Differential Susceptibility of Tracheal Contraction to Nonadrenergic Noncholinergic Relaxation

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ABSTRACT
Studies of nonadrenergic noncholinergic inhibitory (NANC) nerve-induced relaxations routinely examine relaxations in airway tissue in which tone has been established. Little is known about the ability of NANC nerve stimulation to prevent airway smooth muscle contraction. The present study compares the capacity of NANC nerve stimulation to prevent or reverse airway smooth muscle contraction. NANC nerves in the trachea from ovalbumin-sensitized guinea pigs were subjected to electrical field stimulation (EFS, 10 Hz, 0.3 ms, 10 V, 35 min) initiated before or after induction of tone with antigen or histamine. In tissues precontracted with histamine or antigen, EFS elicited a rapid relaxation which peaked within the first 5 min and stabilized by 20 to 35 min. The peak relaxation was smaller in tissues precontracted with antigen, an effect that was not prevented by tissue treatment with a nitric oxide synthase inhibitor. In contrast, the stabilized level of NANC relaxation did not differ between histamine- or antigen-contracted tissues. Activation of NANC nerves prior to induction of tone also resulted in inhibition of the contractile actions of histamine and antigen. However, the stabilized level of tone induced by the contractile agonist added after initiation of EFS was greater than the stabilized tone caused by EFS in tissues already contracted with the same agonist. Relaxations elicited by S-nitrosoglutathione were reduced in antigen-precontracted tissues whereas vasoactive intestinal peptide-induced relaxations were similar in antigen- and histamine-precontracted tissues. Results of this study suggest that NANC nerve activation is more effective at relaxing established airway smooth muscle tone than at preventing airway smooth muscle contraction. Further, the results suggest that the difference in NANC activity in antigen-precontracted tissues cannot be ascribed solely to reductions in the nitric oxide-dependent component of the response.

Nonadrenergic noncholinergic inhibitory nerves represent a primary inhibitory innervation of the airways (Diamond and Altiere, 1996). Although the specific neurotransmitter(s) of these nerves remain to be established with certainty in all species, VIP and NO are implicated in NANC responses in the guinea pig trachea (Ellis and Farmer, 1989; Tucker et al., 1990; Li and Rand, 1991; Said, 1991). Investigation of NANC nerve-mediated relaxation focuses on determining the magnitude of EFS-induced reversal of tone. This procedure generally involves application of a contractile agonist such as histamine. Previous studies have determined that relaxant agonists are less efficacious in preventing airway smooth muscle contraction than in reversing established smooth muscle contraction (Hay et al., 1986, 1988; Gustafsson and Persson, 1991). It is unknown whether airway smooth muscle contraction demonstrates similar differential susceptibility to NANC nerve-induced relaxations.

NANC bronchodilator responses have been demonstrated in vivo. In humans in which bronchomotor tone has been elevated by application of a bronchoconstrictor agonist, NANC responses can be activated reflexively by mechanical stimulation of the larynx (Michoud et al., 1988) or by inhalation of an irritant, such as capsaicin (Lammers et al., 1989). Similar experiments have been conducted in experimental animals (Inoue et al., 1989). NANC responses are usually studied after tone has been induced in the airways. Szarek and colleagues (1986) demonstrated NANC bronchodilator responses by laryngeal stimulation in bronchoconstricted cats, but blockade of NANC nerves failed to modulate of serotonin-induced bronchoconstriction. Based on these results, it may be speculated that NANC nerves are more effective at reversing established bronchoconstriction than at preventing bronchoconstriction.

The present study assesses the relative ability of NANC nerve activation to reverse established contraction compared with the ability to prevent impending contraction of the guinea pig isolated trachea. In addition, alterations in the activities of the putative mediators of NANC inhibitory re-

ABBREVIATIONS: NANC, nonadrenergic noncholinergic inhibitory; KHS, Krebs-Henseleit solution; EFS, electrical field stimulation; VIP, vasoactive intestinal peptide; NO, nitric oxide; NNA, N(ω)-nitro-L-arginine.
responses, VIP and NO, in antigen-contracted tissues are investigated.

