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Regulation of guanosine 3′:5′-cyclic monophosphate in ovine tracheal epithelial cells

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1 Guanosine 3′:5′-cyclic monophosphate (cyclic GMP) is an important second messenger mediating the effects of nitric oxide (NO) and natriuretic peptides. Cyclic GMP pathways regulate several aspects of lung pathophysiology in a number of airway cells. The regulation of this system has not been extensively studied in pulmonary epithelial tissue.

2 We have studied the production of cyclic GMP by suspensions of ovine tracheal epithelial cells in response to activators of soluble guanylyl cyclase (sodium nitroprusside (SNP) and S-nitroso-N-acetyl-penicillamine (SNAP) and particulate guanylyl cyclase (atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and *E. coli* heat stable enterotoxin (STa)).

3 Both $10^{-7}-10^{-3}$ M and $10^{-7}-10^{-3}$ M SNAP generated a concentration-dependent marked elevation in cyclic GMP production when incubated with 10^{-3} M 3-isobutyl-1-methylxanthine (IBMX) (both greater than $25\times$ baseline values with highest drug concentration).

4 The increase in production of cyclic GMP in response to 10^{-6} M SNP and 10^{-5} M SNAP was markedly inhibited by both 5×10^{-5} M haemoglobin (102% and 92% inhibition) and 5×10^{-5} M methylene blue (82% and 84% inhibition).

5 The increase in cyclic GMP in response to 10^{-3} M SNP was measured following co-incubation with the phosphodiesterase inhibitors $10^{-7}-10^{-3}$ M IBMX, $10^{-7}-10^{-4}$ M milrinone and $10^{-7}-10^{-4}$ M SKF 96231. Only $10^{-4}-10^{-3}$ M IBMX significantly increased cyclic GMP levels.

6 Cyclic GMP production was also significantly elevated from baseline by 10^{-5} M ANP, 10^{-5} M BNP, 10^{-5} M CNP and 200 iu ml^{-1} of *E. coli* STa toxin in the presence of 10^{-3} M IBMX. Increases with these natriuretic peptides and STa toxin were smaller in magnitude (2–4 fold) than those seen with SNP and SNAP. CNP was the most potent of the natriuretic peptides studied suggesting type B membrane bound guanylate cyclase is the predominant form expressed.

7 These results suggest that ovine tracheal epithelial cells contain active guanylyl cyclases. The more marked response to SNP and SNAP than to natriuretic peptides suggests that soluble guanylyl cyclase predominates.

Keywords: Cyclic GMP; guanylyl cyclase; nitric oxide; natriuretic peptides; tracheal epithelial cells; phosphodiesterase inhibitors

Introduction

Nitric oxide (NO) is an important molecule in cellular signalling. NO elicits a cellular response in a number of airway cells and roles have been postulated for NO in a range of pulmonary diseases including pulmonary hypertension (Cailes *et al*., 1995), asthma (Hogman *et al*., 1993) and pulmonary infections (Kharitonov *et al*., 1995). NO increases intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels via one of a family of guanylyl cyclases converting guanosine 5′-triphosphate (GTP) to cyclic GMP. Guanylyl cyclase exists in two forms, soluble and membrane bound (particulate) which are stimulated by distinct agonists. Soluble guanylyl cyclase (GC_S) is activated by NO and NO donors, in contrast to membrane-bound guanylyl cyclases (GC_M) which are plasma membrane receptors for the natriuretic peptides and related hormones. Four classes of GC_M have been described (Wong & Garbers, 1992) which can be distinguished in part by their binding affinity for natriuretic peptides and related compounds. GC-A preferentially binds atrial natriuretic peptide (ANP) but also binds brain natriuretic peptide (BNP), GC-B preferentially binds C-type natriuretic peptide, GC-C is mainly found in the gastrointestinal tract and binds the heat-stable bacterial enterotoxin STa as well as the endogenous peptide hormone guanylin. The specificity of the fourth member of this class, retinal GC (retGC) has been less well characterized.

