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Comparison of mucus flow rate, radiolabelled glycoprotein output and smooth muscle contraction in the ferret trachea *in vitro*

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1 The concentration-response curves for rate of mucus output, labelled-glycoprotein output and smooth muscle contraction in response to methacholine, phenylephrine and salbutamol were determined in the ferret trachea *in vitro*.

2 The potencies of methacholine and phenylephrine are both in order: smooth muscle contraction, glycoprotein output, rate of mucus output.

3 At lower concentrations methacholine is more potent than is phenylephrine on smooth muscle contraction, glycoprotein output and rate of mucus output.

4 Concentration-response curves for salbutamol show very little change in rate of mucus output but a large increase in glycoprotein output.

5 It is concluded that the glycoprotein output induced by salbutamol may come from a source different from those induced by methacholine and phenylephrine.

Introduction

There are many studies of the control of tracheal mucus secretion, in a variety of different animals and models. One widely used method is to radiolabel the mucus glycoproteins and to wash out and measure the labelled products. This method has been used in a number of species both *in vitro* (Baker *et al.*, 1977; Phipps *et al.*, 1982; Corrales *et al.*, 1984) and *in vivo* (Gallagher *et al.*, 1975). Another method collects undiluted, uncontaminated mucus from the ferret whole trachea *in vitro* (Robinson *et al.*, 1983; Kyle *et al.*, 1986; 1987; Webber & Widdicombe, 1987a, b; Kyle & Widdicombe, 1987); the volume of the output and therefore the rate of output can be determined. However, there are large and significant differences in the responses to certain drugs measured by the two methods. Salbutamol (β_2-adrenoceptor agonist), the neuropeptides vasoactive intestinal peptide (VIP), substance P and eledoisin-related peptide (ERP), and some prostaglandins greatly increase the output of radiolabelled glycoprotein, but have only small effects (if any) on the rate of mucus output (*loc. cit.*). This difference could be for a number of reasons: species differences, some experiments are *in vivo* whilst others are *in vitro*, and the labelled glycoproteins may come from a source other than that responsible for the volume output.

Therefore it was decided to characterize and compare the responses to methacholine, phenylephrine and salbutamol in terms of rate of mucus output and radiolabelled glycoprotein output *in vitro* in the same species, the ferret and to produce concentration-response curves, which are lacking from virtually all previous studies, thus making it difficult to compare quantitatively the results from different experiments. The ferret whole trachea *in vitro* also allows smooth muscle contractions to be measured at the same time as rate of mucus output (Kyle *et al.*, 1986; Kyle & Widdicombe, 1987).

Methods

The whole trachea in vitro

Ferrets of either sex were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}; Sagatal, May & Baker) intraperitoneally. The trachea was cut free at the larynx and cannulated with a special perspex

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cannula designed for collection of mucus samples. The animal was then killed with an overdose of sodium pentobarbitone (Euthatal, May & Baker) given by cardiac injection and the whole trachea down to the carina was cut free from the adjacent tissue. The carinal end of the trachea was cannulated with a polyethylene cannula and the whole trachea was mounted laryngeal end down in a water-jacketed organ bath at 37°C and bathed with Krebs-Henseleit solution, to which drugs could be added.

The upper cannula was attached to a pressure transducer (Furness Controls) for measurement of pressure changes due to alterations in smooth muscle tone. A fine polyethylene collecting catheter was inserted into the lower perspex cannula for withdrawal of secretions. Sample collections were made every 15 min during drug periods and every 30 min during control periods. The clearance time for substances to run through the length of the trachea was less than 5 min (Kyle *et al.*, 1987). Drug administration was continued for 4 × 15 min, or until the secretion rate had declined. If there was no change in rate of mucus output caused by the drug, the next drug was administered immediately. If there was a change in rate of mucus output then at least 1 h was left between doses during which time the rate had returned to steady control values.

The volume of the sample was estimated by the difference in the weight of the catheter full of secretion and the empty dried catheter after washing out the secretion, and the results are expressed as $\mu l\,min^{-1}$. The change in rate of mucus output is calculated as the difference between the drug-induced rate of output and the control rate immediately before the beginning of the drug period.

The secretion and contraction results are the mean values from 4–8 experiments on different animals, except for methacholine ($10^{-9}\,mol\,l^{-1}$), which are from two experiments. Each concentration was given only once in an experiment, and to prevent exhaustion of the submucosal glands the whole range of concentrations was not examined in a single experiment. Therefore the results could not be normalised against a maximum value.