**Methods**

Male guinea pigs (200–250 g) were sensitized to ovalbumin by the method of Andersson (1980). Ovalbumin was adsorbed to aluminum hydroxide (10 mg/ml) and administered by a single intraperitoneal dose (40 μg/kg). Fourteen to twenty-one days later, animals were anesthetized with pentobarbital (65 mg/kg i.p.). The trachea and lungs were removed en bloc and placed in KHS containing 3 μM indomethacin, and four tracheal ring segments (4–5 mm in length) were obtained from each animal. Each ring segment was mounted on stainless steel tissue hooks between platinum plate electrodes in 15-ml glass-jacketed organ baths containing KHS maintained at 37°C and gassed with 5% CO2 in O2 under an initial load of 3 g. Tissues were allowed to equilibrate for 60 min, during which time the bathing solution was exchanged at 10 min intervals. At the end of the equilibration period, the response to a maximal concentration of acetylcholine (1 mM) was recorded. After washout of acetylcholine and re-establishment of stable base-line tone, tissues were treated with atropine (1 μM) to prevent neurally mediated cholinergic responses. Two experimental protocols were then followed:

**Precontracted tissues.** Two ring segments were precontracted with histamine (1 μM) or ovalbumin (1 ng/ml). Fifteen minutes thereafter (at which time the contractile response had reached a plateau), EFS (10 Hz, 0.3 ms pulse duration, 10 V amplitude) was applied for a period of 35 min. On termination of EFS, tissues were allowed to recover tone. In additional experiments, tissues were exposed to the NO donor, GSNO (20 μM), or VIP (1 μM) rather than to EFS. In a final series of experiments, a frequency-response curve to EFS (0.1–100 Hz, 0.3 ms pulse duration, 10 V amplitude, 5 s period at 60 s intervals) was established in tissues precontracted with either histamine (1 μM) or ovalbumin (1 ng/ml).

**Postcontracted tissues.** Two ring segments were subjected to EFS (10 Hz, 0.3 ms pulse duration, 10 V amplitude) for a 35 min period. Five minutes after commencing EFS, histamine (1 μM) or ovalbumin (1 ng/ml) was applied to the tissue. On termination of EFS, tissues were allowed to recover tone.

In all experiments, propranolol (1 μM) and guanethidine (10 μM) were present continuously in the bathing solution to abolish adrenergically mediated inhibitory responses. Relaxations evoked by EFS were defined operationally as being nonadrenergic noncholinergic (NANC) in nature. On termination of EFS, tissues were allowed to recover tone. The tone of precontracted and of postcontracted tissues was measured immediately before termination of EFS (when the relaxation response had stabilized) and after recovery of tissue tone after termination of EFS (fig. 1, inset). Induced tone immediately before EFS also was measured in precontracted tissues. All responses were expressed as percent of response to 1 mM acetylcholine. In several experiments, the time course of changes in tone was monitored in precontracted and postcontracted tissues.

Throughout the study, paired preparations were used so that four adjacent segments of trachea were obtained from each animal; two were subjected to EFS after tone had been established and the other two had tone induced after EFS had been initiated. To identify the contribution of NO to EFS-induced NANC responses, tracheal segments were incubated with the NO synthase inhibitor, NNA (0.1 mM), for 30 min before application of histamine or ovalbumin. This concentration of NNA has previously prevented acetylcholine-induced, NO-dependent relaxations in rabbit blood vessels (Altiere and Thompson, 1992). All responses were measured isometrically with Grass FT.03c force displacement transducers and displayed on a Maclab 8/Macintosh computer system. Tissues were stimulated with square wave pulses generated by a Grass S-88 stimulator in series with a multichannel signal conditioner ( Stimu-Splitter, Med Lab Instruments, Fort Collins, CO). In frequency-response studies, EFS (5 s period) elicited rapid relaxation which did not recover to pre-stimulation levels before the subsequent stimulation. Accordingly, the frequency-response curve should be considered as pseudocumulative. The following pharmacological agents were used: acetylcholine perchlorate, atropine sulfate, histamine dihydrochloride, NNA, ovalbumin (grade V), propranolol hydrochloride, porcine vasoactive intestinal peptide (Sigma Chemical Company, St. Louis, MO), guanethidine monosulfate (Ciba Geigy, Summit, NJ). All drugs were dissolved and diluted in KHS.

