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Directional Ca^{2+} effect on stimulation of mucin secretion from chicken trachea *in vitro*

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Chicken tracheal mucosa *in vitro* transported and incorporated radioactive precursors into mucins, which were secreted at a steady rate into the tracheal lumen. Secretion of mucins labelled with ^{35}S and ^{3}H after pulse-labelling of the mucosal layer with $Na_2{}^{35}SO_4$ and D-[1-^{3}H]glucosamine as precursors was an energy-dependent process, as it was strongly inhibited by the action of respiratory-chain inhibitors, an uncoupler of oxidative phosphorylation, a metabolic blocker and a temperature shift from 41°C to 5°C. On the other hand, both cholinergic and parasympathomimetic agents considerably increased the secretion of dual-radiolabelled mucins when applied on the submucosal side of the trachea. The effect of Ca^{2+} was directional, since only high submucosal (3.6 or 18 mM) or low luminal (zero or 0.18 mM) Ca^{2+} massively enhanced the secretion of radiolabelled mucin compared with the mucin output measured under physiological Ca^{2+} conditions (1.8 mM). Whereas application of ionophore A23187 on either side of the trachea significantly increased mucin output, its presence in the appropriate tracheal compartment and under appropriate Ca^{2+} conditions further accentuated the output of radiolabelled mucins. Addition of acetylcholine under appropriate conditions also had an additive effect on the Ca^{2+}-stimulated secretion of mucins. Ca^{2+} stimulation of mucin secretion appears to be dependent on the metabolic integrity of the mucosal cells. Mucins secreted in response to high submucosal and low luminal [Ca^{2+}] appear to consist of a number of different types of glycoproteins, as judged from their ion-exchange-chromatographic behaviour.

In recent years much interest has been shown in the study of mucin secretion from tracheal systems. Early investigations on cat and goose trachea *in vivo* have revealed the effects of a variety of drugs like cholinergic and adrenergic agents and their agonists, irritants and anaesthetic agents and of direct nerve stimulation on the mucin output, together with their biochemical analysis (Phipps *et al.*, 1977; Richardson *et al.*, 1978; Kent, 1978). A direct implication of Ca^{2+} in eliciting a physiological response similar to that resulting from the action of cholinergic agents or nerve stimulation in the tracheal system was first reported by Balfre & Richardson (1978), who observed that changes in [Ca^{2+}] of the perfusion medium altered the electrical potential differences in chicken tracheal preparations *in vitro*. More recent studies on isolated chicken tracheal mucosal cells *in vitro* have shown a direct involvement of Ca^{2+} in the secretion of elevated amounts of common exportable glycoproteins as well as of additional unique glycoproteins (N. Mian, J. L. Padron, A. J. Pope, C. E. Anderson & P. W. Kent, unpublished work).

Although these observations indicated that any alteration in [Ca^{2+}], whether higher or lower than the physiological concentration (1.8 mM), enhanced mucin output, they did not elucidate how both high and low extracellular [Ca^{2+}] produced similar responses. Since isolation of mucosal cells from the luminal lining of the trachea exposes their basolateral and luminal sides to the incubation medium, it was proposed that perhaps the two types of membrane system respond differently to high and low extracellular [Ca^{2+}]. By using a technique *in vitro* that offers collection of mucin secreted in the lumen from the whole intact tracheal mucosa (Balfre & Richardson, 1978; Mian *et al.*, 1982*a*), the present study was undertaken to further investigate the mechanism(s) by which mucosal cells respond to high and low [Ca^{2+}], resulting in enhanced mucin output.

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Materials and methods

Materials

Male and female chickens (12–30 weeks) were obtained from Orchard Farm, Little Kingshill, Bucks., U.K. D-[1-^{3}H]Glucose (sp. radioactivity 3–10Ci/mmol), D-[1-^{3}H]galactose (sp. radioactivity 5–20Ci/mmol), D-[1-^{3}H]glucosamine hydrochloride (sp. radioactivity 2–5Ci/mmol), L-[1-^{3}H]-fucose (sp. radioactivity 1Ci/mmol), $^{133}BaCl_2$ (1–10mCi/mg) and $Na_2{}^{35}SO_4$ (25–40Ci/mg) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Ionophore A23187 was kindly provided by The Lilly Research Centre, Windlesham, Surrey, U.K. Gentamicin, penicillin, streptomycin and fungizone were obtained from Flow Laboratories, Irvine, Aryshire, Scotland, U.K. Antimycin A was purchased from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals, AnalaR or laboratory-grade reagents, were obtained from BDH Chemicals, Poole, Dorset, U.K., or Sigma.