Cyclic GMP pathways are important in cell signalling in many epithelia outside the lung. Cyclic GMP regulate transepithelial ion transport in kidney and colonic epithelial cells (Light *et al*., 1990; Argenzio & Armstrong, 1993) as well as increasing mucus secretion in gastric epithelial cells (Brown *et al*., 1993). Less is known about the role of cyclic GMP in airway epithelial physiology although studies in rabbit, human and bovine airway epithelia have suggested that natriuretic peptides and NO may regulate ciliary beat frequency (Tamaoki *et al*., 1991; Geary *et al*., 1995; Sisson 1995). Surprisingly, in view of the potential importance of cyclic GMP pathways in lung pathophysiology, no previous studies have comprehensively examined the regulation of this system in tracheal or bronchial epithelium. The purpose of the present study was to use a number of pharmacological tools to characterize the soluble and particulate guanylyl cyclase/cyclic GMP signalling systems in tracheal epithelial cells.

Methods

Epithelial cell preparation

We studied sheep tracheal epithelium as it shares several physiological properties with human tracheal epithelium (Graham *et al*., 1992) and the large size makes it suitable for biochemical assays. We obtained sheep trachea immediately after death from a local abattoir and placed them in ice-cold Krebs-Henseleit solution of composition (mM): Na^+ 145.0, Cl^- 126.0, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 26.0, PO_4^- 1.2, SO_4^- 1.2 and glucose 5.6; gassed with 95% O_2–5% CO_2.

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Specimens with macroscopically traumatized mucosa were discarded. The tracheal mucosal surfaces were washed with phosphate-buffered saline (pH 7.4) and the epithelial cells removed by gently scraping with a scalpel blade. Cytological confirmation of epithelial cell phenotype was obtained by immunohistochemistry. Cell preparations stained for cytokeratin (NMF 11, Dako Ltd, High Wycombe, U.K.) revealed approximately 95% of cells staining positive (Figure 1a). Staining by use of antibodies directed against actin and desmin, and with a nonsense antibody was negative (Figure 1b). The cells were washed, centrifuged at 120 *g* for 10 min at 4°C (Centra-4R, I.E.C., Dunstable, Beds., U.K.) and resuspended three times in Dulbecco's modified Eagle's medium (DMEM) containing 2×10^{-2} M N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer.

Protocols

The epithelial cells of between 7 and 12 tracheae were pooled for each experiment in order to obtain sufficient tissue to perform the number of assays required. Cell suspensions were divided into 0.5 ml aliquots and incubated with the pharmacological agents or vehicle controls at 37°C for 1 h. This time course was chosen following preliminary time course experiments shown in Figure 2. Between 4 and 6 replicatees were performed for each experiment.

Initial experiments were performed to study the effect of 10^{-3} M 3-isobutyl-1-methylxanthine (IBMX), a non-selective phosphodiesterase inhibitor on cyclic GMP formation. The effect of IBMX addition was investigated over a range of concentrations (10^{-7} M – 10^{-3} M). The effect of 10^{-7} M – 10^{-4} M milrinone (a type III/IV specific phosphodiesterase inhibitor, Shahid *et al*., 1991) and 10^{-7} – 10^{-4} M SKF 96231 (a type V specific phosphodiesterase inhibitor, Murray *et al*., 1991) on cyclic GMP levels was also studied. Solubility precluded studying these compounds at a concentration of 10^{-3} M.

Sodium nitroprusside (SNP) 10^{-7} – 10^{-3} M and S-nitroso-N-acetyl-penicillamine (SNAP) 10^{-7} – 10^{-3} M, both NO donors were used to test activation of GC_S. The effect of 5×10^{-5} M lyophilized sheep haemoglobin which binds free NO (Gibson & Roughton, 1965) was studied on 10^{-6} M SNP and 10^{-5} M SNAP induced cyclic GMP production. The effect of 5×10^{-4} M methylene blue, an inhibitor of guanylyl cyclase (Tamaoki *et al*., 1991) on cyclic GMP production induced by 10^{-6} M SNP and 10^{-5} M SNAP was also studied. Both the haemoglobin and methylene blue were pre-incubated with the cells for 30 min before the addition of SNP or SNAP.

