competition with a known ligand as in (radio)immunoassays will never allow affirmation of identity (of whatever is being investigated) with the known ligand or antigen involved. At best such methods can lead to affirmation of nonidentity, with the known ligand or antigen involved. At risk, they give a statistical security about certain relationship of whatever is being investigated with the spatial structure of the antigenic determinant of the antigen used in eliciting the immune globulins involved.

On a more constructive side our studies have led to the unexpected recognition of several homologies in the amino acid sequences of physiologically unrelated molecules such as a pituitary hormone and the constant chain of an immunoglobulin.

Note added in proof: After this manuscript was submitted for publication there appeared the report by Houck *et al.* (*19*) in which they conclude having obtained, in extracts of human placenta, a high-molecular-weight immunoreactive β-endorphin and two smaller molecules reactive in the radioimmunoassay and in a receptor-binding assay. The report by Houck *et al.* is a good example of the questions raised here. Other than the radioimmunoassay, there is no characterization of the high-molecular-weight material to justify its being referred to as the possible precursor of β-endorphin.

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A Nonadrenergic Vagal Inhibitory Pathway to Feline Airways

Abstract. *In cats anesthetized with chloralose-pentobarbital and artificially ventilated, electrical stimulation of the caudal end of the cut cervical vagus nerve has a biphasic effect on the bronchoconstriction induced by an intravenous infusion of serotonin. The response consists of a brief augmentation of bronchoconstriction followed by relatively prolonged bronchodilation. After muscarinic receptor blockade with atropine, vagal stimulation causes only bronchodilation. Vagally mediated bronchodilation is not affected by beta adrenergic blockade with propranolol, alpha adrenergic blockade with phenoxybenzamine, or adrenergic neuronal blockade with guanethidine, but is abolished by autonomic ganglionic blockade with hexamethonium. These findings support the conclusion that a nonadrenergic inhibitory nervous system is present in the pulmonary airways of the cat and that the system is supplied by preganglionic fibers in the cervical vagus nerves.*

Classical concepts of the nervous regulation of mammalian airway smooth muscle emphasize the importance of excitatory cholinergic nerves and inhibitory adrenergic nerves (*1*). However, the recent discovery of a third type of innervation in airway smooth muscle has brought about a revision of these traditional concepts. Several studies have demonstrated an inhibitory nervous system in the trachealis muscle of the guinea pig (*2–4*) and baboon (*5*) that is neither adrenergic nor cholinergic. An analogous system has been identified in isolated human airways from the trachea to the smallest bronchi (*6*). Because the transmitter has not been identified, the system is known as the nonadrenergic inhibitory system.

Although it has been postulated that nonadrenergic inhibitory fibers derive from the cranial parasympathetic outflow and travel to the lungs in the vagus nerves (*2*), direct evidence for such a pathway is lacking. We report here the results of a series of experiments in which we measured lung mechanics, electrically stimulated the cervical sympathetic and parasympathetic nerves under controlled physiological conditions, and administered selective pharmacologic blocking agents to demonstrate the presence of a nonadrenergic vagal inhibitory pathway to feline pulmonary airways in vivo.

We anesthetized 52 adult female cats intraperitoneally with α-chloralose (80 mg/kg) and sodium pentobarbital (5 mg/kg), cannulated the trachea, and applied positive-pressure ventilation (frequency, 30 breaths per minute; tidal volume, 13 cm^3/kg). Blood pressure was recorded from the femoral artery with a strain-gauge manometer (Statham P23 ID) and heart rate was monitored with a standard limb-lead electrocardiograph. To facilitate intravenous drug administration, we inserted polyethylene catheters into the right saphenous and brachial veins. All experiments were performed with the animals supine. Body temperature was maintained at 36° to 39°C by a heat-exchanging pad.

Airflow was determined by connecting

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a pneumotachograph (Fleisch 00) to the tracheal cannula and measuring the pressure drop across the device with a differential strain gauge (Statham PM 15). Tidal volume was determined by electrical integration of the airflow signal. We pushed a 12-gauge stainless steel needle into the interpleural space through one of the intercostal ligaments and measured transpulmonary pressure with another strain gauge (Statham PM 5) connected differentially to the needle and a side arm of the tracheal cannula. Lung resistance to airflow was measured by electrical subtraction, and dynamic lung compliance was determined as the ratio of tidal volume to transpulmonary pressure at moments of zero flow by using a respiratory analyzer (Hewlett-Packard 8816A). Arterial blood pressure, airflow, tidal volume, transpulmonary pressure, lung resistance, and dynamic lung compliance were recorded continuously during the experiments with a polygraph (Grass model 7).