Statistical comparisons were performed by analysis of variance for repeated measures and post hoc unpaired Student’s t-tests, with differences of P < .05 being considered significant.

![Graph](image)

**Fig. 1.** Magnitude of NANC responses in precontracted and postcontracted tissues. Sensitized guinea pig trachea were contracted with histamine or antigen (denoted by filled dot of inset) before (i.e., precontraction; upper panel of inset) or after (i.e., precontraction; lower panel of inset) initiation of 35 min of EFS (10 Hz, 0.3 ms pulse duration, 10 V amplitude). After termination of EFS, tissue tone was allowed to recover or stabilize. Tissue tone was measured immediately before commencement of EFS (in precontracted tissues only) (A), immediately before termination of EFS (B) and at the peak of recovered tone (C) and expressed as a percentage of the response to 1 mM acetylcholine. Data represent the means ± S.E.M. from four to eight experiments. *P < .05, unpaired Student’s t test, compared with tone in postcontraction tissues treated with the same agonist.
Results

Ovalbumin elicited concentration-dependent contractions in tracheae from sensitized animals. The selected concentrations of ovalbumin- and histamine-evoked contractile responses of similar magnitude (≈55–80% maximal acetylcholine response) which stabilized within 15 min of the addition of agonist. Prolonged EFS elicited a rapid relaxation response in precontracted tissues which peaked within the first 5 min and stabilized at a lower magnitude in the ensuing 30 min. The magnitude of the rapid peak relaxation was smaller in ovalbumin-precontracted tissues than in those contracted with histamine (fig. 2). However, the magnitude of the stabilized relaxation response (30–35 min after EFS onset) was not different between ovalbumin- and histamine-precontracted tissues (fig. 2). After terminating EFS, tissues recovered tone to levels which were similar to those before initiation of EFS (fig. 1). In the frequency-response studies, the magnitude of EFS-induced relaxations in tissues precontracted with histamine were similar to those precontracted with ovalbumin (fig. 3). In other experiments, EFS was initiated 5 min before addition of histamine or ovalbumin administration, i.e., postcontracted. EFS exerted no significant effects on tone during this period (data not shown). Discontinuation of EFS resulted in the tissues contracting further, with magnitudes of contraction similar to those observed before and after EFS application in the precontraction experiments (fig. 1). Comparison of the magnitude of the tone of tissues immediately before cessation of EFS (i.e., the level of tone when tissues are contracted and NANCi responses had stabilized; point B in fig. 1 inset) demonstrated that NANCi nerve-induced relaxations were greater in precontracted than in postcontracted tissues (fig. 1).

Preincubation of tissues with the NO synthase inhibitor, NNA, resulted in significant inhibition of NANCi responses during the first 15 min in histamine- and ovalbumin-precontracted tissues (fig. 4). Nevertheless, the magnitude of the NANCi response during the first 14 min of EFS in NNA-treated, ovalbumin-precontracted tissues was significantly less than that in NNA-treated, histamine-precontracted tissues. However, no differences existed between the relaxations in these tissues after the relaxation response had stabilized, i.e., 20 to 35 min after EFS initiation (fig. 4).

To examine which components of the NANCi responses were repressed by ovalbumin, the time course of the relaxant effects of the two putative mediators, NO and VIP, were examined in histamine- and ovalbumin-precontracted tissues. The NO donor, GSNO, induced rapid relaxation in precontracted tissues which peaked 2 to 3 min after application to the bathing solution. The relaxant effect was significantly reduced in tissues contracted with ovalbumin during the early time period (2–15 min) after GSNO application (fig. 5). No differences were apparent in the magnitude of the...
response in the latter stages of relaxation (20–35 min) when the response had stabilized (fig. 5). VIP provoked a more slowly developing relaxation which peaked 15 to 20 min after application (fig. 5). There were no differences in the magnitude or time course of the relaxant response in tissues precontracted with histamine or ovalbumin.

