In Vivo Demonstration of Nonadrenergic Inhibitory Innervation of the Guinea Pig Trachea

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ABSTRACT To determine if electrical stimulation of autonomic nerves could excite nonadrenergic inhibitory motor pathways in the guinea pig respiratory system in vivo, we studied the effects of electrical stimulation of the cervical vagi and sympathetic nerve trunks on pressure changes (P_p) within an isolated, fluid-filled cervical tracheal segment which reflected changes in trachealis muscle tone. We preserved the innervation and circulation of the segment as evidenced by a rise in P_p with vagus nerve stimulation and a fall in P_p with intravenous isoproterenol.

In five atropine-treated animals, stimulation of the cut vagi or sympathetic nerve trunks resulted in a mean fall in P_p of 7.9 and 8.2 cm H_2O, respectively. Treatment with propranolol attenuated the response to sympathetic stimulation but not vagal stimulation. To determine if these relaxation responses were mediated by an adrenergic or nonadrenergic mechanism, we studied an additional five animals that had been treated with 6-hydroxydopamine to destroy adrenergic nerve endings. In 6-hydroxydopamine, atropine, and propranolol-treated animals, sympathetic nerve stimulation decreased P_p only 0.65 cm H_2O, confirming the elimination of adrenergic nerve influences, whereas vagus nerve stimulation decreased P_p 17.7 cm H_2O. After sectioning the recurrent laryngeal nerves, the mean decrease in P_p during vagus nerve stimulation was only 3.2 cm H_2O. These findings demonstrate the presence of nonadrenergic inhibitory nerves in the guinea pig trachea in vivo. They further show that nonadrenergic inhibitory nerve effects are elicited during electrical stimulation of the vagus nerves and that interruption of the recurrent laryngeal nerves diminishes the magnitude of these effects.

This work was presented in part at the Annual Meeting of the Federation of American Societies for Experimental Biology, Dallas, Tex., April 1979.

Dr. Chesrown is the recipient of a Parker B. Francis Foundation Research Fellowship.

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Received for publication 2 July 1979 and in revised form 19 September 1979.

INTRODUCTION

A large body of evidence from in vitro studies indicates that smooth muscle from a number of organs is relaxed by two distinct types of autonomic nerves: sympathetic or adrenergic nerves and nonadrenergic noncholinergic inhibitory nerves (1–5). Studies in vivo suggest that nonadrenergic inhibitory nerves are responsible for most of the neural inhibition of smooth muscle tone in segments of the gastrointestinal tract (1, 6). For example, Goyal and Rattan (6, 7) demonstrated that electrical stimulation of the cervical vagi relaxed the lower esophageal sphincter entirely through a nonadrenergic inhibitory nerve mechanism. Although a number of investigators have shown nonadrenergic inhibitory nerves in airways in vitro (2–5), it is unknown whether nonadrenergic noncholinergic inhibitory nerve function can be demonstrated in the airways in vivo. To address this question, we examined the relaxation responses of the guinea pig trachea in vivo during electrical stimulation of the cervical vagal and sympathetic nerve trunks. In this report, we demonstrate the presence of nonadrenergic noncholinergic inhibitory nerves in the airways in vivo, and further show that their effects are elicited during electrical stimulation of the vagus nerves.

METHODS

We studied 10 male Hartley strain guinea pigs between 389 and 505 g body wt. They were anesthetized initially with

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. · 0021-9738/80/02/0314/07 $1.00
Volume 65 February 1980 314–320

a mixture of chloralose (50 mg/kg) and urethane (500 mg/kg) given intraperitoneally, and adequate anesthesia was maintained by supplemental doses of 10 and 100 mg/kg of each agent, respectively. To administer drugs, an intravenous catheter (0.65 mm o.d., 0.38 mm i.d.) was placed in a forelimb vein through a skin window and threaded centrally. The animal was then placed in the supine position on a warm surface (37°C). The trachea was exposed through a midline cervical incision and cannulated through an incision in the cricothyroid membrane. The tracheal cannula was then connected to a constant volume rodent ventilator (model 681, Harvard Apparatus Co. Inc., Millis, Mass.). We identified and bilaterally isolated the cervical vagal and sympathetic nerve trunks and recurrent laryngeal nerves with loose ligatures. To allow sampling of arterial blood for measurement of $PaCO_2$, PaO_2, and pH, we placed a catheter in a carotid artery and continuously perfused it at a rate of 0.02 ml/min with heparinized isotonic saline (10 U/ml).

