The aim of this study was to evaluate the influence of pretreatment with the phosphodiesterase-4 inhibitor rolipram on pulmonary resistance, influx of inflammatory cells, and histamine concentration in bronchoalveolar lavage fluid (BALF) during an experimental asthmatic reaction induced in ovalbumin (OA)-sensitized guinea pigs, challenged with OA inhalation. The experiment was performed in three groups of guinea pigs: two experimental groups, pretreated with rolipram or dexamethasone, and a control group without any pretreatment. Lung resistance (LR) was continuously recorded under suppression of spontaneous breathing during early asthmatic reaction. BALF was obtained before and at three time points up to 24 hr after the challenge. In the untreated, control animals a transient, significant increase in neutrophils, total and CD4+ lymphocytes, macrophages, eosinophils, and in histamine concentration in BALF was noted. Pretreatment with rolipram significantly reduced LR, eosinophils infiltration, and histamine release into the bronchoalveolar space during the early asthmatic reaction. These effects were generally comparable with those of dexamethasone, except that dexamethasone also reduced the influx of neutrophils into BALF.

Key words: dexamethasone, experimental asthma, guinea pigs, phosphodiesterase-4, rolipram

INTRODUCTION

Phosphodiesterase-4 (PDE-4) is an enzyme specific for cAMP that belongs to the family of multiple phosphodiesterase isoenzymes (PDE-1 – PDE-11). PDE-4
hydrolyzes the 3’ phosphoric bond altering cyclic nucleotides into the 5’ nucleotide form. cAMP acts as an intermediary (in addition to cGMP) in physiological responses to hormones, neurotransmitters, or drugs and plays a key regulatory role in all types of cells engaged in the pathophysiology of asthma.

The increase in cAMP concentration inhibits release of mediators of allergic reactions from mastocytes and basophiles (1), degranulation and creation of reactive oxygen species in neutrophils and eosinophils (2), proliferation of lymphocytes T (3), and generation of key cytokines such as TNF-α, IL-2, IFN-γ, IL-4 (4). cAMP and cGMP promote relaxation of smooth muscles in the respiratory tract using a classic transduction pathway involving protein kinases (5). cAMP also promotes the secretion of Cl⁻, HCO₃⁻, and Na⁺ from epithelial cells (6) and encourages and synchronizes the movement of cilia (7). The increase in cAMP content enables to maintain the integrity of the endothelial barrier, which protects against increased permeability. Moreover, cAMP inhibits the expression ELAM-1 and VCAM-1 that are of vital importance in recruitment of inflammatory cells in the respiratory tract (8).

In the human respiratory tract, in both trachea and peripheral airways, the presence of phosphodiesterase-like PDE-1, PDE-2, PDE-3, PDE-4, and PDE-5 has been observed. Both biochemical and functional analysis of PDE isozymes suggested that PDE-3 and PDE-4 regulate cAMP content in respiratory smooth muscles (9). It also appears from recent studies that that inhibition of the activity of the latter two isoenzymes could be used in treating respiratory tract diseases (10).

Theophylline has been the archetype, nonselective PDE inhibitor since long used for asthma. Despite its attractive therapeutic profile, suitability of this drug has been limited by a range of side effects occurring in the gastrointestinal system, cardiovascular system, and central nervous system (11). Theophylline actively inhibits PDE activity in respiratory tissues, except the lung tissue, which is a key limiting factor in its therapeutic attractiveness. Currently, research is going on selective phosphodiesterase inhibitors, which show little side effects, such as roflumilaste, cilomilaste, milirone, sildenafil (12, 13).

Activity of almost all PDE isoenzymes has been identified in smooth muscles of the respiratory tract in many animal species (14), but PDE-4 inhibitors show the widest spectrum of inflammatory activity. These inhibitors decrease bronchospasm induced with antigens, hypersensitivity of airways, and permeability of lung microvessels in, among others, guinea pigs (15). They also prevent bronchial constriction evoked by administration of histamine or leukotriene D4 (16).

The objective of the present study was to evaluate the influence of rolipram, a PDE-4 inhibitor, on the course of an experimental asthmatic reaction in the guinea pig and to compare it with the action of dexamethasone. To this end, we analyzed the content of bronchoalveolar lavage fluid (BALF) in the early (EAR) and late asthmatic reaction (LAR); lung resistance (LR) was evaluated only during EAR.
MATERIAL AND METHODS

The study was approved by the Ethics Committee of Warsaw Medical University in Warsaw, Poland.

