

Inhibition of adenosine 5'-monophosphate- and methacholine-induced bronchoconstriction in asthma by inhaled frusemide

R. Polosa, L.C.K. Lau, S.T. Holgate

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ABSTRACT: Recent studies have shown that inhaled frusemide exerts a protective effect against various bronchoconstrictor stimuli in asthma including exercise, fog and allergen. Since mast cell activation seems to be a component of bronchoconstriction by these stimuli it is possible that inhibition of mediator release accounts for some or all of the inhibitory effects of frusemide in asthma. Since inhaled adenosine 5'-monophosphate (AMP) is another stimulus that produces bronchoconstriction by augmenting mast cell mediator release, we have investigated the ability of this drug to antagonise the airway effects of inhaled AMP and methacholine in a randomized, placebo-controlled, double-blind study of 12 asthmatic subjects. Inhaled frusemide (~28 mg) administered 5 min prior to challenge increased the provocation concentration of inhaled AMP and methacholine required to reduce forced expiratory volume in one second (FEV₁) by 20% from baseline from 30 to 96 mg·ml⁻¹ ($p < 0.01$) and from 1.1 to 1.8 mg·ml⁻¹ ($p < 0.01$), respectively. The protection that frusemide afforded against AMP was significantly greater than that against methacholine ($p < 0.05$). These data suggest that inhaled frusemide may serve as a functional antagonist against a smooth muscle spasmogen, such as methacholine, possibly by augmenting prostanoic generation. Its more potent activity against AMP and other bronchoconstrictor stimuli, that are considered to involve mast cell mediators, suggests an additional action on mast cell functions possibly at the level of the Ca²⁺/Mg²⁺-ATPase.

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Dept of Immunopharmacology, Medicine 1, Southampton General Hospital, Tremona Road, Southampton, UK.

Correspondence: Dr R. Polosa, Dept of Immunopharmacology, Medicine 1, D Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO9 4XY, UK.

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Frusemide, an inhibitor of Na-K-Cl co-transport, has been proposed as a novel prophylactic drug for the treatment of provoked asthma when the drug is administered by inhalation but not orally [1, 2]. Although the mechanism of action of inhaled frusemide at the bronchial mucosa level is not known, at least two studies have demonstrated that it protects asthmatic airways against the immediate bronchoconstrictor responses produced by exercise [1] and ultrasonically nebulized water [2]. Recent data generated by the same group has also shown that inhaled frusemide attenuates both the early and late phase bronchoconstrictor responses following allergen challenge [3, 4]. Since a component of bronchoconstriction produced by all three provocants is dependent upon mediator release from mast cells [5-7], it is possible that frusemide exerts its inhibitory effect on these cells.

To test this hypothesis we have investigated the effects of inhaled frusemide on the airways response of asthmatic subjects to inhaled adenosine 5'-monophosphate (AMP), which is also believed to act largely through

augmenting mast cell degranulation [8], and to methacholine which provokes bronchoconstriction directly by stimulating specific muscarinic receptors on airways smooth muscle [9]. The study was performed in a randomized placebo-controlled and double-blind fashion in which the effect of inhaled frusemide or placebo was observed on the position of forced expiratory volume in one second (FEV₁) concentration-response curves produced by inhaled AMP and methacholine.

Methods

Subjects

Twelve asthmatic subjects (7 male) with a mean (\pm SEM) age of 35.4 \pm 4.0 yrs, participated in the study (table 1). All were nonsmokers and all except one were atopic as defined by positive skin prick tests (>2 mm wheal response) to two or more common aero-allergens

Table 1. - Characteristics of subjects studied

Subject No.	Sex	Age yrs	Baseline FEV ₁ % predicted	Atopy**	PC ₂₀ methacholine mg·ml ⁻¹
1	F	22	110	+	2.39
2	M	44	71	+	0.31
3	M	23	88	+	1.75
4	M	52	74	+	0.40
5	F	29	127	+	1.42
6	M	19	115	+	9.61
7	F	41	92	+	0.25
8	F	42	114	+	1.31
9	M	36	91	+	0.52
10	M	27	80	+	0.55
11	M	65	93	-	0.38
12	F	25	94	+	0.86
Mean		35.4	95.8		0.88*
SEM		4.0	4.9		(0.25-9.61)

*: geometric mean (range); **: atopic, positive (+) immediate skin tests to two or more allergens; FEV₁: forced expiratory volume in one second; PC₂₀ provocation concentration producing a 20% fall in FEV₁; F: female; M: male.

