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Pharmacodynamic evaluation of RP3128, a novel and potent CRAC channel inhibitor in guinea pig models of allergic asthma

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ABSTRACT

The increase in intracellular Ca^{2+} levels through the activation of Ca^{2+} release-activated Ca^{2+} (CRAC) channels is essential for mediating a wide scale of immune cell responses. Emerging evidence indicates an involvement of abnormal CRAC channel activity in human diseases such as certain types of immunodeficiency, autoimmunity and allergic disorders. This objective of this study was to evaluate the therapeutic potency of a novel CRAC channel inhibitor, RP3128, in experimental models of allergic asthma using guinea pigs.

Ovalbumin-induced allergic airway inflammation was determined upon acute and long-term (14 days) oral administration of RP3128. *In vivo* changes in specific airways resistance (sRaw) and amplitude of isometric contraction (mN) of ASM (*in vitro*) were estimated to evaluate bronchodilatory effect upon acute and long-term administration of RP3128 or salbutamol. Exhaled nitric oxide (eNO), immunohistochemical and histological analysis of cellular infiltration in airways tissue, and levels of cytokines in plasma as well as bronchoalveolar lavage fluid (BALF), were determined using Bio-Plex® 200 System (BIO-RAD, USA). Ciliary beat frequency (CBF, in Hz) was estimated using a high-speed video camera and LabVIEW™ Software. Additionally, the impact of RP3128 and budesonide on mucociliary clearance was determined.

Acute and long-term administration of RP3128 resulted in significant bronchodilation. Long-term administration of RP3128 exceeded the bronchodilatory effect of salbutamol and significantly decreased eNO and cytokine levels in plasma and BALF, which together with histological and immunohistochemical analysis validated its anti-inflammatory effect compared to budesonide. Data demonstrate the therapeutic potential of RP3128 in respiratory diseases causally associated with allergic inflammation.

1. Introduction

Asthma represents a chronic disorder associated with a substantial medical cost. Asthmatics are limited in their physical, emotional, social, and professional ability, that are further exaggerated if symptoms are not adequately controlled (Braman, 2006). Asthma is also associated with a rise in atopic sensitization and serves as a predisposing factor for several other allergic conditions such as eczema and rhinitis (von Kobyletzki et al., 2012). Current therapies for asthma are symptomatic and fraught with a myriad of side effects. For majority of asthmatics, corticosteroid anti-inflammatory therapy is still the preferred treatment (Kips and Pauwels, 2001). However, this approach requires life-time therapy and a subset of patients remain symptomatic despite optimal treatment creating a clear unmet medical need. Targeting calcium influx through calcium release-activated calcium (CRAC) channels represents a novel approach for combating asthma.

Calcium release-activated calcium (CRAC) channels belong to the class of store-operated channels (SOC) that are present on the plasma membrane of non-excitable cells (lymphocytes and mast cells) and mediate immune responses (Feske et al., 2015). Activation of CRAC channels *via* depletion of endoplasmic reticulum stores results in alteration in calcineurin/ NFAT-dependent expression of several cytokines including interleukin-2 (IL-2). While calcineurin inhibitors are potent immunosuppressive agents, they are limited by nephrotoxicity and neurotoxicity (Yuan et al., 2015). Because localization of the CRAC channels is restricted to non-excitable cells, inhibition of calcium influx by altering CRAC

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channel activity is expected to be an effective strategy for the treatment of autoimmune and inflammatory diseases devoid of side effects. Furthermore, studies in subjects with non-functional CRAC channels suggest that inhibition of this target both safe and efficacious.

Abnormal CRAC channel activity is associated with several diseases including asthma, rheumatoid arthritis, thrombosis, cancer, severe combined immunodeficiency (SCID), and inflammatory bowel disease (Parekh, 2010). The contribution of CRAC channels to asthma stems for several studies in pre-clinical models wherein these channels have been shown to regulate mast cell activation and subsequent downstream effects (Di Capite et al., 2011). Drugs targeting CRAC channels could therefore be of immense clinical benefit.

The objective of this study was to determine the therapeutic potential of RP3128, a novel, small molecule heteroaryl amide compound comprising of a 3,5-disubstitued-1H-pyrazol-1-yl moiety (PCT application no WO2011042797), in airway inflammation. Because CRAC channels are known to regulate the functionality of immune cells relevant in asthma, we hypothesized that intervention with specific inhibitors would attenuate disease progression in animal models. Data demonstrated a significant reduction in CRAC channel mediated calcium influx and associated biomarkers that translated into a potent anti-allergic effect *in vivo*.

2. Material and methods

2.1. Chemicals

Citric acid (AC) p.a., histamine, acetylcholine, methacholine, salbutamol, aluminum hydroxide, budesonide and chicken ovalbumin were purchased from Sigma Aldrich (Lambda Life, Slovakia). Budesonide was prepared as a suspension in 1% TWEEN 80 (in 0.9% saline) according to the manufacturer's instructions. RP3128 compound was triturated first with Tween 80 (1% v/v) and added to 0.5% (w/v) methylcellulose for further trituration. All the other above-mentioned drugs were dissolved in 0.9% saline.

2.2. Animals

The experiments were approved by the Institutional Ethics Committee of Jessenius Faculty of Medicine, registered in Institutional Review Board/Institutional Ethic Board Office (IRB 00005636) in accordance with Slovakian and European Community regulations for the use of laboratory animals and follow the criteria of experimental animal's welfare (decision No. 611/2010).

Adult male Trik guinea pigs, weighing 150–350 g were obtained from the breeding facility Velaz, Prague, Czech Republic (VELAZ s.r.o. Praha/34081/2008-10001) and were housed in an approved animal holding facility. Animals were held in quarantine for a week following which they were shifted to experimental cages and housed for an additional 2–3 days prior to initiation of experiments. Animals were divided into the following groups, each consisting of 10 animals per group:

1. **Negative controls** – sensitized animals received solvent used in studies at a dose of 1 ml/ kg bw., perorally (p.o.) either as a single dose or once daily in the long-term studies. One such group, without any treatment was used for *in vitro* airway smooth muscle (ASM) reactivity tests, in which direct effect of RP3128 on isolated ASM was evaluated.
2. **Positive controls** – sensitized guinea pigs were treated with salbutamol 10 mg/kg bw. intraperitoneally (i.p.), or budesonide (3 mg/ml by inhalation for 5 min) either as a single dose or daily dosing for 14 days.
3. **Experimental groups** – sensitized animals were treated with RP3128 at doses 1, 3 and 10 mg/kg for the acute studies or at 3 mg/kg for the 14-day chronic study. The dose of 3 mg/kg for the long-term study was selected based on results from the acute phase.

