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Effects of the nitric oxide/cGMP system compared with the cAMP system on airway mucus secretion in the rat

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Abstract

Mucus secretion of the airways is under the control of a variety of intracellular second messenger systems. Cyclic nucleotides such as cGMP, coupled to the recently discovered nitric oxide system, and cAMP are of outstanding interest in this respect. The present study used the modified Ussing chamber technique and mucins labelled with $^{35}SO_4$ to investigate mucus secretion in the rat trachea to clarify the contribution of these different second messenger systems to the control of mucin secretion.

A variety of drugs affecting either the generation or the breakdown of the respective cyclic nucleotides were used. Neither drugs interfering with nitric oxide synthase nor the phosphodiesterase isoenzyme responsible for cGMP breakdown nor cGMP analogues were able to affect mucus secretion. In contrast, stimulation of adenylate cyclase or inhibition of the respective phosphodiesterase resulted in a potent increase of mucus secretion. In conclusion, we failed to show the involvement of the nitric oxide/cGMP system, whereas the cAMP system seems to be a very efficient regulator of mucus secretion in the rat trachea.

Keywords: Mucus secretion; Nitric oxide (NO); Cyclic nucleotide; Second messenger; Trachea, rat

1. Introduction

Today, it is widely accepted that nitric oxide (NO) plays an important role in airway function. This system might be involved in airway diseases such as asthma and chronic bronchitis (Barnes and Belvisi, 1993). NO is endogenously produced in a variety of cells and organs including the respiratory tract. At least two enzymes exist, a constitutive and an inducible isoform of the enzyme NO synthase (NOS), both of which convert L-arginine to L-citrulline. Upon release, NO usually acts via an increase of intracellular cGMP by activating soluble guanylate cyclase (Lei et al., 1993). NOS immunoreactivity has been shown in different cell types of rat and human lung (Kobzik et al., 1993), and NOS-containing nerve fibres have been found to supply airway smooth muscle, blood vessels and submucosal glands in the human respiratory tract (Fischer and Hoffmann, 1996). NO acts as a potent bronchodilator in the airways and thus is an important neurotransmitter of the inhibitory nonadrenergic noncholinergic system (Kannan and Johnson, 1992; Lei et al., 1993). Furthermore, it is a potent pulmonary vasodilator (Pepke-Zaba et al., 1991), controls plasma extravasation (Kuo et al., 1992; Erjefalt et al., 1994) and ciliary beat frequency (Jain et al., 1993).

Mucus hypersecretion is a common feature of various diseases of the respiratory tract, such as asthma or chronic bronchitis and chronic obstructive lung disease (Vestbo et al., 1996). Airway mucus secretion is regulated by a complex network of mediators with stimulatory and inhibitory effects including neuropeptides, purines, enzymes from neutrophils and lipid mediators (Ramnarine and Rogers, 1994; Marin, 1994; Wagner and Von Wichert, 1991). There have been only a few studies investigating the role of NO with conflicting results. On the one hand, NO inhibited both basal and neurogenic mucus secretion in ferret trachea in vitro (Ramnarine et al., 1996) and on the

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other hand, it had a stimulatory role in the regulation of mucus secretion in isolated submucosal glands from feline trachea (Nagaki et al., 1995).

The aim of the present study was to elucidate the effect of the NO/cGMP system on mucus secretion in the rat trachea using the modified Ussing chamber technique and $^{35}SO_4$ as a marker of mucus glycoproteins (mucins). Additionally, we compared the effect of the NO/cGMP system with that of the cAMP system, which is assumed to play a critical role in several cellular responses. Therefore, we characterized the effects of the NO donors isosorbide dinitrate and sodium nitroprusside, the NO precursor L-arginine, the NOS inhibitor *N*(G)-nitro-L-arginine methyl ester as well as the cyclic nucleotide analogues Db-cGMP and 8-Br-cGMP, the inhibitor of the cGMP-specific phosphodiesterase-isoenzyme zaprinast, and atrial natriuretic peptide (ANP). In addition, we compared these agents with drugs affecting the cAMP system, namely, the adenylate cyclase activator forskolin, Db-cAMP, 8-Br-cAMP, vasoactive intestinal peptide (VIP), and the inhibitor of the cAMP-specific phosphodiesterase rolipram. We also investigated two inhibitors of phosphodiesterase isoenzymes (9-*erythro*-(2-Hydroxy-3-*n*-nonyl) adenine hydrochloride (MEP1) as a phosphodiesterase II and vinpocetine as a phosphodiesterase I inhibitor) since the effect of these two isoforms on mucus secretion is unknown so far (Wagner et al., 1996).