Human 28-atrial natriuretic peptide (ANP) 10^{-5} M, human 32-brain natriuretic peptide (BNP) 10^{-5} M, C-type natriuretic peptide (CNP) 10^{-5} M and heat-stable *E. coli* enterotoxin (STa) 200 iu ml^{-1} were used to test activation of GC_M. As natriuretic peptides may be inactivated by enkephalinases present in lung epithelium (Johnson *et al*., 1985), these experiments were performed in the presence of 10^{-6} M phosphoramidon, an inhibitor of these enkephalinases.

Cyclic GMP measurement

Cyclic GMP was extracted by adding 1 ml of ice-cold 0.1 M hydrochloric acid to 0.5 ml of cell suspension followed by sonication for 30 s at half power (SP 518, Ultrasonics Ltd., Shipley, Yorks., U.K.). The resulting suspension was centrifuged at 1500 *g* for 30 min at 4°C (DPR-6000, Damon/IEC Ltd., Dunstable, Beds., U.K.). The supernatant was removed and freeze-dried (SB9, Lab Plant Ltd., Huddersfield, Yorks., U.K.) before the measurement of cyclic GMP content. The pellet was re-suspended in phosphate-buffered saline (pH 7.4) and protein estimated by the method of Lowry *et al*. (1951) with bovine serum albumin as standard. Cyclic GMP was measured with a commercially available ELISA kit (RPN 226, Amersham Ltd, Little Chalfont, Buckinghamshire, U.K.). The samples were first acetylated with a mixture of acetic anhydride and triethylamine to increase the sensitivity of the assay to 9 fmol 100 μl^{-1}. The coefficient of variation of the assay was 11%. All samples were assayed in duplicate.

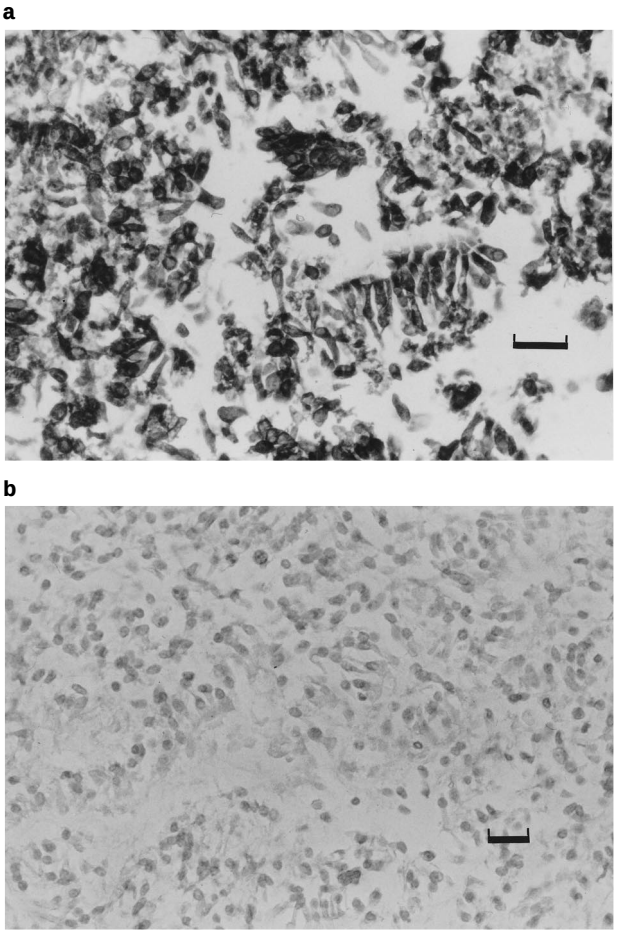

Figure 1 Photomicrograph (a) shows ovine tracheal epithelial cells stained with horseradish-peroxidase labelled anti-cytokeratin antibody showing approximately 95% of cells are positively stained denoting epithelial cell phenotype. Photomicrograph (b) shows no staining with nonsense antibody control. Scale bar = 20 μm.

