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Effects of inhaled glaucine on pulmonary responses to antigen in sensitized guinea pigs

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Abstract

The alkaloid (*S*)-(+)-1,2,9,10-tetramethoxyaporphine (glaucine) is a phosphodiesterase 4 inhibitor with bronchodilator and anti-inflammatory activity in vitro. In this study, we examined the in vivo effects of glaucine on an animal model of asthma. In ovalbumin sensitized guinea pigs, inhaled glaucine (10 mg ml^{-1}, 3 min) inhibited the acute bronchoconstriction produced by aerosol antigen (antigen response was 256 ± 42 and 95 ± 14 cm H_2O l^{-1} s^{-1} in control and glaucine-treated animals, respectively; $P < 0.05$). Pretreatment with glaucine (10 mg ml^{-1}, 10 min inhalation, 30 min pre- and 3 h post-antigen exposure) markedly reduced airway hyperreactivity to histamine, eosinophil lung accumulation, and increased eosinophil peroxidase activity in bronchoalveolar lavage fluid 24 h after exposure of conscious guinea pigs to aerosol antigen. In addition, inhaled glaucine (5–10 mg ml^{-1}, 3 min) inhibited the microvascular leakage produced after inhaled antigen at all airway levels. These data support the potential interest of phosphodiesterase 4 inhibitors in asthma treatment.

Keywords: Glaucine; Phosphodiesterase 4 inhibitor; Sensitized, guinea pig; Airway hyperreactivity; Airway eosinophil infiltration; Airway microvascular leakage

1. Introduction

Asthma is an inflammatory disease characterized by reversible bronchial obstruction and airways hyperreactivity, eosinophil accumulation, and plasma exudation. Much interest is currently being directed at the inflammatory component of asthma. To this respect, the cyclic nucleotide phosphodiesterase isoenzyme type 4 has been identified as a target for therapeutic intervention since selective phosphodiesterase 4 inhibitors combine anti-bronchoconstrictive and anti-inflammatory properties (Torphy, 1998).

(*S*)-(+)-1,2,9,10-Tetramethoxyaporphine (glaucine) is an alkaloid isolated from the plant *Glaucium flavum* Crantz (Papaveraceae) that has been used as an antitussive (Dierckx et al., 1981; Constant et al., 1983). Glaucine is a tetrahydroisoquinoline derivative, structurally related to papaverine. Different authors (Kukovetz and Poch, 1970; Van Inwegen et al., 1979) postulated that the mechanism of action of many isoquinoline derivatives including papaverine, involves inhibition of phosphodiesterase. Papaverine is a non-selective inhibitor of phosphodiesterase isoenzymes but interestingly glaucine was found to be a relatively potent and selective inhibitor of soluble phosphodiesterase 4 isolated from bovine aorta, human bronchus, and human neutrophils (Ivorra et al., 1992; Cortijo et al., 1999). This finding is interesting since few natural products have been described as selective inhibitors of phosphodiesterase isoenzymes. In addition, a very low ratio between the potencies at the phosphodiesterase 4 catalytic site and at the high-affinity [^{3}H]rolipram binding site in rat brain has been recently reported for glaucine (Cortijo et al., 1999). Since second generation phosphodiesterase 4 inhibitors which do not preferentially interact with the high affinity binding site should induce less emesis (Torphy, 1998), the finding of a similar profile for glaucine suggests a low potential emetic effect of this alkaloid. In fact, vomiting has not been reported as an adverse effect during past clinical use of glaucine (Dierckx et al., 1981; Gastpar et al., 1984).

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Apart from phosphodiesterase 4 inhibition, glaucine is endowed with other pharmacological activities such as non-selective α-adrenoceptor antagonism and Ca^{2+} entry blockade (Ivorra et al., 1992; Orallo et al., 1993). These activities would not be detrimental in asthma. Thus, calcium channel blockers received attention as potential anti-asthma drugs (Barnes, 1985) as well as α-adrenoceptor antagonists (Barnes et al., 1981; Black and Armour, 1986). Glaucine relaxes guinea pig and human isolated airways (Kasé et al., 1983; Cortijo et al., 1999), and inhibits acetylcholine- and histamine-induced contraction of guinea pig airways in vitro and in vivo (Kasé et al., 1983). Functional responses of human neutrophils and eosinophils are inhibited also by glaucine (Cortijo et al., 1999). Although not tested in asthmatics, glaucine shows a trend towards increase of airways specific conductance in man (Constant et al., 1983).