2.3. Antigen-induced airway hyperresponsiveness

Sensitization of animals using ovalbumin was performed based on the method described by Franova et al. (2013). Briefly, Al $(OH)_3$ adsorbed ovalbumin was administered intraperitoneally and subcutaneously (day 1 of sensitization) and intraperitoneally (day 3). Allergen challenges (1–2 min by inhalation) were performed on days 9, 12, 15, 18, and 20 in a double chamber whole-body plethysmograph box for small laboratory animals (HSE type 855, Hugo Sachs Elektronik, Germany). Twenty four hours after the last allergen provocation, compounds were administered either as a single dose (acute studies) or once daily for a 14-day period (chronic studies).

2.4. Evaluation of Airway Smooth Muscle (ASM) reactivity under in vivo conditions

In vivo airway smooth muscle reactivity was evaluated using a double chambered body plethysmograph box for laboratory animals. Values of specific airway resistance (sRaw) calculated by Pennock et al. (1979) and their changes were regarded as an indicator of *in vivo* airway reactivity. The sRaw is proportional to phase difference between nasal and thoracic respiratory airflow. Changes in sRaw were measured under basal conditions (to test the impact on hyperreactivity of the airways) and 1 min after a 30 s exposure to acetylcholine (0.3 M/l), histamine, and methacholine (both in concentration of 10^{-6} M/l) in groups treated with salbutamol, vehicle, or RP3128. An interval of 1 min was provided between exposure to the bronchoprovoking agent and measurement of sRaw during which fresh air was insufflated into the nasal chamber.

2.5. Evaluation of ASM reactivity under in vitro conditions

Changes in ASM reactivity following cumulative doses of contractile mediators (acetylcholine and histamine) was tested by the well described organ tissue bath method (Sutovska et al., 2013a). Briefly, guinea pigs were killed by transversal interruption of neck spinal cord. Consequently, the respiratory organs were removed. Four strips (two of tracheal and two of pulmonary smooth muscle) obtained from each animal were placed into organ bath chambers filled with Krebs-Henseleit´s buffer (in nM: NaCl 112.9, KCl 4.7, $CaCl_2$ 2.8, $MgSO_4$ 0.5, $NaHCO_3$ 24.9, glucose 11.1) saturated with pneumoxide (95% O_2+5% CO_2), held at a temperature of 36 ± 0.5 °C, and maintained at pH 7.5 ± 0.1. Single strips were fixed onto the sliding arm and the other end was bound by a thin thread to the hook of a transducer (Experimetria Ltd., Hungary). The tension was used to monitor the intensity of contractile responses. The amplitude of isometric contraction (mN) of tracheal and pulmonary smooth muscle in response to cumulative doses of acetylcholine and histamine (10^{-8}–10^{-3} M/l) was used for evaluation of ASM reactivity.

Pulmonary and tracheal strips of negative control group were pre-contracted using acetylcholine or histamine ($c = 10^{-5}$ M/l). Contractile amplitude was recorded as the response following addition of cumulative doses of RP3128 (10^{-8}–10^{-3} M/l) directly into the chamber.

2.6. Measurement of exhaled nitric oxide (eNO) under in vivo conditions

Changes in eNO values were used as an indicator of the anti-inflammatory effect of RP3128. Animals were placed into an offline

sampling chamber connected with NIOX Flex Offline Start Kit 04-1210-F (Aerocrine AB, Sweden), and breathed NO- free air for 5 min. Subsequently, the exhaled gas (flow rate 5 ml s^{-1}) was analyzed for 7 s. NIOX uses a high sensitivity and high specificity chemiluminescence gas analyzer with an integrated software, to accurately measure NO molecules at very low concentrations (particle per billion, ppb).

2.7. Assessment of cytokine level under in vitro conditions

Blood from guinea pigs heart was collected immediately after transversal spinal cord interruption. Bronchoalveolar lavage was collected by flushing with warm saline (37 °C) in a volume based on the body weight of the animal (10 ml/kg). Serum or supernatant from bronchoalveolar lavage fluid (BALF) were obtained by centrifugation of samples at the centrifugal force of 2054 g for 5 min and 277 g for 2 min, respectively.

Cytokines were measured using a Th1/Th2 Human Cytokine (Bio-Rad, USA) panel on a Bio-Plex® 200 System. Capture antibody–coupled beads were first incubated with standards, samples, or controls followed by incubation with biotinylated detection antibodies. After washing away the unbound biotinylated antibodies, the beads were incubated with a reporter streptavidin-phycoerythrin (S-P) conjugate. Following the removal of S-P excess, the beads were passed through the Bio-Plex 200 suspension array reader equipped with two lasers, one (532 nm excitation) for analytes quantification and s (635 nm excitation) for cytokine identification All washes were performed using a Bio-Plex Pro wash station. Data (concentrations in pg/ml) were obtained using a high-speed digital processor and the Bio-Plex Manager™ 6.0 software l.

2.8. Evaluation of Ciliary Beat Frequency (CBF) under in vitro conditions

Experiments were carried out under standard laboratory conditions (ambient temperature was maintained at 21–24 °C and humidity at 55 ± 10% using LG multi type air-conditioner, LG Neo plazma, Slovakia). The temperature of the microscopic glass slide and the saline (natrium chloratum 0.9%) used as a nutritive medium for cilia, was kept in a range of 37–38 °C.

Following transversal interruption of the male guinea pigs neck spinal cord, transverse access to the trachea was made approximately in the middle of its normal length. Ciliated samples were obtained using a cytology brush that was dipped into saline and gently rotated on the mucosal surface of the trachea. Tracheal brushings were immediately placed into saline solution and were processed for microscopy.

Microscopic preparations were examined using phase contrast inverted biological microscope (Kvant model IM1C, Slovakia), 3 min after brushing the tracheal cilia. Ciliary cell beating was recorded using a digital high speed video camera (Basler A504kc; Basler AG, Germany) at a frame rate of 256–512 fps (frames per s). Approximately, 10–12 video recordings of the same microscopic preparation were performed at 1 min intervals. Duration of each recording was approximately 5–10 s. Video recordings were analysed using LabVIEW™ Software to generate a ciliary region of interest (ROI), intensity variation in selected ROI, and intensity variance curve. Curve was then analysed according to the Hargaš et al. (2011) method using the fast Fourier transform (FFT) algorithm. The median of frequency (Hz) for each ROI and their arithmetic means were referred as definite CBF value for each microscopic preparation.