Chemicals

SKF 96231 (2-(2-propoxyphenyl)-6-purinone) was a kind gift from SmithKline Beecham Pharmaceuticals Ltd., Welwyn, U.K. Unless otherwise stated all other chemicals were obtained from Sigma-Aldrich Company Limited, Poole, Dorset, U.K. All reagents were dissolved in DMEM-HEPES with the exception of IBMX, milronone and SKF 96231 which was first dissolved in ethanol, to give a final concentration of 2% v/v ethanol in the bathing solution. Ethanol was added to the bathing solutions of control experiments not involving these reagents to give a final ethanol concentration of 2% v/v. All concentrations of reagents shown refer to the final concentration in the cell suspension.

Statistical analyses

Results are shown as means ± s.e.mean. The significance of drug effects was assessed by one way analysis of variance. A P value of less than 0.05 was regarded as significant.

Results

Effect of IBMX on basal cyclic GMP levels

After 4 h incubation cyclic GMP levels in cells incubated with 10^{-3} M IBMX (473 ± 144 fmol mg^{-1} protein) did not differ significantly from cells incubated with ethanol control (218 ± 40) ($n=4$, $P=0.14$, Figure 2).

Effect of IBMX on stimulated cyclic GMP levels

As seen in Figure 2 the presence of 10^{-3} M IBMX increased cyclic GMP production in response to 10^{-3} M SNP. At 2 h cyclic GMP levels were 10416 ± 811 fmol mg^{-1} protein in the presence of 10^{-3} M IBMX compared with 2868 ± 352 fmol mg^{-1} protein with SNP alone ($P<0.001$, $n=4$). In view of this all subsequent experiments were carried out in the presence of 10^{-3} M IBMX unless otherwise stated.

Effect of activators of GC_S on cyclic GMP levels

SNP and SNAP, in combination with 10^{-3} M IBMX, both generated marked concentration-dependent elevations in cyclic GMP levels (Figure 3a and b). SNP increased cyclic GMP levels from a control level of 195 ± 29 fmol mg^{-1} protein to 5099 ± 1051 fmol mg^{-1} protein (10^{-3} M SNP) ($P<0.001$, $n=6$), with an EC_{50} of 1.9×10^{-5} M. SNAP increased cyclic GMP levels from a control level of 45 ± 7 fmol mg^{-1} protein to 9491 ± 1499 fmol mg^{-1} protein (10^{-3} M SNAP) ($P<0.001$, $n=6$), with an EC_{50} of 2.0×10^{-4} M.

The increase in cyclic GMP production in response to SNP and SNAP was inhibited by the addition of 5×10^{-4} M methylene blue or 5×10^{-5} M Hb (Figure 4). The increase in cyclic GMP production in response to 10^{-6} M SNP was inhibited 82% by methylene blue from 1744 ± 279 to 291 ± 57 fmol mg^{-1} protein ($P=0.01$, $n=5$), and 102% by Hb from 1744 ± 279 to 136 ± 23 fmol mg^{-1} protein ($P=0.01$, $n=5$). The increased cyclic GMP production in response to 10^{-5} M SNAP was inhibited 84% by methylene blue from 2156 ± 216 to 478 ± 70 fmol mg^{-1} protein ($P=0.001$, $n=5$) and 92% by haemoglobin from 2156 ± 216 to 524 ± 83 fmol mg^{-1} protein ($P=0.001$, $n=5$).