(house dust, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, mixed grass pollen, mixed tree pollen, cat fur, dog hair, mixed feathers, *Aspergillus fumigatus*, *Candida albicans* - Bencard, Brentford, Middlesex, UK.). All patients had baseline forced expiratory volume in one second (FEV₁) >65% of their predicted values or >2.0 l. None was receiving oral corticosteroids, theophylline, or sodium cromoglycate on a regular basis. Bronchodilators were withheld for 8 h prior to each visit to the laboratory, although subjects were allowed to continue inhaled corticosteroids as usual. None of the subjects was studied within 4 wks of an upper respiratory tract infection or exacerbation of their asthma. Subjects gave written, informed consent and the study was approved by the Southampton University and Hospitals Ethical Committee.

Bronchial provocation

Pulmonary function was measured before and during the provocation as the forced expiratory volume in one second (FEV₁) using a dry wedge spirometer (Vitalograph®, Buckinghamshire, UK.), the higher of two consecutive measurements being used for analysis. On each challenge day, methacholine (Sigma Chemical Co., Poole, Dorset, UK), and AMP (Sigma Chemical Co., St. Louis, USA.) were made up freshly in 0.9% (w/v) sodium chloride to produce a range of increasing doubling concentrations of 0.03-64 mg·ml⁻¹ (0.2-327 mmol·l⁻¹ and 0.39-400 mg·ml⁻¹ (4.48-1,151.4 mmol·l⁻¹), respectively. The solutions were administered as aerosols generated from a starting volume of 3 ml in a disposable Inspiron mini-nebulizer (C.R. Bard International, Sunderland, UK.) driven by compressed air at 8 l·min⁻¹. Under these conditions, the nebulizer has an output of 0.48 ml·min⁻¹ and generates an aerosol with a

mass median particle diameter of 4.7 µm [10]. Wearing a noseclip, subjects inhaled the aerosolized solutions in 5 breaths from end-tidal volume to full inspiratory capacity via a mouthpiece [11]. All the bronchial provocations were carried out at the same time of day.

Study design

The study was divided into 2 phases. In the first phase, subjects attended the laboratory on two separate occasions at least 48 h apart to undertake concentration-response studies with inhaled AMP and methacholine in the absence of any drug treatment. On the first occasion, after 15 min rest, three baseline measurements of FEV₁ were made at intervals of 3 min followed by inhalation of 0.9% sodium chloride and repeat FEV₁ measurements at 1 and 3 min, the higher value being recorded. Provided FEV₁ did not fall by >10% of the baseline value, an AMP concentration-response study was carried out. After administration of each AMP concentration, FEV₁ was measured at 1 and 3 min. Increasing doubling concentrations of AMP were inhaled at 5 min intervals until FEV₁ had fallen by >20% of the post-saline value (PC₂₀).

On the second occasion, a bronchial provocation test with inhaled methacholine was undertaken in a similar manner to that described for AMP, FEV₁ measurements being recorded 1 and 3 min after inhalation of each concentration of methacholine and the corresponding PC₂₀ values derived.