Biosynthetic radiolabelling of tracheal secretions and collection of samples under control and experimental conditions

Birds were killed by intravenous injection (60mg/kg body wt. via a wing vein) of sodium pentobarbital (Sagatal; May and Baker, Dagenham, Essex, U.K.). The trachea was exposed, and its cranial and caudal ends were cannulated with flanged glass cannulae, dissected free of connective tissue and then suspended in a vertical glass organ bath filled with avian physiological buffer maintained at 41°C with O_2/CO_2 (19:1) being bubbled continuously. The avian physiological buffer contained 137mM-NaCl, 2.68mM-KCl, 0.105mM-$MgCl_2$, 1.8mM-$CaCl_2$, 11.6mM-$NaHCO_3$, 0.4mM-NaH_2PO_4, 4.4mM-glucose, gentamicin (50μg/ml), streptomycin (100μg/ml), fungizone (2.5μg/ml) and penicillin (100 units/ml) and pH was maintained at 7.2 by bubbling continuously with O_2/CO_2 (19:1). The tracheal lumen was washed with avian physiological buffer before use. To biosynthetically radiolabel tracheal mucins, the tracheal lumen was filled with buffer solution containing $Na_2{}^{35}SO_4$ (50μCi/ml) and ^{3}H-radiolabelled sugars (50μCi/ml) for 1h. The tracheal lumen was emptied by flushing out the luminal contents and was refilled with buffer solution free of radioactively labelled precursors. The secretion samples were collected at 10min intervals over a period of 120min by removing the luminal content and refilling it with the fresh buffer solution. Depending on the experimental requirements, buffer solutions containing different concentrations of Ca^{2+} or Mg^{2+} or other test materials were used both in the luminal and submucosal compartments of the tracheal preparation for different time intervals with bridging controls. Luminal secretion samples were extensively dialysed at 4°C against water before radioactivity measurements and freeze-drying. Secretion samples that showed the presence of dislodged mucosal cells on phase-contrast-microscopic examination were discarded.

The osmolarity of buffer solutions containing various amounts of Ca^{2+} or Mg^{2+} was adjusted to the osmolarity of normal physiological buffer by altering NaCl concentration. La^{3+}-containing solutions were prepared in 5.8mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, gassed with O_2. The ionophore A23187 was dissolved in dimethyl sulphoxide. The concentration of dimethyl sulphoxide in the final buffer solution was 50μl/100ml.

Chemical and radioactivity estimation and ion-exchange chromatography

N-Acetylneuraminic acid and uronic acid were determined by the resorcinol method of Svennerholm (1957) and by the carbazole method of Bitter & Muir (1962) respectively. The sulphate content was measured by the method of Mende & Whitney (1978). The carbohydrate composition of the samples was analysed by g.l.c. after methanolysis by the method of Clamp *et al.* (1971). The presence of carbohydrate residues in secretion samples was examined by the periodic acid/Schiff reaction by the method of Mantle & Allen (1978). Estimations of the radioactivities of the samples and the ion-exchange chromatography were performed as described previously (Mian *et al.*, 1982*a*).

Calculation of results

Changes in secretion of ^{35}S and ^3H radioactivities and in periodic acid/Schiff-positive material were calculated by comparing the output during the test period with that in the preceding pre-stimulus or pre-inhibition control period and are expressed as mean percentage changes (Δ%) $\pm$ S.E.M. The significance of effects produced by different interventions was tested by Student's *t* test or by the Mann–Whitney U test (Siegel, 1956).

Results

Chicken tracheal mucosa incorporated a variety of sugars and sulphate into secretory mucins (Fig. 1). A lower degree of consistency in the secretion profile of ^{3}H-labelled mucins using D-[1-^{3}H]glucose compared with D-[1-^{3}H]glucosamine as a precursor can be attributed to the extensive metabolic use of glucose, whereas glucosamine appears to be a specific precursor for glycoprotein biosynthesis. Under physiological conditions, tracheal mucosa secreted dual-radiolabelled macromolecules and periodic acid/Schiff-positive material at a steady rate

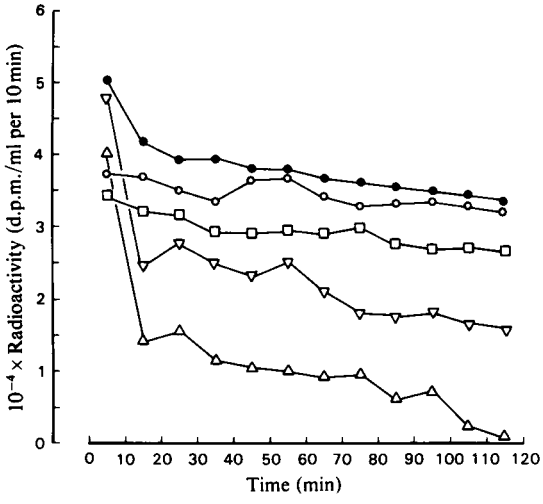

Fig. 1. *Secretion profile of radiolabelled macromolecules after pulse labelling of chicken trachea in vitro with different radiolabelled sugars and* $Na_2{}^{35}SO_4$

Tracheal preparations *in vitro* were pulse-labelled with radioactively labelled precursors (50μCi/ml) for 1h. The lumen was then emptied and washed with physiological buffer. The secretion samples were collected at 10min intervals for 2h using physiological buffer. Samples were extensively dialysed for 96h against water containing 0.02% NaN_3 before estimation of their radioactivities. The radioactively labelled precursors used were $Na_2{}^{35}SO_4$ (●), D-[1-^{3}H]glucosamine (○), L-[1-^{3}H]-fucose (□), D-[1-^{3}H]galactose (▽) and D-[1-^{3}H]-glucose (△). Values plotted are averages of three experiments.

