of the evoked response. The mean response of the same 13 control tissues was 329 ± 72 cpm/mg wet weight. Since the the relative error is 22 %, the precision is not increased over method i above. Furthermore, since tissue weight remains approximately constant during the course of an experiment, the absolute peak height divided by tissue weight is also not reproducible with successive stimuli.

iii. Fractional Release

To allow for the progressively decreased baseline and electrically evoked release of ^{3}H, we considered two additional methods of quantifying responses. The evoked response could be expressed as a proportion of the total quantity of ^{3}H present in the tissue at the time of stimulation. We measured the total cpm remaining in each tissue at the end of the experiments after disaggregating each tissue in sodium hydroxide (1.0 N for 48 h). We added the tissue content of ^{3}H to the sum of the cpm recovered from the onset of stimulation to the end of the experiment. This total represented the ^{3}H in the tissue immediately before stimulation. The fractional release was calculated as the absolute evoked response (absolute peak height) divided by the total ^{3}H in the tissue at the

onset of stimulation. The mean response of the same 13 tissues was 0.152 ± 0.03 %. The relative error is 21 %, and thus the method is neither more precise nor reproducible than methods i or ii above.

Additionally, to justify this method, we must assume that the observed secretory rate is proportional to the total amount of ^{3}H present in the tissue, i.e., that secretion follows a single exponential. However, after stimulation, there are at least two exponential processes (FIGS. 5.2, 5.3; TABLE 5.2). Furthermore, although DMI reduced evoked release by 71 % (FIG. 5.9), it did not reduce baseline release, indicating that some of the tissue ^{3}H is not mobilized by nerve stimulation. Therefore, the tissue content of ^{3}H represents both releasable and non-releasable ^{3}H, and we cannot justify using this fractional release method to estimate the evoked responses.

iv. Relative Peak Height

To avoid comparing evoked ^{3}H release with the non-releasable ^{3}H in tissues, we used a different kind of correction. The peak height of the evoked ^{3}H release was compared to the baseline release at the same time. We calculated the relative peak height, $ER_{(rel)}$, by dividing the absolute peak height by the the pre-stimulus baseline point (FIG. 5.4). The unweighted mean response of the 13 control tissues to a single period of electrical stimulation was 797 ± 70 (SEM) % of baseline. The relative standard error is 9 %, which is the smallest of any of the methods for estimating the evoked response. Additionally, we found that the estimates of successive evoked

responses were more reproducible than for any of the other methods. The maximum likelihood estimates for the means of the second and third responses of the 13 control tissues were 109 and 111 % respectively, of the first response. Neither of these is significantly different from the first response ($p>0.4$). The findings that this method not only is the most precise, but also the most reproducible suggests that the method most closely reflects the true behavior of the system. We therefore used it for presentation of all data on ^{3}H efflux except those obtained from the experiments with high concentrations of either desmethylimipramine or tetrodotoxin.

APPENDIX II:

ESTIMATING STANDARD ERRORS OF ^3H RELEASE

The nonlinear least squares regression program provides the standard errors of the estimates for each of the exponential parameters. These standard errors measure the uncertainty with which exponential parameters are estimated from the raw data. Since we calculate evoked responses from these parameters, we must propigate this uncertainty through the calculations to the final result. In general, for a function Z=f(x,y) with known standard errors of x and y, e_x and e_y, an estimate of the standard error of Z, e_Z, is:

$$e_Z = \sqrt{(\frac{\delta Z}{\delta x})^2 e_x^{\,2} + (\frac{\delta Z}{\delta y})^2 e_y^{\,2}}$$

Equation 1,

assuming that the covariance of the estimates of x and y is negligible. The individual standard errors of x and y are weighted for their relative contributions to the function by $\frac{\delta Z}{\delta x}$ and $\frac{\delta Z}{\delta y}$, respectively. Specifically, for the sum function $Z = x + y$, $\frac{\delta Z}{\delta x} = 1$, and $\frac{\delta Z}{\delta y} = 1$. Therefore, an estimate of the standard error of Z, e_Z, is:

$$e_Z = \sqrt{e_x^{\,2} + e_y^{\,2}}.$$

The peak height of the response to electrical stimulation is the sum of the intercept parameters of the exponential terms ($A_1 + A_2$) (see FIG. 5.4). The peak height minus the baseline secretory rate just

before stimulation (B), is the absolute evoked response $ER_{(abs)}$. An estimate of the standard error of the absolute peak height, $e_{ER_{(abs)}}$ is:

$$e_{ER_{(abs)}} = \sqrt{e_{A_1}^2 + e_{A_2}^2 + e_B^2}$$

Equation 2.