In the second phase of the study, subjects attended the laboratory on four occasions, not less than 72 h apart, to undertake concentration-response studies with inhaled AMP and methacholine after nebulized frusemide or matched nebulized vehicle placebo. These were administered double-blind and in random order 5 min

prior to challenge. On each occasion, after 15 min rest, three baseline measurements of FEV₁ were recorded at intervals of 3 min, followed by inhalation of nebulized frusemide (Lasix, Hoechst AG, Frankfurt AM Main, W. Germany) in a concentration of 10 mg·ml⁻¹ or nebulized vehicle consisting of 0.9% sodium chloride adjusted to the same pH and tonicity as the frusemide (pH 8.31, osmolarity 290 mOsm·kg⁻¹). The aerosol solutions were generated from a starting volume of 4.0 ml in an Inspiron mini-nebulizer driven by compressed air at 8 l·min⁻¹, and inhaled to dryness by deep tidal breathing over a 7–8 min time period. The same nebulizer was used for all studies on all subjects. The dose of frusemide delivered to the mouth was calculated by weighing and on the 6 occasions was 28±2.5 mg (mean±SEM).

Five min after inhaling the frusemide or vehicle placebo, a concentration-response study with one of the two agonists was performed. On each occasion, three post-drug measurements of FEV₁ were recorded at 3 and 5 min, followed by inhalation of nebulized 0.9% sodium chloride and repeat measurements of FEV₁ at 1 and 3 min. Provided FEV₁ did not fall by >10% of the post-drug baseline value, the concentration-response study was undertaken. After inhaling each agonist concentration, FEV₁ was measured at 1 and 3 min, the higher value being recorded. Increasing doubling concentrations of agonist were then inhaled at 5 min intervals until FEV₁ had fallen by >20% of the post-saline value or the highest concentration had been administered.

Data Analysis

Figures refer to the mean±SEM unless otherwise stated, and the *p*<0.05 level of significance was accepted. Pre- and post-treatment baseline values of FEV₁ prior to bronchial challenges were compared within each study day using Student's *t*-test for paired data and between study days by two-factor analysis of variance (ANOVA).

The fall in FEV₁ following each concentration of agonist was expressed as a percentage of the higher of the two post-saline baseline FEV₁ recordings. The percentage fall in FEV₁ was plotted against the cumulative concentration of bronchoconstrictor agonist on a logarithmic scale, and the provocation concentration required to produce a 20% decrease in FEV₁ from the post-saline baseline value (PC₂₀) determined by linear interpolation.

The repeatability of the challenge procedure with inhaled methacholine and AMP in these patients was determined according to the method described by ALTMAN and BLAND [12], of plotting the difference against the mean of the logarithmically transformed PC₂₀ values obtained on the placebo and open study days. The mean and standard deviation (SD) of the mean difference between these values were then derived and used to calculate the coefficient of repeatability between the results of the two study days.

The slopes of the AMP and methacholine concentration-response curves were determined by least squares linear regression analysis and compared between post-placebo and post-frusemide study days using Student's *t*-test for paired data. AMP and methacholine PC₂₀ values were logarithmically transformed to normalize their distribution and compared by means of two-factor ANOVA. Concentration ratios for the protective effect of frusemide against bronchoprovocation with each agonist were calculated by dividing the PC₂₀ value obtained after administration of active drug by that obtained after placebo. The relative potency of frusemide in protecting against bronchoconstriction induced by the two agonists was analysed by comparing the concentration ratios using the Wilcoxon signed rank test.

Relationship between PC₂₀ AMP and PC₂₀ methacholine values and the respective concentration ratios after frusemide were investigated by least squares linear regression analysis.

Table 2. – Effects of inhaled frusemide and placebo on baseline FEV₁ values

Subject No.	Methacholine study days			AMP study days		
	Baseline	Placebo	Frusemide	Baseline	Placebo	Frusemide
1	3.75	4.00	4.15	4.00	3.95	3.85
2	1.95	2.00	1.95	2.10	1.95	2.05
3	4.80	4.55	4.30	4.30	4.30	4.35
4	2.75	2.50	2.70	2.70	2.40	2.80
5	4.80	5.00	4.95	4.70	4.90	4.80
6	5.15	5.10	5.25	5.30	5.00	5.05
7	2.45	2.50	2.75	2.60	2.40	2.50
8	2.70	2.60	2.65	2.70	2.60	2.70
9	3.55	3.70	3.55	3.45	3.65	3.65
10	3.40	3.50	3.60	3.50	3.45	3.55
11	2.20	2.25	2.25	2.30	2.20	2.20
12	3.15	3.30	3.30	3.40	3.15	3.15
Mean	3.39	3.42	3.45	3.42	3.33	3.38
SEM±	0.31	0.31	0.30	0.29	0.30	0.29

FEV₁: forced expiratory volume in one second in litres; AMP: adenosine 5'-monophosphate.