We separated the cervical vagosympathetic nerves into their two component bundles and verified the identity of each by testing for a vagal effect on heart rate and for a sympathetic effect on pupil size. The nerves were then ligated, sectioned, and moistened with warm physiological saline. We placed the caudal ends of the nerves on bipolar platinum electrodes and, in the case of the sympathetics, advanced the electrodes close to the thoracic outlet in an effort to reach preganglionic fibers that, in the cat, are known to loop up the sympathetic trunk before descending into the thorax (*7*). The nerves were stimulated for 5 seconds at supramaximal voltage (30 V) with 1-msec rectangular pulses of various frequencies from a stimulator (Grass model S44).

Initially, we stimulated the vagus and sympathetic nerves alternatively and observed the effects on airway mechanics while the animals were in the control condition. Right- and left-sided stimulation produced similar responses. In 11

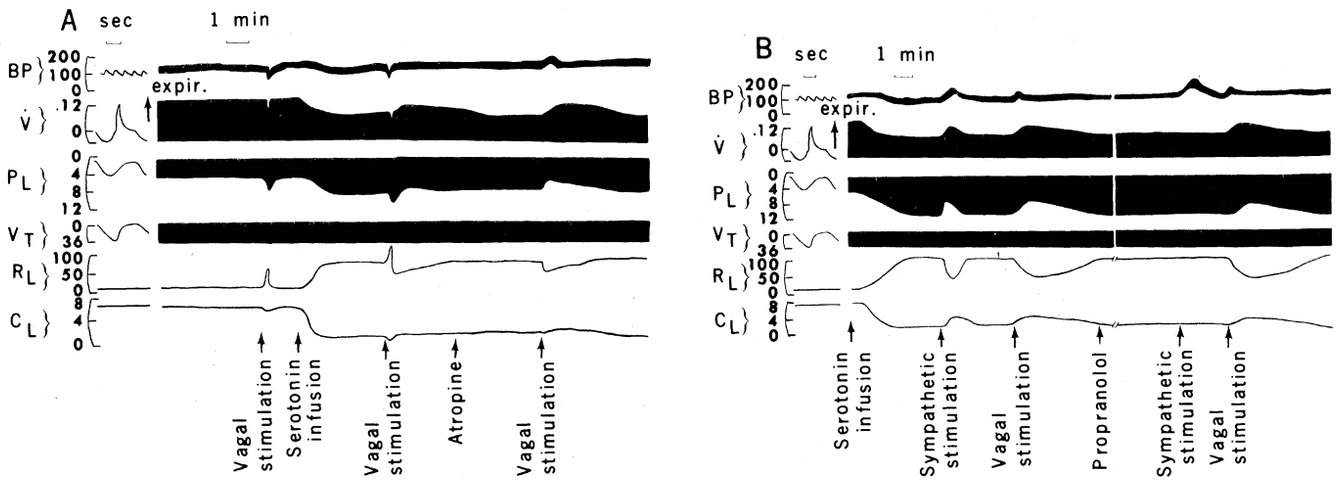

Fig. 1. Oscillographic records from two representative experiments. (A) Physiological responses to electrical stimulation of the distal end of the cut cervical vagus nerve on three successive occasions (arrows): (i) just prior to beginning an infusion of serotonin, (ii) under steady-state conditions during the infusion, and (iii) after cholinergic blockade with atropine (0.5 mg/kg) during the infusion. (B) Effects of beta adrenergic blockade with propranolol (0.5 mg/kg) on the physiological responses to electrical stimulation of the caudal end of the cut cervical sympathetic or vagus nerve under steady-state conditions during an infusion of serotonin. The animal was atropinized before the start of the infusion. Note the differences in the time course of changes in lung mechanics produced by sympathetic vis-à-vis vagal stimulation. Abbreviations: *BP*, systemic arterial blood pressure (millimeters of mercury); $\dot{V}$, airflow rate (liters per second); P_L, transpulmonary pressure (centimeters of water); V_T, tidal volume (milliliters); R_L, lung resistance (centimeters of water per liter per second); C_L, dynamic lung compliance (milliliters per centimeter of water).

