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NERVOUS CONTROL OF MUCIN SECRETION INTO HUMAN BRONCHI

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SUMMARY

1. Pieces of ferret trachea and human bronchi were mounted in Ussing chambers and given [^{35}S]sulphate as a radiolabelled precursor of mucous glycoproteins (mucins). The output of ^{35}S bound to macromolecules was studied as an index of mucin secretion.

2. In the ferret trachea, electrical field stimulation increased the rate of mucin secretion. Tetrodotoxin (10^{-7} M or 10^{-6} M) abolished this effect.

3. Pilocarpine (25 μM) stimulated the output of mucins from human bronchus. Atropine (10^{-5} M) abolished this effect.

4. Electrical field stimulation of human bronchus stimulated mucin secretion. Tetrodotoxin (10^{-6} M) abolished this effect.

5. Field stimulation in the presence of either atropine (10^{-5} M) or atropine with *l*-propranolol (10^{-5} M) and phentolamine (10^{-5} M) caused no stimulation of mucin secretion rate.

6. Some bronchi were treated with noradrenaline (10^{-5} M) for 1 h to allow the adrenergic nerves to take up transmitter. Even in these, atropine prevented the effect of field stimulation.

7. We conclude that activity in cholinergic nerves can stimulate mucin secretion in the bronchi in man. Our results provide no evidence that the adrenergic nerves or non-adrenergic, non-cholinergic nerves have a direct action on bronchial secretory cells in man.

INTRODUCTION

The nervous regulation of airway mucus secretion has been studied extensively in the cat and ferret, and in both these species muscarinic and α- and β-adrenoceptor agonists stimulate the release of mucus glycoproteins (mucins) (Gallagher, Kent, Passatore, Phipps & Richardson, 1975; Peatfield & Richardson, 1982; Borson, Charlin, Gold & Nadel, 1984). Electrical stimulation of nerves supplying the tracheal secretory cells of these species also augments mucin secretion and there is evidence for the involvement of cholinergic, adrenergic and non-adrenergic, non-cholinergic nerves in the response (Peatfield & Richardson 1982, 1983, Borson *et al.* 1984). In man, both cholinergic and adrenergic nerve fibres approach the bronchial mucus glands (Meyrick & Reid, 1970; Pack & Richardson, 1984). Both muscarinic agonists and α-and β-adrenoceptor agonists increase mucin release from human bronchi

in vitro (Sturgess & Reid, 1972; Phipps, Williams, Richardson, Pell, Pack & Wright, 1982; Pack, Williams, Phipps, Richardson & Rich, 1984). We have undertaken the present study to determine whether bronchial secretion in man is under neural control and, if so, which receptors are involved. A brief report of some of these results has been published (Baker, Peatfield & Richardson, 1984).

METHODS

The method used has been described in detail previously (Phipps *et al.* 1982), so will only be outlined here. All experiments were carried out *in vitro*, some with ferret trachea but the majority with human main-stem, lobar or segmental bronchus.

Four ferrets of either sex were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (Sagatal, May and Baker; (60 mg kg^{-1})). The chest was opened and the whole trachea excised and transferred to a wax bath containing ice-cold Krebs–Hanseleit solution to which glucose (1 mg ml^{-1}) had been added, and which was gassed with 5 % CO_2 in O_2. Adventitious tissue was dissected from the external surface of the trachea which was then cut longitudinally and opened. Three pieces from each trachea were mounted between the two halves of modified Ussing chambers (internal diameter, 9 or 13 mm). The output from a stimulator was connected to two pairs of the six pins that pierced the tissue (Borson *et al.* 1984; Pack *et al.* 1984).