In vitro ^{35}S-labelled tracheal explants

Male ferrets were anaesthetized with sodium pentobarbitone ($45\,mg\,kg^{-1}$ i.p.; Nembutal, Abbott). The whole trachea was excised from the larynx to the carina and was dissected free from surrounding connective tissue in a bath containing medium 199 with added $NaHCO_3$ ($26.2\,mmol\,l^{-1}$), indomethacin ($56.0\,\mu mol\,l^{-1}$), penicillin ($100\,ku\,l^{-1}$) and streptomycin ($100\,ku\,l^{-1}$) at 10°C and bubbled with 95% O_2/5% CO_2. The trachea was cut longitudinally through the posterior membrane and cut transversely into ten pieces which were then incubated for 2 h in a shaking water-jacketed organ bath at 37°C containing medium 199 with $6.7\,mCi\,l^{-1}$ $Na_2^{35}SO_4$ and bubbled with 95% O_2/5% CO_2. After incubation each explant was placed in a plastic container with 5 ml non-radioactive medium 199 at 37°C. Every 30 min the bathing solution was replaced. The first three washings were discarded; previous experiments had shown that activity is high in the first washings but comes down to a stable baseline after 90 min. Subsequent washings were transferred to dialysis bags, and the container was washed with 1 ml of medium 199 which was also transferred to the dialysis bag.

After 3 h the drugs were added to eight of the ten tracheal pieces for 30 min, the other two pieces acting as controls. The samples were dialysed three times against Na_2SO_4 ($0.21\,mmol\,l^{-1}$) and sodium azide ($0.15\,mmol\,l^{-1}$) to remove unbound label. Bound label was measured with liquid scintillation counting, and the radioactive output during the drug period was expressed as a percentage of the output in the immediately preceding control period; the outputs in the drug and control periods were corrected by subtracting the mean percentage changes in outputs of bound $^{35}SO_4$ in the two control tissues which had not been exposed to drug. Each result is a mean value from 4–9 pieces of trachea from two or more ferrets, each piece of tissue being exposed to only one concentration of one drug.

Drugs and solutions

The drugs used were: methacholine chloride (Sigma), phenylephrine hydrochloride (Sigma), salbutamol sulphate (Ventolin, Allen & Hanbury) and indomethacin (Berk). All drugs were dissolved in Krebs-Henseleit solution or medium 199 and prepared fresh on the day of the experiment. The Krebs-Henseleit composition was ($mmol\,l^{-1}$): NaCl 120.8, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. Medium 199 is a standard culture medium for cell and tissue preparations (Parker, 1967).

Results

Control values

The rates of mucus output at the beginning of the experiments was zero or near zero ($0.061 \pm 0.018\,\mu l\,min^{-1}$, mean ± s.e. mean, $n = 21$). During the course of experiments control outputs before drug applications changed little from the initial values, means being 0.052 ± 0.028 ($n = 21$), 0.034 ± 0.014 ($n = 21$) and $0.053 \pm 0.017\,\mu l\,min^{-1}$

($n = 16$) for controls before the second, third and fourth drug applications respectively. Changes in rates of mucus output due to drugs were usually large compared to control values, and are expressed as absolute changes, since percentage changes of controls at or near zero are uninformative.

The output of ^{35}S-labelled glycoprotein decayed approximately exponentially after the 3 h equilibration period, the initial mean output being 0.22 ± 0.017 Bq min^{-1} ($n = 16$); the control output 1 h later after the tissue had been tested with a drug was 0.16 ± 0.0091 Bq min^{-1} ($n = 16$). Results are expressed as percentages of controls, as done in previous studies (see Discussion).

The ferret tracheal preparation has virtually no resting tone in its smooth muscle, so drugs such as salbutamol that might relax contracted smooth muscle were ineffective. Before each drug application the pressure in the trachea was set at atmospheric and changes are expressed in absolute values.

Methacholine

A concentration range of 10^{-9} to 10^{-4} mol l^{-1} was examined on rate of mucus output and smooth muscle contraction (tracheal pressure) (Figure 1). The steep part of the contraction curve was between 10^{-8} and 10^{-6} mol l^{-1}, and for rate of mucus output between 10^{-7} and 10^{-5} mol l^{-1}. Thus methacholine was more potent on smooth muscle contraction than on rate of mucus output. The maximum contraction and rate of mucus output stimulated by methacholine occurred at concentrations greater than 10^{-4} mol l^{-1}. The maximum pressure change caused by contraction of smooth muscle was greater than 137 mmH_2O and the maximum rate of mucus output was greater than 3.3 μl min^{-1}.