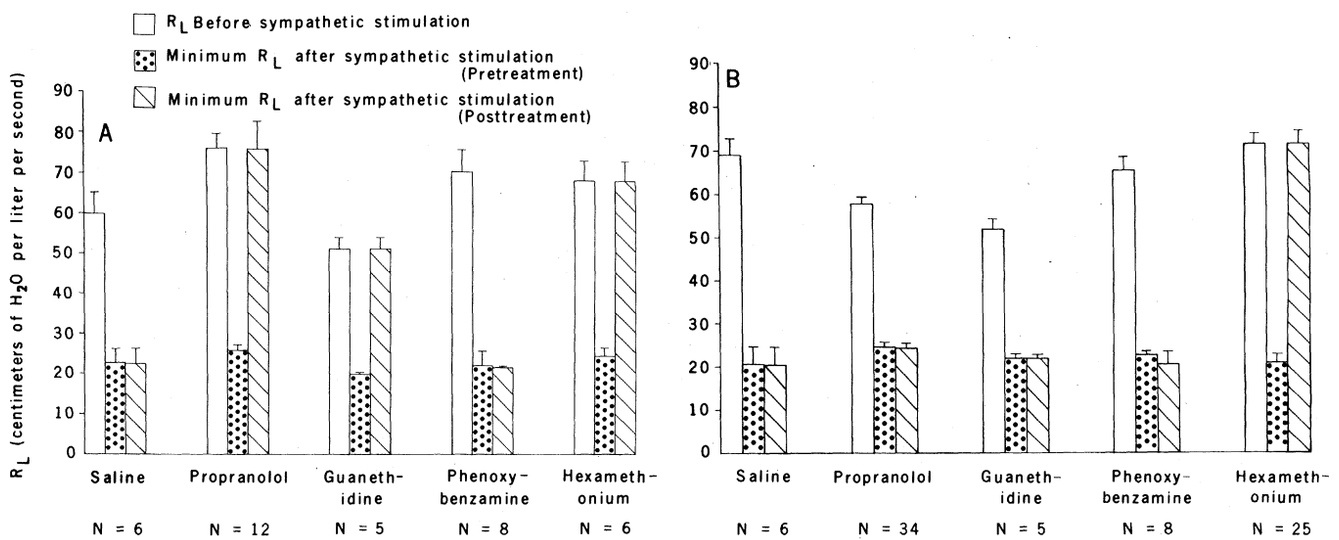

Fig. 2. Effects of pharmacologic blocking agents on the decrease in lung resistance (R_L) produced by stimulation of (A) the cervical sympathetic nerves or (B) the cervical parasympathetic nerves during an infusion of serotonin in atropinized cats.

cats, vagal stimulation at 30 pulses per second caused transient bronchoconstriction characterized by decreased flow and increased transpulmonary pressure. Pulmonary resistance increased 407 ± 20 percent, and dynamic long compliance decreased 21 ± 5 percent. Sympathetic stimulation with the same stimulation parameters had no detectable effect on airway mechanics, indicating a lack of tonus or a failure to stimulate efferent fibers innervating the bronchial musculature. To test the former possibility, we elevated bronchial muscle tone with an intravenous infusion of serotonin creatinine sulfate (10 to 20 μg/kg-min). A steady state was achieved within 5 minutes, at which time lung resistance had increased 382 ± 25 percent and dynamic lung compliance had decreased 74 ± 6 percent. Electrical stimulation of the cervical sympathetic nerves now had a distinct bronchodilating effect. The serotonin-induced change in lung resistance was reversed 77 ± 4 percent, and the change in dynamic lung compliance was reversed 50 ± 4 percent.

In subsequent experiements, a serotonin infusion was used to augment airway smooth muscle tone before nerve stimulation; vagal stimulation in 20 animals so infused produced a biphasic response consisting of brief bronchoconstriction followed by relatively prolonged bronchodilation. During the constriction phase, pulmonary resistance increased an additional 111 ± 5 percent above the baseline level and dynamic lung compliance fell an additional 11 ± 3 percent. During the relaxation phase, the effects of serotonin on lung resistance and dynamic compliance were reversed 57 ± 3 and 25 ± 5 percent, respectively. When the original degree of bronchoconstriction returned, we administered atropine sulfate (0.5 mg/kg) and repeated the vagal stimulation 5 minutes later. Cholinergic blockade completely abolished the constriction phase of the response but had no substantive effect on the relaxation phase (Fig. 1A). This reaction pattern was highly reproducible; indeed, in the presence of atropine, vagal stimulation caused bronchodilation in every animal studied.

A specific interaction between serotonin and efferent vagus nerves was demonstrated in dog lungs (*8*): vagal effects on airway smooth muscle were potentiated by serotonin aerosol but not by acetylcholine or histamine. In view of this, we wondered whether the bronchodilation response to vagal stimulation is specific for serotonin-induced elevations in lung resistance. Accordingly, we used histamine (10 to 60 μg/kg) in place of serotonin to increase the tone of the airway musculature of five atropinized animals, and achieved a steady-state increase in lung resistance of 85 ± 10 percent. Supramaximal vagal stimulation at 30 pulses per second completely reversed the histamine-induced increase in lung resistance. Hence it can be concluded that vagally mediated bronchodilation in cats does not require a specific interaction involving serotonin.