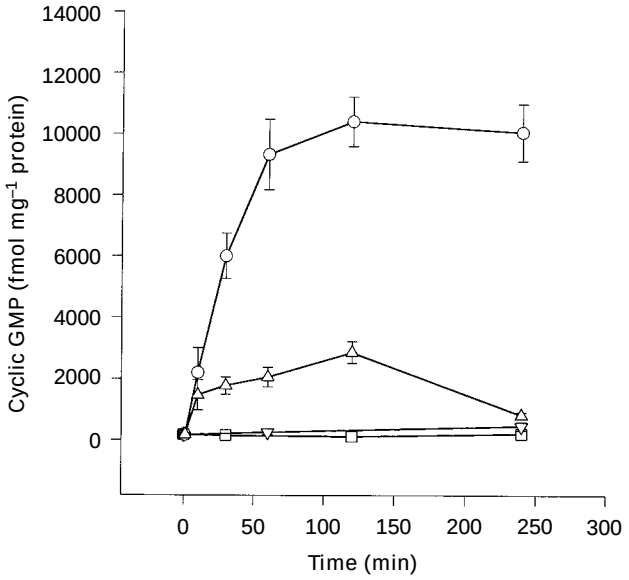

Figure 2 Timecourse of cyclic GMP production by ovine tracheal epithelial cells in response to: (○) 10^{-3} M sodium nitroprusside (SNP) and 10^{-3} M 3-isobutyl-1-methylxanthine (IBMX), (△) 10^{-3} M SNP alone, (▽) 10^{-3} M IBMX alone, (□) ethanol control. Data represent the mean (±s.e.mean, vertical lines) of 4 replicates, where error bars are not shown they lie within the data point.

Effect of activators of GC_M on cyclic GMP levels

All three natriuretic peptides produced small but significant elevations in cellular cyclic GMP level (Figure 5). After 1 h incubation cyclic GMP levels increased from a baseline of 336 ± 62 fmol mg^{-1} protein to 1060 ± 56 fmol mg^{-1} protein with 10^{-5} M ANP ($n=4$, $P<0.001$), to 1087 ± 27 fmol mg^{-1} protein with 10^{-5} M BNP ($n=3$, $P<0.001$), to 1426 ± 178 fmol mg^{-1} protein with 10^{-5} M CNP ($n=4$, $P=0.006$), and to 707 ± 99 fmol mg^{-1} protein with 200 iu STa toxin ($n=4$, $P=0.04$).

Comparison of phosphodiesterase isoenzyme specific inhibitors

Cyclic GMP levels following incubation with 10^{-4} M milrinone (32 ± 13 fmol mg^{-1} protein), 10^{-4} M SKF 96231 (47 ± 24 fmol mg^{-1} protein), and 10^{-3} M IBMX (48 ± 15 fmol mg^{-1} protein) did not differ from control levels (46 ± 16 fmol mg^{-1} protein) ($n=4$, Table 1). In cells stimulated with 10^{-3} M SNP addition of up to 10^{-4} M milrinone (271 ± 42 fmol mg^{-1} protein), 10^{-4} M SKF 96231 (307 ± 89 fmol mg^{-1} protein) or 10^{-5} M IBMX (284 ± 32 fmol mg^{-1} protein) did not increase cyclic GMP levels compared with SNP alone (337 ± 72 fmol mg^{-1} protein). Addition of 10^{-4} M IBMX (909 ± 122 fmol mg^{-1} protein) and 10^{-3} M IBMX (1482 ± 186 fmol mg^{-1} protein) significantly increased cyclic GMP levels compared to SNP alone (337 ± 72 fmol mg^{-1} protein) (both $P<0.001$, $n=4$).