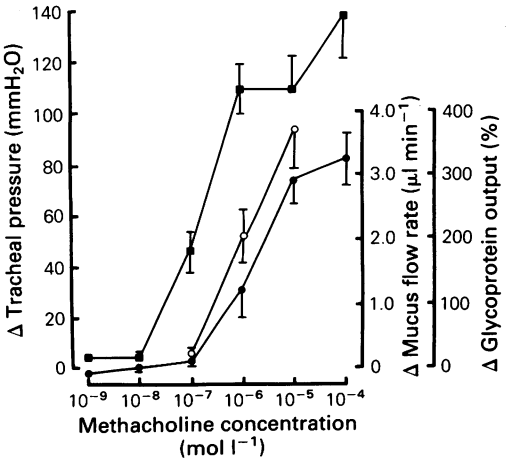

Figure 1 Concentration-response curves for methacholine-induced rate of mucus output (●), radiolabelled glycoprotein output (○), and smooth muscle contraction (■, assessed as pressure change) in ferret trachea *in vitro*. Values are means with s.e. means shown by vertical lines.

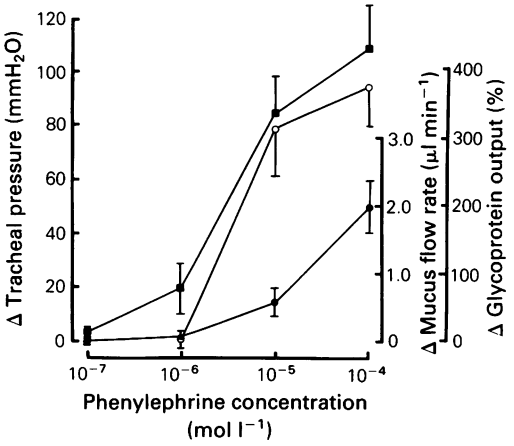

Figure 2 Concentration-response curves for phenylephrine-induced rate of mucus output (●), radiolabelled glycoprotein output (○), and smooth muscle contraction (■, assessed by pressure change) in ferret trachea *in vitro*. Values are means with s.e. means shown by vertical lines.

The concentration-response curve for ^{35}S-labelled glycoprotein output was similar to that for rate of mucus output over the range 10^{-7} to 10^{-5} mol l^{-1} (Figure 1).

Phenylephrine

The steep part of the curves occurred between 10^{-6} and 10^{-5} mol l^{-1} for smooth muscle contraction and between 10^{-5} and 10^{-4} mol l^{-1} for rate of mucus output (Figure 2). The contraction of the smooth muscle approached a maximum at above 10^{-4} mol l^{-1}, where the pressure was greater than 108 mmH_2O. The concentration-response curve for rate of mucus output showed no sign of reaching a maximum at 10^{-4} mol l^{-1}, with a value about 2.0 μl min^{-1}.

The labelled glycoprotein output concentration-response curve was very steep between 10^{-6} and 10^{-5} mol l^{-1} and to the left of that for rate of mucus output (Figure 2). It appeared to be reaching a maximum at 10^{-4} mol l^{-1}.

Salbutamol

A concentration range of 10^{-6} to 10^{-3} mol l^{-1} failed to promote a significant change in the rate of mucus output (Figure 3). At 10^{-6} mol l^{-1} there was a rela-

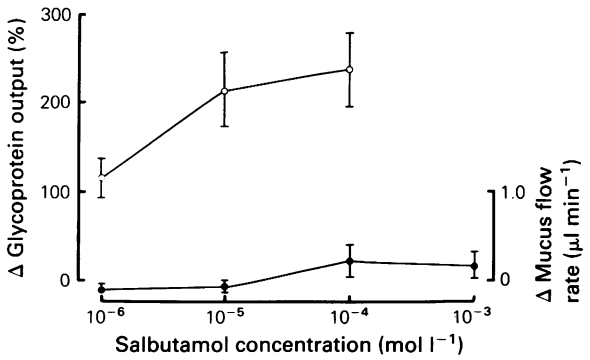

Figure 3 Concentration-response curves for salbutamol-induced rate of mucus output (●) and radiolabelled glycoprotein output (○) in ferret trachea *in vitro*. Values are means with s.e. means shown by vertical lines.

tively large increase (over 100%) in ^{35}S-labelled output, and this appeared to be reaching a maximum at 10^{-4} mol l^{-1} (Figure 3).

Discussion

In models where radiolabelled glycoprotein is removed by washing the trachea or tracheal pieces, there is always a basal output of glycoprotein. This basal output is unaffected by tetrodotoxin or by adrenoceptor or cholinoceptor antagonists in the ferret trachea labelled with $^{35}SO_4$ in modified Ussing chambers (Borson *et al.*, 1984); it is hardly inhibited by atropine or propranolol in the cat trachea *in vivo* (Gallagher *et al.*, 1975; Peatfield & Richardson, 1983) or in the ferret trachea *in vitro* (B. Wilffert, unpublished results). However, in the air-filled whole trachea there is little or no basal rate of output of mucus (this paper; Kyle *et al.*, 1987; Kyle & Widdicombe, 1987; Webber & Widdicombe 1987a, b). The epithelium stripped from the trachea can produce a relatively large output of radiolabelled material (Borson *et al.*, 1984). Thus it is possible that the washing removes glycoproteins from the luminal surface of the epithelium which are not usually part of the collectable volume of secretion under normal physiological conditions, such as when the lumen is air-filled.