2. Materials and methods

2.1. Chemicals

The organ culture medium, M 199 in Earle's balanced salt solution, was obtained from Gibco (Eggenstein, Germany). $Na_2{}^{35}SO_4$ for radiolabelling glycoproteins was from Amersham (Braunschweig, Germany). Cellulose dialysis tubing (12–14 kDa molecular mass cut-off) was from Serva (Heidelberg, Germany). Atrial natriuretic peptide (rat) and vasoactive intestinal peptide (rat) were obtained from Bachem (Heidelberg, Germany), 9-*erythro*-(2-Hydroxy-3-*n*-nonyl) adenine hydrochloride (MEP1) was from Biolog (Bremen, Germany), sodium nitroprusside, isosorbide-dinitrate, forskolin, L-NAME, L-arginine, 8-Br-cAMP, 8-Br-cGMP, dibutyryl-cAMP, dibutyryl-cGMP and phosphor-amiden were from Sigma (Deisenhofen, Germany). KT 5823 was bought from Calbiochem Novabiochem (Bad Soden, Germany). Vinpocetine was received as a gift from Thiemann (Waltrop, Germany), and rolipram and zaprinast were gifts from Byk-Gulden (Konstanz, Germany) (see Table 1 for details).

2.2. Animals

Male Sprague–Dawley rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany) with an average weight of 400 g were kept in a light- and temperature-controlled room and had free access to water and a rat standard diet (Altromin, Lage, Germany). These rats were specific pathogen-free.

2.3. Studies on tracheal mucus secretion (Ussing chamber technique)

Rats were anaesthesized by an intraperitoneal injection of 70 mg/kg body weight pentobarbital (Nembutal®). The trachea was excised (cranially from the larynx, caudally to the bifurcation) through a ventral collar midline incision and median sternotomy and immediately transferred to an organ bath filled with medium 199 equilibrated with carbo-

Table 1
List of applied drugs

Drug	Abbreviation	Mode of action	Concentration range tested
Forskolin		Adenylate cylase activator	100 nM–1 μM
Isosorbide dinitrate	ISDN	NO donor	100 nM–1 μM
Sodium nitroprusside	SNP	NO donor	100 nM–1 μM
L-arginine		NO precursor	1 nM
N(G)-methyl-L-arginine methyl ester	L-NAME	NO synthase inhibitor	100 μM
vasoactive intestinal peptide	VIP	Neuropeptide	1 μM
atrial natriuretic peptide	ANP	Neuropeptide	1 μM
8-Bromo-cyclic adenosine-mono-phosphate	8-Br-cAMP	Non-hydrolysable cAMP analogue	1 mM
8-Bromo-cyclic guanosine-mono-phosphate	8-Br-cGMP	Non-hydrolysable cGMP analogue	1 mM
Dibutyryl-cyclic adenosine-mono-phosphate	Db-cAMP	Non-hydrolysable cAMP analogue	1 mM
Dibutyryl-cyclic guanosine-mono-phosphate	Db-cGMP	Non-hydrolysable cGMP analogue	1 mM
KT 5823		Inhibitor of protein kinase G	1 μM
Rolipram		Inhibitor of phosphodiesterase IV	1 nM–100 μM
Zaprinast		Inhibitor of phosphodiesterase V	1 nM–100 μM
9-*erythro*-(2-Hydroxy-3-*n*-nonyl) adenine hydrochloride	MEP1	Inhibitor of phosphodiesterase II	1 nM–100 μM
Vinpocetine		Inhibitor of phosphodiesterase I	1 nM–100 μM
Phosphoramidon		Inhibition of neutral endopeptidase	10 μM