In view of these findings, we decided to extend the pharmacological characterisation of glaucine by examining the in vivo effects of this alkaloid in an experimental model of allergic asthma. The inhalation route was selected for the administration of glaucine to minimize its systemic effects including haemodynamics and respiratory depression (Kasé et al., 1983; Orallo et al., 1993). Therefore, the aim of the present study was to examine the effects of aerosolized glaucine on the antigen-induced bronchoconstriction and airway hyperreactivity, eosinophil infiltration, and eosinophil peroxidase activity in bronchoalveolar lavage fluid, and airway microvascular leakage elicited in actively sensitized guinea pigs. Phosphodiesterase 4 activity and cyclic AMP levels in guinea pig airways were also studied to provide evidence of glaucine working through this specific mode of action.

2. Methods

2.1. Sensitization protocol, animal preparation, and assessment of the effects of glaucine on antigen-induced bronchoconstriction

Male Dunkin–Hartley guinea pigs (Interfauna Iberica, Barcelona, Spain) ranging in weight from 400 to 500 g were used. They were housed six per cage under standard conditions (22 ± 2°C, 40–60% humidity, and a 12-h light [7:00–19:00]–dark cycle), and used after adaptation for at least 1 week with free access to food and water. All protocols were in accordance with guidelines for animal care in Spain. Animals were actively sensitized as previously reported (Ortiz et al., 1996). In brief, animals received two intraperitoneal injections of 0.5 ml of sterile 0.9% (w/v) sodium chloride in distilled water (saline) containing 20 μg ovalbumin and 100 mg of aluminium hydroxide $Al(OH)_3$ given 24 h apart. This procedure has previously been shown to result in the development of both immunoglobulin E- and immunoglobulin G_1-type antibodies (Andersson, 1980; Banner et al., 1996b). Experiments were carried out 28–31 days after sensitization.

Sensitized guinea pigs were anaesthetised with urethane (1.5–2 g kg^{-1}, i.p.). The jugular vein was cannulated for intravenous drug administration. The trachea was cannulated and animals were mechanically ventilated with room air by means of a Ugo Basile ventilator at a rate of 60 breaths min^{-1} with a stroke volume of 1 ml 100 g^{-1} body weight. A heated (37°C) pneumotachograph (Fleish 000) was positioned in the ventilator circuit to measure inspiratory and expiratory flow rate. The inspiratory limb also contained a DeVilbiss ultrasonic nebulizer to allow the generation of aerosols, which could be carried into the airways with inspired air. A side arm from the tracheal cannula was attached to the positive port of a differential pressure transducer (Celesco model LCVR) and used to measure pulmonary inflation pressure while the negative port of the transducer was attached to a cannula inserted in the intra-pleural space to measure intra-thoracic pressure. The difference between these two pressures was the transpulmonary pressure. Arterial blood pressure was measured by a transducer (Spectramed Statham P23XL) connected to a saline-filled cannula inserted into the carotid artery. Body temperature was maintained at 37 ± 0.5°C by a heated blanket. Signals for airflow, transpulmonary pressure, and arterial blood pressure were amplified (Pulmonary Monitoring System, PMS 800, Mumed, London, UK) and fed via an analogue to digital converter to a personal computer. Lung resistance (R_L; cm H_2O l^{-1} s^{-1}) and dynamic compliance (C_{dyn}; ml cm H_2O^{-1}) were calculated according to the method of Amdur and Mead (1958) by use of PMS dual software but only data of R_L were analysed as reported by Advenier et al. (1972).

After 10 min stabilisation, animals received glaucine (1, 5 or 10 mg ml^{-1}, 3 min, i.e. 180 tidal breaths) by inhalation, and 30 min later, they were challenged with inhaled antigen (5 mg ml^{-1}, 30 s). The dose of inhaled glaucine was derived from studies with other phosphodiesterase inhibitors administered by inhalation or intratracheally (Raeburn et al., 1994; Ortiz et al., 1996). The dose of antigen was selected from other studies (Daffonchio et al., 1987) as a dose which produces submaximal bronchoconstriction with minimal systemic effects. Control animals were equally treated but received drug vehicle instead of glaucine. To calculate the inhaled dose of glaucine, we measured the output from the nebulizer for 3 ml of saline in its chamber and with airflow of 0.3 l min^{-1} obtaining a value of 0.067 ± 0.004 ml min^{-1} ($n = 5$) which is consistent with other values previously reported (Sakamoto et al., 1993; Ortiz et al., 1996). Thus, the inhaled dose of glaucine (10 mg ml^{-1}, 3 min) was calculated as ~2.0 mg/animal) The pulmonary response to antigen was expressed as change from baseline in absolute values of lung resistance.

