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Adenosine-induced secretion in the canine trachea: modification by methylxanthines and adenosine derivatives

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1 Adenosine alone at 0.1 and 1.0 mg per tracheal segment stimulated mucus secretion by 52% and 88%, respectively, compared to baseline ($P < 0.0001$).

2 The site of the potent secretagogue effect of adenosine in canine trachea was consistent with A_2 activation.

3 A_2 site activation and enhanced secretion were also induced by N-ethylcarboxamide adenosine and dipyridamole.

4 N^6-R-phenylisopropyl adenosine (PIA) and 2',5'-dideoxyadenosine (ddAdo) inhibited the adenosine-induced secretion (35% and 42%, respectively). However, when PIA or ddAdo were administered in conjunction with the potent phosphodiesterase inhibitor, methylisobutylxanthine (MIX), the effects of PIA were potentiated and the effects of ddAdo were reversed, yielding stimulation (A_2) and antagonism (A_1) of secretion, respectively.

5 8-Phenyltheophylline by aerosol was a very potent antagonist of the secretagogue effect of adenosine (70% inhibition; $P < 0.00001$).

Introduction

Adenosine and antagonists of adenosine modified at the purine and ribose moieties have been implicated as having a regulatory function in a variety of cell types and tissues in bronchial airways. Activation of adenylate cyclase in rat mast cells modulated (both stimulated A_2 and inhibited A_1) histamine release through R-site-active (purine modified) and P-site-active (ribose modified) adenosine analogues (Holgate *et al.*, 1980). Adenosine, but not guanosine, was a potent, fast-acting bronchoconstrictor in both allergic and non-allergic asthmatic subjects (Cushley *et al.*, 1983) suggesting it had a specific pharmacological effect. In isolated animal tracheal and bronchial airway preparations with artificially increased tone, adenosine caused relaxation (Fredholm *et al.*, 1979). However, in man, aerosolized adenosine caused a potent bronchoconstriction (Pauwells & Vander-Straeten, 1983). The reason for this species difference in respiratory smooth muscle response to adenosine is not known.

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It is assumed that in tissues where adenosine shows a biological effect, this effect is due to activation of adenylate cyclase at the external or internal membrane receptor. However, with the availability of adenosine derivatives modified at the purine and ribose moieties, it is possible to pinpoint the site of action of adenosine (Wolff *et al.*, 1981; Fredholm, 1982). The role of adenosine as an autacoid in the lung and airways is not known, but it has been suggested that adenosine antagonism is a mechanism behind the bronchodilator effects of xanthines (Fredholm, 1980a; Marquardt *et al.*, 1978). With the recent demonstration of the potent secretagogue effect of adenosine in a canine tracheal preparation (Johnson *et al.*, 1985) it was of interest to determine whether adenosine acted through A_2 site activation resulting in secretion. This study extends these findings and pinpoints the site of the potent secretagogue effect of adenosine by use of methylxanthines, N-ethylcarboxamide adenosine (NECA), N^6-R-phenylisopropyl adenosine (PIA), 2',5'-dideoxyadenosine (ddAdo), and dipyridamole to antagonize and potentiate mucus secretion in the trachea.

Methods

Tracheal hillock measurement

Thirty-two mongrel dogs (weight 18–30 kg) were anaesthetized with a mixture of chloralose (100 mg kg^{-1}) and urethane (500 mg kg^{-1}) intravenously, and their respiratory muscles were paralysed with gallamine triethiodide (20–40 mg kg^{-1}, i.v.). An endotracheal tube was tied into the lower trachea and connected to a Harvard respirator (model 607A) that delivered room air at a constant tidal volume and frequency. To monitor ventilation level, two polyethylene catheters were connected to the tracheal cannula: one catheter was connected to a medical gas analyser (Beckman model LB-2) that continuously measured the content of the expired gas for determination of end-tidal CO_2, the other was connected to a pressure transducer (Statham, model PMI 131TC + 2.5-350) for measurement of airway pressure. A polyethylene catheter was tied into the femoral artery of each dog and connected to a pressure transducer (Statham, model P23ID) for measurement of blood pressure; this catheter also allowed collection of arterial blood samples for measurements of blood gas tensions by a blood gas analyser (Corning, model 175). A second polyethylene catheter was tied into the femoral vein for injection of drugs. All physiological measurements were recorded and monitored on a polygraph (Grass Instruments, model 7D).

For close arterial injection of the adenosine, a small polyethylene catheter was inserted into the muscular branch of the cranial thyroid artery (CTA), so as not to obstruct blood flow from the carotid artery. Adenosine or NECA were administered at a standardized rate and flushed with saline; doses were diluted in 1.0 ml of 0.9% saline (pH 7.4). Localized blood pressure changes across the vascular bed of the cervical trachea were measured by placing two catheters, connected to two Statham pressure transducers (model P23ID), into the CTA. The difference in pressure in the injected side versus the control side was corrected for saline injection. It was not possible to measure flow, therefore, resistance could not be calculated.