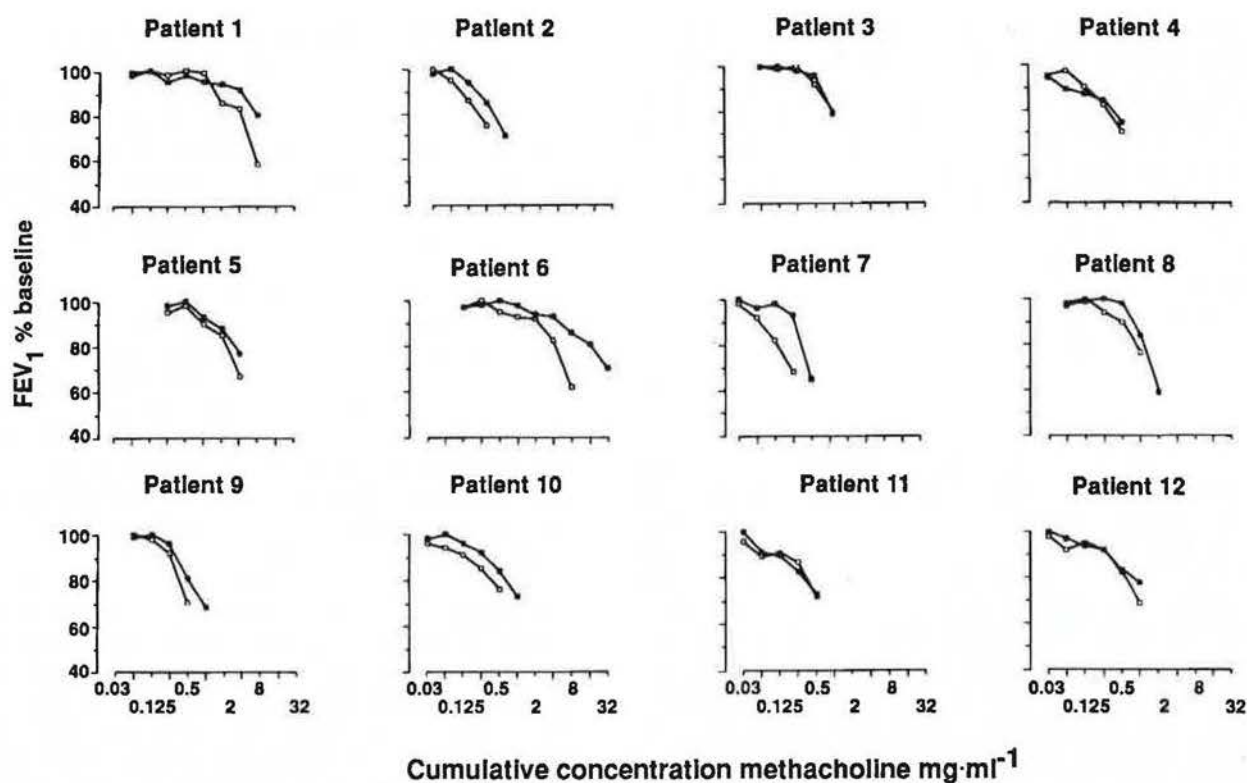


Fig. 1. - Effect of inhaled placebo (□) and frusemide ~28 mg (■) on the concentration-related falls in FEV₁ produced by inhaled methacholine in 12 asthmatic subjects. FEV₁: forced expiratory volume in one second.