For a quotient function $Z = \frac{x}{y}$, $\frac{\delta Z}{\delta x} = \frac{1}{y}$ and $\frac{\delta Z}{\delta x} = \frac{-x}{y^2}$. An estimate of the standard error for Z, e_Z, is:

$$e_Z = \frac{1}{y}\sqrt{e_x^2 + (Z)^2 e_y^2}$$

Equation 3,

The relative evoked response $ER_{(rel)}$, is the absolute evoked response divided by the pre-stimulus baseline, B. An estimate of the standard error of the relative peak height, $e_{ER_{(rel)}}$, is:

$$e_{ER_{(rel)}} = \frac{1}{B}\sqrt{e_{ER_{(abs)}}^2 + ER_{(rel)}^2 e_B^2}$$

Equation 4.

Data were calculated and stored as the baseline, absolute peak height, and relative peak height $ER_{(rel)}$, each point with its net standard error.

APPENDIX III:

STATISTICAL ANALYSIS OF ^{3}H RELEASE

Calculations (APPENDIX II) showed that the standard errors of the estimates of relative evoked response, $e_{ER_{(rel)}}$, of 13 control tissues to a single electrical stimulus was often 15 % of the evoked response. This is 46 % of the standard deviation of the 13 $ER_{(rel)}$ themselves (32 %). The presence of this significant estimation uncertainty (relative to overall standard deviation) prevents us from calculating a precise sample mean, $\overline{X}$, from n data points x_i by using the usual formula,

$$\overline{X} = \left(\sum_{i=1}^{n} x_i\right)/n,$$

because the to use the formula, we must assume equal precision for each datum. Furthermore, we cannot compare two different samples using "t" tests or conventional analysis of variance, because to use either of these methods, we must assume that the data points are known with the same degree of precision.

Therefore, to determine appropriately weighted mean values of the peak heights under different experimental conditions, we used the method of maximum likelihood [15]. Each estimated datum (peak height) x_i can be thought of as a function of the sample mean, X, the error of the estimate, ϵ_{x_i}, and an individual component η_{x_i} that represents the deviation of the i^{th} true peak height from the mean due to random biological variation:

$$x_i = X + \eta_{x_i} + \epsilon_{x_i}.$$

Equation 5.

If we assume that both random terms (η_{x_i} and ϵ_{x_i}) are normally distributed, and that e_{x_i} as determined in APPENDIX II, approximates the true error ϵ_{x_i}, we can estimate the mean, X, and the biological variance, $\mathfrak{m}^2$, by minimizing the negative log likelihood function, -2 ln L, with respect to X and $\mathfrak{m}^2$:

$$-2 \ln L = \sum_{i=1}^{n} \left| \frac{(X - x_i)^2}{\mathfrak{m}^2 + e_{x_i}^2} + \ln(\mathfrak{m}^2 + e_{x_i}^2) \right|$$

Equation 6.

To compare groups thought to have two different means, the likelihood ratio test can be used. It involves computing the minimum values of -2 ln L for two models. First, for the "full" model, we calculated the value of -2 ln L, called -2 ln L_f, for the pooled data from both groups allowing each group a separate mean value (i.e., the samples are different). Next, for the "reduced" model, we calculated the minimum value of -2 ln L, called -2 ln L_r, for the pooled data allowing only a single common mean for both groups (i.e., the groups are the same). Then twice the negative log likelihood ratio,

$$\Delta = 2 \ln L_r - 2 \ln L_f$$

Equation 7,

is distributed as CHI^2 with 1 degree of freedom [15]. Mean results

are presented with the estimate of biological variance (m^2).

Analysis as above of responses of the 13 control tissues showed that the second and third responses to electrical stimulation were 109 ± 17 and 111 ± 14 % respectively, of the first response. Physostigmine decreased the evoked response by 37 ± 6 %. For comparison, the unweighted mean values of the same tissues showed that the second and third responses were 105 ± 12 % and 100 ± 13 %, respectively of the first response. Additionally, physostigmine caused 34 ± 11 % inhibition. The finding that the maximum likelihood estimates are nearly the same as the unweighted mean estimates suggests that all of the estimation errors were of similar magnitude.

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