The mucosal surface of the upper trachea was exposed by cutting along the anterior midline of the upper two-thirds of the extrathoracic trachea; the cut edges were pulled apart widely, and the secretions that formed were removed by wiping the mucosa with small pieces of tissue paper soaked in saline (0.9%). To visualize secretions coming from the submucosal gland duct openings, powdered tantalum (an inert metal, 2–5 μm in size) was sprayed until it formed a uniform layer over the mucosal surface. The rate of baseline secretion was not affected by the tantalum layer and did not change over the couse of 6 h, but the tantalum layer prevented the normal ciliary dispersion of secretions from the submucosal gland duct openings; these secretions, therefore, caused elevations ('hillocks') in the tantalum layer.

To quantitate the number of hillocks formed in a 1.2 cm^2 field, 1–2 cm from the larynx, their images were recorded with a videocamera (Hitachi CCTV model HV-17TU) mounted on a dissecting microscope (Zeiss Opmi-1) with a beam splitter. The camera was connected to a videotape recorder (Sony, model VO-2610) and a time signal generator (Panasonic WJ-810). The appearance of hillocks and the time signal were recorded simultaneously on videotape, the recorded images were photographed from a television monitor (Sony Trinitron KV 1914), and the number of hillocks were counted for a 1 to 2 min period. Only hillocks larger than 0.1 mm in diameter were counted. The response to adenosine was determined by counting the number of baseline hillocks in each of the dogs during a 2 min control period and comparing this value with a similar 2 min period in the same microscopic field immediately after adenosine or NECA injection (0.1. and 1.0 mg in 1.0 ml saline) into the CTA.

In other studies, the tracheal hillock method of quantitation has been correlated with [^{3}H]-glucosamine incorporation into high molecular weight glycoprotein by collecting, weighing, and dialysing mucus, and counting the radioactivity in the non-dialysable portion. A correlation coefficient between hillock count and specific activity (c.p.m. mg^{-1}) of nondialysable glycoproteins in control and acetylcholine-induced secretion was $r = 0.86$ in six repeat assays.

Pharmacological antagonism of adenosine

When MIX, PIA, or ddAdo were tested for their effect on secretion, the results were compared to the control response, and to the response after adenosine alone at 0.1 or 1.0 mg given into the CTA for a 1 and 2 min period. MIX (1.0 mg), ddAdo (0.5 mg) and PIA (0.5 mg) were administered in the CTA 5 min before adenosine. All agonists and antagonists were made up in 0.9% saline and given in 1.0 ml. Dipyridamole 0.5 mg kg^{-1} in saline was given into the femoral artery 20 min before the adenosine.

Aerosol administration of 8-phenyltheophylline

8-Phenyltheophylline was completely insoluble in saline, and was therefore suspended in 50% dimethylsulphoxide (DMSO) and 15 breaths of a 1% solution were aerosolized directly into the endotracheal tube 5 min before the adenosine challenge via the CTA. The 50% DMSO vehicle alone had no effect on the 2 min hillock count.

Drugs and chemicals

Chloralose, urethane, adenosine, and 8-phenyltheophylline were purchased from Sigma Chemical Corp., St Louis, MO. 2′,5′-Dideoxyadenosine was purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. N^6-R-phenylisopropyl adenosine was purchased from Boehringer Mannheim, Indianapolis, IN, U.S.A. N-ethylcarboxamide adenosine was a gift from Byk Gulden, Lomberg Chemische Fabrik, GmbH, Konstanze, W. Germany. Dipyridamole (Sigma, St Louis, MO), dimethylsulphoxide (Fisher, Fairlawn, N.J.), $NaNO_2$ (Mallinckrodt, St Louis, MO) and methylisobutylxanthine (P-L Biochemicals, Milwaukee, WI) were also used.

Statistical analysis

Mucous secretion rates associated with baseline and adenosine treatment were obtained on each of the 32 dogs. All dogs received both doses of adenosine. Some animals received repeated doses of adenosine during the experimental procedure.

Mucus secretion was represented by the number of hillocks per cm^2. These counts were taken at 1 and 2 min after treatment. To examine the effect of various doses of adenosine on mucus secretion, the number of hillocks compared to baseline was calculated for each time after treatment. These percentage increase values were averaged across times-after-treatment and then over time periods to obtain a single percentage increase value for each treatment-dog combination. Summary statistics were calculated from hillock numbers per time interval for each dose group. The increases associated with each treatment group were then compared to zero and to each other by Student's *t* test. Differences in blood pressure across the trachea were compared by one-way analysis of variance (ANOVA).