Animals and Immunization

The experiment was carried out in two phases. Tests referring to BALF analysis were carried out on Hartley-Dunkin guinea pigs (males, weighing 250-300g). The animals came from the Charles-River laboratory and were parasite free. The measurement of pulmonary resistance was carried out in tri-color guinea pigs of either sex (weighing 250-300g), obtained from the Laboratory Animal Breeding Center (Dlugosiodlo-Jagiel, Poland).

In both phases of the experiment, we used similar immunization and administration of clemastin dexamethasone and roliprame schemes. The guinea pigs were immunized with ovalbumin (OA) in the concentration 10µg/ml with aluminium hydroxide used as an adjuvant according to a method by Iijima (17) in which 1000 µg of albumin was dissolved in 3 ml H₂O, 1.35 ml 1M NaHCO₃ was added, and next 3 ml 0.2M KAl(SO₄)₂ x 12 H₂O was slowly dropped to the mixture while stirring continually. Finally, 100 ml H₂O was added. The suspension was left at room temperature for 15 min, centrifuged at 300 x g for 15 min at 4°C. Then, the supernatant was decanted and rejected and the sediment was washed three times in PBS and suspended in 100ml PBS to obtain antigen colloid suspension - Al(OH)₃. Immunization included two injections of 1ml of the suspension at a 2-wk interval: 0.25 ml intraperitoneally and the rest subcutaneously (5 injections of 0.15 ml) in the nape of the neck, and in the region of axillary and inguinal lymph nodes. The allergic challenge, consisting of OA inhalation, was carried out on the 10th day after the second injection.

Drug injections were made intraperitoneally; dexamethasone (Dexaven; Jelfa, Jelenia Góra, Poland), in a dose of 1.2 mg/kg, was given twice: 20 h and 1 h 20 min before the OA challenge; Rolipram, 1mg/kg, was given 30 min before OA inhalation, and Clemastinum, an antihistaminic H₁ receptor antagonist (18), 2 mg/kg, was injected in all animals 1.5 h before OA challenge to avoid systemic anaphylaxis.

Lung resistance (LR)

Animals were anesthetized with a mixture of ketamine/xyalzine (40mg/ kg and 5mg/kg, respectively, i.p.). Next, trachea and jugular vein were surgically accessed, trachea was cannulated, and pancuronium bromide, 1.5 mg/kg, was injected into the jugular vein, and the animlas were artificially ventilated. Initially, animals inhaled a PBS aerosol and next a solution of 0.5% albumin in PBS for 2 min each, using an ultrasound nebulizer Thomex L-2 (Medbryt; Warsaw, Poland) with 1.2 ml/min flow rate. LR was continuously recorded (under suppression of spontaneous breathing at a volume of 3ml and frequency of 100 breaths/min) during 12 min after OA inhalation, and the highest LR values were analyzed. The reference for the LR after the allergic challenge was the LR value obtained after initial, 2-min inhalation of PBS, taken as 100%. From a 100 measurements of LR values the median was calculated for each minute.

BALF

In the analysis of the BALF content, a 2-min inhalation of 0.5% albumin in PBS using a blow inhalator with a nozzle – Type D-2 (Medbryt; Warsaw, Poland) was applied. Five guinea pigs were subjected to the inhalation procedure simultaneously in a 17dm³ chamber. Bronchoalveolar lavage was carried out in accordance with the Iijima procedure (17). Guinea pigs were anaesthetized with Vetbutal.
in a dose of 50 mg/kg (Biowet, Pu³awy, Poland). Lungs were lavaged twice in 10 ml PBS, with 10 IU of heparin/1 ml. On average, BALF recovery amounted to 16.0 ± 1.3 ml. After filtration through gauze, BALF was centrifuged at 300 x g for 15 min at 4°C. Cell sediment was suspended in 0.8 ml of the RPMI 1640 medium (Invitrogen Corporation, UK). In the suspension, the following were determined:

- total number of cells - using Bürker’s chamber
- cell differentiation – cell suspension smears were stained using May-Grünwald- Giems (Pappenheim’s method) and microscopically evaluated for the percentage of lymphocytes, macrophages, neutrophils, and eosinophils, by the analysis of 300 cells with the exclusion of epithelial cells.
- T-helper to T-suppressor lymphocytes (CD4/CD8) ratio, using specific antibodies and flow cytometry. Antibodies of Serotec (Serotec, Düsseldorf, Germany): anty-CD8 (no MCA752F) and anti-CD4 (no MCA 749F) were used. In each analysis, cells stained with mouse isotype IgG conjugate were used as a negative control. The samples were analyzed using a FACS Celibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). The cells were collected by CellQuest software (Becton-Dickinson, San Jose, CA, USA) and a minimum 6000 events were acquired.
- Histamine concentration was evaluated in BALF supernatant using a radioimmunoenzyme assay - Histamine RIA kit (Immunotech, Marseilles, France).