To complete isolation of an innervated tracheal segment, we transected the lower cervical trachea taking care to preserve the continuity of the recurrent laryngeal nerves. We cannulated the thoracic trachea through this opening, connected the cannula to the ventilator, and connected a side arm from this cannula to a differential pressure transducer (Sanborn, model 268B, Hewlett Packard Medical Systems, Waltham, Mass.) to measure respiratory system pressure relative to atmosphere (P_{resp}).[1] We next cannulated the caudal end of the isolated tracheal segment with a length of polyethylene tubing (PE-240) (Fig. 1). The cannula within the cephalad end of the tracheal segment, which had been previously connected to the ventilator, was then connected to a liquid pressure transducer (model 1280, Hewlett Packard Co., Palo Alto, Calif.). This transducer was positioned at the level of the trachea to avoid hydraulic pressure effects. The transducer, cannulae, and tracheal segment were next flushed and filled with warmed (37°C) isotonic saline and the distal cannula clamped such that the transtracheal pouch pressure (P_p) was equal or nearly equal to zero. When the pouch was opened to administer drugs, care was taken to close at a P_p of 0 cm H_2O to prevent pouch distention.

To monitor changes in smooth muscle tone in a structure known (in other species) to be innervated by nonadrenergic inhibitory nerves, we measured pressure changes within the lower esophageal sphincter (P_{LES}), using a liquid-filled, perfused catheter system (8). A closed-end catheter with a small side hole, 1.0 cm from the end, was passed through the mouth into the stomach and connected to a liquid pressure transducer (model 1280, Hewlett-Packard Co.). Isotonic saline was continuously infused at 0.01 ml/min, and the catheter was slowly withdrawn from the stomach until a zone of high pressure (5–30 cm H_2O above gastric pressure) was entered. All pressures (P_{resp}, P_p, and P_{LES}) were recorded continuously on a multichannel physiological recorder.

The cervical vagus nerves were cut and the distal ends placed on bipolar electrodes, and electrically stimulated as indicated below. The cervical sympathetic nerve trunks were not cut but placed intact on bipolar stimulating electrodes and electrically stimulated. All nerve stimulations were made with 5-V pulses of 2 ms duration at 15 Hz (model S44, Grass Instrument Co., Quincy, Mass.). Each stimulation was continued for 60–90 s. Succinylcholine (10–30 μg, i.v.) was given intermittently 1–3 min before stimulation to induce skeletal muscle paralysis during stimulations. Succinylcholine had no effect on P_p or P_{resp}.

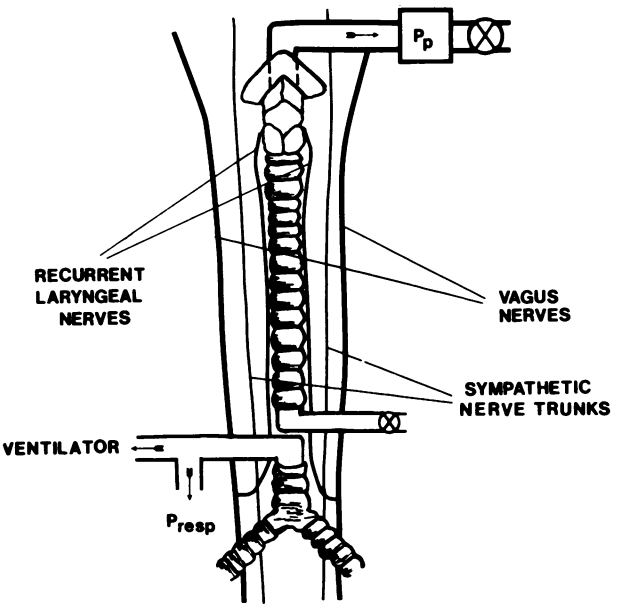

FIGURE 1 Tracheal pouch neuroanatomic relationships.