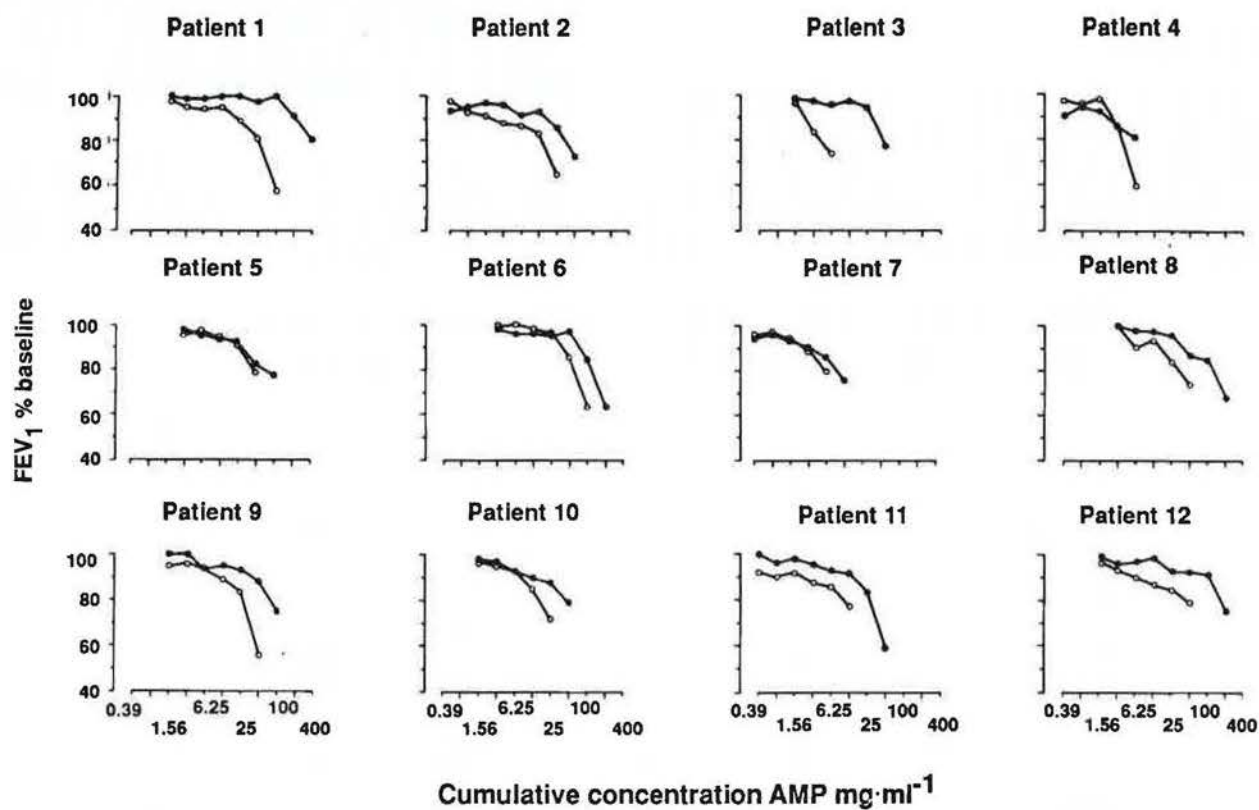


Fig. 2. - Effect of inhaled placebo (○) and frusemide ~28 mg (●) on the concentration-related falls in FEV₁ produced by inhaled adenosine 5'-monophosphate (AMP) in 12 asthmatic subjects. FEV₁: forced expiratory volume in one second.