2.2. Assessment of the effects of glaucine on airway hyperreactivity, eosinophil infiltration, and eosinophil peroxidase levels

A separate group of sensitized conscious guinea pigs were exposed to antigen aerosol as described by Farmer et al. (1992). Animals were placed in a clear plastic chamber (approximate volume, 4 l) which was connected to the output of a DeVilbiss ultrasonic nebuliser. The nebuliser chamber was filled with ovalbumin (0.1% in saline) or saline solution. The output from the nebuliser was approximately 8–10 ml h^{-1} and the duration of the antigen challenge was 60 min. The time course of airway hyperreactivity in antigen exposed guinea pigs has been examined by Havill et al. (1990) and the response at 24 h was selected on this basis. Twenty-four hours after exposure to the aerosol, airway reactivity was determined from dose–response curves to histamine obtained in animals anaesthetized and instrumented as indicated above. After 10 min stabilisation period, four successive administration of histamine (2, 5, 10 and 25 $\mu g\ kg^{-1}$, i.v.) were given at time intervals ($\sim$6–10 min) sufficient to allow lung function values to return spontaneously to baseline after each challenge. The pulmonary responses to histamine were expressed as changes from baseline in absolute values of lung resistance.

After measurement of airway reactivity, animals were killed by an overdose of urethane. Bronchoalveolar cells of guinea pigs were collected in 10 successive lavages using 5 ml aliquots of sterile saline with heparin 10 IU ml^{-1} at room temperature injected and recovered through a polyethylene tracheal cannula. Cell suspensions were concentrated by low speed centrifugation, and the cell pellet resuspended. Total cell counts were made in a haemocytometer. Differential cell counts were determined from cytospin preparations by counting 300 cells stained with May–Grünwald–Giemsa. Because the yield of the injected fluid was equivalent in all experimental groups ($\geq$ 85%), the results are expressed as cell number ml^{-1}. The remaining lavage fluid was centrifuged at $200 \times g$ for 15 min at 4°C, and 1 ml fractions of the supernatant were collected and stored at -20°C until the determination of eosinophil peroxidase as described below.

The levels of free eosinophil peroxidase in the supernatant from bronchoalveolar lavage fluid was determined as a marker of eosinophil activation according to the method of Strath et al. (1985) with modifications (Cortijo et al., 1999). In brief, 100 μl of the substrate solution consisting of 0.1 mM *o*-phenylenediamine dihydrochloride in 0.05 M Tris–HCl containing 0.1% Triton X-100 and 1 mM H_2O_2 were added to 100 μl of the samples in microplate wells. The plates were left at 37°C for 30 min before stopping the reaction by the addition of 50 μl of 4 M sulphuric acid. The optical density was measured at 492 nm using a Microplate Autoreader (EL309, Bio-Tek Instruments). The eosinophil peroxidase levels are expressed in peroxidase units ml^{-1} as determined from comparison with a standard curve for horse radish peroxidase (0–20 mU ml^{-1}).

The experimental groups were as follows: negative control group, i.e. sensitized animals receiving drug vehicle and exposed to aerosol saline; positive control group, i.e. sensitized animals subsequently exposed to aerosol antigen and receiving drug vehicle; and treated groups, i.e. sensitized animals exposed to antigen and treated twice with glaucine (1, 5 or 10 mg ml^{-1}, 10 min) 30 min before and 3 h after antigen challenge. The doses of glaucine were selected as indicated above, and the dose regime was chosen as outlined by Lagente et al. (1994). Cell counts and eosinophil peroxidase levels were determined only for negative and positive control groups and for glaucine (10 mg ml^{-1}).

To calculate, approximately, the inhaled dose (in mg) of glaucine, the following formula was used (Karlsson et al., 1990): [(respiratory volume in ml $min^{-1} \times$ output in mg $min^{-1} \times$ deposition)/airflow in ml min^{-1}] $\times$ exposure time in min, where respiratory volume is $2.10 \times$ (body weight in g)$^{0.75}$ as outlined by Guyton (1947), output is the amount of drug leaving the nebuliser (drug concentration in mg $ml^{-1} \times$ volume delivered in ml min^{-1}), and deposition in airways was estimated as 20% (Schlesinger, 1985). Thus, the inhaled dose after a 10-min exposure (guinea pig weight $\sim$ 500 g) to an aerosol of solution (10 mg ml^{-1}), with an output from nebuliser of 0.067 ml min^{-1} (see above), and airflow of 300 ml min^{-1} can be calculated to be $\sim$ 1.0 mg/animal.