Urethane, α-chloralose, histamine diphosphate, isoproterenol hydrochloride, atropine sulfate, propranolol hydrochloride, carbamylcholine chloride, and 6-hydroxydopamine were purchased from Sigma Chemical Co., St. Louis, Mo., and succinylcholine chloride from Burroughs Wellcome Co., Research Triangle Park, N. C. All drug doses were calculated as weight of the free base. Divided doses of intravenous 6-hydroxydopamine (6-OHDA) were given to each of five animals under light ether anesthesia. The first dose of 50 mg/kg was given 7–9 d before the experiment, and a second dose of 100 mg/kg was given 1–2 d before the experiment (3).

At the end of most experiments, we confirmed the integrity of the circulation to tracheal segments prepared as described by observing either an increase in P_p after a single intravenous dose of 30 μg histamine or a decrease in P_p after a single large dose of 1.0 mg isoproterenol hydrochloride.

In this experimental model, we used changes in P_p as an index of changes in trachealis muscle tone. Contraction of the trachealis muscle will result in an increase in P_p above atmospheric pressure. Because there was probably some degree of tone in the trachealis muscle under the conditions of our experiments when the tracheal segment was closed, we interpreted the fall in P_p below atmospheric pressure to result from outward recoil of the cartilaginous rings when relaxation of the trachealis muscle occurred. To examine the range of P_p resulting from maximal contraction or relaxation, we prepared three additional animals for measurement of P_p in the manner described. We induced maximal contraction by topical application of carbamylcholine (20 μM), which resulted in an increase in P_p of 19.7±4.1 cm H_2O (mean±SE), and maximal relaxation by topical application of isoproterenol (1 mM), which resulted in a decrease in P_p of 13.0±1.5 cm H_2O (mean±SE) below atmospheric pressure.

There are several limitations to this experimental model. The amount of tone within the trachealis muscle is unknown at the time the segment is closed when P_p equals atmospheric pressure. It is also undefined how changes in P_p relate quantitatively to changes in trachealis muscle

[1] *Abbreviations used in this paper*: 6-OHDA, 6-hydroxydopamine; P_{LES}, pressure within the lower esophageal sphincter; P_p, pouch pressure; P_{resp}, respiratory system pressure.

tone. Further, if the system is opened during the experiment, allowing zero P_p to reflect a new level of trachealis muscle tone, subsequent changes in P_p cannot be quantitatively compared to earlier measurements. To avoid some of these difficulties in interpretation, we have compared the effects of a given intervention on P_p to preintervention levels within each animal, thus allowing each animal to serve as its own control. Statistical analysis of results was made using Student's *t* test for paired variates.

RESULTS

Guinea pigs without 6-OHDA treatment. In each of five animals, we initially confirmed that excitatory parasympathetic innervation of the tracheal segment was intact by observing an increase in P_p during brief (5–15 s) electrical stimulation of the intact vagus nerves. The mean increase in P_p with this stimulation was 11.0±3.2 cm H_2O (mean±SE). The increase was statistically significant, $P < 0.05$. There was no change in P_p with bilateral cervical vagotomy.

After atropine administration (5.0 mg/kg, i.p.), electrical stimulation of the cut vagi resulted in a significant ($P < 0.01$) decrease in P_p of 7.9±1.7 cm H_2O (mean±SE). The results from each animal before and after atropine treatment are summarized in Fig. 2. Electrical stimulation of the sympathetic trunks also significantly ($P < 0.05$) decreased P_p (Fig. 3). On the average, the magnitude of the decrease, 8.2±2.8 cm H_2O (mean±SE) was similar to that observed during vagal stimulation. Because the decrease in P_p observed during sympathetic nerve stimulation was probably mediated by a β-adrenergic mechanism, we administered the β-adrenergic receptor antagonist, propranolol, (10 mg/kg, i.p.) (9, 10) in an attempt to block this inhibitory activity. After propranolol, baseline P_p increased over 10–20 min in three of five animals and was unchanged in the other two. In spite of the increased P_p after propranolol, the subsequent relaxation responses induced by electrical stimulation of the sympathetic nerves were attenuated, but their magnitude remained of statistical significance, $P < 0.05$ (Fig. 3). In contrast, the relaxation responses observed during stimulation of the vagus nerves were not attenuated by the β-adrenergic receptor antagonist (Fig. 4). In an attempt to increase the effectiveness of the β-adrenergic receptor blockade, we next flushed and filled the tracheal segments of these five animals with 10 μM propranolol. Again significant ($P < 0.01$) relaxation responses induced by sympathetic nerve