2.9. Immunohistochemical and histological analysis of lung tissue

The immunohistochemical method described previously (Sutovska et al., 2013b) was used to detect mast cell tryptase in lung tissue. Briefly, formalin- fixed paraffin-embedded tissue samples were cut into 4 μm sections and stained. Slides were baked in an oven at 59 °C for 2 h and were then treated in PT Link system (DAKO). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Immunohistochemical reaction was performed using mouse anti-mast cell tryptase (DAKO, S640, Clone AA1, Kit K 0609 LSAB system) for 20 min. Reaction was visualized by incubation with chromogen (3, 3′ diaminobenzidine) for 2–3 min and counterstained with Meyers' haematoxylin. Standard haematoxylin-eosin (HE) staining was used to detect eosinophils in pulmonary tissue samples.

All double-blind labeled microscopic slides were assessed by two independent observers. Cases with discrepancy between observer's results were noticed and were repeatedly assessed by both observers together using dualhead microscope. In every processed specimen, the degree of eosinophils and mast cells infiltration was semiquantitatively determined using 4-degree scaling. According to this scale, *negative* sample was determined by degree of infiltration 0 (no eosinophils or tryptase-positive cells) or 1 (mild infiltration) and *positive* sample was defined as degree of infiltration 2 (moderate infiltration) or 3 (diffuse infiltration).

2.10. Statistics

Data obtained are depicted as means ± S.E.M. Statistical analysis was performed using one-way ANOVA (with Bonferroni post hoc test) or Student's t-test, as appropriate. The Fisher's exact test was used to evaluate the immunohistochemical features. A $P < 0.05$ was deemed to be significant.

3. Results

3.1. RP3128 and airways defense reflexes and non-reflexive mechanisms

3.1.1. The influence of acute administration on airway reactivity

The changes in **sRaw *in vivo*** due to three bronchoprovoking agents–citric acid, histamine and methacholine, each producing bronchoconstriction by different mechanisms involved in asthma pathogenesis (Sutovska et al., 2007), were recorded upon acute administration of RP3128. In this part of experiments, RP3128 compound was tested at 3 different doses (1, 3 and 10 mg/kg bw). The response was determined 2 and 4 h post-administration. Salbutamol was administered by i.p. route. *N* value in graphs represents the baseline data before any drug treatment.

Acute treatment with RP3128 at a dose 3 mg/kg suppressed the citric acid and histamine-induced sRaw values, however only the decrease in citric acid-induced airway reactivity was statistically significant (Fig. 1). Furthermore, RP3128 was significantly more effective in suppression of citric acid-induced sRaw than positive control drug salbutamol. RP318 was ineffective in its ability to suppress sRaw induced by methacholine. Although doses of 1 and 10 mg/kg RP3128 reduced sRaw values induced by different bronchoconstrictors, the effect was not statistically significant.

In vitro tests evaluated the effect of orally administered RP3128 on acetylcholine and histamine-induced pulmonary smooth muscle contractility in isolated ASM. Suppression of acetylcholine-induced contractions was significantly higher compared with vehicle at all doses of RP3128 tested and similar to salbutamol (Fig. 2). Reduced amplitude of histamine-induced contractions was observed only for the 3 mg/kg dose of RP3128 with relaxation

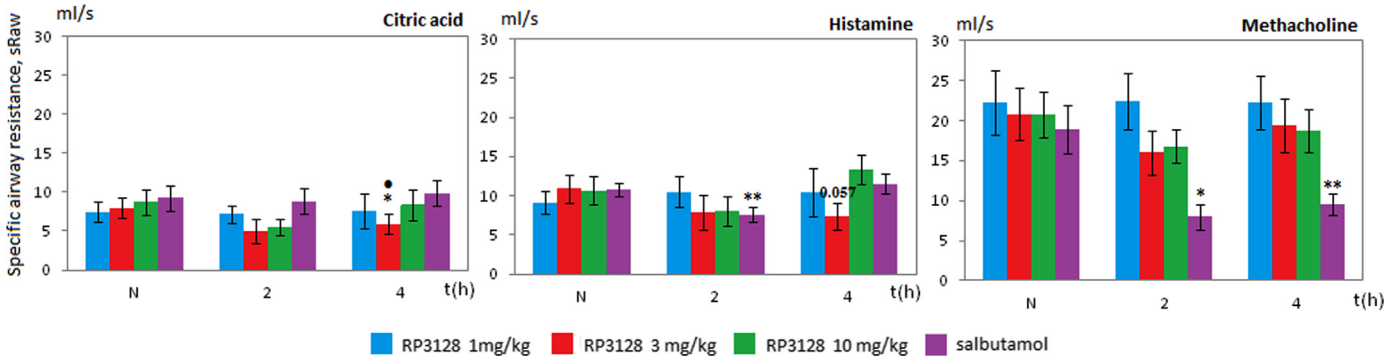

Fig. 1. The changes in specific airways resistance (sRaw) induced by short-time inhalation of AC, histamine and methacholine measured in sensitized animals before and after perorally administered different doses of RP3128. The effect was compared with effect of positive control drug salbutamol. $^{*}P<0.05$, $^{**}P<0.01$ vs N; $^{\bullet}P<0.05$ vs salbutamol.

being significantly higher to vehicle and salbutamol (Fig. 2).

To confirm relaxing effect of RP3128 on ASM, we tested the effect of cumulative doses of RP3128 on pulmonary and tracheal strips pre-contracted using acetylcholine or histamine. Both salbutamol and RP3128 significantly effectively suppressed initial amplitude of acetylcholine/histamine-induced contraction (data not shown). Administration of RP3128 resulted in a significant reduction in histamine and acetylcholine-induced pulmonary smooth muscle contractile responses compared to salbutamol tested under similar conditions (Fig. 3).

3.1.2. *The influence of long-term administration on airway reactivity*

The dose of RP3128 (3 mg/kg bw.) was selected for long-term (LT) experiments based on results from the acute studies. Influence of long-term oral administered RP3128 on airway reactivity *in vivo* was determined 24 h after the last dose. Long-term treatment of ovalbumin-sensitized animals with RP3128 significantly reduced basal as well as citric acid, histamine, or methacholine-induced values of hyper-reactivity confirming the bronchodilatory properties of the compound (Fig. 4). Responses were significantly lower when compared with the control drug, salbutamol (SAL).

In vitro reactivity of isolated tracheal (Fig. 5) and pulmonary (Fig. 6) was reduced in experimental as well as positive control group. Reduction in reactivity of pulmonary strips corroborated with the data from acute experiments.