2.3. Assessment of the effect of glaucine on microvascular leakage after antigen challenge

Preparation of animals and experimental protocols were derived from Advenier et al. (1992) with modifications (Ortiz et al., 1996). The sensitization, anaesthesia, and instrumentation procedures were as noted above. After 10 min stabilisation, the animals were given glaucine (1, 5 or 10 mg ml^{-1}, 3 min) or its vehicle, which was followed 30 min later by the injection of Evans blue dye (20 mg kg^{-1}, i.v.), and 1 min later, aerosol antigen was administered (5 mg ml^{-1}, 30 s). This dose of antigen was selected from other studies (Hui et al., 1991) as having marginal systemic effects. Five minutes after antigen inhalation, the animals were hyperinflated with twice the tidal volume by manually blocking the outflow of the ventilator. Then, the animals were disconnected from the ventilator in order to collect the tissues for measurement of airway microvascular permeability. Animals pretreated with aerosol drug vehicles and then receiving antigen or its vehicles were used as controls. The effect of drugs in animals receiving antigen vehicle was also tested. At the end of the experiments, lower portion of trachea, main bronchi, proximal and distal intrapulmonary airways were collected. In addition, samples from oesophagus and bladder were taken to

check for systemic extravasation. Then, the Evans blue dye was extracted and quantified as previously described (Ortiz et al., 1993).

2.4. Assessment of the effects of glaucine on phosphodiesterase 4 activity and cyclic AMP content in guinea pig airways

2.4.1. Phosphodiesterase 4 activity in guinea pig trachea

These experiments were carried out as previously outlined by Harris et al. (1989) with modifications (Cortijo et al., 1999). Tissues were homogenized in 5 vol of ice-cold buffer A (composition in mM: [bis(2-hydroxyethyl)imino]-tris(hydroxymethyl)methane (bis-Tris), 20; sodium acetate, 50; benzamidine, 2; ethylenediaminetetraacetic acid, 2; β-mercaptoethanol, 5; and phenylmethylsulphonylfluoride, 0.05; pH 6.5). The homogenate was centrifuged ($15\,000 \times g$, 10 min) and the supernatant injected into a Mono-Q HR 5/5 column (Pharmacia) attached to a Fast Protein Liquid Chromatography system. The phosphodiesterases were eluted against a sodium acetate gradient (50–1000 mM). Fractions of 0.5 ml were collected, analyzed and stored as previously described (Cortijo et al., 1999). Cyclic nucleotide phosphodiesterases were assayed following the procedure of Thompson and Strada (1984). The standard incubation mixture contained, in a final volume of 400 μl, 40 mM Tris–HCl, 5 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 1 μM [^{3}H]-labeled/unlabeled cyclic AMP (~200 000 d.p.m.) and phosphodiesterase inhibitors as appropriate. The assay was initiated by adding 100 μl of the enzyme solution to the standard incubation mixture and the reaction was carried out at 30°C for 20 min. Since phosphodiesterase 3 and 4 activities coelute at high ionic strength (Harris et al., 1989), the inhibition assays for glaucine (0.5–100 μM) were run in aliquots of fractions obtained at 0.6–0.7 M sodium acetate, and in the presence of 10 μM 5-(4-acetimidophenyl)pyrazin-(1*H*)-one (SKF94120), a selective phosphodiesterase 3 inhibitor, as previously reported (Cortijo et al., 1999).

2.4.2. Cyclic AMP content in guinea pig trachea

These experiments were carried out essentially as previously described (Cortijo et al., 1999). Tissues were equilibrated in Krebs solution gassed with 5% CO_2 in O_2 at 37°C (pH 7.4) for 90 min, and then exposed for 30 min to glaucine (10 μM) or its vehicle followed by addition of forskolin (300 nM) or its vehicle for 10 min. Next, tissues were rapidly removed, blotted, snap-frozen in liquid nitrogen, and stored at −80°C. The tissues (~0.5 g in 1 ml of cold 10% trichloroacetic acid) were homogenized (6 × 10 s bursts) and centrifuged ($600 \times g$ for 15 min at 4°C). The soluble fraction was stored at −20°C until assay for cyclic AMP content. The residual precipitation was used for the measurement of protein content (Lowry et al., 1951). The amount of cyclic AMP was estimated by enzyme immunoassay kit (RPN 225; Amersham Life Sciences, UK) following the instructions of the manufacturer without acetylation.

2.5. Statistical analysis of results; drug sources

Data are presented as mean ± S.E.M. Statistical analysis of results was carried out by analysis of variance (ANOVA) followed by Bonferroni test or by Student's *t*-test as appropriate (GraphPad Software, San Diego, CA, USA). Significance was accepted when $P < 0.05$.

The following drugs were used: Evans blue dye, formamide, forskolin, (+)-glaucine, histamine dihydrochloride, horse radish peroxidase type I, *o*-phenylenediamine dihydrochloride, ovalbumin (Grade V, fatty acid-free), Triton X-100, and urethane were all purchased from Sigma-Aldrich Química (Madrid, Spain). All other drugs and chemicals were from the same sources previously stated (Cortijo et al., 1999). Water purified on a Milli-Q (Millipore Iberica, Madrid, Spain) system was used throughout. Glaucine was dissolved in distilled water (in vitro experiments) or saline (in vivo experiments) immediately before use. Evans blue dye was dissolved in isotonic saline (20 mg ml^{-1}) and filtered through a 5 μm Millipore membrane.