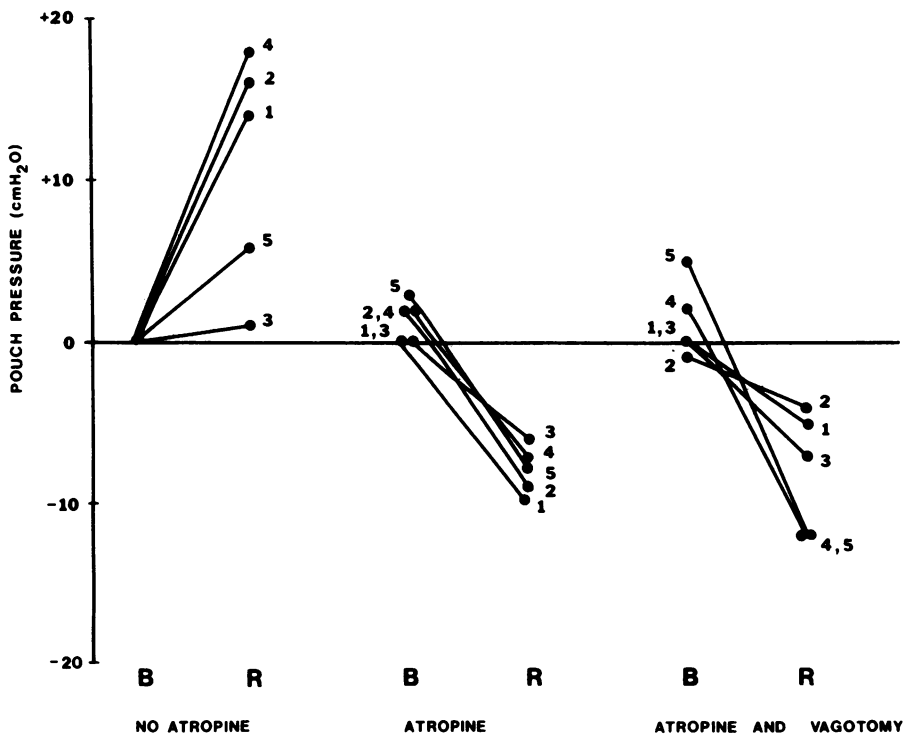

FIGURE 2 Effects of electrical stimulation of the vagus nerves on tracheal pouch pressure before and after treatment with atropine and before and after atropine and vagotomy in guinea pigs. Data from each animal are indicated by a number (1–5). B = base-line value, R = maximal response during stimulation. The magnitude of the increase in P_p during vagal stimulation is of statistical significance ($P < 0.05$), as is the decrease in P_p after atropine ($P < 0.01$) and atropine and vagotomy ($P < 0.05$).

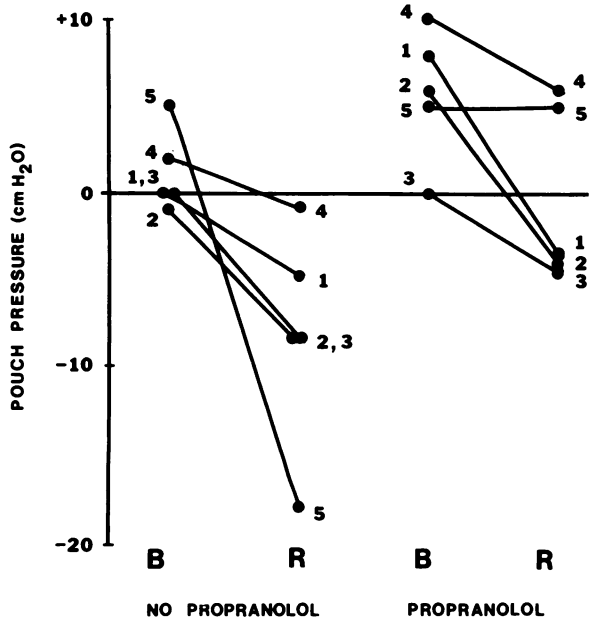

FIGURE 3 Effects of stimulation of cervical sympathetic nerve trunks on tracheal P_p in five atropine-treated guinea pigs before and after intraperitoneal treatment with propranolol. Numbers are the same as in Fig. 2. B = base-line value, R = maximal response during stimulation. The magnitude of the decrease in P_p is of statistical significance ($P < 0.05$) both before and after propranolol treatment.