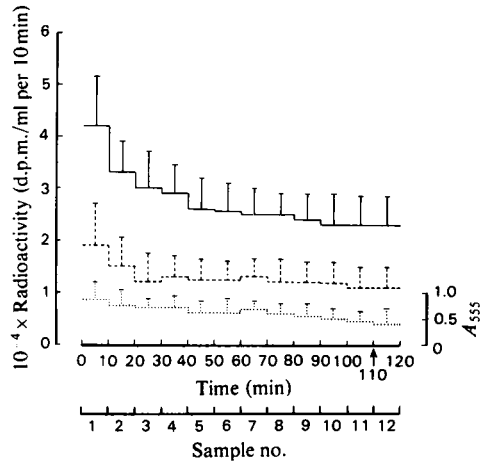

Fig. 2. *Secretion of periodic acid/Schiff-positive material and dual-radiolabelled macromolecules after simultaneous pulse-labelling with* D-[1-^{3}*H*]*glucosamine and* $Na_2{}^{35}SO_4$ *of chicken trachea in vitro*

Tracheal preparations *in vitro* were pulse-labelled with $Na_2{}^{35}SO_4$ and D-[1-^{3}H]glucosamine, 50μCi/ml each, for 1h and secretion samples were collected and dialysed as described in Fig. 1 before being subjected to radioactivity measurements and the periodic acid/Schiff test. Results are means ± S.E.M. of nine experiments. ——, ^{35}S radioactivity; – – – –, ^{3}H radioactivity; · · · ·, A_{555} (periodic acid/Schiff test measurement).

over the 2h experimental period (Fig. 2). Administration of metabolic inhibitors such as KCN, dinitrophenol or antimycin A into the tracheal lumen, although producing an outburst of secretion in the first 10min sample, subsequently caused a sharp decline in the secretion of dual-radiolabelled macromolecules (Fig. 3). On the other hand, a temperature shift from 41°C to 5°C progressively inhibited the secretion without producing any initial outburst in mucin output (Fig. 3). Treatment with puromycin, although showing a little change in the mucin output during the first 30min of its application, subsequently suppressed the mucin output to a considerably lesser extent compared with that caused by metabolic inhibitors (Fig. 3). Administration of acetylcholine on either side or both sides of the chicken trachea elicited an increase in the output of dual-radiolabelled and periodic acid/Schiff-positive macromolecular material (Fig. 4). Withdrawal of acetylcholine reduced the secretion output and repeated washing with physiological buffer restored the secretion to the expected baseline level (Fig. 4). Acetylcholine and pilocarpine in enhancing mucin output and atropine in depressing mucin output had a greater effect when applied on the submucosal side rather than on the luminal side (Table 1). 2-Deoxyglucose, irrespective of the site of application, suppressed mucin output (Table 1). Although an outburst of mucin output was observed on the first application of antimycin A, La^{3+} and EGTA on the luminal side, their subsequent treatment, irrespective of the site of application, caused depression in mucin output (Table 1). On the other hand, ionophore A23187, whether applied on the luminal side or on the submucosal side, enhanced mucin output (Table 1). The addition of 2.1mM-Mg^{2+} (20 times the normal concentration) or its omission from the luminal or submucosal side had hardly any effect on mucin output (Table 1). Agents that either stimulated or inhibited secretory activity when applied on either side of the trachea were found to produce an additive effect if administered simultaneously on both sides of the trachea. Secondly, even after withdrawal of agents such as acetylcholine, pilocarpine and ionophore A23187, their stimulatory effect apparently persisted in the immediate poststimulus sample.

Table 1. *Effect on the secretion of ^{35}S- and ^{3}H-radiolabelled macromolecules and of periodic acid/Schiff-positive material from chicken trachea in vitro after luminal and/or submucosal application of a variety of agents*

Pulse-labelled chicken tracheal preparations *in vitro*, as described in the text, were treated with the agents listed below for a 10 min period each time. The site of administration of the test agent at the time of collection of the luminal secretion sample is also noted in the Table. From each trachea 12 samples were usually collected at 10 min intervals and the sample numbers mentioned in the Table refer to the third, sixth and ninth samples, as shown in Fig. 2. The luminal and submucosal compartments of the trachea were exposed to physiological buffer except when a test solution was added to the appropriate side(s) for a 10 min period. The third and sixth samples were collected with the test agent present on the luminal and submucosal side respectively and the ninth sample was collected when both luminal and submucosal sides were exposed to the test agent. All other samples, before or after treatment were collected under control conditions. The secretion of ^{35}S- and ^{3}H-radiolabelled macromolecules ('^{35}S' and '^{3}H' respectively in the Table) and of periodic acid/Schiff-positive (PAS+ive) material in the test sample was compared with the preceding control sample and the results are expressed as mean percentage change (Δ%). Values are mean percentage changes (Δ%) ± S.E.M. of the numbers of observations noted in the Table. The significance of the effects of various interventions was determined by Student's *t* test or Mann–Whitney's U test. Changes that were not significant with the controls are referred to as NS.