3. Results

3.1. Effects of glaucine against antigen-induced bronchoconstriction

Challenge of sensitized animals with aerosol antigen (5 mg ml^{-1}) provoked an acute rise in lung resistance with a peak in about 2–3 min. Inhalation of glaucine (5 or 10 mg

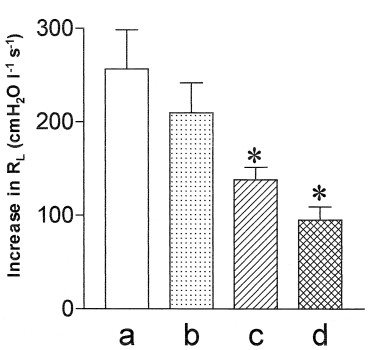

Fig. 1. Effects of inhaled glaucine on the increase in pulmonary resistance (R_L) produced by aerosol administration of antigen (5 mg ml^{-1}, 30 s) to sensitized guinea pigs. Thirty minutes before antigen challenge, control animals received inhaled saline (a), and treated animals were given inhaled glaucine at 1 (b), 5 (c), or 10 (d) mg ml^{-1} for 3 min. The baseline values for pulmonary resistance were 163 ± 6, 176 ± 9, 167 ± 11, 155 ± 12, and 179 ± 12 cm H_2O l^{-1} s^{-1} in the control and low, middle and high dose-treated groups, respectively, with no statistically significant difference found among these values. Columns are mean ± S.E.M. ($n = 5$). * $P < 0.05$ compared with control (a).

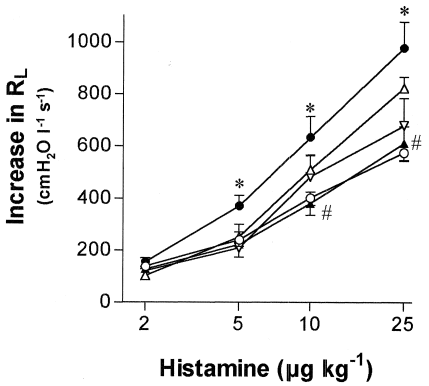

Fig. 2. Effect of inhaled glaucine on airway hyperreactivity expressed as the increase in pulmonary resistance (R_L) produced by histamine (2, 5, 10 and 25 μg kg^{-1}, i.v.), 24 h after exposure of conscious sensitized guinea pigs to an aerosol of antigen (0.1%, 60 min). Negative control group: saline-treated guinea pigs exposed to the vehicle for antigen (○; $n=7$); positive control group: saline-treated guinea pigs challenged with antigen (●; $n=7$). Treated groups received inhaled glaucine for 10 min, 30 min before and 3 h after antigen challenge. The dose administered was 1 (△; $n=5$), 5 (▽; $n=5$), or 10 (▲; $n=7$) mg ml^{-1}. The baseline values for pulmonary resistance were 157±8, 167±10, 177±13, 165±11, and 173±9 cm H_2O l^{-1} s^{-1} in the negative control, positive control, low, middle and high dose-treated groups, respectively, with no statistically significant difference found among these values. Points are means±S.E.M. of n experiments as indicated. *$P<0.05$ vs. negative control; #$P<0.05$ vs. positive control.

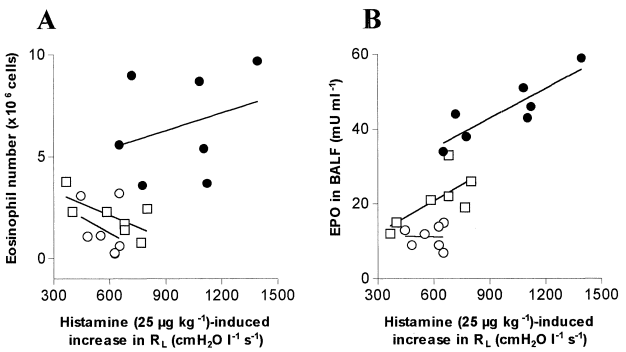

Fig. 3. Correlation analysis between individual values for airway responsiveness, expressed as the increase in pulmonary resistance (R_L) in response to histamine (25 μg kg^{-1}, i.v.), and the eosinophil number (panel A) or eosinophil peroxidase (EPO) levels in bronchoalveolar lavage fluid (BALF) (panel B). Negative control group: saline-treated guinea pigs exposed to the vehicle for antigen (○; $n=7$); positive control group: saline-treated guinea pigs challenged with antigen (●; $n=7$); treated group: glaucine (10 mg ml^{-1})-treated guinea pigs challenged with antigen (□; $n=7$). Correlations between histamine responsiveness and eosinophil number were not significant. Correlation between histamine responsiveness and eosinophil peroxidase reached significance only for positive control group ($r=0.758$, $P=0.01$).

ml^{-1}) 30 min before the antigen challenge significantly inhibited the antigen response (Fig. 1). A lower dose of glaucine failed to produce statistically significant inhibitory effects on antigen-induced bronchoconstriction. No significant change in arterial blood pressure was observed by inhaled glaucine up to 10 mg ml^{-1} (data not shown).