A total of ninety-six pieces of bronchus were obtained from lungs freshly removed from forty male and eleven female patients with bronchial carcinoma (median age = 63 years: range, 36–75). The patients had received either atropine (0·5 mg intramuscularly) or hyoscine (6 $\mu g/kg^{-1}$ body weight intramuscularly) as part of their pre-medication before the operation. So far as could be ascertained macroscopically, none of the tissues was invaded by carcinoma. They were transported to the laboratory in ice-cold Krebs–Henseleit solution that had been gassed with 5 % CO_2 in O_2, and were prepared and mounted in the same manner as the ferret tracheas.

Each half-chamber was filled with 15 ml Krebs–Henseleit solution, circulated by bubbling with 5 % CO_2 in O_2, and maintained at 37 °C. At the beginning of each experiment 74 MBq sodium [^{35}S]sulphate (Amersham PLC) were added to the fluid bathing the submucosal side of each tissue. Samples were collected from the luminal side of the tissue at timed intervals and replaced with fresh Krebs–Henseleit solution. The samples were dialysed exhaustively against distilled water containing 0·01 % (w/v) sodium azide and 0·01 % (w/v) sodium sulphate to disperse the non-covalently bound radiolabel. They were then either dialysed against 6 M-urea or sonicated for 30 s with an ultrasonicator (Soniprobe, Dawe Instrument) to disperse blebs of undissolved mucus. The amount of bound ^{35}S in each sample was assayed by scintillation counting and a quench correction made by the external channels ratio method to determine the number of becquerels in each sample.

Field stimulation was applied as 0·5 or 2·0 ms pulses of 50 V at 10 Hz for 10 min of a 15 min collection period, usually during the periods starting 2·5, 3·5 and 4·5 h after the addition of the radiolabel. In some experiments no stimuli were given so that the changes in ^{35}S release at these times could be calculated and used as control results. Antagonists were added to the fluid bathing both the luminal and submucosal surfaces in some experiments, usually at the beginning of the experiment. Fluid on the luminal side was replaced after each collection. The drugs used were: tetrodotoxin (Sigma), atropine sulphate (Sigma), *l*-propranolol hydrochloride (ICI) and phentolamine mesylate (Rogitine, Ciba). In fourteen tissues noradrenaline acid tartrate (Levophed, Winthrop) was added to the solution bathing each half of the tissue for the first two collection periods. It was then washed out and fresh radioactive sulphate was added to the submucosal half chamber. In these experiments, atropine sulphate (Sigma) was present on both sides of the tissue throughout. Electrical stimulation was applied as described, or, at the same stage in the experiment, a control sample was taken. Pilocarpine hydrochloride (Sigma) was added to the submucosal and luminal sides of seventeen human tissues for 15 min starting 4·5 h after the beginning of the experiment.

Changes in secretory rate are expressed as the mean percentage change (Δ^{35}S %) and the range. Changes were calculated by comparing the output during the test period with the output during the preceding control period. We used an unpaired *t* test to assess the significance of a difference between two groups of data. For data groups between which multiple comparisons were made, we

performed a one-way analysis of variance and then carried out several comparisons between pairs of grouped data using the method of least significant difference (Snedecor & Cochran, 1967). To reduce the variability of the results we transformed the data by first converting the percentage changes to ratios and then taking the logarithm. P values are for two-tailed tests except where stated otherwise.

RESULTS

The effect of field stimulation on ferret trachea

Electrical field stimulation at 50 V, 10 Hz for a duration of 0·5 ms caused a 364% mean increase in ^{35}S-labelled mucin output (range: +66 to +549%, $n = 9$), significantly greater than the changes that occurred in control tissues from the same