To examine the change in the adenosine effect after an agonist or antagonist, the hillock increase values were calculated as before; however, in this case they were not averaged over the time-after-treatment.

Results

Adenosine-induced enhancement of secretion

Intra-arterial injection of 0.1 or 1.0 mg adenosine into the CTA gave statistically significant increases of 52% and 88% over the control response, respectively (Table 1). The response was essentially complete in 2 min. The doses of adenosine or NECA were also randomized to assess a cumulative effect. There appeared to be no effect of high dose followed by low dose. Each injection was separated by 20 min. NECA at 0.1 and 1.0 mg increased secretion by 43% and 64%, respectively ($P<0.05$). Dipyridamole (0.5 mg), an inhibitor of adenosine re-uptake, potentiated the res-

Table 1 Modification of the secretagogue effect of adenosine by antagonists* of adenylate cyclase in canine trachea

Condition	*Dose*	*Number of dogs*	*2 min Hillocks* (± s.e.mean)	*% change*	P *value*†
Control		32	117 ± 17		
Adenosine	0.1 mg	32	178 ± 5.5	+ 52ʃ	<0.00001
	1.0 mg	32	221 ± 8.2	+ 88	<0.00001
NECA	0.1 mg	3	168 ± 23	43ʃ	0.019
	1.0 mg	3	193 ± 20	64	0.003
Adenosine	0.1 mg				
+ MIX	1.0 mg	5	181 ± 9	+ 2§	NS
+ ddAdo	0.5 mg	8	104 ± 12	− 42	<0.00001
+ MIX + ddAdo		2	224 ± 15	+ 26	0.053
+ PIA	0.5 mg	6	116 + 13	− 35	<0.0001
+ MIX + PIA		3	99 ± 17	− 44	0.0002
Adenosine	1.0 mg				
+ dipyridamole	0.5 mg	4	278 + 16	+ 26¶	0.0157
+ 8-phenyltheophylline 1% aerosol		2	67 ± 2	− 70	<0.00001

*NECA = N-ethylcarboxamide adenosine; MIX = methylisobutylxanthine; ddAdo = 2′,5′-dideoxyadenosine; PIA = N^6-R-phenylisopropyl adenosine.
†Student's *t* test; $P<0.05$ considered significantly different. NS, not significant.
ʃCompared to control.
§Compared to adenosine 0.1 mg alone.
¶Compared to adenosine 1.0 mg alone.

ponse to adenosine by 26% ($P = 0.015$), when it was given 20 min before adenosine (1.0 mg).

Injection of adenosine lowered femoral blood pressure in a dose-related fashion. Measurement of blood pressure changes across the tracheal vascular bed showed that the stimulation of secretion was probably not due to an effect on local blood pressure. The vasoconstrictor phenylephrine increased blood pressure and mucus secretion by 28% and 150%, respectively ($P < 0.025$); changes in blood pressure across the trachea were also statistically higher (28%) with the vasoconstrictor. In three dogs, comparison of the effect of adenosine (1.0 mg) on tracheal blood pressure with that of another vasodilator sodium nitrite ($NaNO_2$) at 10 mg showed that although both $NaNO_2$ and adenosine are vasodilators ($NaNO_2$ 10 mg, CTA lowered blood pressure across the tracheal by 45 mm; 15 mmHg systolic and diastolic, respectively), only adenosine caused an increase in secretion rate (180%) while having a modest effect on tracheal blood pressure.

Effects of PIA and ddAdo

N^6-R-phenylisopropyl adenosine (PIA), and A_1 site agonist, inhibited adenosine, but was slightly more active when combined with MIX (35% inhibition when used alone, 44% inhibition when used together, $P = 0.0002$: Table 1. 2′5′-Dideoxyadenosine (ddAdo), a reputed A_2 agonist, reduced the effect of adenosine when used alone (42%, $P < 0.00001$), but the antagonism was reversed in the presence of MIX. When MIX was given with ddAdo and both drugs preceded adenosine, this inhibition was turned into stimulation of the effect of adenosine: 42% inhibition alone and 26% stimulation when both were administered ($P = 0.053$).

Effect of methylxanthines

Of the potent phosphodiesterase (PDE) inhibitors, only 8-phenyltheophylline was able to antagonize significantly the secretagogue effect of adenosine when given alone. Aminophylline at comparable doses (data not shown) was unable to block the effect of adenosine. MIX alone did not antagonize adenosine; however, when combined with PIA or ddAdo, MIX potentiated the effects of the A_1 activator (PIA) and reversed the effects of the A_2 activator (ddAdo). Aerosolized 8-phenyltheophylline was a very potent antagonist (70% inhibition; $P < 0.00001$) of adenosine's stimulant effect (Table 1).