3.2. Effects of glaucine on antigen-induced airway hyperreactivity and eosinophil accumulation and eosinophil peroxidase activity

Twenty-four hours after antigen challenge, guinea pigs exhibited airway hyperreactivity to histamine. Glaucine (10 mg ml^{-1}) completely inhibited the hyperreactivity to histamine whilst the effects of the lower doses tested of this compound failed to reach significance (Fig. 2). The heightened airway reactivity to histamine induced by antigen exposure was unrelated to haemodynamic changes since the concentration-dependent transient falls in blood pressure in response to histamine did not significantly differ between the different groups of animals studied (data not shown).

Eosinophil numbers, as well as macrophage and total cell counts in bronchoalveolar lavage fluid, increased significantly 24 h after antigen challenge of sensitized animals (Table 1). Glaucine (10 mg ml^{-1})-treated guinea pigs showed a significant reduction of total cell count. This reduction was due mainly to a marked decrease in eosinophil accumulation (Table 1).

There was also a significant increase in the amount of eosinophil peroxidase in bronchoalveolar lavage fluid supernatant recovered from immunized animals challenged with antigen (45.0 ± 3.1 mU ml^{-1}, $n=7$) compared with saline-challenged guinea pigs (11.3 ± 1.1 mU ml^{-1}; $n=7$; $P<0.05$ from negative control). In sensitized guinea pigs challenged with antigen but pretreated with glaucine (10 mg ml^{-1}), the eosinophil peroxidase values were signifi-

Table 1
Total and differential cell counts in bronchoalveolar lavage fluid recovered from sensitized guinea pig lungs 24 h after exposure to an aerosol of antigen or vehicle (saline). The animals were distributed into a negative control group (saline-treated animals exposed to saline), a positive control group (saline-treated animals exposed to antigen), and a drug treated group of animals which received inhaled glaucine (10 mg ml^{-1} for 10 min), at 30 min before and 3 h after antigen exposure. Results are expressed as means ± S.E.M. of cells ×10^5 ml^{-1} ($n=7$ per group)

Experimental group	Total cell count	Eosinophil	Neutrophil	Macrophage	Lymphocyte
Negative control	8.23 ± 0.86	1.40 ± 0.47	0.22 ± 0.05	6.17 ± 10.70	0.45 ± 0.04
Positive control	20.44 ± 2.21[a]	6.53 ± 0.97[a]	0.36 ± 0.08	12.61 ± 1.68[a]	0.94 ± 0.14
Glaucine-treated	13.33 ± 1.29[b]	2.10 ± 0.36[b]	0.30 ± 0.07	10.34 ± 1.08	0.59 ± 0.07

[a] $P<0.05$ vs. negative control.
[b] $P<0.05$ vs. positive control.

cantly diminished (21.1 mU ml^{-1}; $n = 7$; $P < 0.05$ from positive control).

The airway reactivity to histamine was not correlated to the eosinophil numbers in bronchoalveolar lavage fluid (Fig. 3A) but the hyperreactivity to histamine observed in the positive control group was significantly correlated with the eosinophil peroxidase values in the bronchoalveolar lavage fluid of these animals (Fig. 3B). This correlation was lost in sensitized guinea pigs challenged with antigen but pretreated with glaucine (10 mg ml^{-1}).

3.3. Effects of glaucine on microvascular leakage after antigen challenge

Animals treated with inhaled glaucine (1–10 mg ml^{-1}) but not receiving extravasation stimuli (i.e. challenged with the vehicle for antigen) showed values of Evans blue dye content in airways (data not shown) similar to those of control animals (i.e. animals treated with drug vehicle and then receiving the vehicle for antigen; Fig. 4). Sensitized animals challenged with aerosol antigen (5 mg ml^{-1}) showed significant extravasation of Evans blue dye at all airway levels (Fig. 4). Challenge with inhaled antigen did not result in significant extravasation in oesophagus and bladder (data not shown), thus confirming that extravasation appeared restricted to airways. Inhaled glaucine (5–10 mg ml^{-1}) was effective to reduce the antigen-induced extravasation at all airway levels (Fig. 4).