gen gas (95% oxygen, 5% carbon dioxide). The connective tissue was removed and the trachea was opened along the paries membranaceus and then mounted between the two halves of a modified Ussing chamber. Seven millilitres of medium 199 in Earl's balanced salt solution equilibrated with carbogen gas at 37°C and pH 7.41 was added to the mucosal and submucosal sides, respectively. To the solution bathing the submucosal side, 50 μCi $Na_2{}^{35}SO_4$ was added and allowed to equilibrate with the tissue for the duration of the experiment. After 2 h, the release of bound $^{35}SO_4$ to the mucosal (luminal) side reached steady state. Afterwards, the luminal solution was collected every 15 min and replaced with fresh medium. The samples to analyze mucus secretion were assayed as explained in the Experimental design section. The perfusate samples from the luminal side were collected in cellulose dialysis tubing (12,000–14,000 Da molecular mass cut-off) and dialysed against distilled water containing excess unlabelled Na_2SO_4 to remove unincorporated $^{35}SO_4$, and sodium azide (10 mg/l) to prevent bacterial degradation. Dialysis was complete when the radioactivity of the dialysis fluid 3 h after the last change was equal to the radioactivity of water used for dialysis. The samples were transferred to plastic vials, mixed with 10 ml of scintillation fluid (Lumagel®), and radioactivity was determined using a liquid scintillation counter. The counts of the samples reflect the mucus secretion rate, since former studies (Wagner et al., 1995a,b,c) using high-performance liquid chromatography (HPLC) and autoradiography have shown that labelled macromolecules represent mucus glycoproteins (mucins) coming from the submucosal glands.

This Ussing chamber method separating submucosal and mucosal solutions is a well established method to measure the macromolecular secretion of glands and epithelium of tracheal preparations. It offers the advantage that the radiolabelled precursor can equilibrate with the submucosal side of the tissue, during which time materials secreted into the lumen can be collected. The method depends on an intact epithelial diffusion barrier, which effectively avoids free diffusion of the precursor. According to our studies and the literature, sulfate, which is present on many of the carbohydrate side chains as a terminal residue, has great advantages over other precursors (e.g. labelled amino acids or sugars), especially because it is not metabolized (Borson et al., 1988). Thus, the output of labelled mucins can be assessed. Dialysis against distilled water containing excess unlabelled SO_4 effectively removes ionically bound unincorporated $^{35}SO_4$, so that an unintentional study of the mere transport of SO_4 can be excluded. Former high-performance liquid chromatography studies of the $^{35}SO_4$-labelled molecules identified them as high-molecular-weight glycoconjugates that are not digested by chondroitinase ABC. Thus, these macromolecules are true glycoproteins.

Our histologic studies have demonstrated that the Sprague–Dawley rats used for these experiments have large submucosal glands embedded between the cartilage rings, with the highest density in the ventral midline. They extend from the larynx at least to the tracheal bifurcation, decreasing in size distalwards. Therefore, our Ussing chambers were positioned just in between this region of highest submucosal gland density over the ventral midline in a longitudinal direction in a standardized manner from the 2nd to the 8th cartilage ring. These specific pathogen-free rats nearly completely lack goblet cells in their superficial epithelium, so that the almost exclusive source of mucus production is the submucosal glands and not cells in the superficial epithelium. Moreover, autoradiographic studies done with ^{35}S-radiolabel have demonstrated the accumulation of the label in the submucosal glands, especially the acini. Nearly no label was found in the cells of the superficial epithelium, which furthermore suggests the origin of the mucin to be the submucosal glands (Wagner et al., 1995a,b,c).

2.4. Experimental design

After 2 h, the secretion of labelled macromolecules reached a steady state. Then, samples were collected every 15 min. The average of the first four samples represented the basal secretion rate (= 100%). Drugs were applied to the mucosal side and fractions were collected 15 min later. Between each application, four fractions of 15 min were collected to allow the system to recover and reach a basal secretion rate again. Dose–response correlations for the drugs tested were performed by luminal application in increasing steps of a factor 10 according to the above-mentioned design. In order to test the viability of the system, each experiment was finished with a stimulation with acetylcholine 1 mM, the most potent secretory stimulus known for this system (about 600% of basal secretion).

2.5. Statistical analysis

Values are expressed in % of basal secretion. Data are presented as means ± S.E.M. Statistical analysis was performed with Student's *t*-test for unpaired samples. Experiments with at least five animals were performed for each experimental protocol.

3. Results

First, we characterized the effect of forskolin, a direct activator of adenylate cyclase. Forskolin showed a clear dose-dependent stimulatory effect on mucus secretion: 100 nM 120.83 ± 5.64%, 1 μM 154.71 ± 14.34%, 10 μM 215.29 ± 16.59%, 100 μM 227.47 ± 13.31%, 1 mM 223.50 ± 15.98% (Fig. 1). ISDN and SNP, both known to stimulate guanylate cyclase by liberating NO and thus elevating the intracellular cGMP level, did not significantly affect the secretion rate in the concentration range

tested: SNP 100 nM 104.22 ± 8.31%, 1 μM 96.35 ± 9.98%, 10 μM 98.85 ± 1.99%, 100 μM 112.93 ± 6.55%, 1 mM 98.36 ± 4.85%; ISDN 100 nM 95.68 ± 6.21%, 1 μM 102.58 ± 13.66%, 10 μM 91.33 ± 12.24%, 100 μM 96.29 ± 5.19%, 1 mM 109.82 ± 7.84% (Fig. 1). Combination with zaprinast (10 μM) did not alter the results significantly (data not shown).