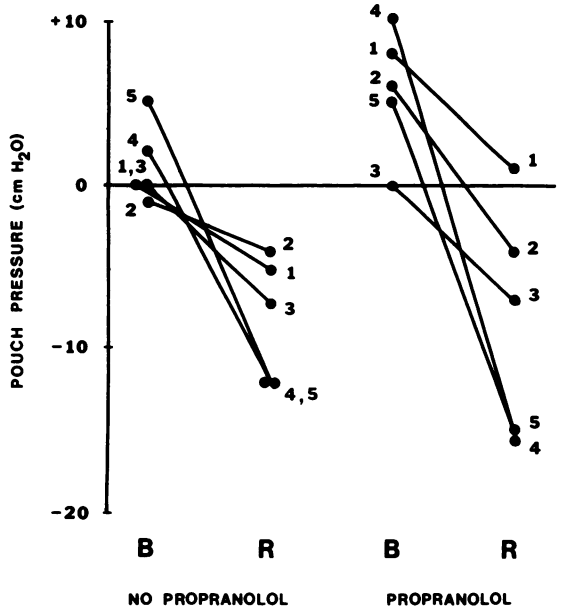

FIGURE 4 Effects of stimulation of the cut vagi on tracheal P_p in five atropine-treated guinea pigs before and after intraperitoneal treatment with propranolol. Numbers refer to the same guinea pigs as in Fig. 2. B = base-line value, R = maximal response during stimulation. The decrease in P_p is of statistical significance, $P < 0.05$, both before and after propranolol treatment.

stimulation occurred with the mean decrease in P_p being 4.2±0.9 cm H_2O. Stimulation of the vagi continued to result in significant ($P < 0.05$) decreases in P_p, the mean decrease was 13.0±3.4 cm H_2O. To be certain that the decreases in P_p observed during stimulation of the vagus nerves did not result from release of a humoral agent, we repeated vagal stimulation in two animals after abruptly stopping the circulation (large right ventriculotomy or air bolus, 20 cm^3). The tracheal relaxations induced by vagus nerve stimulation immediately after stopping the circulation were similar to those observed with the circulation intact.

In contrast to P_p, P_{resp} increased significantly ($P < 0.01$) during each stimulation of the vagus nerves, both before (30.8±3.8 cm H_2O) and after (17.0±4.7 cm H_2O) atropine. Sympathetic stimulation had no effect on P_{resp} unless P_{resp} had been elevated by vagus nerve stimulation. In this circumstance, electrical stimulation of the sympathetic nerves resulted in a fall in P_{resp}.

Technically adequate recordings of P_{LES} were successfully made in three of five animals. In each of these three, vagus nerve stimulation caused an abrupt decrease in P_{LES} to near atmospheric levels. This response was not altered by either atropine (5.0 mg/kg, i.p.) or propranolol (10 mg/kg, i.p.). Stimulation of the intact sympathetic nerve trunks did not change P_{LES} in one of these animals and decreased it to near atmospheric in the other two. In these two animals, the decrease in P_{LES} was not changed after propranolol administration.

Guinea pigs treated with 6-OHDA. As noted, the tracheal relaxation during sympathetic nerve stimulation was attenuated but not abolished by propranolol treatment. We could not, therefore, be sure from these data whether this nerve-induced relaxation was due to an incomplete β-adrenergic blockade or to noncholinergic inhibitory nerves. To help resolve this issue, we studied an additional five guinea pigs that had been treated with 6-OHDA, a drug which is taken up into norepinephrine containing adrenergic nerve terminals and damages them, resulting in a chemical sympathectomy (3, 11). The five animals treated with this drug were otherwise prepared for physiological measurements in an identical manner as the animals not given 6-OHDA. After administration of atropine (5.0 mg/kg, i.p.), electrical stimulation of the distal end of the cut vagi resulted in a significant fall in P_p, 4.7±1.1 cm H_2O; however, in these animals, sympathetic nerve stimulation had inconsistent and statistically insignificant effects (Fig. 5). The tracheal segment was next flushed and filled with 10 μM propranolol in saline. This intervention resulted in a marked increase in P_p, over 10–20 min in each animal, most likely reflecting