Radiolabelled precursors are incorporated into proteoglycans as well as glycoproteins in human, canine (Coles *et al.*, 1982) and rabbit explants, where they may be largely products of the cartilage and connective tissue (Gallagher & Kent, 1975). They may be present in secretions washed from normal human airways *in vivo* (Bhaskar *et al.*, 1985) and canine airways *in vitro* (Bhaskar *et al.*, 1986), although the latter result is disputed (Ellis & Stahl, 1973). It is not known if ferret tracheal explants produce proteoglycans. Thus labelled macromolecules may come from sources other than the submucosal glands.

The ferret is a useful experimental animal for investigating tracheal mucus secretion. It has a trachea 7.5 cm long which contains few goblet cells in the epithelium but a large number of submucosal glands which are relatively evenly distributed throughout its length (Jacob & Poddar, 1982; Robinson *et al.*, 1986). The output of mucus can be measured directly without washings. We have expressed changes in output in absolute terms rather than percentages, since control rates of output were near zero. The control outputs were small compared with provoked secretions due to methacholine or phenylephrine. We have not used drug antagonists in these experiments. Others have shown that mucus production from the ferret trachea, measured by micropipetting ducts of submucosal glands or by observation of 'hillock' formation, and with the same concentrations of agonists as we have used, is appropriately blocked: methacholine by atropine, phenylephrine by phentolamine, and terbutaline by propranolol (Borson *et al.*, 1980; 1984; Ueki & Nadel, 1981).

The concentrations of drugs used in our experiments are similar to those used in a large number of other studies assessing the rate of mucus and glycoprotein outputs (*loc. cit.* in Introduction and Discussion). Threshold values were derived for all responses including those for smooth muscle contraction, except for the effect of salbutamol on glycoprotein output; here the smallest concentration (10^{-6} mol l^{-1}) more than doubled glycoprotein output with no effect on mucus flow rate. For most of the responses we did not increase drug concentration sufficiently to establish maxima, since we thought it unjustifiable to exceed 10^{-4} to 10^{-3} mol l^{-1}, so concentration-response curves could not be normalised and analysed mathematically.

Our results show fairly close agreement between the positions of the concentration-response curves of methacholine and phenylephrine for measurements of rate of mucus output and change in radiolabelled glycoprotein output. However, the curves obtained by the two methods for salbutamol are very different. Relatively high concentrations of salbutamol fail to induce any significant changes in the rate of mucus output, whilst there is a large concentration-dependent change in ^{35}S-labelled glycoprotein output. The increase in ^{35}S-labelled glycoprotein output from the trachea can be induced by salbutamol in other species and models (Peatfield & Richardson, 1982; Phipps *et al.*, 1982). Thus the secretion induced by salbutamol must contain a large quantity of glycoproteins (or similar macromolecules) in a small volume of fluid. Studies with the tantalum-covered 'hillock' method of measuring the volume of mucus output of ferret tracheal submucosal glands (Borson *et al.*, 1980; Robinson, 1984) agree well with the findings in the whole trachea model; β_2-adrenoceptor agonists stimulate only a small increase in volume output. However micropipette measurements show increased volume output from single submucosal glands in the cat *in vivo* with terbutaline (Ueki & Nadel, 1981; Leikauf *et al.*, 1984).

In dogs, output of labelled glycoprotein from tracheal explants *in vitro* in response to methacholine is concentration-dependent over a similar range to that in ferrets (Coles *et al.*, 1984a, b). The maximal effect occurs at a slightly lower concentration than that for ferrets and the percentage increase in glycoprotein output is much higher in our studies. These differences may be species-dependent, or because different labels ($^{35}SO_4$ and [^{14}C]-glucosamine) were used or because glycoprotein was measured by precipitation for the dogs.

The whole trachea *in vitro* has the advantage that smooth muscle contractions can be measured simultaneously with rate of mucus output. There is little resting tone in the preparation so that relaxations cannot be measured unless the muscle is pharmacologically precontracted. The agents used for this might influence mucus secretions, so the effect of salbutamol as a relaxant was not examined. The concentration-response curves show that methacholine and phenylephrine are more potent at lower concentrations on smooth muscle contraction than on rate of mucus output. Methacholine is more potent at lower concentrations on both the rate of mucus output and smooth muscle contraction than is phenylephrine.

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