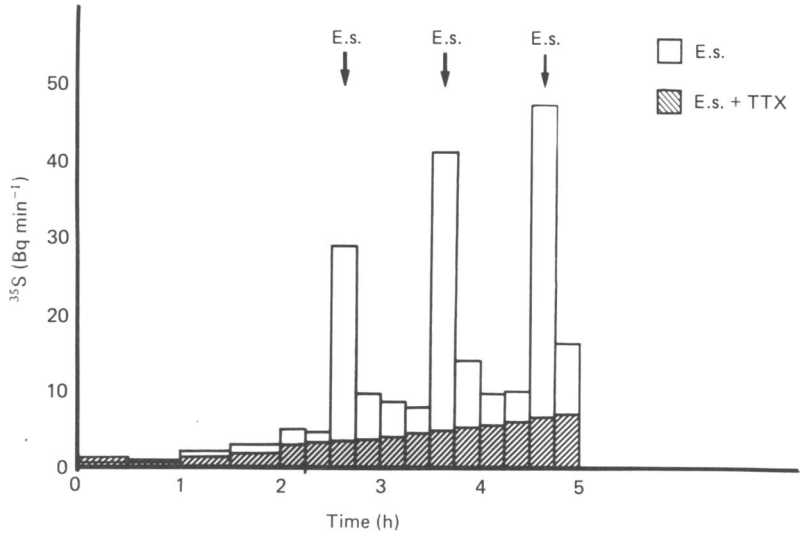

Fig. 1. Graph of the rate of output of mucin-bound radioactivity from two pieces of trachea from the ferret against time. The open bars represent output from the tissue stimulated electrically (50 V, 10 Hz, 0·5 ms for 10 min) at the times indicated (E.s.). The hatched bars represent output from the tissue stimulated likewise but to which tetrodotoxin (TTX) at a final concentration of 10^{-6} M, had been added at the beginning of the experiment. Tetrodotoxin completely abolished the effect of electrical field stimulation.

animals at the same stage in the experiment. ($\Delta^{35}S = +6\%$, range: −28 to +24%, $n = 9$, $P < 0{\cdot}001$). Administration of tetrodotoxin (10^{-7} M or 10^{-6}M) at least 1h before the first period of stimulation to a third set of explants from the same animals virtually abolished the effect of field stimulation ($\Delta^{35}S = +8\%$, range: −1 to +16%, $n = 9$, $P < 0{\cdot}001$) (Fig. 1).

To explants from one animal pulses were applied at 2 ms duration. This increased ^{35}S-labelled mucin output by +1403% (range: +892 to +1659%, $n = 3$), compared with +12% (range: +7 to +15%, $n = 3$) in the control tissue. Tetrodotoxin (10^{-7}M) appeared to block the effect of field stimulation: $\Delta^{35}S = 36\%$, range: −24 to +96%, $n = 3$.

The effect of field stimulation on human bronchi

Electrical stimulation of human bronchial tissue at 50 V, 10 Hz and a duration of 0·5 ms increased ^{35}S-labelled mucin output by 50%. This was significantly greater than the 17% increase in unstimulated control tissue (see Table 1 for probabilities). Tetrodotoxin (10^{-7}M) added at the beginning of the experiment (2·5 h before the first stimulation) completely blocked the effect of electrical stimulation ($\Delta^{35}S = +18\%$). An example of two experiments is shown in Fig. 2 and all the individual results are displayed in Fig. 3.

TABLE 1. Table of probability values (P) for comparison between the mean mucin-bound ^{35}S release in response to the stimuli given by the horizontal and vertical headings. P values are for two-tailed tests. N.s. = $P > 0.05$

	n	E.s. 54	E.s.+TTX 33	E.s.+APP 40	E.s.+A 32
Controls	48	<0·005	N.s.	N.s.	N.s.
E.s.	54	—	<0·05	<0·05	<0·05
E.s.+APP	40	—	—	—	N.s.

E.s. = electrical stimulation.
E.s.+TTX = electrical stimulation in the presence of tetrodotoxin (10^{-6} M).
E.s.+APP = electrical stimulation in the presence of atropine (10^{-5} M), *l*-propranolol (10^{-5} M) and phentolamine (10^{-5} M).
E.s.+A = electrical stimulation in the presence of atropine (10^{-5} M).