**Discussion**

Results of the present study demonstrate that the magnitude of the inhibitory effect of NANCi nerve stimulation on tone of the guinea pig trachea depends on the sequence of nerve activation relative to induction of tone in the tissue. Initiation of NANCi responses after tone had been established resulted in greater functional antagonism of the induced tone than when NANCi nerves were activated before induction of tone. Notably, this effect occurred independently of the agent used to contract the tracheal smooth muscle insofar as the precontraction versus postcontraction difference in functional antagonism was observed in tissues in which tone had been induced with either histamine or antigen.

Undem and colleagues (1989) demonstrated previously that continuous EFS of the guinea pig trachea initiated before antigen application resulted in suppression of the antigen-induced contractile response. The nature of the inhibitory effect appeared to involve functional antagonism at the level of the smooth muscle cell, rather than modulation of mediator release. EFS did not affect antigen-induced release of histamine or leukotrienes, the primary mediators of the antigen-induced contraction in the guinea pig trachea (Undem et al., 1989). Although the parameters of stimulation in the present study (10 Hz, 0.3 ms pulse duration) were less intense than those used by Undem and co-workers (1989) (16 Hz, 1 ms pulse duration), the degree of inhibition of the antigen-induced contraction attributable to NANCi nerve activation was remarkably similar at approximately 20%.

The time course of NANCi responses in sensitized tissues precontracted with histamine or antigen also was evaluated in the present study. The magnitude of the NANCi responses in histamine- and antigen-precontracted tissues were similar when stimulation periods were brief (5 s, as in the frequency-response experiments) or after prolonged periods of stimulation when relaxation responses had stabilized, i.e., 25 to 30 min. By contrast, NANCi responses were diminished in antigen-precontracted tissues (relative to those precontracted with histamine) during intermediate stimulation periods (30
s to 15 min). The underlying cause of the reduced relaxations induced by NANCi nerve activation may be related to neurotransmitter metabolism or the nature of the contractile agonist.

Mast cell-derived proteases have been reported to degrade VIP (Caughey et al., 1988; Lilly et al., 1994), one of the putative mediators of NANCi responses in the guinea pig trachea. In vivo, NANCi and VIP-induced bronchodilator responses are attenuated by a protease-dependent mechanism in the antigen-challenged cat (Miura et al., 1992). Such proteases released from antigen-activated mast cells would be predicted to attenuate relaxations mediated by VIP or other relaxant peptides from NANCi nerves (Caughey et al., 1988; Franconi et al., 1989; Tam and Caughey, 1990). On the other hand, antigen-induced contraction of the sensitized guinea pig trachea is mediated by histamine and other mediators, primarily leukotrienes (Muccitelli et al., 1987; Undem et al., 1989). Accordingly, an alternative mechanism for the reduced activity of NANCi nerve activation may reflect a diminished capacity of NANCi transmitter(s) to functionally antagonize contractile mediators released in addition to histamine.

To investigate these possibilities, relaxations induced by a single concentration of VIP or of the NO donor, GSNO, were evaluated in tissues precontracted with either antigen or histamine. The time course and magnitude of the VIP-induced relaxation in tissues precontracted with antigen were similar to those seen in histamine-precontracted tissues, arguing against a role for mast cell-derived proteases in abrogating VIP-induced relaxant responses in the sensitized guinea pig trachea. The absence of a difference in the relaxation responses also fails to provide support for the notion that contractile mast cell mediators (apart from histamine) alter the relaxant effects of VIP. In examination of the NO donor, the magnitude of the GSNO-induced relaxant response was shown to be suppressed in antigen-precontracted tissues. Notably, the relaxation was reduced in the intermediate period after GSNO addition, i.e., 2 to 15 min.