We have shown (Wagner et al., 1996) that the type IV phosphodiesterase inhibitor rolipram is a potent stimulator of mucus secretion: 1 nM 109.81 ± 5.34%, 10 nM 133.46 ± 11.09%, 100 nM 184.71 ± 19.52%, 1 μM 187.33 ± 15.65%, 10 μM 192.08 ± 8.44%, 100 μM 222.63 ± 23.93% (Fig. 2). In contrast, the cGMP-specific PDE inhibitor zaprinast (type V inhibitor) was without effect in the same concentration range. MEP1, a PDE isoenzyme type II (usually termed cGMP-dependent phosphodiesterase) inhibitor, induced a slight increase at the highest concentration, which is considered to be non-specific: 100 nM 102.67 ± 5.71%, 1 μM 101.98 ± 4.25%, 10 μM 100.55 ± 5.51%, 100 μM 138.01 ± 10.39%. Vinpocetine, a PDE type I (named Ca^{2+}/calmodulin-dependent phosphodiesterase) inhibitor, stimulated mucus secretion at a high concentration: 100 nM 105.86 ± 2.98%, 1 μM 110.1 ± 3.46%, 10 μM 113.63 ± 4.94%, 100 μM 146.82 ± 9.01%.

Forskolin and rolipram (together at 10 μM each) stimulated mucus secretion to 199.72% ± 9.75, this being not significantly different from the effect obtained for each drug alone.

Application of non-hydrolysable, membrane-permeable analogues of the cyclic nucleotides on the mucosal side of the trachea at a concentration of 1 mM yielded the following results: dibutyryl-cAMP 130.2 ± 3.4%, 8-Br-cAMP 131.6 ± 3.4%; dibutyryl-cGMP 101.1 ± 2.2%, 8-Br-cGMP 110.7 ± 4.6%. Combination of the cGMP analogues with zaprinast did not change the results (data not shown). Neither the NO precursor L-arginine (100.9 ± 3.8% at 1 mM) nor the NO synthase inhibitor L-NAME (102.5 ± 5.6% at 100 μM) alone affected mucus secretion. Additionally, KT 5823, an inhibitor of proteinkinase G, did not show any effect on basal mucus secretion (101.23 ± 8.9% at 1 μM).

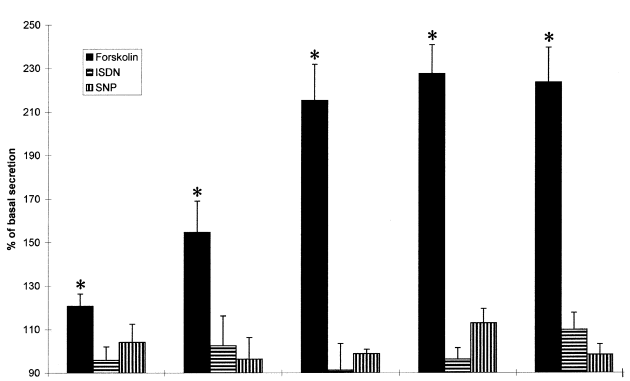

Fig. 1. Effects of the NO donors ISDN and SNP and the adenylate cyclase activator forskolin on mucus secretion.

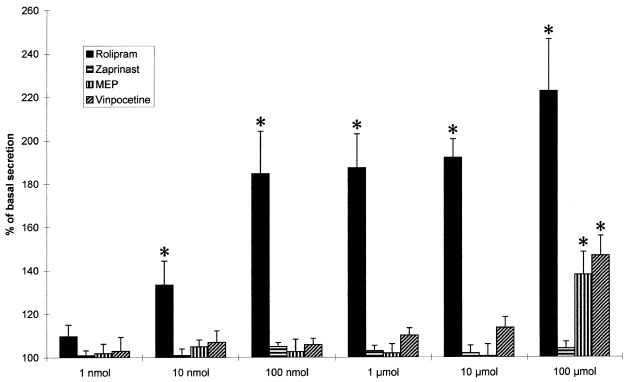

Fig. 2. Effects of selective phosphodiesterase inhibitors on mucus secretion.