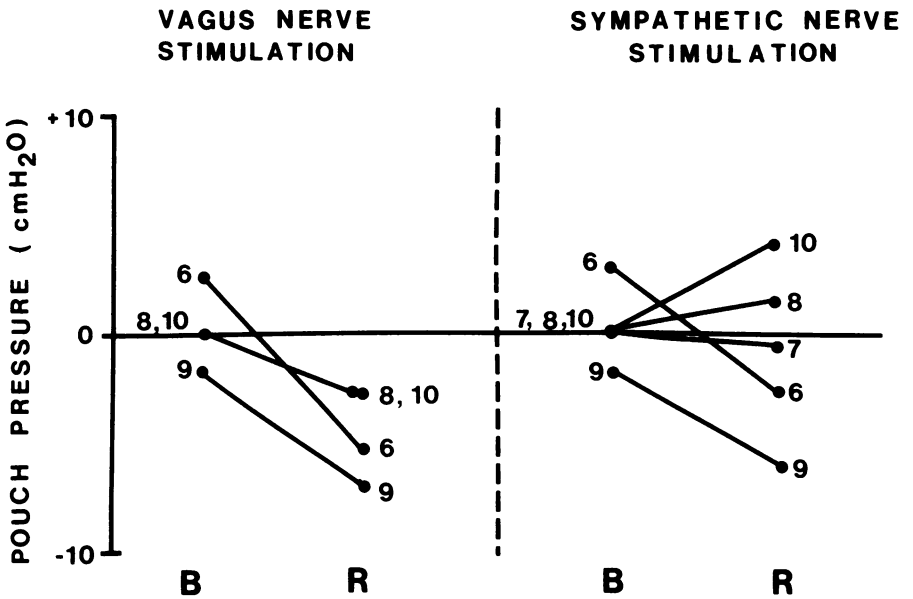

FIGURE 5 Effects of stimulation of the cut vagi and intact sympathetic nerve trunks stimulation on tracheal P_p in five guinea pigs treated with 6-OHDA and atropine, animal numbers 6–10. Vagal stimulation in animal 7 was not done. B = base-line value, R = maximal response during stimulation. The decrease in P_p during vagal stimulation was of statistical significance, $P < 0.05$, whereas the magnitude of the change in P_p during sympathetic stimulation was not.

blockade of the β-adrenergic effects of circulating catecholamines from the adrenal glands, which are not destroyed by 6-OHDA, because their terminals contain mostly epinephrine, not norepinephrine. Even with the increased base-line trachealis muscle tone resulting from propranolol treatment, subsequent sympathetic nerve stimulation resulted in no significant change in P_p (Fig. 6), confirming that we had abolished the sympathetically mediated relaxation with 6-OHDA, and indicating that nonadrenergic inhibitory nerves were not stimulated during sympathetic nerve stimulation. However, with base-line P_p increased after propranolol, stimulation of the distal ends of the cut vagus nerves resulted in a significant ($P < 0.02$) fall in P_p (Fig. 6). The magnitude of this decrease was 17.5±3.2 cm H_2O, which is significantly ($P < 0.05$) greater than the decrease in P_p with vagal stimulation before propranolol was placed in the pouch.

We next sectioned both recurrent laryngeal nerves in each of these animals. The tracheal relaxation induced during stimulation of the vagi was significantly ($P < 0.05$) attenuated after this intervention (Fig. 7).

Treatment with 6-OHDA did not alter the inhibitory effects of vagus nerve stimulation on P_{LES} in the four animals in which P_{LES} was measured. In each of these animals, as in the animals without 6-OHDA treatment, vagus nerve stimulation after treatment with atropine (5.0 mg/kg) still significantly ($P < 0.01$) increased P_{resp} (31.5±2.1 cm H_2O). Stimulation of the sympathetic nerve trunks in the 6-OHDA-treated animals did not change P_{resp} even when P_{resp} had been increased by prior vagus nerve stimulation.