3.1.3. *Effect of long-term administration of RP3128 on CBF*

The CBF of ovalbumin-sensitized negative control group was significantly elevated in comparison with healthy unsensitised animals. Neither RP3128 nor budesonide affect CBF (Fig. 7)

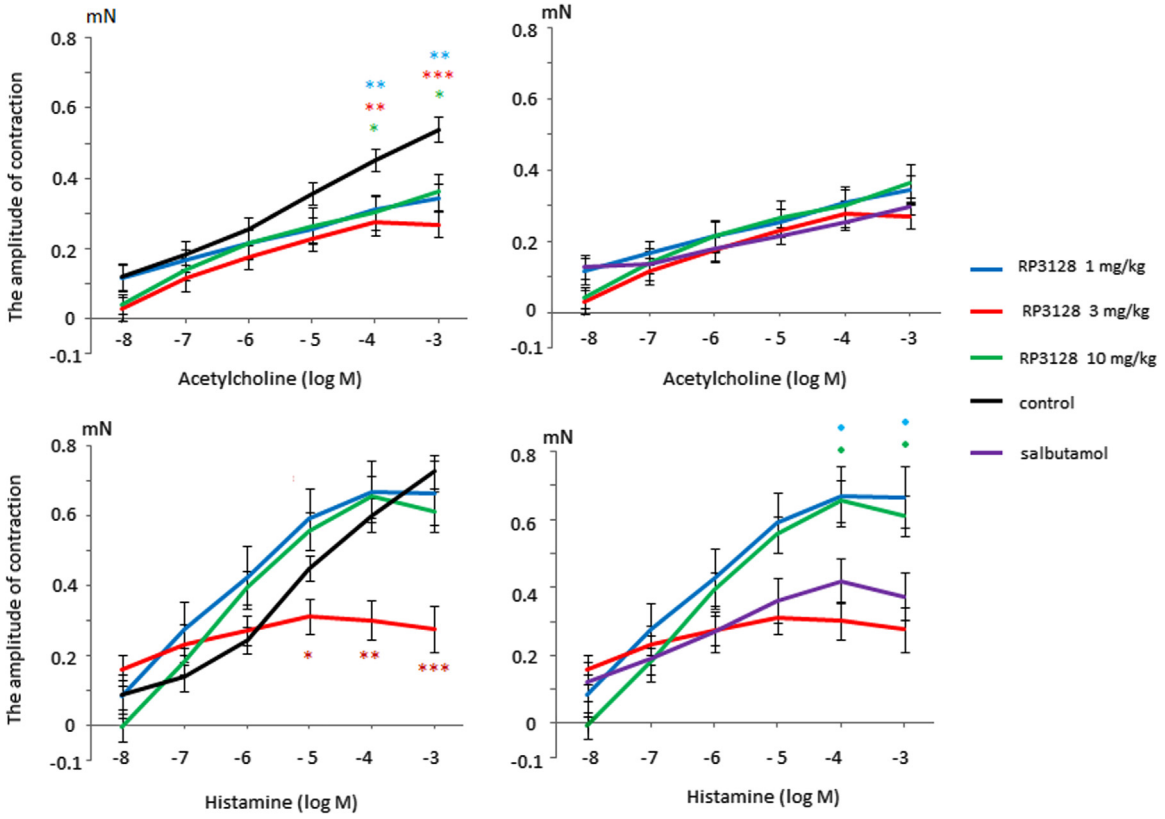

Fig. 2. The contractile response of pulmonary smooth muscle (mN) on cumulative doses of acetylcholine or histamine (10^{-8}–10^{-3} M/l) added to isolated strips to bath chamber. The lung tissue was obtained of animals treated by different doses of RP3128 compound (2 h after perorally administered agent). The effect of the experimental drug was compared to vehicle (control) and salbutamol tested under the same conditions. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs control; $^{\bullet}P<0.05$ vs salbutamol.

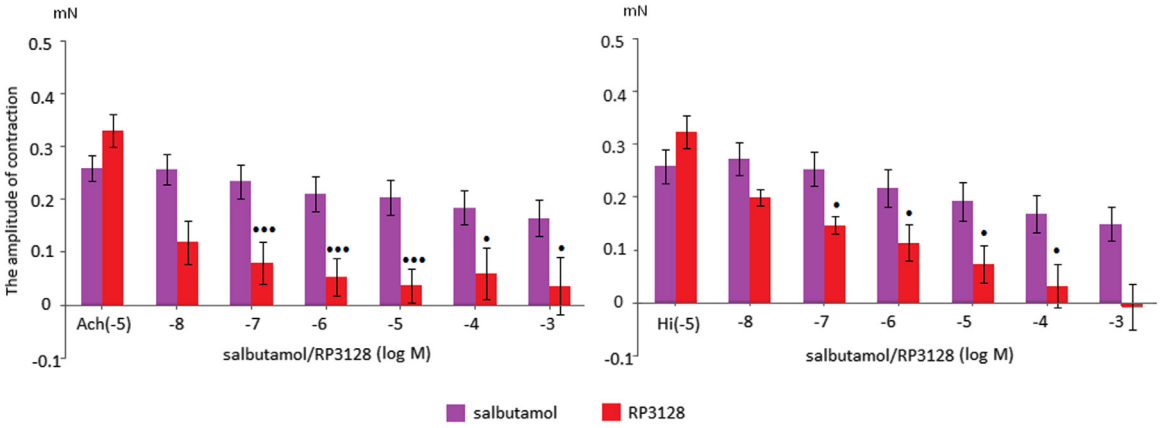

Fig. 3. The contractile response of pulmonary smooth muscle (mN) precontracted by acetylcholine (left) or histamine (right) on cumulative doses of RP3128 (10^{-8}–10^{-3} M/l) added to bath chamber. The effect of the experimental drug was compared to salbutamol. $^{\bullet}P<0.05$, $^{\bullet\bullet}P<0.01$ and $^{\bullet\bullet\bullet}P<0.001$ vs salbutamol.

indicating that the compounds did not influence mucociliary clearance in an experimental model of allergic asthma.

3.2. RP3128 and allergic inflammation of the airways

3.2.1. Influence of RP3128 on eNO

Allergic inflammation of airways, a common feature in the pathogenesis of asthma, is associated with increased levels of exhaled NO. Exhaled NO is measured in clinical practice to diagnose the intensity of airway inflammation. As shown in Fig. 8, long-tern oral administration of RP3128 caused a significant reduction in eNO with changes being comparable to budesonide (BUD).