In the presence of atropine, *l*-propranolol and phentolamine (each 10^{-5}M), electrical stimulation increased mucin output by 29%, indistinguishable from unstimulated controls but significantly less than the effect of stimulation without antagonists. When atropine alone was given, the increase on electrical stimulation was 17%, significantly less than the effect of electrical stimulation in the absence of antagonists but not significantly different from either the control changes or the response to electrical stimulation in the presence of atropine, phentolamine and *l*-propranolol (Table 1).

The effect of pre-treatment with noradrenaline on field stimulation

In experiments in which noradrenaline (10^{-5}M) was added to the fluid bathing each side of the tissue for the first hour of the experiment and in which atropine (10^{-5}M) was present throughout, the effect of electrical field stimulation ($\Delta^{35}S = +20\%$, range = -44 to $+100\%$, $n = 21$) was no different from control samples taken from the same tissues ($\Delta^{35}S = +21\%$, range = -46 to $+83\%$, $n = 21$).

The effect of pilocarpine on human bronchi

Addition of pilocarpine at a final concentration of 25 μM to the submucosal and luminal sides of the tissue increased ^{35}S output by 255% (range: -8 to $+1367\%$, $n = 9$). Pilocarpine had no such effect when given in the same manner and at the equivalent time in some of the experiments where the tissues had been pre-treated with atropine, phentolamine and *l*-propranolol ($\Delta^{35}S = +38\%$, range -30 to $+193\%$, $n = 8$; $P < 0.05$, 1-tailed test).

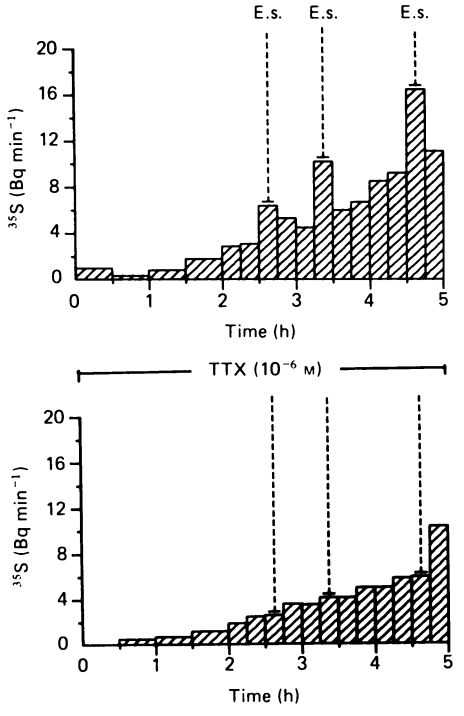

Fig. 2. Graph of the output of mucin-bound radioactivity from two pieces of bronchus from the same human lung against time. The upper trace represents output from the tissue stimulated electrically (E.s.) at the times indicated. The lower trace represents output from the tissue stimulated likewise but to which tetrodotoxin (TTX), at a final concentration of 10^{-6} M, had been added at the beginning of the experiment. Tetrodotoxin completely abolished the effect of electrical field stimulation.

DISCUSSION

We have shown that electrical field stimulation of ferret trachea caused a large increase in radiolabelled mucin output and that addition of tetrodotoxin to the Ussing Chambers completely blocked this effect. These results confirm those of Borson *et al.* (1984) and demonstrate that the electrical stimulus excited nerves within the tissue which then activated secretory cells. The secretory cells did not respond directly to electrical stimulation.

Electrical field stimulation of human bronchial explants also increased mucin output. This increase was inhibited by addition of tetrodotoxin (10^{-6} M) 2·5 h beforehand. This result shows that with the stimulus parameters used (50 V, 10 Hz, 0·5 ms) electrical stimulation caused release of mucins by a neural mechanism alone.