The following peptides tested were used at a concentration of 1 μM, which is the most effective concentration for peptidergic influences in this system (Wagner et al., 1998). VIP induced a stimulation of 169.0 ± 17% of basal secretion, whereas ANP failed to affect secretion (99.2 ± 5.8%) both alone and in combination with the neutral endopeptidase inhibitor phosphoramidon (97.2 ± 13.9%).

4. Discussion

The present study clearly demonstrates a potent stimulatory effect of elevated intracellular cAMP levels on mucus secretion from isolated rat trachea. Activation of the adenylate cyclase by forskolin dose dependently increased the output of mucus glycoproteins. This result is in good agreement with previous studies demonstrating a remarkable stimulatory effect of phosphodiesterase inhibitors, which increase cyclic nucleotide levels by limiting their breakdown, and of peptides activating adenylate cyclase. At least in the rat trachea, phosphodiesterase isoenzyme type IV, which is specific for cAMP, seems to be the predominant isoenzyme that accounts for the stimulatory effect of PDE inhibition since only the PDE IV inhibitor rolipram and the mixed PDE III/IV inhibitor zardaverine stimulated mucin output, whereas motapizone (PDE III inhibitor) and zaprinast (PDE V inhibitor) were without any effect (Wagner et al., 1996). This is now further confirmed by our results that extend the cited study. Inhibition of PDE type I by vinpocetine and of type II by MEP1 failed to elicit significant mucus secretion within the concentration range regarded to be isoenzyme specific. Interestingly, the maximum effect, an about two-fold increase of basal secretion, that could be elicited by forskolin at 100 μmol was nearly the same as the maximum effect induced by the PDE IV inhibitor rolipram at 100 μmol and could not be potentiated further by combination of the two drugs. Obviously, secretion evoked by the cAMP pathway

is limited at this point. The comparatively weak but still significant effect of the membrane-permeable and not hydrolysed cAMP-analogues Db-cAMP and 8-Br-cAMP is most likely due to the diffusion barrier of the epithelium as the trachea was used as a whole organ. Therefore, these drugs might only in part reach the submucosal glands from where mucus derives. Vasoactive intestinal peptide was able to stimulate mucus secretion in a dose-dependent manner, but was much less potent than, e.g. the tachykinins (Wagner et al., 1995a,b,c). VIP belongs to a family of peptides which are characterized by a high degree of sequence homology including peptide histidine isoleucine (PHI)/peptide histidine methionine (PHM), pituitary adenylate cyclase-activating polypeptide (PACAP)-27 and -38, helodermin, helospectin I and II. The stimulatory effect of these peptides on mucus secretion in the rat airways is most likely receptor-coupled (Wagner et al., 1998). The VIP receptor has been identified in the airways of the rat (Ichikawa et al., 1995) and even in the human trachea in the surroundings of submucosal glands (Fischer et al., 1992). In most systems investigated, these receptors are coupled with the cAMP-dependent pathway of signal transduction (Ulrich et al., 1998) which, together with the above mentioned results, supports the idea of a stimulatory effect of the cAMP system.

Conversely, in our study neither isosorbide dinitrate nor sodium nitroprusside, which liberate NO, were able to affect mucus secretion in isolated rat trachea. Even zaprinast as a cGMP-specific PDE V inhibitor alone or in combination with the NO donors had no effect. Additionally, inhibition of the NO synthase by L-NAME or application of the substrate for NO synthase, L-arginine, did not influence basal mucus secretion. The exogenously applied analogues Db-cGMP and 8-Br-cGMP in concentrations up to 1 mM did not evoke any effect. As identical derivatives as for the cAMP analogues were used, this cannot be explained by a low membrane permeability or hydrolysis by phosphodiesterases, because combining them with zaprinast did not alter the result. Additionally, KT 5823, an inhibitor of protein kinase G, did not affect mucus secretion in the rat trachea. ANP, which has been shown to be present in the airways as part of the complex peptidergic system (Baraniuk and Kaliner, 1990), acts through a special receptor that is coupled to the particulate guanylate cyclase, thus elevating intracellular cGMP (Hamad et al., 1996). ANP alone and in combination with the neutral endopeptidase inhibitors thiorphan or phosphoramidon, to prevent its breakdown, did not alter mucus secretion, suggesting that ANP had no major impact on the regulation of mucus secretion in the airways, in contrast to its properties concerning airway smooth muscle relaxation (Hamad et al., 1996).