Table 3. - Effects of inhaled frusemide and placebo on airway methacholine and AMP responsiveness

Subject No.	PC ₂₀ methacholine mg·ml ⁻¹			PC ₂₀ AMP mg·ml ⁻¹		
	Baseline	Placebo	Frusemide	Baseline	Placebo	Frusemide
1	2.39	7.99	15.31	81.07	87.29	784.38
2	0.31	0.30	0.57	5.86	26.57	62.11
3	1.75	1.56	2.41	13.46	6.12	85.42
4	0.40	0.50	0.61	4.61	5.98	11.33
5	1.42	4.06	5.83	56.95	80.10	109.14
6	9.61	7.81	31.13	121.11	97.50	208.83
7	0.25	0.21	0.62	6.54	7.45	13.32
8	1.31	0.98	1.52	71.64	59.34	202.66
9	0.52	0.53	0.86	64.70	49.70	148.20
10	0.55	0.64	1.09	32.15	27.09	85.74
11	0.38	0.52	0.45	11.39	14.08	48.06
12	0.86	0.94	1.31	32.05	96.88	344.95
Geometric mean	0.88	1.06	1.81	25.41	30.02	95.97
Range	0.3-9.6	0.2-8.0	0.5-31.1	4.6-121.1	6.0-97.5	11.3-784.4

PC₂₀: provocation concentration producing a 20% fall in forced expiratory volume in one second;
AMP: adenosine 5'-monophosphate.

Results

There were no significant differences in post-placebo or post-frusemide baseline values of FEV₁ between any of the study days (table 2). After placebo and frusemide both AMP and methacholine provoked concentration dependent falls in FEV₁, with slopes that did not depart significantly from parallel. For the 12 subjects studied, the geometric mean (range) concentrations of methacholine and AMP required to produce a 20% decrease in FEV₁ after placebo were 1.06 (0.21-7.99) and 30.02 (5.98-97.50) mg·ml⁻¹, respectively, (figs 1 and 2) (table 3). Thus, on a molar basis, AMP was approximately 16 fold less potent than methacholine in reducing FEV₁.

The challenge procedure in this group of patients was found to be repeatable for both methacholine and AMP, there being a coefficient of repeatability of 1.3 doubling concentration for methacholine and of 1.7 doubling concentration for AMP, respectively. The inhalation test was repeatable to within a single doubling dilution in 10 of the 12 subjects receiving methacholine and in 9 of the 12 inhaling AMP.

Inhaled frusemide had a small but significant protective effect against the fall in FEV₁ produced by methacholine (fig. 1), the geometric mean (range) PC₂₀ increasing from 1.06 after placebo to 1.81 (0.45-31.13) mg·ml⁻¹ after frusemide ($p < 0.01$). The same dose of inhaled frusemide was more effective in protecting against the AMP provoked fall in FEV₁ (fig. 2). Geometric mean (range) PC₂₀ increasing from 30.02 after placebo to 95.97 (11.33-784.38) mg·ml⁻¹ ($p < 0.01$). Thus, when expressed as concentration ratios frusemide afforded a 1.7 and 3.2 fold protection of the airways against methacholine and AMP, respectively, which were significantly different ($p < 0.05$).

No significant relationship could be found between the concentration ratios for either AMP or methacholine and

the baseline levels of airways responsiveness measured as the PC₂₀ methacholine. Similarly, no significant correlation was found between the baseline PC₂₀ AMP and the concentration ratios for AMP and methacholine. However, a significant relationship was observed between the baseline PC₂₀ methacholine and the baseline PC₂₀ AMP ($r = 0.75$, $p < 0.01$, $n = 12$).

Discussion

The present study demonstrates that frusemide administered in an inhaled dose of approximately 28 mg significantly protected the airways of asthmatics against bronchoconstriction provoked by methacholine and AMP, respectively. The protection afforded by frusemide was twice as great against AMP than for methacholine suggesting that more than one mechanism is involved. In providing protection against a variety of bronchoconstrictor stimuli such as exercise [1], fog [2], and allergen [3, 4], in addition to AMP and methacholine the inhibitory effects of these drugs involve mechanisms common to all these stimuli and reflects functional antagonism.