3.2.2. Cytokine levels in BALF and plasma

Long-term administration of RP3128 and budesonide significantly decreased levels of IL-4, IL-5, IL-13 and TNF-α in **plasma** (Fig. 9) and **BALF** (Fig. 10) in comparison with sensitized vehicle-treated control group of guinea pigs. Except for IL-5, the plasma and BALF levels of cytokines in experimental group were almost similar to the unsensitised control group. In plasma, RP3128 was more effective at suppressing IL-4 and TNF-α compared to budesonide while the effect on IL-5 and IL-13 was less pronounced. Furthermore, all BALF cytokines of the experimental group were significantly lower than levels measured in guinea pigs treated by budesonide.

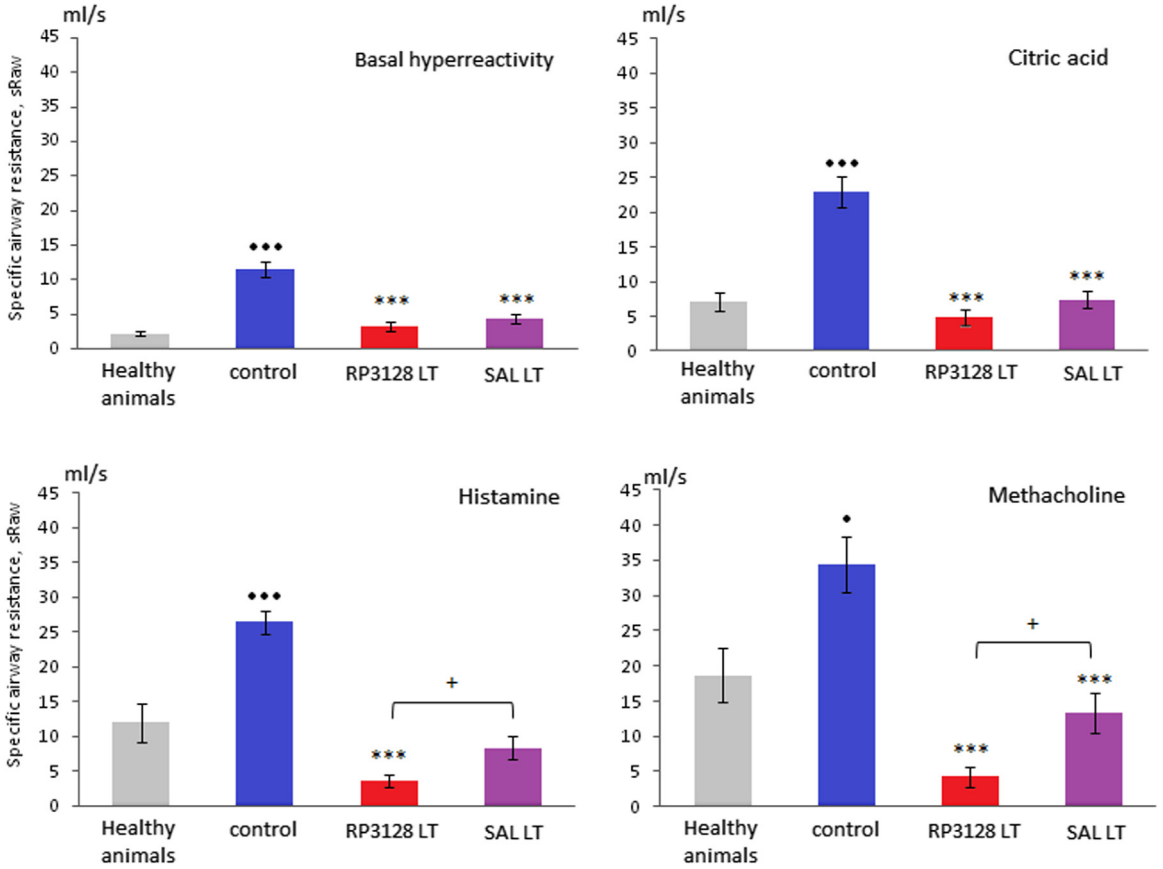

Fig. 4. The changes in specific airways resistance (sRaw) measured under the basal conditions (basal hyperreactivity) and induced by short-term inhalation of AC, histamine and methacholine in sensitized animals after perorally, long-term administered RP328 compound (RP LT, daily dose 3 mg/kg bw.) compared with effect of vehicle (control) and positive control drug, beta-2 agonist salbutamol (SAL LT). $^{***}P<0.001$ vs control; $^{\bullet}P<0.05$ and $^{\bullet\bullet\bullet}P<0.001$ vs healthy animals; $^{+}P<0.05$ vs salbutamol.

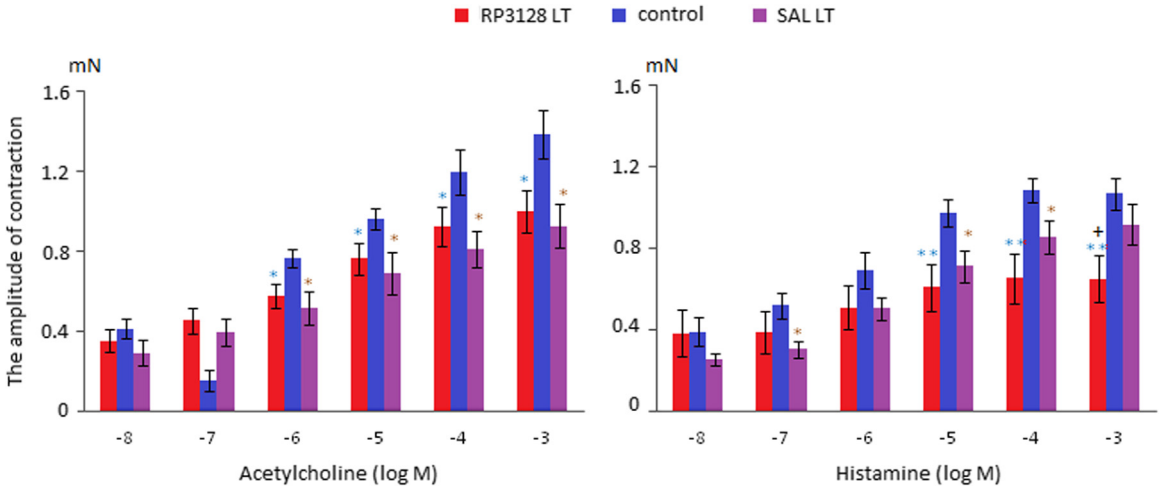

Fig. 5. The contractile response of tracheal smooth muscle (mN) on cumulative doses of acetylcholine and histamine (10^{-8}–10^{-3} M/l) added to isolated strips to bath chamber. The tracheal reactivity of animals treated long-term by RP3128 (RP LT) was compared to contractile response of control groups treated by vehicle (control) and salbutamol (SAL LT). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs control (*t*-test); $^{+}P<0.05$ vs SAL LT (one-way ANOVA, Bonferroni post-hoc test).