**Experimental set-up**

For both BALF content evaluation and RL measurements, the animals were randomly selected into three equal experimental groups: a group treated with rolipram, another one treated with dexamethasone, and a control group in which asthmatic reactions occurred without any drug treatment.

**Data analysis**

A Mann Whitney U test was applied for the statistical analysis. P ≤ 0.05 was taken as being indicative of statistically significant changes. A commercial Statistica 5.1 package was used for statistical data elaboration.

**RESULTS**

*Fig. 1* illustrates changes in the LR profile during the course OA inhalation in the control, dexamethason-treated, and rolipram-treated groups of animals. In the control group, LR significantly increased from the 6th min of the inhalation onward. The increase was absent in both dexamethasone and rolipram groups; the difference in the inhibitory effects on the OA-induced LR growths of the two drugs was insignificant.

Both rolipram and dexamethasone inhibited the release of histamine that was present in the control, untreated animals in response to the OA challenge (*Fig. 2*). The inhibition was nearly total and comparably significant for the two drugs up to 6-8 h of the challenge (*Fig. 2*).

The influence of dexamethasone and rolipram on the content of cells in BALF after the inhalation challenge with OA in the experimental groups of animals studied is shown in *Fig. 3*. Rolipram caused significant, selective inhibition of
Fig. 1. Changes in lung resistance after ovalbumin challenge.

Fig. 2. Histamine content before and 1.5 h, 6-8 h, 12 h, and 24 h after ovalbumin challenge.

Fig. 3. Cellular content in BALF before and 1.5 h, 6-8 h, 12 h, and 24 h after ovalbumin challenge.
eosinophils influx compared with the control, untreated group. The selectivity of rolipram’s effect on the eosinophils is confirmed by the fact that a total number of leucocytes recovered from BALF at all the time points of the OA challenge studied did not differ between the control and rolipram groups, and there was even a significant increase in the influx to BALF of macrophages, at 1.5 h, and neutrophils, at 6-8 h, in the rolipram group. Unlike rolipram, dexamethasone inhibited the influx to BALF of all types of cells. Moreover, this inhibition was evident even before the OA challenge, and with one exception of neutrophils (at 6-8 h) it included all the cells at each time point tested.

In the control group, OA inhalation challenge resulted in increased Th/Tc ratio. Rolipram caused the Th/Tc ratio to increase further at 6-8 h and 24 h of the experiment. In animals pretreated with dexamethasone, the value of Th/Tc ratio remained unchanged after the allergenic challenge (Fig. 4).

**DISCUSSION**

The main finding of the present study is that pretreatment with rolipram counteracted the increase in lung resistance evoked by allergenic challenge. The study corroborates the results obtained by other authors who tested the influence of rolipram on the respiratory tract in both *in vivo* and *in vitro* animal models. Rolipram inhibits albumin-induced bronchospasm and the early asthmatic reaction (19) and in *in vitro* conditions causes relaxation of fragments of trachea (20). Single injection of rolipram in a dose of 1.0 mg/ kg before the allergenic challenge is as efficient in inhibiting the increase in lung resistance in EAR as are two injections of dexamethasone of 1.2mg/kg each. Concentrations of glucocorticoids used by other authors to inhibit asthmatic reactions in guinea pigs varied considerably; for dexamethasone, administered intraperitoneally, the dose varied from 0.25 mg/kg to 20 mg/kg (21-23). Glucocorticoids are administered before or, less frequently, after the allergenic challenge. In our study, selection of the dose and scheme of dexamethasone administration was inspired by data from other studies (24) and by our own experience with the
application of dexamethasone in guinea pigs subjected to pulmonary allergic reaction (25). We have found that a greater single dose of dexamethasone (4 mg/kg) increases considerably the permeation of protein to guinea pigs’ airways (unpublished data). Due to methodological discrepancies a detailed discussion with the results of other authors is difficult. However, in general opinion, glucocorticoids, since long used in the treatment of asthma and other allergic diseases, remain the drugs of reference in the evaluation of effectiveness of new medications.

Clemastine, a first generation H₁ receptor antagonist (18), was used in the present study to prevent systemic anaphylaxis, which was essential to carry out the experiment. However, the interaction of histamine, released by degranulation of mastocytes, with HR₁ receptors mediates a variety of effects, such as vasodilation, bronchial smooth muscle contraction, and several immune-inflammatory and effector functions (26). The pulmonary effects of histamine-H₁ receptor interaction are to be altered on the background of H₁ blockade by clemastine, which could have influenced the results of the allergic reaction modeled in our study.