Site of application of test agent at the time of collection of luminal secretion		Luminal			Submucosal			Luminal and submucosal		
Sample number		Third			Sixth			Ninth		
Agent		^{35}S	^{3}H	PAS+ive material	^{35}S	^{3}H	PAS+ive material	^{35}S	^{3}H	PAS+ive material
Pilocarpine (1 μg/ml)	Mean Δ(%)	+28	+19	+31	+98	+65	+68	+106	+87	+78
	±S.E.M. (%)	6	5	9	8	6	12	16	8	15
	n	6	6	6	8	8	8	6	6	6
	P	<0.05	<0.05	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Acetylcholine (1 μg/ml)	Mean Δ(%)	+38	+31	+40	+78	+70	+100	+110	+95	+110
	±S.E.M. (%)	8	7	8	12	8	15	20	15	20
	n	6	6	6	6	6	6	6	6	6
	P	<0.05	<0.05	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Atropine (14 μM)	Mean Δ(%)	−30	−45	−55	−94	−96	−78	−96	−95	−86
	±S.E.M. (%)	14	15	13	14	8	13	15	10	15
	n	6	6	6	7	7	7	7	7	7
	P	<0.05	<0.05	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2-Deoxyglucose (5 mM)	Mean Δ(%)	−10	−15	−5	−25	−40	−10	−40	−50	−27
	±S.E.M. (%)	5	10	5	10	15	5	10	8	7
	n	6	6	6	6	6	6	6	6	6
	P	NS	NS	NS	<0.05	<0.05	NS	<0.001	<0.001	<0.05
Antimycin A (10 μM)	Mean Δ(%)	+40	+30	+35	−65	−75	−49	−90	−95	−96
	±S.E.M. (%)	7	15	9	10	15	18	12	10	13
	n	7	7	7	7	7	7	7	7	7
	P	<0.05	<0.001	<0.05	<0.001	<0.001	<0.05	<0.001	<0.001	<0.001
$LaCl_3$ (2 mM)	Mean Δ(%)	+38	+28	+40	−35	−30	−60	−80	−70	−110
	±S.E.M. (%)	8	6	10	5	6	9	20	15	23
	n	8	8	8	8	8	8	8	8	8
	P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.001	<0.001	<0.001	<0.001
EGTA (2 mM)	Mean Δ(%)	+30	+25	+19	−70	−60	−85	−80	−85	−113
	±S.E.M. (%)	5	6	9	10	15	17	16	18	19
	n	7	7	7	7	7	7	7	7	7
	P	<0.05	<0.05	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ionophore A23187 (20 μM)	Mean Δ(%)	+110	+80	+95	+120	+75	+110	+190	+130	+140
	±S.E.M. (%)	25	16	17	20	15	18	20	25	26
	n	8	8	8	8	8	8	8	8	8
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
$MgCl_2$ (2.1 mM)	Mean Δ(%)	+6	+5	−6	+7	+4	−5	+6	+5	−7
	±S.E.M. (%)	5	4	3	5	4	4	5	4	4
	n	7	7	7	7	7	7	7	7	7
	P	NS	NS	NS	NS	NS	NS	NS	NS	NS
$MgCl_2$ (zero)	Mean Δ(%)	+5	+6	+5	−6	+5	+7	−6	−7	−5
	±S.E.M. (%)	4	3	4	4	4	5	4	5	4
	n	3	3	3	3	3	3	3	3	3
	P	NS	NS	NS	NS	NS	NS	NS	NS	NS

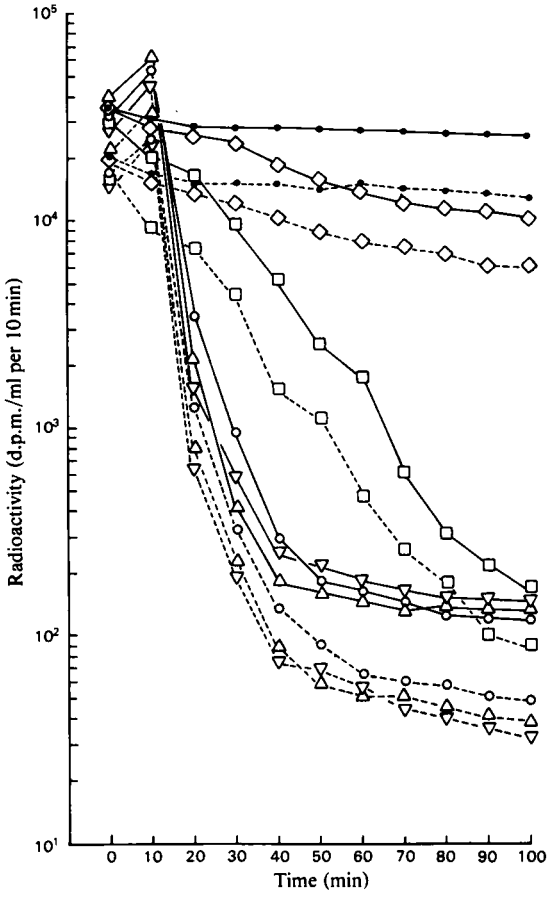

Fig. 3. *Effect of metabolic inhibitors, puromycin and of temperature shift on the secretion of ^{35}S- and ^{3}H-radiolabelled macromolecules from chicken trachea in vitro*

Tracheal preparations *in vitro* were simultaneously pulse-labelled with $Na_2{}^{35}SO_4$ (50μCi/ml) and D-[1-^{3}H]glucosamine (50μCi/ml) for 1h at 41°C under physiological conditions. The tracheal lumen was emptied and washed with physiological buffer. Secretion samples were then collected at 10min intervals using physiological buffer (●), buffer solution containing 2.68mM-KCN (○), 5mM-dinitrophenol (△), 10μM-antimycin A (▽), 0.1mg of puromycin/ml (◇) or physiological buffer under conditions when the tracheal preparation was transferred to an organ bath maintained at 5°C (□). All samples were extensively dialysed against water in the presence of 0.02% NaN_3 before radioactivity measurements. Values are averages of three experiments. ——, ^{35}S radioactivity; – – – –, ^{3}H radioactivity.