In the kidney, Na⁺ is absorbed in a two-step process that first involves Na⁺ entry across the luminal face of the tubular epithelium by diffusion through Na⁺-selective channels. After entering the cell, Na⁺ is subsequently extruded toward the submucosal (basolateral) space by the frusemide-sensitive Na⁺/K⁺ pump (Na⁺-K⁺-ATPase). Cl⁻ accompanies Na⁺ in the absorptive direction driven by the electrical gradient established by active Na⁺ transport, so that NaCl is absorbed. Frusemide acts as a diuretic by reducing salt reabsorption in the thick ascending limb of the loop of Henle [13-15] via the inhibition of the Na⁺-K⁺-ATPase responsible for the Na-K-Cl co-transport across the tubular epithelium [16, 17]. When present at the basolateral side, frusemide

inhibits Cl^- flux by a number of epithelia, possibly by an action on a linked $\text{Na}^+\text{-Cl}^-$ entry process [18], supporting the view that the drug may act by blocking the $\text{Na}^+\text{-K}^+\text{-ATPase}$. Two recent studies [1, 2] have suggested that the bronchoprotective properties of inhaled frusemide may be related to the activity of the drug on ion and water translocation across airway epithelium. However, since the drug inhibits Cl^- flux only when added to the basolateral side of the epithelium [19–21] whereas in these experiments the drug reached the epithelium from the luminal face of the bronchial mucosa, it is unlikely that the inhibitory effect of the drug on the epithelial cell $\text{Na}^+\text{-K}^+\text{-ATPase}$ accounts for its effect in reducing provoked bronchoconstriction. However, since epithelial disruption and increased permeability [22, 23] occur in asthma it is possible that a small amount of frusemide may gain access to the lamina propria and basolateral surface of the epithelium. In addition, the fact that an oral dose of frusemide (40 mg) failed to protect against the various bronchoconstrictor stimuli does not exclude an effect on the luminal aspect of the epithelium since with other drugs such as salbutamol, differences in potency between inhaled and oral routes may be as high as 1,000 fold [24]. Another property of "loop" diuretics is inhibition of Cl^- transport by alternative mechanisms active at the basolateral membrane [25, 26] possibly involving a decrease in passive Cl^- permeability at the luminal face of the epithelium [26].

Drugs such as frusemide may also produce some of their effect in the kidney by the secondary production of endogenous vasodilator substances [27]. These are likely to be prostaglandins (PGs) since the vascular effect of frusemide in hypervolaemic dogs is blocked by indomethacin [28]. In humans, frusemide causes an increase in the plasma concentration of free arachidonic acid [29] and increases the urinary excretion of PGs [30]. SULLIVAN and PATRICK [31] found that aortae from rats given intravenous frusemide had an increased capacity to produce PGI_2 and in anaesthetized rats, GERKENS and SMITH [32] have demonstrated that frusemide may release prostanoids which inhibit constrictor responses in the peripheral vasculature. Taken together, these data provide convincing evidence for frusemide's capacity to generate eicosanoids with functional effects. If the same applies to human bronchial epithelium, this could account for some of the inhibition of the bronchoconstriction produced by methacholine, AMP and other stimuli since both PGE_2 and PGI_2 are potent functional antagonists through their capacity to stimulate adenylate cyclase in the airways [33–35].

Human airway tissue [36] and pulmonary vascular endothelial cells [37] are rich sources of PGI_2 and PGE_2 . In asthma, inhaled PGI_2 affords short-term protection against several stimuli such as exercise [38], nebulized distilled water (fog) [38], PGD_2 [35] and methacholine [35], without having any consistent effect on basal airway calibre. By using an *in situ* blood perfused rat mesentery model, JACKSON and CAMPBELL [39] have suggested that the low level of PGE_2 generation which results from frusemide administration, functionally antagonizes the vasoactive effects of angiotensin II or

sympathetic stimulation without having any direct vasodilator action itself. Release of PGE_2 and/or PGI_2 in response to inhaled frusemide could antagonize the constrictor effects of AMP, and to a lesser extent of methacholine, without changing FEV_1 as an index of baseline airway calibre.