DISCUSSION

A number of investigators have demonstrated the presence of nonadrenergic inhibitory nerves in isolated airway smooth muscle in vitro (2–5). In general, these investigators have used electrical field stimulation of isolated tissues in the presence of atropine and propranolol to demonstrate nonadrenergic inhibitory nerve activity. Our studies in vivo employing electrical stimulation of the sympathetic and vagus nerve trunks were prompted by their findings, but we found it necessary to use 6-OHDA treatment to remove adrenergic nerve inhibitory effects. Our findings confirm the presence of these nonadrenergic inhibitory fibers in vivo and clearly delineate their anatomic pathways to the trachealis muscle.

In these experiments, we have demonstrated in vivo that respiratory smooth muscle tone in the guinea pig can be inhibited by two distinct neural mechanisms. As might be expected, electrical stimulation of the sympathetic nerve trunks induced tracheal relaxation which was attenuated by a competitive β-adrenergic receptor antagonist and was abolished completely by treatment with 6-OHDA, a drug which—in the doses

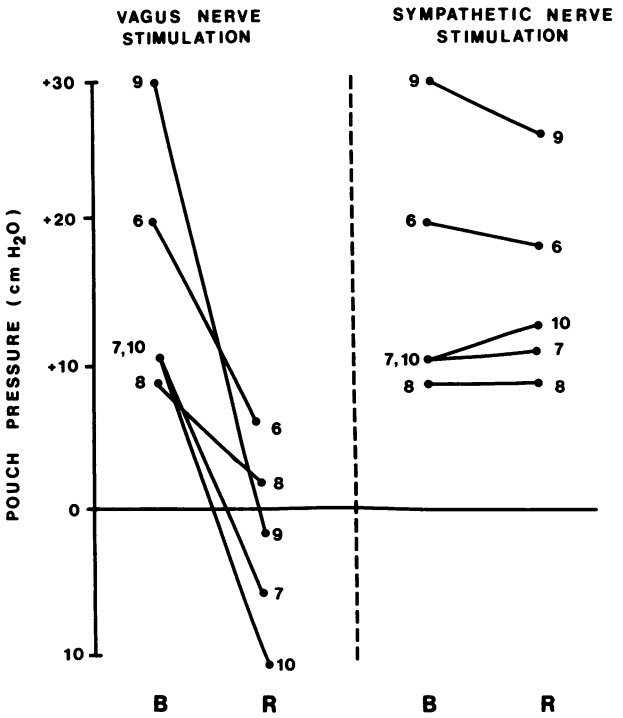

FIGURE 6 Effects of stimulation of the cut vagi and sympathetic nerve trunks on tracheal P_p in five guinea pigs treated with 6-OHDA, atropine, and propranolol. Animal numbers are the same as in Fig. 5. B = base-line values, R = maximal response during stimulation. The decrease in P_p during vagal stimulation was of statistical significance, $P < 0.02$, whereas the change in P_p during sympathetic stimulation was not.

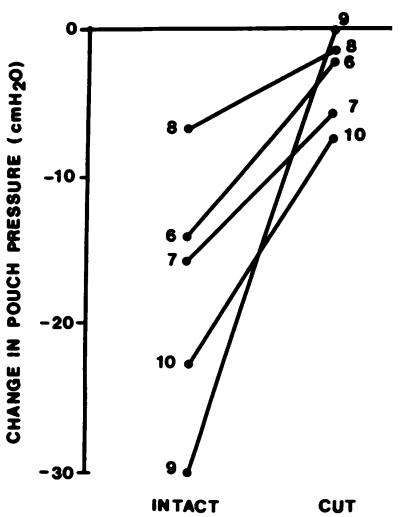

FIGURE 7 Effect of stimulation of the cut vagi on tracheal P_p before and after sectioning the recurrent laryngeal nerves in five guinea pigs treated with atropine, propranolol, and 6-OHDA. The numbers correspond to those in Figs. 5 and 7. The magnitude of the effect lost with recurrent laryngeal nerve section was of statistical significance, $P < 0.05$.