Directional Ca^{2+} effect on stimulation of secretion from chicken trachea in vitro

Application of high Ca^{2+} (3.6 or 18.0mM) on the luminal side or of low Ca^{2+} (zero or 0.18mM) on the submucosal side of the trachea did not affect secretion of dual-radiolabelled and periodic acid/Schiff-positive macromolecular products. However, administration of high Ca^{2+} on the submucosal side or of low Ca^{2+} on the luminal side caused a significant increase in the mucin output (Fig. 5 and Table 2). Simultaneous perfusion of the luminal and submucosal sides of the trachea with low- and high-Ca^{2+} buffers respectively appeared to have an accumulative effect on the enhanced mucin output (Table 2). Replacement of these buffer solutions with physiological buffer on the appropriate side of the trachea did not immediately return the secretion to its baseline level (Fig. 5). The secretion of dual-radiolabelled and periodic acid/Schiff-positive macromolecular products in the post stimulus sample was significantly higher than that in the prestimulus sample ($\Delta^{35}S = +55 \pm 8\%$, $\Delta^{3}H = +45 \pm 6\%$, Δ periodic acid/Schiff-positive material $= +80 \pm 15\%$; $n = 32$; $P < 0.001$); repeated washing-out with physiological buffer was required to restore secretion to its baseline level (Fig. 5). Addition of ionophore A23187 on the appropriate side of the trachea was found to accentuate the low

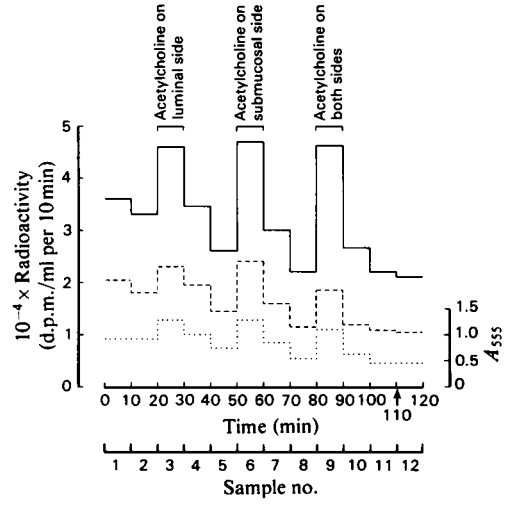

Fig. 4. *Effect of acetylcholine on the secretion of dual-radiolabelled macromolecules and periodic acid/Schiff-positive material from chicken trachea in vitro*

Tracheal preparations *in vitro* were simultaneously pulse-labelled with D-[1-^{3}H]glucosamine (50μCi/ml) and $Na_2{}^{35}SO_4$ (50μCi/ml) for 1h. Secretion samples were collected using physiological buffer in the absence or presence of acetylcholine (1μg/ml). Acetylcholine solutions were applied on the luminal, submucosal or on both sides of the trachea for 10min periods at different time intervals during the experiments. Values are averages of three experiments. ——, ^{35}S radioactivity; – – – –, ^{3}H radioactivity; · · · ·, A_{555}.

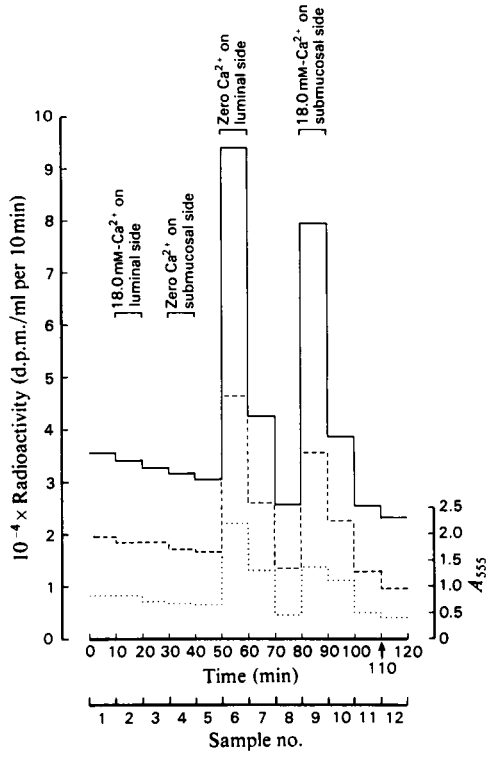

Fig. 5. *Directional Ca^{2+} effect on the secretion of dual-radiolabelled macromolecules and periodic acid/Schiff-positive material from chicken trachea in vitro*

Tracheal preparations *in vitro* were simultaneously pulse-labelled with D-[1-^{3}H]glucosamine (50μCi/ml) and $Na_2{}^{35}SO_4$ (50μCi/ml) under physiological conditions for 1h. Secretion samples were then collected using physiological buffer or buffer containing no Ca^{2+} or 18.0mM-Ca^{2+}. The osmolarity of these solutions was adjusted by altering NaCl concentrations. These solutions were applied for a 10min period on the luminal or submucosal or on both sides of the trachea as indicated in the Figure. The bridging control samples were collected using physiological buffer on both sides of the trachea. When one side of the trachea was exposed to a test solution the other side was perfused with physiological buffer. Results are averages of three experiments. ——, ^{35}S radioactivity; – – – –, ^{3}H radioactivity; · · · ·, A_{555}.