Our results showed that, after administration of the muscarinic antagonist atropine, electrical stimulation had no effect. The dose of atropine used was sufficient to block the action of pilocarpine. The surgical operation would have severed the axons connecting the paravertebral sympathetic ganglia to the adrenergic nerve endings in the bronchial wall in man, so by the time of the first period of field stimulation (about 3·5 h from the operation) enough noradrenaline might have been lost to enfeeble neurotransmission. Since human bronchial tissue obtained previously

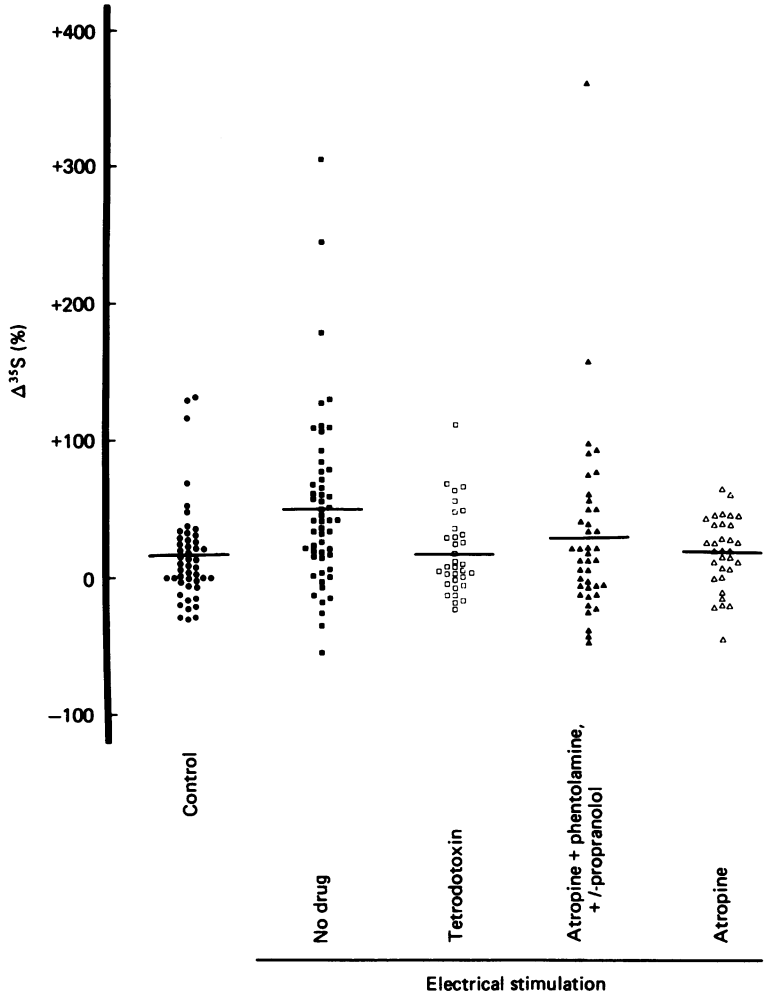

Fig. 3. Plot of percentage change in the rate of output of mucin-bound radioactivity in the stimulated period over the preceding collection period without stimulation for each of five conditions: controls, in which no stimuli were applied, and electrical field stimulation either alone or in the presence of various antagonists. The horizontal lines mark the mean of each group. Compared with controls, electrical stimulation increased mucin output. The effect was blocked in each of the three series of experiments with different blocking agents.

in the same manner incorporated L-DOPA L-β-3, 4-dihydroxyphenylalanine and 5-hydroxydopamine into their nerve endings (Pack & Richardson, 1984), we thought that pre-treatment with noradrenaline should replenish any lost stores of transmitter. However, our results showed that even after incubation with noradrenaline, field stimulation of human bronchial tissue in the presence of atropine had no effect on radiolabeled mucin secretion. We therefore conclude that if adrenergic transmitters were released during electrical field stimulation, then compared with cholinergic transmitters, any direct effect on secretory cells was insignificant. Human bronchial