Taken together, these results are consistent with the diminished NANCi response in antigen-precontracted tissues being caused by a reduced capacity of the NO component to reverse contractions elicited by mast cell-derived mediators. If this were the case, inhibition of NO production during NANCi nerve activation would prevent the difference in the magnitude of the NANCi responses in antigen- and histamine-precontracted tissues. However, in the present study, inhibition of NO synthase attenuated the NANCi response in both antigen- and histamine-precontracted tissues. This is consistent with the purported role for NO in the NANCi neural response in the guinea pig trachea (Tucker et al., 1990; Li and Rand, 1991). Because the responses remained smaller in magnitude during the intermediate period of EFS in antigen-precontracted tissues than those precontracted with histamine, it may be concluded that selective inhibition of the NO component is not entirely responsible for diminished NANCi responses in antigen-precontracted tissues. These considerations notwithstanding, the present results emphasize the need for caution in interpreting results with a specified duration of nerve stimulation, particularly in tissues in which multiple transmitters may be mediating the response.

Through observation of the effects of a NO synthase inhibitor and α-chymotrypsin on the time course and magnitude of NANCi responses in the guinea pig trachea, Tanigata and Uchiyama (1996) proposed that NO mediated the early stages of the relaxation and VIP the latter stages of the relaxation. The present studies provide circumstantial support for this contention. First, NO synthase did not affect the magnitude of NANCi responses during the latter periods of EFS, i.e., after 15 min stimulation. Second, the magnitude of the NANCi response during the latter period of EFS was similar in tissues precontracted with either antigen or histamine. This corresponds well with the lack of difference in the magnitude of the relaxation induced by exogenously applied VIP in antigen- and histamine-precontracted tissues.

A reduced ability of relaxant agonists to prevent isolated airway smooth muscle contraction rather than to reverse established contraction has been reported previously in the guinea pig trachea for salbutamol inhibition of carbachol- and leukotriene-induced contractions (Hay et al., 1986, 1988) and for terbutaline and theophylline interactions with carbachol- and histamine-evoked increases in airway smooth muscle tone (Gustafsson and Persson, 1991). Similar results were obtained in the present study. The magnitude of the relaxation induced by prolonged NANCi nerve stimulation was reduced when EFS was initiated before antigen administration (postcontraction) than when applied after activation of antigen (precontraction). Such an effect could be explained by EFS enhancing antigen-induced release of mediators from tracheal mast cells. Arguing against this possibility, however, is the observation that NANCi nerve activation fails to affect antigen-induced release of histamines or leukotrienes from the superfused, sensitized guinea pig trachea (Undem et al., 1989). In addition, the same pattern of NANCi functional antagonism (i.e., precontraction vs. postcontraction differences) was observed when histamine was used to induce airway smooth muscle tone. Had facilitation of mediator release by NANCi nerve activation been underlying this phenomenon, one would have predicted the magnitude of contraction induced by ovalbumin to be greater than that evoked by histamine in the presence of NANCi nerve stimulation. This was not the case. Finally, putative transmitters of tracheal NANCi nerves, VIP and NO, have been reported to inhibit rather than enhance mast cell mediator release (Tippins et al., 1987; Masini et al., 1994a, b).

A more plausible mechanism involves differential modulation of contraction-dependent intracellular signal transduction pathways in tracheal smooth muscle cells by NANCi nerve activation. For example, smooth muscle contractions have been separated into two components: an initial phasic stage, involving generation of contraction, and a tonic phase, which corresponds to the maintenance of contraction. Both phases appear to use different pools of calcium (Rodger, 1992). As such, it is conceivable that differential susceptibility of cellular pools of calcium to NANCi nerve activation may underlie the observed effects. Additional experiments investigating modulation of intracellular calcium by NANCi nerve activation during phasic and tonic components of agonist-induced contraction are necessary to address this possibility.

In summary, the present results indicate that guinea pig tracheal contraction is differentially susceptible to NANCi nerve activation such that the inhibitory effect of NANCi nerve stimulation is more profound when airway smooth muscle is already contracted. Differences in the ability of
NANCi nerves to relax antigen- vs. histamine- precontracted preparations do not appear to be a consequence of diminished VIP or NO relaxant activity.

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References


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