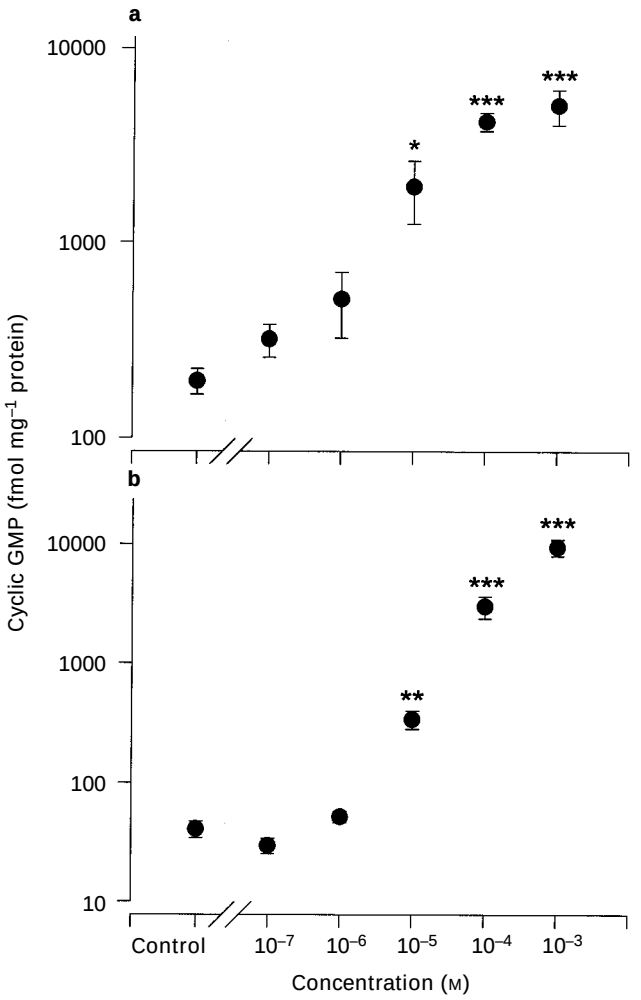

Figure 3 Cyclic GMP levels in ovine tracheal epithelial cells following 1 h incubation with (a) 10^{-7}–10^{-3} M sodium nitroprusside and (b) 10^{-7}–10^{-3} M S-nitroso-N-acetyl-penicillamine both in addition to 10^{-3} M IBMX. Data represent the mean of 6 replicates; vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared with control.

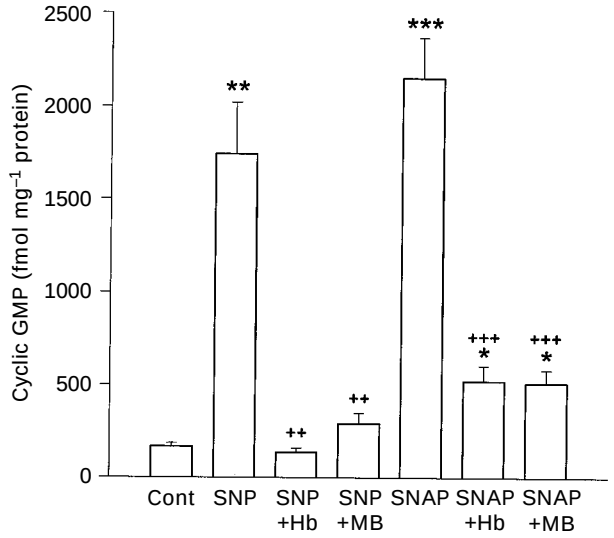

Figure 4 The effect of 5×10^{-5} M haemoglobin (Hb) and 5×10^{-4} M methylene blue (MB) on cyclic GMP levels in ovine tracheal epithelial cells following 1 h incubation with 10^{-6} M sodium nitroprusside (SNP) and 10^{-5} M S-nitroso-N-acetyl-penicillamine (SNAP). Data represent the mean of 5 replicates; vertical lines show s.e.mean. $*P<0.05$, $**P<0.01$, $***P<0.001$ when compared to control. $^{++}P<0.01$, $^{+++}P<0.001$ when compared to SNP or SNAP alone.