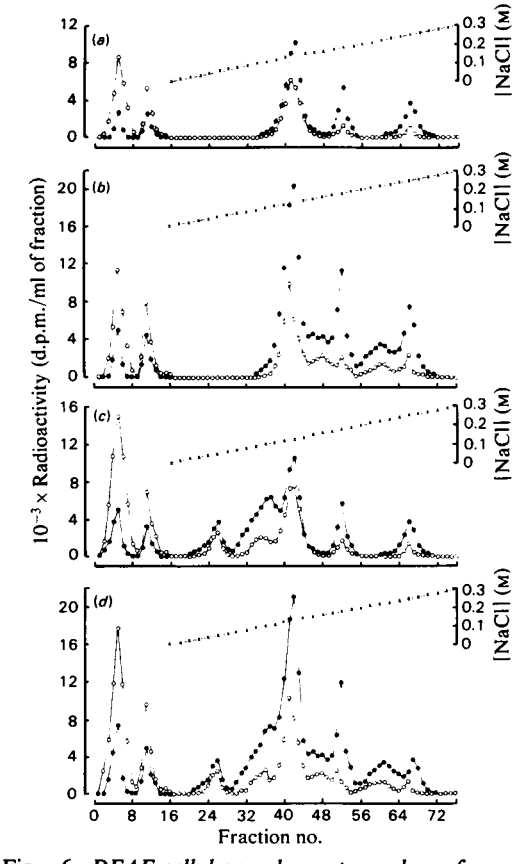

Fig. 6. *DEAE-cellulose chromatography of normal secretions and those produced in response to the directional Ca^{2+} effect from chicken trachea in vitro*

Secretion samples were extensively dialysed against water and then freeze-dried. The resulting materials were solubilized and dialysed against 20mM-Na_2HPO_4/NaH_2PO_4 buffer, pH 7.2, containing 10mM-NaCl and applied to DE-52 DEAE-cellulose columns (1.5cm × 8.0cm) equilibrated with the same buffer. The columns were eluted with 40ml of the same buffer, followed by 150ml of a linear gradient (0–0.3M) of NaCl in the same buffer. Fractions (2.5ml) were collected at a flow rate of 15ml/h. ●, ^{35}S radioactivity; ○, ^{3}H radioactivity; ×, [NaCl], measured by conductivity. Recoveries of ^{35}S and ^{3}H radioactivities and of the actual amount of material were approx. 97%. (*a*) Fractionation profiles of secretions produced under physiological conditions; (*b*) secretions produced in the presence of 18mM-Ca^{2+} on the submucosal side; (*c*) secretions produced in the presence of zero Ca^{2+} on the luminal side; (*d*) secretions produced in the presence of 18mM-Ca^{2+} on the submucosal and zero Ca^{2+} on the luminal side of the trachea.

luminal and high submucosal Ca^{2+} effect in enhancing secretion output (Table 2). Similarly, the directional Ca^{2+} effect and the acetylcholine effect were found to be accumulative on the secretion output (Table 2). On the other hand, whereas initial exposure of the luminal side to atropine or antimycin A appeared to suppress the low luminal Ca^{2+} effect on the secretion output (Table 2), subsequent treatments with atropine on the submucosal side as well as on both the luminal and submucosal sides simultaneously further decreased the directional Ca^{2+} effect. In this manner, repeated treatments with antimycin A not only completely abolished the

Table 2. *Effect of various secretion-stimulatory and -inhibitory agents and of Mg^{2+} on the stimulated secretion of ^{35}S- and ^{3}H-radiolabelled macromolecules and of periodic acid/Schiff-positive (PAS+ive) material as a result of the directional Ca^{2+} effect*

All details are as given in Table 1 except that the concentrations of Ca^{2+} in the control or test solutions perfusing luminal or submucosal side of the trachea were altered as noted below. Before and after exposure to the test solution, both sides of the trachea were perfused with normal physiological buffer containing 1.8 mM-Ca^{2+}. The results are expressed as described in the legend to Table 1.