3.2.3. *The influence of RP3128 on infiltration of pulmonary tissue by mast cells and eosinophils*

Manifest eosinophilia and tryptase positivity (Tables 1 and 2) were evidenced in pulmonary sections of ovalbumin-sensitized vehicle-treated guinea pigs (control) in comparison with unsensitised animals (healthy animals). Infiltration of lung tissue by eosinophils and mast cells were significantly suppressed in animals upon long-term treatment with RP3128 or budesonide. Although RP3128 showed distinct suppression of pulmonary eosinophilia, activity was lower compared to budesonide. Lower effect of RP3128 on eosinophils functions also corresponded with the higher IL-5 levels in BALF.

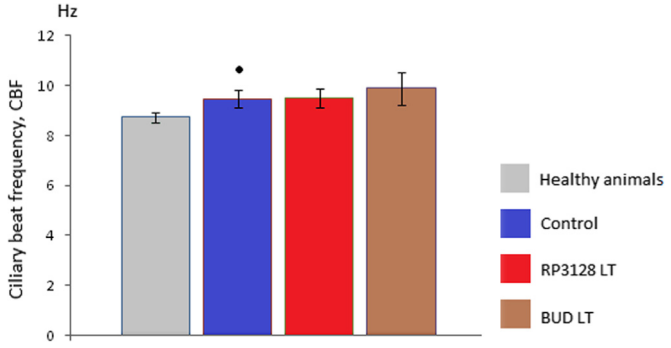

Fig. 7. The influence of allergen (ovalbumin) on CBF. The changes in this parameter evaluated in experimental (RP3128), negative control (control) and positive control (BUD LT) groups of animals as the response on long-term administration of novel CRAC channels modulator, vehicle and budesonide. $^{\bullet}P<0.05$ vs healthy animals.

4. Discussion

Store-operated Ca^{2+} entry (SOCE) is an important Ca^{2+} influx pathway in many non-excitable cells that is regulated by the filling state of ER intracellular Ca^{2+} stores (Feske, 2010). Emptying of ER $Ca2^{+}$ results in activation of plasma membrane Ca^{2+} channels that mediate sustained Ca^{2+} influx which is required for many cell functions as well as refilling of Ca^{2+} stores. The CRAC channel is the best characterized SOC channel with well-defined electrophysiological and molecular properties that modulates both innate and adaptive immune reactions thereby playing an important role in T-cell mediated diseases including allergies and transplant rejection (Feske et al., 2006; McCarl et al., 2009; Picard et al., 2009). Studies conducted in severe combined immunodeficiency (SCID) patients (Thompson et al., 2009) attest the CRAC channel as a potential interventional in treatment of T-lymphocyte and mast cell-mediated autoimmune or allergic disorders.

RP3128 is a novel, small molecule heteroaryl amide compound comprising of a 3,5-disubstitued-1H-pyrazol-1-yl moiety, that

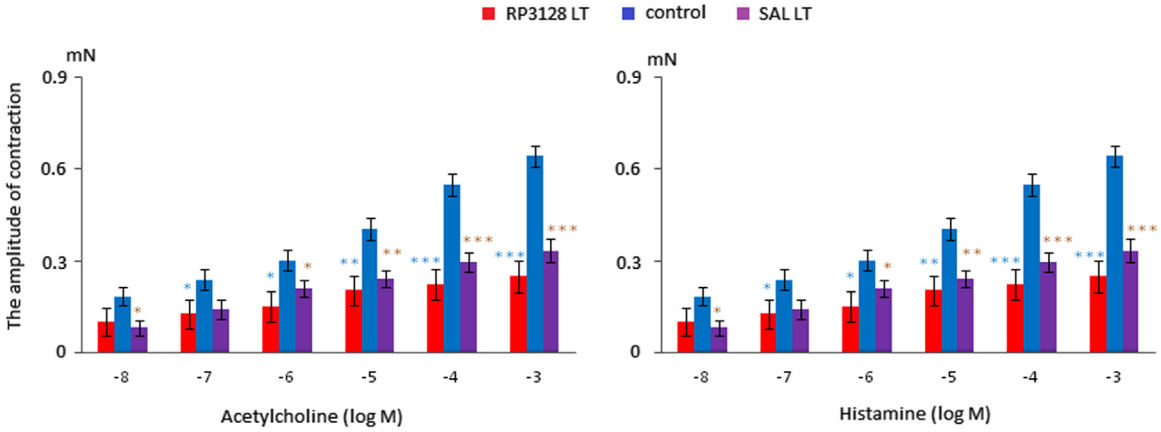

Fig. 6. The changes in pulmonary smooth muscle (mN) contractile response on cumulative doses of acetylcholine and histamine (10^{-8}–10^{-3} M/l) registered in animals treated long-term by RP3128, vehicle and salbutamol. For further explanation see also Fig. 5.

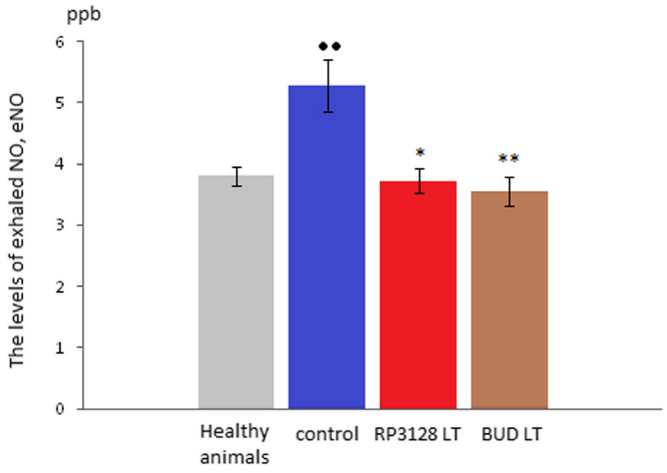

Fig. 8. The changes in values of exhaled NO (eNO) measured in unsensitized guinea pigs (Healthy animals), negative control group treated by vehicle (control), experimental group of animals treated long-term by RP3128 compound (RP LT) and positive control group received long-term budesonide (BUD LT). $^{••}P<0.01$ vs healthy animals; $^{*}P<0.05$ and $^{**}P<0.01$ vs control.

possesses CRAC channel inhibitory activity. Previous reports (Vakkalanka et al., 2013) have demonstrated that RP3128 significantly inhibited I_{CRAC} (103 nM) as well as calcium entry into Jurkat cells (40 nM) besides reducing IL-4 (< 400 nM) and IL-5 (< 250 nM) release from human whole blood and PBMC and IgE-induced RBL-2H3 cell degranulation (24 nM). Oral administration of RP3128 in guinea pigs resulted in a dramatic reduction in eosinophil infiltration in an acute model of PAF-induced allergic asthma (ED50=0.4 mg/kg/p.o) as well as in an experimental model of ovalbumin-induced chronic airway inflammation (ED50=0.6 mg/kg/p.o).