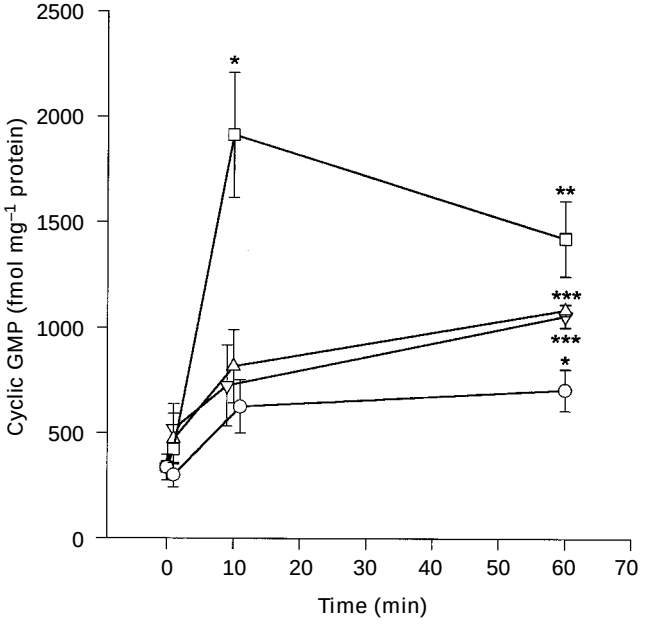

Figure 5 Cyclic GMP levels in ovine tracheal epithelial cells following incubation with (▽) 10^{-5} M ANP, (△) 10^{-5} M BNP, (□) 10^{-5} M CNP and (○) 200 iu STa toxin. Data represent the mean of 4 replicates; vertical lines show s.e.mean. $*P<0.05$, $**P<0.01$, $***P<0.001$ when compared to unstimulated levels.

Discussion

This is the first study to compare the production of cyclic GMP by activators of soluble and particulate guanylyl cyclase in tracheal epithelium. Our study showed that ovine tracheal epithelial cells contain active guanylyl cyclase. There was some variation in baseline cyclic GMP production between experiments which may reflect either inter-animal variation or variability due to the extraction procedure. Marked increases in cyclic GMP production were seen with SNP and SNAP. The magnitude of the increases produced by both agents was comparable. Some variation in responses to NO donors was observed between experiments, for example the magnitude of the rise in cyclic GMP levels with 10^{-6} M SNP between Figure 3a and Figure 4. This result is likely to be due to intrinisic tissue variability. The fact that SNP and SNAP induced increases in cyclic GMP levels were blocked by haemoglobin and methylene blue suggests that these agents activate guanylyl cyclase via NO donation. These results suggest that soluble guanylyl cyclase is abundant in tracheal epithelium.

Table 1 Cyclic GMP levels (fmol mg^{-1} protein) in ovine tracheal epithelial cells following incubation for 1 h with 10^{-4} M milrinone, 10^{-4} M SKF 96231 and $10^{-5}-10^{-3}$ M 3-isobutyl-1-methylxanthine (IBMX), with and without 10^{-3} M sodium nitroprusside

	Concentration	*−Sodium nitroprusside*	*+Sodium nitroprusside*
Control	–	45±16	337±72
Milrinone	10^{-4} M	32±13	271±42
SKF 96231	10^{-4} M	47±24	307±89
IBMX	10^{-5} M	–	284±32
IBMX	10^{-4} M	–	909±122*
IBMX	10^{-3} M	48±15	1482±186*

Data represent the mean±s.e.mean of 4 replicates. $*P<0.001$ compared to 10^{-3}M sodium nitroprusside alone.

ANP, BNP, CNP and STa toxin produced much smaller rises in cyclic GMP than NO donors. This suggests either that these cells have less membrane bound guanylyl cyclase activity, that the agents used were not biologically active in this species or that the concentrations used were not sufficient. Lack of biological activity is unlikely as α-human ANP has been previously shown to be active in the sheep (Silberbach *et al.*, 1994), as has CNP-22 (Charles *et al.*, 1995) and STa toxin (Zamora *et al.*, 1994). Human BNP 32 has been shown to be biologically active in other mammalian models (Takagi & Araki, 1992). The concentrations of natriuretic peptides required to elevate cyclic GMP (10^{-5} M) were 10^2 and 10^6 fold greater than have previously been shown to be active in human cultured nasal epithelial cells (Geary *et al.*, 1993) and rabbit tracheal epithelial cells (Tamaoki *et al.*, 1991), respectively. Our results suggest that GC_M is present in much smaller quantities than GC_S in ovine tracheal epithelial cells. The order of potency CNP > ANP = BNP > STa toxin is consistent with type B > type A > type C GC_M sub-classes being present.