The obvious failure of the NO/cGMP system to influence mucus secretion under basal conditions in the airways is in partial contrast to the results obtained by others. Ramnarine et al. (1996) used ferret tracheas in vitro and a similar Ussing chamber technique as in our experiments and found that the NOS inhibitor *N*(G)-monomethyl-L-arginine (L-NMMA) potently stimulated the output of $^{35}SO_4$-labelled mucus glycoproteins whereas an NO donor, FK 409 ((±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide), significantly inhibited basal secretion. Furthermore, neurogenic mucus secretion induced by electrical field stimulation was potentiated by L-NMMA and inhibited by FK 409, whereas cholinergically induced secretion was not influenced by these two compounds. They concluded that NO might be directly implicated as an inhibitory neurotransmitter in airway mucus secretion.

Nagaki et al. (1995, 1996), however, reported the opposite effect, namely, that the NOS inhibitor L-NAME significantly inhibited acetylcholine and bradykinin-induced secretion from isolated submucosal glands from feline trachea using trichloroacetic acid precipitable [^{3}H]-glycoconjugates as a secretory marker. Furthermore, the NO donor ISDN induced a significant increase in mucus secretion in human airways. Thus, they assumed endogenous NO to have a stimulatory action on mucus secretion in the airways.

Of course, different species were used and this may explain these differences. However, the ferret as well as the cat and the Sprague–Dawley rat used in our study are suitable models for investigating secretion from submucosal glands since they contain only very few, if any, goblet cells under specific pathogen-free conditions (Jefferey, 1994). Thus, an unintentional interference with secretion from goblet cells can be excluded. For example, when investigating isolated tracheal epithelial cells from the guinea-pig, a species where goblet cells are the primary source of mucus, Adler et al. (1995) found mucus secretion induced by histamine, platelet activating factor (PAF) and tumor necrosis factor alpha (TNF-α) to be blocked by the NOS-inhibitor *N*(G)-monomethyl-L-arginine (L-NMMA). As goblet cells and submucosal glands are under different control, it is not possible to extrapolate these findings from one experimental model to another.

The present study has shown that, independent of receptor-mediated effects, the signal transduction pathway involving cAMP is a potent stimulus for secretion and that this action can be reproduced by limiting the breakdown of cAMP as well as by increasing its production. The second messenger system involving cGMP, which is in part coupled to the neurotransmitter nitric oxide via an activation of soluble guanylate cyclase (Lei et al., 1993), however, completely failed to affect mucus secretion under basal conditions. Whether there is a role for NO under prestimulated conditions with peptidergic agonists or in altered pathophysiology caused by inflammatory processes remains to be elucidated.

NO has important functions in the airways, generally of an inhibitory character as part of the nonadrenergic noncholinergic (NANC) system. It inhibits NANC bronchoconstriction (Lei et al., 1993) and suppresses airway

plasma extravasation (Erjefalt et al., 1994), which argues in favour of NO having a critical role in the maintenance of airway tone, whereas an imbalance in the production of NO may lead to disorders such as asthma (Barnes and Belvisi, 1993). Indeed, asthmatics have increased levels of NO in exhaled air and within bronchial epithelium (Kharitonov et al., 1994), probably as a result of induction of inducible nitric oxide synthase (iNOS) associated with the inflammatory process (Robbins et al., 1994). In asthma, mucus hypersecretion is a common symptom, and in ferret and feline airways NO seems to be involved in the regulation of mucus secretion, but results are conflicting (Ramnarine et al., 1996; Nagaki et al., 1995, 1996). Our study with isolated rat trachea failed to detect effects of the NO/cGMP pathway, in contrast to the cAMP signal transduction system, which evoked potent stimulation. Other second messenger systems involving intracellular calcium or protein kinase C have also been shown to potently influence mucus secretion in the isolated rat trachea (Bredenbröker et al., 1998). Thus, it seems rather unlikely that there is an essential role for NO in the control of submucosal glands in the airways, in contrast to its role in the regulation of smooth muscle tone. Mucus secretion is a critical part of asthma pathophysiology. Therefore, the development of new therapeutic strategies, such as drugs affecting NO metabolism or isoenzyme-specific PDE inhibitors, should carefully consider their impact on the secretory function of the airways.

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