$[Ca^{2+}]$ (mM) ...		Luminal, 0, submucosal, 1.8			Luminal, 1.8, submucosal, 18.0			Luminal, 0 submucosal, 18.0		
Site of application of test agent ...		Luminal			Submucosal			Luminal and submucosal		
Sample number ...		Third			Sixth			Ninth		
Agent		^{35}S	^{3}H	PAS+ive material	^{35}S	^{3}H	PAS+ive material	^{35}S	^{3}H	PAS+ive material
Control	Mean Δ(%)	+215	+190	+240	+206	+170	+190	+317	+308	+275
	±S.E.M. (%)	15	16	25	16	18	22	18	20	25
	n	20	20	20	20	20	20	20	20	20
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ionophore A23187 (20 μM)	Mean Δ(%)	+305	+250	+278	+310	+230	+212	+405	+360	+335
	±S.E.M. (%)	25	26	15	28	15	18	18	16	19
	n	11	11	11	11	11	11	11	11	11
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Acetylcholine (1 μg/ml)	Mean Δ(%)	+230	+210	+205	+350	+210	+265	+460	+390	+375
	±S.E.M. (%)	20	25	29	15	25	28	15	18	16
	n	9	9	9	9	9	9	9	9	9
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Atropine (14 μM)	Mean Δ(%)	+105	+60	+68	+55	+45	+40	+45	+30	+35
	±S.E.M. (%)	25	16	18	17	13	8	6	5	7
	n	6	6	6	6	6	6	6	6	6
	P	<0.001	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05	<0.05
Antimycin A (10 μM)	Mean Δ(%)	+65	+40	+36	−35	−27	−28	−90	−85	−68
	±S.E.M. (%)	16	9	15	14	8	10	17	15	18
	n	7	7	7	7	7	7	7	7	7
	P	<0.001	<0.01	<0.05	<0.05	<0.05	<0.05	<0.001	<0.001	<0.001
$MgCl_2$ (2.1 mM)	Mean Δ(%)	+4	−2	−4	+192	+150	+178	+195	+160	+139
	±S.E.M. (%)	15	10	5	25	28	29	15	22	29
	n	9	9	9	9	9	9	9	9	9
	P	NS	NS	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

directional Ca^{2+} effect but also inhibited the secretion otherwise expected under control conditions in the absence of antimycin A (Table 2). Administration of Ca^{2+}-free buffer containing 2.1 mM-Mg^{2+} into the tracheal lumen completely abolished the low luminal Ca^{2+} effect (Table 2). However, the presence of 2.1 mM-Mg^{2+} in the buffer solution containing 18 mM-Ca^{2+} on the submucosal side of the trachea did not influence the high submucosal Ca^{2+} effect in enhancing secretion output from the trachea (Table 2).

Heterogeneity among dual-radiolabelled macromolecular products secreted as a result of the directional Ca^{2+} effect

Chemical analysis of secretions collected under normal and experimental Ca^{2+} conditions showed a considerable variation in the carbohydrate and ester sulphate residues (Table 3). However, no uronic acid was detected by using the modified carbazole method of Bitter & Muir (1962). The percentage amounts of different carbohydrate residues were consistently lower in the secretion samples collected under altered Ca^{2+} conditions compared with those under normal Ca^{2+} conditions. On the other hand, ester sulphate/*N*-acetylglucosamine and ester sulphate/*N*-acetylneuraminic acid ratios were slightly higher for secretion samples collected under altered Ca^{2+} conditions compared with similar ratios for samples collected under normal Ca^{2+} conditions (Table 3). Fractionation of these secretions on ion-exchange chromatography, although showing that these were mixtures of different types of neutral and acidic macromolecular components, also revealed that samples collected under altered Ca^{2+} conditions varied in the relative proportions of their

Table 3. *Chemical composition of secretion samples collected under different experimental conditions*
Secretion samples collected under normal and experimental conditions as listed below were extensively dialysed and freeze-dried before chemical analysis. *N*-Acetylneuraminic acid and ester sulphate residues were measured chemically as described in the text. All other sugar residues were analysed by g.l.c. The amounts of sugars and of ester sulphate residues were calculated as mg/100mg dry wt. of mucin and values are expressed as means ± S.E.M. Two determinations on five separate samples were made in each series of experiments. Molar ratios of monosaccharides and of ester sulphate residue were calculated by taking *N*-acetylglucosamine as 1.0.

	Normal Ca^{2+} (1.8mM)		High submucosal Ca^{2+} (18.0mM)		Low luminal Ca^{2+} (0mM)		High submucosal Ca^{2+} (18.0mM) + low luminal Ca^{2+} (0mM)	
	(mg/100mg)	(molar ratio)	(mg/100mg)	(molar ratio)	(mg/100mg)	(molar ratio)	(mg/100mg)	(molar ratio)
Fucose	8.9 ± 0.8	0.52	6.2 ± 0.5	0.44	5.6 ± 0.6	0.47	5.0 ± 0.7	0.42
Galactose	10.4 ± 0.7	0.61	9.0 ± 0.6	0.64	8.0 ± 0.7	0.66	7.9 ± 0.8	0.67
N-Acetylgalactosamine	10.4 ± 1.0	0.61	9.6 ± 0.8	0.67	8.1 ± 0.6	0.67	8.2 ± 0.9	0.70
N-Acetylglucosamine	17.1 ± 1.1	1.00	12.9 ± 1.2	1.00	12.1 ± 0.9	1.00	11.8 ± 0.8	1.00
Mannose	4.5 ± 0.6	0.27	3.4 ± 0.5	0.24	3.1 ± 0.6	0.26	2.8 ± 0.5	0.24
Glucose	3.5 ± 0.5	0.21	3.2 ± 0.6	0.23	2.7 ± 0.4	0.23	2.8 ± 0.4	0.24
N-Acetylneuraminic acid	3.9 ± 0.6	0.23	3.0 ± 0.4	0.22	2.9 ± 0.6	0.24	2.7 ± 0.5	0.23
Ester sulphate	5.3 ± 0.7	0.31	6.2 ± 0.8	0.44	4.4 ± 0.5	0.36	5.3 ± 0.4	0.45

constituent glycoproteins compared with the normal secretion samples (Fig. 6). Secretions produced in response to low luminal and high submucosal Ca^{2+} effects appeared to contain different types of glycoproteins, in addition to those present in the normal secretions, the major differences being in the number and type of acidic glycoproteins (Fig. 6).