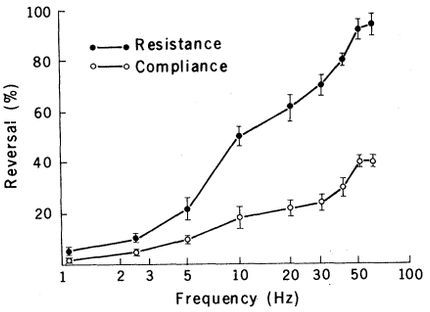

Fig. 3. Effects of stimulus frequency on vagally mediated reversal of serotonin-induced lung resistance and compliance changes in atropinized cats. Each point represents the mean (± standard error) of the results of 17 experiments.

Relaxations produced by vagal stimulation were easily distinguishable from the more evanescent relaxations caused by sympathetic stimulation. Nevertheless, we used various adrenergic blocking agents in a series of experiments designed to eliminate the possibility that efferent adrenergic fibers were involved in the dilation response to vagal stimulation. An infusion of serotonin was used to augment bronchial muscle tone, and physiological criteria were used to establish the efficacy of each pharmacologic intervention before and after nerve stimulation.

With the tone of the airway musculature increased and the animals atropinized, we recorded two or more dilation responses to sympathetic or parasympathetic nerve stimulation; the frequencies used gave responses that were approximately 80 percent of the maximum. We then administered the beta adrenergic receptor blocking agent *dl*-propranolol hydrochloride (0.5 mg/kg) or the adrenergic neuronal blocking agent guanethidine sulfate (2 mg/kg). The adequacy of the beta adrenergic blockade was confirmed by absence of bronchodilation in response to a previously effective dose of isoproterenol hydrochloride (20 ng/kg); the adequacy of the adrenergic neuronal blockade was confirmed by loss of the carotid sinus reflex. Both propranolol and guanethidine were effective in negating the bronchodilation previously attendant on stimulation of the cervical sympathetic nerves. However, neither drug inhibited the dilation response to vagal stimulation. Figure 1B shows the results of a representative experiment with propranolol.

In further experiments, phenoxybenzamine hydrochloride (2 mg/kg) was used to block alpha adrenergic receptors. Phenoxybenzamine prevented the pressor response to a bolus injection of norepinephrine bitartrate (1 μg/kg) but did not affect the bronchodilation produced by sympathetic or vagal nerve stimulation. On the other hand, interruption of autonomic ganglionic transmission with hexamethonium chloride (2 mg/kg) markedly suppressed both sympathetic and vagal bronchodilation. On the basis of these pharmacologic studies (summarized in Fig. 2), it is evident that a nonadrenergic, multineuronal pathway serving a bronchodilation function in the pulmonary airways is present in the cervical vagus nerves of the cat.

We endeavored to locate the airways involved in vagally mediated bronchodilation by comparing the relative effects of nerve stimulation on lung resistance and dynamic compliance. We reasoned that changes in resistance would reflect involvement of large conducting airways and that changes in compliance would reflect involvement of small peripheral airways (respiratory bronchioles and alveolar ducts). Although such anatomical inferences from physiological measurements are not exact, they do provide a reasonable estimate of the probable site of an airway response (*9*). Infusion of serotonin caused lung resistance to rise and compliance to fall, indicating generalized constriction of the bronchial tree. Depending on the stimulus frequency, vagal stimulation reversed these changes to various degrees. At any given frequency, resistance changes were more completely reversed by vagal stimulation than were compliance changes (Fig. 3). For example, a 50 percent mean reversal of resistance change was achieved with a stimulus frequency of ten pulses per second; at this frequency the mean reversal of compliance change was only 18 percent. The difference between the reversal of resistance and compliance changes was significant at each frequency ($P < .05$, Student's t-test). These results imply that vagal inhibitory fibers are preferentially distributed to the conducting airways.

Vagally mediated bronchodilation usually persisted for several minutes after termination of the electrical stimulus. Since a prolonged response is not typical of synaptic transmission at other autonomic effector organs, we considered the

possibility that neurotransmitters or neurohormones released from the abdominal viscera may have circulated to the bronchial muscle and contributed to the maintenance of bronchodilation. Accordingly, we made an incision through the abdomen and diaphragm in three cats and sectioned the right and left vagus nerves 2 cm proximal to the esophageal hiatus. These animals continued to respond to stimulation of the cervical vagus nerves with prolonged bronchodilation. Thus it would appear that the common mechanisms for rapidly terminating neurotransmitter action, such as enzymatic degradation and neuronal uptake, are poorly developed or inoperative in the nonadrenergic system of airway smooth muscle control. It is also possible that this system operates through a mechanism fundamentally different from the usual forms of synaptic transmission; for example, a neuroparacrine control system in which chemical mediators are released from neurosecretory fibers and slowly diffuse through the extracellular space to reach their targets (*10*).