The objective of the current studies was to evaluate the pharmacodynamic properties of RP3128 in a well-validated guinea pig model of allergic asthma. This animal model of allergic airway inflammation was described previously by Franova et al. (2013). Among rodents, distribution, proportion and function of airways receptors in guinea pigs are similar to human airways and are therefore preferred in experiments involving airway inflammation (Muccitelli et al. 1987). Repetitive exposure of guinea pigs to allergen leads to complex of changes that almost mimic the asthma phenotype in human, e.g. airway hyperreactivity, increased cough response, and allergic inflammation of the airways characterized by typical histological features and corresponded changes in cytokines and mediators. We explored the anti-inflammatory and bronchodilatory effects of RP3128 on typical symptoms of allergic asthma in relevant *in vivo* and *in vitro* studies.

Acute and long-term oral administration of RP3128 displayed significant ability in attenuating airway reactivity *in vivo* and *in vitro*. Specific airway resistance (sRaw) was registered to evaluate airway reactivity. The measurement of sRaw is widely used by pediatric pulmonologists and therapeutic decisions are based on this approach. According to Mahut et al. (2009), sRaw is very sensitive parameter appropriate to detect mild levels of airway obstruction. Acute administration of RP3128 (3 mg/kg bw.) significantly decreased acetylcholine -induced sRaw, while long-term treatment of sensitized animals reduced basal hyper-reactivity as well. The apparently lower efficacy at 10 mg/kg compared to the 3 mg/kg dose group could possibly be related to pharmacodynamic interaction between the inducing agent (histamine) and RP3128. Unfortunately, the mechanism behind this was not explored in this study.

Increased airway reactivity in asthma usually correlates with severity of airway inflammation, a process that most likely involves a complex interaction among mast-cells, T-cells and eosinophils. Results are consistent with the *in vitro* profile described for RP3128 profound effects on immune cell functions (Vakkalanka et al., 2013).

Acute as well as long-term administration of RP3128 resulted in a marked reduction in ASM reactivity. While acute experiments confirmed a significant relaxing effect of RP3128 on acetylcholine and histamine-induced contractions of pulmonary smooth muscle strips only, long-term treatment resulted in reduction in contractions of pulmonary as well as tracheal smooth muscle tissue. It is generally accepted that asthma is a disease of small diameter intraparenchymal bronchi and therefore the responses seen with RP3128 on pulmonary tissue should be considered as a highly

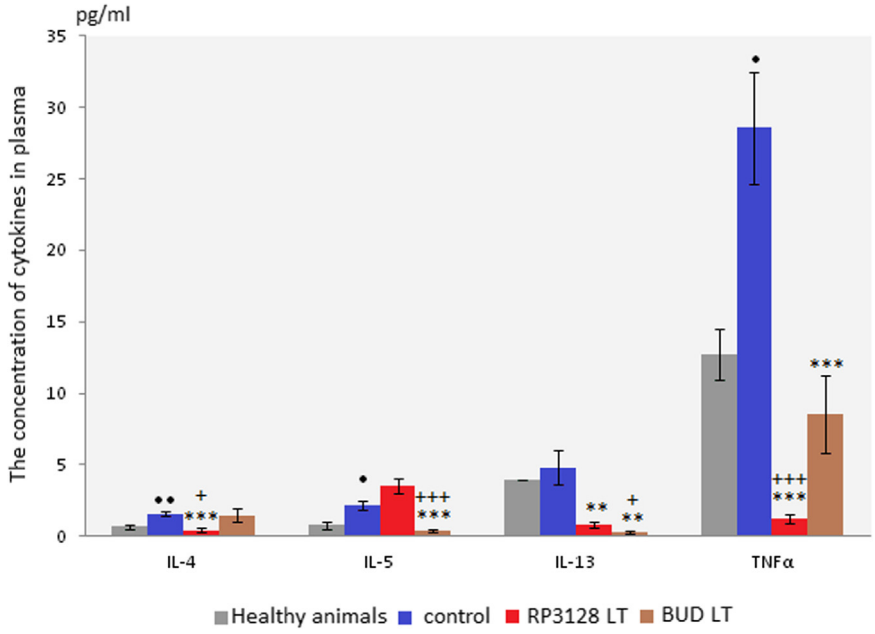

Fig. 9. The comparison of changes in levels of cytokines IL-4, IL-5, IL-13 and TNF-α measured in plasma obtained from unsensitized guinea pigs (Healthy animals), ovalbumin-sensitized negative control (control), animals treated long-term by RP3128 (RP LT) and budesonide (BUD LT). $^{**}P<0.01$ and $^{***}P<0.001$ vs control; $^{•}P<0.05$ and $^{••}P<0.01$ vs Healthy animals; $^{+}P<0.05$, $^{+++}P<0.001$ vs budesonide.