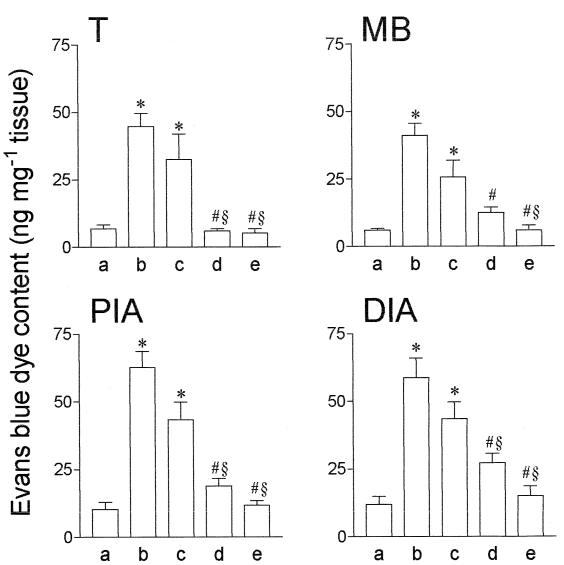

Fig. 4. Effect of inhaled glaucine on aerosol antigen (5 mg ml^{-1}, 30 s)-induced airway extravasation in guinea pigs. Extravasation of Evans blue dye was measured in guinea pig trachea (T), main bronchi (MB), proximal (PIA), and distal (DIA) intrapulmonary airways. In the control group, guinea pigs were treated with drug vehicle (saline) and then exposed to aerosol antigen vehicle (saline) (a). Saline-treated animals then exposed to antigen reacted with marked extravasation at all airway levels (b). A dose-related inhibition of the antigen-induced extravasation was observed in animals treated with glaucine at 1 (c), 5 (d), or 10 (e) mg ml^{-1} for 3 min. Columns are means ± S.E.M. of five animals for each group. $^{*}P < 0.05$ compared to (a); $\#P < 0.05$ compared to (b); $\S P < 0.05$ compared to (c).

3.4. Effects of glaucine on phosphodiesterase 4 activity and cyclic AMP levels

Glaucine produced a concentration-dependent inhibition of phosphodiesterase 4 activity isolated from guinea pig trachea with a $-\log IC_{50}$ value of 5.32 ± 0.05 ($n = 5$). The cyclic AMP hydrolytic activity in the same fractions was virtually abolished (≥ 90% inhibition) by the selective phosphodiesterase 4 inhibitor rolipram (10 μM) which indicates that this activity corresponds to a phosphodiesterase 4 type.

Glaucine (10 μM) did not significantly increase the basal levels of cyclic AMP in guinea pig trachea (11.7 ± 1.3 and 13.5 ± 1.6 pmol mg^{-1} protein in the absence and presence of glaucine; $n = 5$). Forskolin (300 nM) significantly increased cyclic AMP content (27.6 ± 1.8 pmol mg^{-1} protein; $n = 5$; $P < 0.05$ from basal values), and glaucine (10 μM) augmented the forskolin-stimulated cyclic AMP accumulation (67.3 ± 4.5 pmol mg^{-1} protein; $n = 5$; $P < 0.05$ from values in the absence of glaucine).

4. Discussion

Glaucine is a selective phosphodiesterase 4 inhibitor endowed with inhibitory activities on human airway smooth muscle and inflammatory cells in vitro (Cortijo et al., 1999). In the present study, we have extended these observations to show that glaucine inhibits phosphodiesterase 4 activity in guinea pig trachea. The presence of a low K_m, rolipram-sensitive, cyclic AMP hydrolytic activity in guinea pig trachea identifiable as phosphodiesterase 4 was demonstrated by Harris et al. (1989). The potency of glaucine as phosphodiesterase 4 inhibitor found in this study is similar to values reported in other tissues (Ivorra et al., 1992; Cortijo et al., 1999). Glaucine did not increase basal cyclic AMP but augmented the cyclic AMP accumulation produced by the adenylyl cyclase activator forskolin. This result is consistent with findings in human bronchus with this alkaloid (Cortijo et al., 1999) and similar to reports for other phosphodiesterase 4 inhibitors in guinea pig trachea (Small et al., 1991). These data provide experimental evidence of glaucine acting through inhibition of phosphodiesterase 4 in this model system. The ability of phosphodiesterase 4 inhibitors to depress the release of inflammatory mediators from guinea pig trachea and guinea pig eosinophils has also been demonstrated (Underwood et al., 1993; Banner et al., 1996a). This profile makes this alkaloid attractive for exploring its anti-asthma activity in vivo. Although with limitations, animal models are convenient for studying the pharmacological activity of potential anti-asthma drugs (Coleman, 1999). We have explored the

in vivo effects of glaucine in immunized guinea pigs, a widely used model of experimental asthma.