Discussion

The bronchial effects of adenosine, particularly its stimulation of secretion, have been largely ignored. There are now reports of its bronchoconstrictor effects in man (Cushley *et al.*, 1983). In both *in vitro* and *in vivo* animal studies, adenosine has shown a bronchorelaxant effect mediated by an extracellular A_2 subtype adenosine receptor and an intracellular purine-sensitive site, both of which mediate relaxation (Brown & Collis, 1982). Rats have been reported to show bronchoconstriction with adenosine that appeared to be both atropine- and aminophylline-sensitive (Pauwells & VanderStraeten, 1983).

Receptors for adenosine have been extensively studied and identified in various bronchial tissue. These studies have implied that the adenylate cyclase, which is often presumed to be activated by adenosine, can be regulated by the nucleoside. Adenosine receptors have been shown on human lung mast cells modulating the release of histamine and slow reacting substance of anaphylaxis (SRS-A) (Holgate *et al.*, 1980). In human tissue there is inhibition of release of both mediators by adenosine, while in the guinea-pig and rat, adenosine enhances mediator release (Vardey & Skidmore, 1982). Finally, adenosine receptors have been located on axon terminals of excitatory neurones, possibly influencing neurotransmitter release (Goodman *et al.*, 1983), and on many blood vessels, possibly mediating vasoconstriction (Lukacsko & Blumberg, 1983).

The activation of adenylate cyclase in the tracheal submucosal gland by nucleosides, leading to the stimulation of mucous glycoprotein release, has recently been shown in the dog (Johnson *et al.*, 1985). Since hyperreactivity of glands and respiratory smooth muscle to a variety of stimuli is a common feature of many bronchial and respiratory disease, it became of interest to see whether adenosine was acting by activation of glands. Further, we wanted to assess if adenosine derivatives and antagonists could block the effect of adenosine. A pharmacological blockade of adenosine by potent methylxanthines would add to the speculation (Fredholm, 1980b) that methylxanthine's bronchiolar relaxant effects are due to antagonism of adenosine receptors and not PDE inhibition. Our data showed potent stimulation of secretion after A_2 site activation by adenosine or NECA.

The recent publication (Bruns *et al.*, 1983) elaborating structure-activity analysis of adenosine receptors by radioligand binding studies, classified A_1 sites as inhibiting adenylate cyclase and having a high affinity for PIA. A_2 sites, on the other hand, are stimulatory to adenylate cyclase and have a high affinity for NECA and ddAdo. In our studies, PIA always inhibited secretion and did not require coadministration of a PDE inhibitor to show this inhibition. 2′,5′-Dideoxyadenosine (ddAdo), however, inhibited secretion without PDE inhibition, and in the presence of potent PDE inhibition, ddAdo stimulated secretion. The

reason for this requirement for PDE inhibition, in addition to A_2 activation by ddAdo to show stimulated secretion, is under investigation at present. It is possible that ddAdo can only stimulate if the metabolic breakdown of cyclic AMP is blocked by PDE inhibitors. Since we did not measure cyclic AMP levels in the tissue following adenosine activation, we cannot be sure that A_2 receptors were activated; however, our data are consistent with activation of the A_2 site.

Finally, in the dog only aerosolized 8-phenyltheophylline, purported to be eight to ten fold more potent than aminophylline (Fain & Malbon, 1979), was able to antagonize adenosine by itself. A similar result with the aerosolized PDE inhibitor theophylline was seen in the antagonism of adenosine-induced bronchoconstriction in man (Cushley *et al.*, 1984). MIX inhibited only in the presence of the A_1 inhibitor PIA.

The possibility that secretagogue effect of adenosine was a result of its effects on blood flow and pressure was considered unlikely because of the comparison of its effect with that of $NaNO_2$, a known potent vasodilator. Localized vascular resistance was not determined, however, pressure changes across the tracheal vascular bed due to phenylephrine vasoconstriction could be shown, and this also led to a potent secretagogue effect. When adenosine was compared with $NaNO_2$, both thought to act by vasodilatation, adenosine demonstrated a potent secretagogue effect and a weak vasodilatation. Sodium nitrate caused a massive blood pressure fall without increasing secretion. Therefore, it is concluded that the mechanism by which adenosine enhances secretion is not related to its vasodilator effect. Further evidence for a separate adenosine effect was gathered when it was shown that the vasoconstrictor phenylephrine, when administered before adenosine, was incapable of antagonizing the secretagogue effect.

These findings indicate that: (1) adenosine may have a modulatory role in hypersecretion of mucus in respiratory diseases, and (2) it may be possible to inhibit unwanted hypersecretion by aerosol administration of adenosine derivatives.

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