The physiological significance of the nonadrenergic inhibitory innervation of the airways is unknown. Possibly it is the remnant of a primitive inhibitory nervous system. Alternatively, it may serve as the dominant inhibitory nervous system in certain mammalian airways or provide redundancy for the inhibitory actions of adrenergic nerves and circulating catecholamines. Richardson and Beland (*6*) found no evidence of adrenergic inhibitory fibers in human bronchial muscle with pharmacological or histochemical techniques, and speculated that the nonadrenergic inhibitory system might function as the principal inhibitory nervous system for smooth muscle in human airways. The tracheobronchial tree and the gastrointestinal tract are both endodermic in origin, and in the latter a nonadrenergic inhibitory system is known to be the most important mechanism controlling the relaxation phase of peristalsis and the function of many sphincters (*11*). When the system is absent, as in humans with Hirschsprung's disease (*12*) or in animals whose intramural ganglion cells have been anoxically destroyed (*13*), the result is loss of inhibitory control and spasm of the gastrointestinal smooth muscle. If the nonadrenergic inhibitory system in airway smooth muscle plays a correspondingly important role in regulating bronchial muscle tone, then, as suggested by Richardson and Bouchard (*4*), a defect in the system could account for the hyperreactive airways of victims of asthma and chronic bronchitis. Such a defect could also account for the common but enigmatic clinical observation that beta adrenergic blocking agents cause bronchoconstriction in asthmatic patients but not in nonasthmatics.

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Inhibition of Cell Division and Growth by a Redox Series of Cyanine Dyes

Abstract. *A series of cyanine dyes used in photography, with reduction potentials from −1.35 to −0.20 volts, were tested for their ability to inhibit mitosis and cell growth in fertilized sea urchin eggs. Low concentrations of dyes with reduction potentials more negative than −1.0 volt generally inhibited mitosis and growth, whereas those with more positive reduction potentials did not. The active dyes penetrated the cell, entered all subcellular compartments, were bound to numerous macromolecules, and inhibited synthesis of macromolecules. Thus mitosis and growth may be retarded with substances that can alter electrochemical activity in cells.*

Electrochemical studies of dyes used in photography to control silver halide responses in emulsions (*1*) have provided a dye series with a wide range of reduction and oxidation potentials. Using this dye series, Gilman (*2*) determined the electrochemical potentials that will shut off the photoelectronic activity of silver halide substrates. The effective control of electronic events achieved with this series led us to the idea that these same dyes might also be used to modify electronic events in living cells. Control of electrochemical potentials in biochemical reactions could provide new ways of limiting abnormal or infectious cell growth in disease.

The electrochemical properties of these cyanine dyes were measured polarographically by the method of Large (*3*). A more negative reduction potential (E_R) indicates greater difficulty in reducing a dye; a more positive oxidation potential indicates greater difficulty in oxidizing the dye. When used on photographic silver halide emulsions, dyes with E_R values more negative than −1.0 V are the most efficient spectral sensitizers, and dyes with less negative E_R values are desensitizers of the normal photographic process (*2*).

Certain cyanine dyes are useful for treating parasitic infections (*4*) and can inhibit respiratory chain reactions involving electron transport (*5*). Still, few attempts have been made to systematically relate the electrochemical properties of cyanine dyes to their biological actions. The study described here was performed to determine whether such a correlation applies to fertilized sea urchin eggs.

A strong relation was found between the E_R of a cyanine dye and its ability to inhibit cell division. Methanolic solutions of 22 dyes at concentrations of 10^{-6} to $10^{-5}M$ were added to suspensions of fertilized eggs of the sea urchins *Arbacia punctulata* and *Lytechinus variegatus* (200,000 eggs per milliliter) 3 minutes after fertilization. A light microscope was used to observe and photograph the ability of the eggs to divide and form the mitotic apparatus.

The dyes were assigned a number from 0 to 5+, with 5+ representing complete mitotic inhibition and 0 indicating none. With few exceptions, the more negative the E_R value of the dye, the greater the inhibition of mitosis (Fig. 1). Dyes 15 and 20 showed anomalous behavior photographically and in our biological model. The inability of dye 5 ($E_R = -1.12$ V) to inhibit mitosis may be

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