Inhaled glaucine given to sensitized animals 30 min before challenge reduced the acute increase in pulmonary resistance produced by aerosol administration of ovalbumin in anesthetized ventilated guinea pigs. The doses of inhaled glaucine (5–10 mg ml^{-1}, 3 min; $\sim 1-2$ mg) necessary to reduce significantly the allergen-induced bronchoconstriction are higher than the corresponding inhaled or intratracheal doses of rolipram and RP73401 (3-cyclopentyloxy-*N*-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide) ($\sim$ 10–500 μM) that inhibit markedly the bronchoconstriction to antigen in actively sensitized guinea pigs (Raeburn et al., 1994; Ortiz et al., 1996). This finding is consistent with differences in their respective potencies as phosphodiesterase 4 inhibitors since the IC_{50} value of glaucine ($\sim$ 3.4 μM; Cortijo et al., 1999) is lower than that of rolipram ($\sim$ 0.6 μM; Cortijo et al., 1993) and RP73401 ($\sim$ 1 nM; Raeburn et al., 1994). Therefore, the preventive effect of glaucine on the antigen-induced acute bronchoconstriction may be related to its ability to inhibit phosphodiesterase 4 activity, and certainly both local and systemic administration of a number of selective phosphodiesterase 4 inhibitors reduce the bronchoconstriction observed immediately after antigen exposure in sensitized guinea pigs (Teixeira et al., 1997; Torphy, 1998).

However, the possible contribution of other mechanisms to the inhibitory effect of glaucine should also be considered. Glaucine is a non-selective α-adrenoceptor antagonist (Orallo et al., 1993). Barnes et al. (1980) reported an increased concentration of α_1-adrenergic receptors in lung homogenates of ovalbumin sensitized guinea pigs. In spite of this finding, there is little evidence of an important role of α-adrenoceptors in allergen-induced asthma (Barnes et al., 1981; Black and Armour, 1986). Alternatively, the effects of glaucine may be attributed to its blocking properties at the benzothiazepine site of Ca^{2+} channels (Ivorra et al., 1992). Diltiazem as well as other calcium antagonists attenuate antigen-induced bronchoconstriction in different animal species (Fanta et al., 1982; Malo et al., 1983; Hertz and Cloarec, 1986; Korach et al., 1987) and showed some activity in asthmatic patients (Barnes, 1985). Therefore, the contribution of these actions to the inhibitory effect of glaucine cannot be ruled out by present experiments.

Inhalation of aerosolized glaucine (10 mg ml^{-1}, 10 min, given 30 min before and 3 h after antigen exposure; total dose $\sim$ 2 mg) by conscious guinea pigs also inhibited the airways hyperreactivity and pulmonary eosinophilia that appears in sensitized animals 24 h after their exposure to antigen aerosol. Lower doses of glaucine appeared not effective. These results are consistent with previous studies that show anti-hyperreactivity effects and inhibition of lung eosinophil influx after systemic administration of selective phosphodiesterase 4 inhibitors (Teixeira et al., 1997; Torphy, 1998). Consistent with other studies, we found also that eosinophil peroxidase activity in bronchoalveolar lavage fluid was increased in sensitized animals 24 h after antigen exposure (Lagente et al., 1994; Banner and Page, 1995) and that eosinophil peroxidase levels but not the eosinophil numbers correlated with airway hyperresponsiveness (Pretolani et al., 1994). Inhaled glaucine diminished eosinophil peroxidase levels in bronchoalveolar lavage fluid, a finding which is in keeping with a similar inhibitory effect showed by Lagente et al. (1994) for systemic administration of other selective phosphodiesterase 4 inhibitors.

In addition, inhalation of glaucine (5–10 mg ml^{-1}, 3 min; $\sim 1-2$ mg) by anaesthetized guinea pigs reduced antigen-induced microvascular leakage at all airway levels of sensitized animals challenged with aerosol antigen. Selective phosphodiesterase 4 inhibitors are endowed with anti-exudative properties in guinea pig airways (Raeburn and Karlsson, 1993; Ortiz et al., 1993, 1996). Therefore, inhibition of phosphodiesterase 4 activity by glaucine may contribute to the inhibition of airway microvascular leakage showed by this compound. However, alternative mechanisms may contribute to the effects of glaucine since the calcium antagonist verapamil has demonstrated an anti-leakage effect in a guinea pig model, in which α-adrenoceptor antagonists were not effective (Boschetto et al., 1989).

In conclusion, inhaled glaucine effectively reduced the antigen-induced bronchoconstriction, airways hyperreactivity, pulmonary eosinophilia and increased eosinophil peroxidase levels, and airway microvascular leakage elicited in actively sensitized guinea pigs. The main mechanism of these inhibitory effects of glaucine is likely to be its established activity as selective phosphodiesterase 4 inhibitor but contribution of other pharmacological properties cannot be excluded. Glaucine has been used for years as a remedy for cough (Dierckx et al., 1981; Gastpar et al., 1984) but its potential use as anti-asthmatic is uncertain since other more potent and selective agents appear advantageous for treating asthma (Torphy, 1998; Barnette, 1999).

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