The lack of elevation of cyclic GMP levels in the absence of IBMX, and the reduction in cyclic GMP levels at 4 h compared with 2 h incubation (Figure 2) suggest that cyclic GMP is rapidly degraded by phosphodiesterases in tracheal epithelium. In our experiments we could only inhibit phosphodiesterase breakdown of cyclic GMP with high concentrations ($10^{-4}-10^{-3}$ M) of IBMX. Concentrations of up to 10^{-4} M milrinone and 10^{-4} M SKF 96231 did not increase cyclic GMP levels. Human cultured bronchial epithelial cells have been shown to contain mainly type IV phosphodiesterase (PDE) (affinity: cyclic AMP≫cyclic GMP) but also quantities of type I (cyclic AMP≤cyclic GMP), type III (cyclic AMP=cyclic GMP) and type V (cyclic GMP≫cyclic AMP) (Kelly *et al.*, 1995; Robichaud *et al.*, 1996). At lower concentrations ($<10^{-4}$ M) milrinone is specific for type III and IV PDE (Shahid *et al.*, 1991), and at $<10^{-5}$ M SKF 96231 is specific for type V PDE (Murray *et al.*, 1991). The lack of effect of milrinone and SKF 96231 suggests that either the above isoenzymes are not present in these cells, or more likely that cell permeability is a problem with these compounds in this tissue.

Previous studies of GC expression in mammalian airway epithelial cells have yielded conflicting results depending on the species and airway generation studied. Evidence for GC_S expression in bronchial epithelial cells was found by Fellybosco

et al. 1994). They transfected human cultured bronchial epithelial cells with an inducible NO synthase gene increasing intrinsic NO production. They found increased levels of cyclic GMP production in these transfected cells compared with non-transfected cells in the presence of IBMX. Functional ANP receptors have previously been demonstrated by ultracytochemical localization on rat bronchiolar epithelium (Rambotti & Sprecca, 1991). Tamaoki *et al*. (1991) found that ANP increases cyclic GMP levels (approximately 2–3 fold increase) in rabbit cultured tracheal epithelial cells, but did not investigate the effect of other natriuretic peptides or NO donors. In human cultured nasal epithelial cells Geary *et al*. (1993) found elevations in cellular cyclic GMP (approximately 30 fold increase) levels in response to SNP and CNP, but not ANP, BNP or STa toxin.

The precise functions of cyclic GMP in airway epithelial cells have not yet been fully evaluated. In non-airway epithelial tissues cyclic GMP regulated several physiological processes including electrolyte transport and mucin secretion. In the gastrointestinal tract cyclic GMP has a major influence on the movement of water and electrolytes across the epithelium (Forte *et al*., 1992; Argenzio & Armstrong 1993), and a similar effect has been shown on tubular absorption in the nephron (Hammond *et al*., 1985; Light *et al*., 1989; 1990). In rat gastric epithelial cells mucin secretion is increased by elevated intracellular cyclic GMP levels (Brown *et al*., 1993).

The effect of cyclic GMP on ion transport has also been studied in pulmonary epithelium. Ion transport as measured by changes in short-circuit current was not influenced by SNP, CNP or 8Br-cyclic GMP (a cell permeable analogue of cyclic GMP) in human cultured nasal epithelium (Geary *et al*., 1995). In rat cultured distal foetal lung epithelium (95% type 2 pneumocytes) O'Brodovich *et al*. (1992) also found no change in short circuit current with ANP and 8Br-cyclic GMP. Cyclic GMP may regulate the ciliary beat frequency of airway epithelial cells although studies have shown conflicting results depending on the species and site examined. In human cultured nasal cells CNP and 8-Br cyclic GMP but not SNP cause an increase in ciliary beat frequency (Geary *et al*., 1995). In contrast ANP decreased ciliary beat frequency in rabbit cultured tracheal epithelial cells (Tamaoki *et al*., 1991). Further studies (Nagaki *et al*., 1995) have suggested that NO may be involved in the control of mucus secretion by human and feline isolated submucosal glands but not intact airway epithelial explants.

In conclusion, our studies demonstrate that guanylyl cyclases are present in sheep tracheal epithelial cells. Pharmacological characterization shows that soluble guanylyl cyclase is the predominant sub-type expressed.

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