The capacity of frusemide to produce a greater protection against the bronchoconstrictor action of AMP than to methacholine suggests an additional, more specific pharmacological action. Based on the known mechanism of action of AMP in augmenting mast cell mediator release, frusemide may attenuate the response through inhibition of mediator secretion. Indeed a loop diuretic sensitive $\text{Ca}^{++}/\text{Mg}^{++}\text{-ATPase}$, located on the outer surface of the mast cell membrane, has been shown to be involved in the coupling of mast cell activation to secretion [40, 41]. Ethacrynic acid, a well-known "loop" diuretic, at a concentration 10^{-6} M produces ~50% inhibition of rat mast cell $\text{Ca}^{++}/\text{Mg}^{++}\text{-ATPase}$ activity which causes a parallel marked inhibition of histamine release induced by allergen, AMP, compound 48/80 and dextran [40–42]. These findings suggest that "loop" diuretics may modulate mast cell responses to various stimuli via inhibition of $\text{Ca}^{++}/\text{Mg}^{++}\text{-ATPase}$, although at the time of writing no published data on the effect of frusemide on mast cell function is available.

In conclusion, we have shown that inhaled frusemide protects the airways of asthmatics against the constrictor effect of AMP and methacholine and, therefore, adds to the accumulating evidence for this drug's efficacy in asthma. Interference with epithelial ion transport probably accounts for the small inhibitory effect against methacholine, while an additional effect on mast cells could account for its increased potency against AMP. Since mast cell activation with histamine release are important components of bronchoconstriction produced by allergen, exercise and hypotonic aerosols, a common inhibitory effect on mast cells could be important. Clearly, further studies are needed to clarify whether frusemide has an inhibitory action against activation of human mast cells *in vitro*, and if so whether this relates to its *in vivo* pharmacology.

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Inhibition de la broncho-constriction induite par l'adénosine 5'-monophosphate et la methacholine dans l'asthme, par inhalation de frusemide. R. Polosa, L.C.K. Lau, S.T. Holgate.
RÉSUMÉ: Des études récentes ont démontré que l'inhalation de frusemide exerce un effet protecteur contre différents stimuli broncho-constricteurs dans l'asthme, notamment contre l'effort, le brouillard et les allergènes. Puisque l'activation des mastocytes semble être un élément broncho-constricteur après emploi de ces stimuli, il est possible que l'inhibition de la libération de médiateurs rende compte en tout ou en partie, des effets inhibiteurs du frusemide dans l'asthme. Puisque l'adénosine 5'-monophosphate par inhalation (AMP) est un autre stimulus provoquant la broncho-constriction en augmentant la libération de médiateurs des mastocytes, nous avons investigué si le frusemide était capable d'exercer un effet antagoniste à l'égard des effets de l'inhalation d'AMP et de methacholine

sur les voies aériennes, et ce dans un essai randomisé, contrôlé par placebo, et en double anonymat, chez 12 sujets asthmatiques. L'inhalation de frusemide (environ 28 mg), administrée 5 minutes avant la provocation, augmente les concentrations d'AMP inhalé et de methacholine nécessaires pour entraîner une réduction du VEMS de 20% à partir des valeurs basales. Cette augmentation est respectivement de 30 à 96 mg·ml⁻¹ (p<0.01) et de 1.1 à 1.8 mg·ml⁻¹ (p<0.01). La protection obtenue par le frusemide contre l'AMP est significativement plus nette que celle obtenue contre la methacholine (p<0.05). Ces observations suggèrent que l'inhalation de frusemide peut servir d'antagoniste fonctionnel contre un spasmogène du muscle lisse, comme la methacholine, peut-être en augmentant la formation de prostanoïdes. Son activité plus marquée à l'égard d'AMP et d'autres stimuli broncho-constricteurs, qui sont considérés comme impliquant des médiateurs mastocytaires, suggère une action complémentaire sur les fonctions mastocytaires, peut-être au niveau de la Ca⁺⁺/Mg⁺⁺-ATPase.

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