In our experiment, the asthmatic reaction was accompanied by a transient increase in the absolute number of lymphocytes in BALF, along with the Th/Tc increase. Rolipram further enhanced the Th/Tc ratio. There are no relevant data in the literature to be compared with in experimental models of asthma. Still, it can be supposed that rolipram influences the recruitment of lymphocytes from the pulmonary vascular bed in a selective manner. The influence of rolipram on the migration and polarization of human T lymphocytes has been reported by other authors, who showed that at low concentrations it inhibits the adhesion of T cells to β-1 and β-2 integrin ligands and of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (27).

In the present study, both dexamethasone and rolipram prevented the increase in lung resistance in EAR and in histamine release, but their influence on the influx of lymphocytes and the Th/Tc ratio was different. In animals pretreated with dexamethasone, the lymphocyte influx was inhibited, whereas the Th/Tc ratio in EAR remained within the range of values present before the allergenic challenge. This finding confirms that lung resistance in EAR is not underlain by lymphocyte activity and is due mainly to the presence of mediators released from mastocytes and basophiles (28).

A decrease in the influx of cells to BALF by glucocorticoides in the course of OA-induced asthmatic reactions in guinea pigs is reported in other studies (22, 23, 29). Rare observations concerning the lack of influence of dexamethasone on the recruitment of eosinophils to the airways (21) have not been confirmed in our study. In contrast to dexamethasone, rolipram inhibited only the influx of eosinophils, whereas it was ineffective in relation to the other cells isolated from airways. The influx of cells to the airways is the result of many factors such as: IL-8, TNF-α for neutrophils, IL-5, GM-CSF, and eotaxin.
for eosinophils, or CD11/18, ICAM-1 for lymphocytes (30). It seems a reasonable assumption with respect to the selective inhibition of eosinophil influx observed in our study that there might be distinctly different adhesion pathways for neutrophils and eosinophils (31).

The inhibiting influence of glucocorticoids on the production of IL-5 has been reported in many experiments on human cells and animal models (32, 33). Agents that increase cAMP level exert a similar effect on IL-5 (32). In a mouse model, administration of rolipram prevents changes in LR and decreases IL-5 level in BALF (33). Moreover, in sensitized guinea pigs aerosolized ovalbumin, IL-5, and eotaxin cooperated in mediating a rapid transfer of eosinophils from bone marrow to lungs in response to the allergenic challenge (34). In patients suffering from asthma, dexamethasone administration decreases mRNA expression for eotaxin-3 (35), which is assumed to be at play in the recruitment of eosinophils (35). No such data exist for the PDE-4 inhibitors as yet.

Permeation of histamine to the bronchoalveolar space, from the degranulation of mucous mastocytes of the respiratory tract as a result of asthmatic reaction, has been extensively described in the medical references. This phenomenon was observed in the present study as well and the dynamics of histamine concentration growth in BALF after the allergenic challenge is in accordance with the observations by others (23). In the present study dexamethasone and rolipram were equally effective in the stabilization of mastocytes. There are discrepancies concerning the glucocorticoid effect on histamine release from pulmonary mastocytes. According to some authors, glucocorticoids do not inhibit histamine release from human mastocytes, unlike that from basophiles (36). Others show that dexamethasone have a weak inhibitory effect on histamine release in primates (37). Literature does not provide data concerning the influence of dexamethasone on histamine release in EAR in guinea pigs. Current data concerning the effectiveness of rolipram in inhibiting histamine production come predominantly from in vitro experiments on human cells (38). Contrary to our present observations, the authors quoted above have found histamine release from mastocytes. Others show that rolipram limits histamine release only from activated basophiles (39). Thus, the issue of rolipram’s effect on histamine release remains unsettled.

If bronchospasm and the influx of eosinophils are considered the most important factors in the allergenic cascade, in our experiments rolipram in a single dose was equally effective in inhibiting the two events as was dexamethasone in a double dose. This finding, however, does not justify drawing a conclusion that a single application of rolipram also is beneficial in inhibiting late asthmatic reactions, as some investigations show that rolipram, unlike dexamethasone, is not equally efficient in limiting these reactions (23).
REFERENCES


Author’s address: P. Nejman-Gryz, Department of Internal Diseases, Pneumology and Allergology, Warsaw Medical University, Banacha 1a St., 02-097 Warsaw, Poland; phone: +48 22 5991560.