mucus glands appear to be innervated by both cholinergic and adrenergic fibres (Meyrick & Reid, 1970; Partanen, Laitinen, Hervonen, Toivanen & Laitinen, 1982; Pack & Richardson, 1984) and they also respond to both muscarinic and adrenoceptor agonists (Sturgess & Reid, 1972; Phipps *et al.* 1982), so it seems likely that sympathetic nerves play some part in the regulation of mucus secretion from human bronchi. The role of the sympathetic innervation may be to modulate the parasympathetic control of secretion by a presynaptic action. The present experiments have not tested this hypothesis. The apparent contradiction that sympathomimetic agonists stimulate human bronchial secretion (Phipps *et al.* 1982) but that we have been unable to demonstrate a nervously mediated adrenergic stimulation of secretory cells is probably a reflexion of the small and very variable response of the tissues to field stimulation. In addition there may be adrenoceptors on secretory cells that are able to respond to applied drugs but which are not accessible to transmitter released from nerve endings. This suggestion has been made previously to explain why phenylephrine increased tracheal secretion from the cat *in vivo*, but thymoxamine, an α-adrenoceptor antagonist, failed to reduce the effect of sympathetic nerve stimulation (Peatfield & Richardson, 1982). In the cat, where adrenergic nerve fibres also lie close to tracheal submucosal glands (Silva & Ross, 1974; Murlas, Nadel & Basbaum, 1980), sympathomimetic drugs promote tracheal mucus secretion (Gallagher *et al.* 1975; Ueki, German & Nadel, 1980) and electrical stimulation of the sympathetic nerves supplying the trachea also increases secretion (Peatfield & Richardson, 1982).

Our results also revealed that, after combined administration of atropine and adrenoceptor antagonists at doses similar to those which block the effects of adrenoceptor agonists (Phipps *et al.* 1982), the response to electrical field stimulation was also indistinguishable from controls. In both the ferret and cat there is evidence of a non-adrenergic, non-cholinergic neural mechanism regulating tracheal secretion (Borson *et al.* 1984; Peatfield & Richardson, 1983). A non-adrenergic inhibitory control of human bronchial muscle has been demonstrated by Richardson & Béland (1976). Peptides have been suggested as being possible neurotransmitters of this mechansim. One such peptide, vasoactive intestinal peptide (VIP), has also been localized to submucosal glands of human airways (Dey, Shannon & Said, 1981) and a number of peptides coexist with classical transmitters in the peripheral nerves supplying the airways of many species (see Schultzberg, Hökfelt & Lundberg, 1982). VIP inhibits mucus secretion from human bronchial explants (Coles, Said & Reid, 1981). If VIP, or any other inhibitory peptide, were released in conjunction with classical stimulatory neurotransmitters during field stimulation, then its action would be masked; the observed response to stimulation might be less than that which would have occurred without peptide release. In our experiments, though, electrical stimulation during combined muscarinic receptor and adrenoceptor blockade did not reveal an inhibition of mucus secretion, so if such co-release does occur, the action of the non-adrenergic, non-cholinergic transmitter on mucus output is minimal; however, our results do not preclude such transmitters from modulating the action of the classical transmitters.

The increase in mucus output from human bronchi in response to electrical field stimulation was both small and highly variable compared with equivalent stimulation

of ferret tracheal explants. We cannot say firmly why this should be but the age or condition of the tissue before resection, the fact that the patients suffered from lung disease or had received atropine pre-medication, or that the handling of the lung during surgery released mediators that raised the basal secretion might explain the paucity of the effect. Human bronchial mucus appears to be more tenacious than that from the ferret trachea and this makes the former more difficult to harvest. We suppose that in the human experiments more mucus is carried over from one period of collection to the next, adding to the variability. The smallness and variability of the response mean that to come to a more quantitative conclusion concerning the interaction of nerves and the relative roles of receptors mediating the nervous control of mucus secretion from human bronchi we would have to perform a huge number of experiments.

The conclusions that emerge from these experiments are that efferent nerves control bronchial mucus secretion in man and that cholinergic nerves form an important pathway for this effect.

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