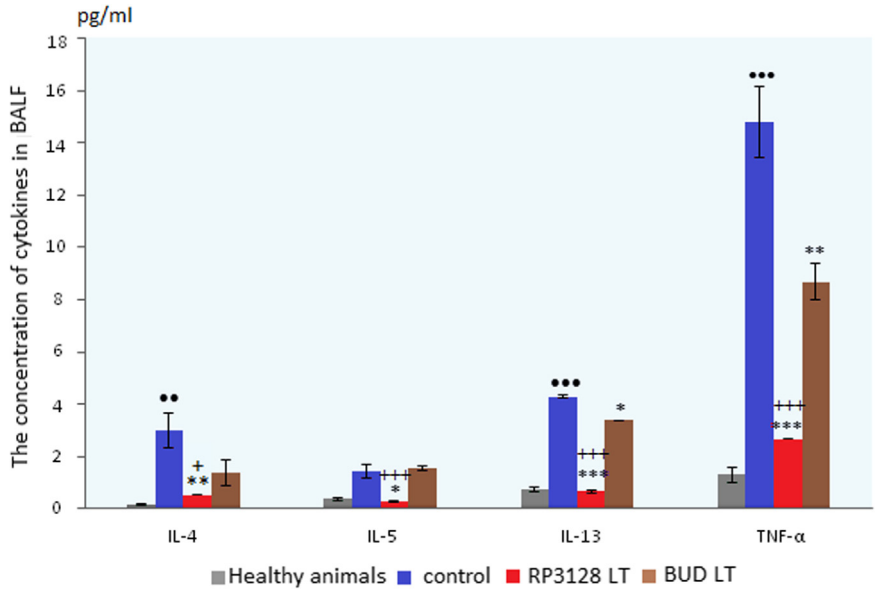

Fig. 10. The changes in levels of IL-4, IL-5, IL-13 and TNF-α assessed in BALF. $^{*}P < 0.05$ vs control; $^{\bullet\bullet\bullet}P < 0.001$ vs Healthy animals; for further explanation see also legend of Fig. 9.

Table 1
The number of negative (degree of infiltration 0 and 1) and positive (degree 2 and 3) samples infiltrated by eosinophils (HE staining) was evaluated in unsensitized control group (Healthy animals), negative control ovalbumin-sensitized group (control), experimental group of animals received long-term RP3128 (RP LT) and positive control group treated by budesonide (BUD LT).

EOSINOPHILS			
	Negative	Positive	*P*
Healthy animals	**8**	**1**	
control	**1**	**7**	c
RP LT	**7**	**1**	a
BUD LT	**10**	**0**	b

a – $P < 0.05$ and b – $P < 0.001$ vs control. c – $P < 0.01$ vs Healthy animals (Fisher's exact test).

Table 2
The number of negative and positive samples infiltrated by mast cells (immunohistochemical evidence of mast cell tryptase). For further explanation see also legend of Table 1.

MAST-CELLS			
	Negative	Positive	*P*
Healthy animals	**9**	**0**	
control	**2**	**6**	b
RP LT	**10**	**0**	a
BUD LT	**8**	**0**	a

a – $P < 0.01$ vs control. b – $P < 0.01$ vs Healthy animals (Fisher's exact test).

favorable finding. Isolated ASM from ovalbumin-sensitized rodents is commonly used for exploring acute effect of agents on primary mast cell responses to allergic stimulation (de Lima and da Silva, 1998; Nishioka et al., 2007). *In vitro* challenge with ovalbumin evokes mast cell degranulation and the release of mediators that induce ASM contractions (Yamaguchi et al., 2006; Rice et al., 2013). The reduction in contraction of pulmonary strip contraction with RP3128 could therefore be potentially attributed to the compound's effect on mast cells. Besides, the reduced contractile response of isolated ASM upon long-term RP3128 administration closely mimicked its anti-inflammatory activity noticed in the *in vivo* and *in vitro* experiments.

Exhaled NO levels were measured to determine the anti-inflammatory effect of RP3128 following repetitive ovalbumin challenges. Elevated eNO levels are associated with allergic inflammation of airways that is reduced upon administration of anti-inflammatory drugs (Sutovska et al., 2013b; Kocmalova et al., 2015). Long term treatment with RP3128 resulted in significantly reduced levels of NO in exhaled air in ovalbumin-sensitized animals. Previous reports in asthmatics have demonstrated an association between enhanced exhaled NO levels, upregulated inducible NO-synthase (iNOS), and airway eosinophilia (Redington, 2006). Eosinophils, T-cells, and mast cells are the major source of iNOS as well as cytokines such as IL-4, IL-5, IL-13 and TNF-α that stimulate overexpression of iNOS in airway epithelial cells (Paoliello-Paschoalato et al., 2005; Benson et al., 2011) and cause asthma progression (Feske et al., 2012). Secretion of these cytokines is driven by transcription factors such as nuclear factor of activated T-cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), and activator protein 1 (AP-1) that require sustained Ca^{2+} influx through CRAC channels (Prakriya, 2009). Our experiments demonstrated that long term administration of RP3128 significantly reduced IL-4, IL-13 and TNF-α in plasma and BALF. Furthermore, histological and immunohistochemical analysis confirmed reduced eosinophilia and mast cell infiltration into lung parenchyma. Taken together, our findings suggest that RP3128-mediated suppression of CRAC channels activity in immune cells led to a decrease in cytokine levels, changes in cellular pattern as well as in reduced eNO. Results are in line with the findings of Yoshino et al. (2007) wherein they demonstrated the suppressive effects of YM-58483, a selective CRAC channel inhibitor on the late phase asthmatic response (bronchoconstriction and lung eosinophil infiltration) in actively sensitized guinea pigs.

The cilia of respiratory epithelium represent relatively vulnerable structures targeted by different stimuli in asthmatic subjects. Serotonin from activated mast cells or TNF-α caused an increase in CBF (Weiterer et al., 2014) and IL-13 induced CBF-inhibitory effect and altered morphology of ciliated cells (Laoukili et al., 2001). According to Braiman and Priel (2001), CBF represents a crucial parameter of mucociliary clearance and ciliary transport efficiency is linearly dependent on CBF. Taylor et al. (2009) suggested that the increase in CBF is a process mainly dependent on Ca^{2+}

accumulated near plasma membrane in which CRAC channels should be possibly involved as a part of intimately interconnected signaling network consisting of Ca^{2+}, calmodulin and the cyclic nucleotide pathways. Although a significant increase CBF was noticed in in ovalbumin-sensitized guinea pigs, neither budesonide nor RP3128 influenced CBF thereby questioning the role of CRAC channels in this phenomenon.

5. Conclusion

In summary, the current studies define a major role for CRAC channels the etiology as well as the pathogenesis of allergic inflammation of airways. Data demonstrate that modulation of CRAC channels activity *via* RP3128 resulted in bronchodilatory effect similar to that of beta2 agonist, salbutamol, and an anti-inflammatory effect similar to that of corticosteroid, budesonide. With a profound anti-inflammatory and bronchodilatory effect, intervention by RP3128 represents a novel approach for the treatment of asthmatics. Importantly, these studies also highlight subtle differences between human and guinea pig response on RP3128 and conclude that the guinea pig is an appropriate species for exploring the role of CRAC channels in allergic airway inflammation *in vivo* as well as *in vitro*.

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