Discussion

The results presented here suggest that mucin secretion as a physiological cellular event is an energy-dependent process. Inhibition of secretion by 2-deoxyglucose, similar to that reported on the mast cells (Douglas & Ueda, 1973), and by a temperature shift from 41°C to 5°C without producing an initial outburst of secretion, can be as a result of progressive suppression of ATP production. Inhibition of mucin secretion by lowering the ambient temperature can also suggest that the physical state of the membranes (Dahl *et al.*, 1979) plays an important role in the membrane fusion steps involved in exocytotic processes (Douglas, 1974). Although inhibition of mucin secretion as a result of the long-term effect of EGTA appears to implicate Ca^{2+} and hence its role in the interaction of plasmalemma and secretory granule membranes, an immediate effect of this chelating agent in eliciting mucin output appears to be an outcome of Ca^{2+} removal from plasmalemma. Similarly, an immediate cellular response in eliciting a burst of mucin secretion on the application of respiratory-chain inhibitors, oxidative phosphorylation uncouplers (Carafoli, 1974) or of La^{3+} (Mela, 1968; Carafoli, 1974) appears to be a result of interference with the energy-linked translocation of Ca^{2+}, whereas subsequent strong inhibition of secretion by these metabolic blockers seems to result from disruption of the energy metabolism of the cell.

The action of Ca^{2+} in enhancing mucin output appears to be selective and directional in the sense that low-Ca^{2+} buffer (0.18mM-Ca^{2+} or no Ca^{2+}) produces a major response when applied on the luminal side and high-Ca^{2+} buffer (3.6mM or 18.0mM) elicits a similar response when applied on the submucosal side of the chicken trachea. The observed cellular response to Ca^{2+} changes appears to be independent of the presence of other stimuli. The present observations thus clarify why isolated tracheal mucosal cells *in vitro*, which have basolateral and luminal surfaces exposed to the incubation medium, respond to both high and low extracellular Ca^{2+} concentrations by producing elevated amounts of mucins (N. Mian, J. L. Padron, A. J. Pope, C. E. Anderson & P. W. Kent, unpublished work). The inhibition of the directional Ca^{2+} effect on mucin output by metabolic blockers or by cholinergic agonists would suggest that perhaps the integrity of cellular metabolism and the Ca^{2+} receptors are essential for the execution of the mandatory steps involved in the Ca^{2+}-stimulated secretory processes. The possibility that the directional Ca^{2+} effect operates through Ca^{2+} mobilization from or into the cell can be envisaged from the observations that (*a*) application of ionophore A23187 on either side of the trachea in the presence of physiological Ca^{2+} concentrations (1.8mM) enhances the mucin output and (*b*) its application on

the appropriate side of the trachea in the presence of high and low/no Ca^{2+} further accentuated the Ca^{2+} effect. Ionophore A23187 has been shown to release secretory products either by introducing Ca^{2+} into cells (Cochrane & Douglas, 1974; Thorn, 1974) or by releasing Ca^{2+} from intracellular bound pools (Berridge & Prince, 1972; Berridge *et al.*, 1974). The directional Ca^{2+} effect also appears to be specific, since in the presence of 1.8mM-Ca^{2+}, omission of Mg^{2+} from the luminal side or addition of 2.1mM-Mg^{2+} (20 times the normal concentration) on the submucosal side produces no change in the mucin output. On the other hand, although the addition of 2.1mM-Mg^{2+} on the submucosal side in the presence of 18mM-Ca^{2+} does not inhibit Ca^{2+}-stimulated mucin output, addition of 2.1mM-Mg^{2+} on the luminal side in the absence of Ca^{2+} abolishes the secretory response, perhaps by interfering with the Ca^{2+} outflow process. Mg^{2+} has been found to antagonize Ca^{2+} effects in other tissues (Douglas, 1968; Rahwan *et al.*, 1973; Rubin, 1974). An alteration in the dynamic equilibrium of cellular Ca^{2+} can be envisaged as a result of the action of ionophore A23187, La^{3+}, EGTA, metabolic inhibitors and blockers, potentiation of such changes in the cell Ca^{2+} by simply altering extracellular Ca^{2+} concentrations cannot easily be understood as yet and needs further direct investigations.

Finally, the differences in the chemical composition and in the number and type of constituent glycoproteins of secretions produced in response to the directional Ca^{2+} effect are of some importance insofar as the biological significance of enhanced secretion of selective glycoproteins is concerned (Mian *et al.*, 1982*a*,*b*).

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