used—destroys many nerve endings containing norepinephrine (3). However, in the presence of atropine, electrical stimulation of the vagus nerves also induced relaxation of the trachealis muscle which was not attenuated by propranolol or 6-OHDA, and most likely reflects activation in vivo of nonadrenergic noncholinergic inhibitory nerves. Our conclusion that we have demonstrated a separate inhibitory mechanism in vivo depends on our being able to determine that we had effectively eliminated adrenergic inhibitory activity in the tracheal pouch. To do so, it was first necessary to demonstrate that we could completely abolish the inhibitory effects of stimulation of the sympathetic trunks on the tracheal pouch. To accomplish this goal, we used 6-OHDA, a compound which effects a chemical sympathectomy by destruction of norepinephrine containing nerve endings. Because the effectiveness of the chemical sympathectomy may vary from organ to organ (11), we used a treatment regimen known to diminish catecholamine fluorescence (3), and further demonstrated that electrical sympathetic nerve stimulation did not relax the tracheal pouch. Thus, we were able to use 6-OHDA-treated animals to explore the possibility of nonadrenergic inhibitory pathways.

In these 6-OHDA-treated animals, electrical stimulation of the cut vagi (after atropine treatment) resulted in a fall in tracheal pouch pressure (Fig. 5), which is consistent with the hypothesis that nonadrenergic inhibitory fibers to the trachea run in the vagus nerves. The fall in pouch pressure was more marked after increasing trachealis muscle tone by filling the tracheal pouch with propranolol (Fig. 6). This suggests that the nonadrenergic inhibitory mechanism is a potent one in the presence of preexisting airway tone. We further found in these five animals that section of the recurrent laryngeal nerves dramatically reduced the inhibitory effects of vagal stimulation. These data indicate that the major pathway for eliciting nonadrenergic inhibitory nerve effects in the airways resides in the vagi and reaches the trachealis muscle via the recurrent laryngeal nerves.

Although we were able to demonstrate inhibition of trachealis muscle tone by vagal stimulation in the presence of atropine, P_{resp} consistently increased with this intervention. This probably resulted from our administering insufficient quantities of atropine to effect an adequate blockade of the excitatory effects of vagal stimulation of the lung. Because we could easily block the excitatory effects of vagal stimulation in the trachea, other possible explanations of our results include a much greater density of excitatory cholinergic innervation in the lung, different affinities of cholinergic receptors in the trachea and lung, or perhaps an intrapulmonary noncholinergic

nonadrenergic excitatory neurotransmitter. Our experiments do not allow us to determine which, if any, of these possible explanations are correct.

In the gastrointestinal tract, nonadrenergic inhibitory activity has been shown to be the major inhibitory mechanism controlling smooth muscle tone (1, 6). We demonstrated that the airway relaxation resulting from vagal stimulation was accompanied by relaxation of the lower esophageal sphincter. The nonadrenergic inhibitory control of this sphincter has been previously demonstrated to be elicited during vagus nerve stimulation (6, 7). Thus, our observations confirm their findings and extend them by demonstrating that the vagal inhibition of both lower esophageal sphincter tone and trachealis muscle tone is not affected by 6-OHDA.

The physiological role of these nerves in the control of airway tone has been a matter of considerable speculation (1, 5). This demonstration of their function in vivo provides further evidence of their possible importance. It is clear that their inhibitory function may vary from species to species. For example, nonadrenergic inhibitory nerves have not been demonstrated in canine airways in vitro (12), and appear to have their major influence in the guinea pig on the trachealis muscle, although they may have more pronounced pulmonary effects in the cat (13). Because nonadrenergic inhibitory activity can be demonstrated in both large and small human airways in vitro (5), it is tempting to speculate that absence of this inhibitory mechanism could play a role in the airways hyperreactivity which characterizes asthma.

ACKNOWLEDGMENTS

The authors thank Dr. Roland H. Ingram for reviewing this manuscript, Ms. Reidun Johansen-Khan for secretarial assistance, and Ms. Diane Saunders for helping with the illustrations.

This work was supported by National Institutes of health grants HL-17382 and 00549, the American Heart Association, and the Parker B. Francis Foundation.

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