 DOI: 10.2478/v10201-011-0022-8

RELATIONSHIP BETWEEN POTASSIUM ION CHANNELS AND AIRWAYS DEFENCE REFLEXES INFLUENCED BY EXPERIMENTALLY INDUCED ALLERGIC INFLAMMATION IN GUINEA PIGS

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Abstract

Previously, we declared important role of ATP-sensitive (K_{ATP}) and calcium-sensitive (BK_{Ca}) potassium ion channels in cough and other defence reflexes of the airways coupled with reactivity of airways smooth muscle (ASM) and suggested their potential use as antitussives and antiasthmatic drugs.

The aim of presented studies was prove whether openers of potassium ion channels, K_{ATP} - pinacidil and BK_{Ca} – NS 1619, inhibit the cough reflex and modulate the ASM reactivity in conditions of experimental allergic inflammation of the airways in guinea pigs and if their influence on airways defence reflexes is changed by developing airways inflammation. Presented studies were realized in 4 partial experimental procedures with unsensitized guinea pigs and animal on 7th, 14th and 21th day of sensitization. Allergic inflammation of airways was induced by repetitive exposure of guinea pigs to ovalbumine and the degree of allergic inflammation was determined by histological analysis of tracheal and pulmonary samples. The cough reflex was induced by 0, 3 M citric acid aerosol for 3 min interval in which total number of coughs was counted. ASM reactivity *in vivo* was expressed as values of specific airway resistance (R.V) calculated by Pennock.

The cough response on the citric acid was significantly increased on 7th and 14th days of sensitization. The experiments showed persistent cough suppressive effect of pinacidil almost similar to codeine. The antitussive activity of NS 1619 remained only on 7th day of sensitization similar to its effect in group of unsensitized animals. Sensitization by ovalbumine gradually increased the values of R.V on bronchoprovoking agent citric acid. Pinacidil suppressed R.V values of both, unsensitized and sensitized animals, more significantly than salbutamol. In unsensitized guinea pigs, NS 1619 significantly reduced R.V values, but allergic inflammation attenuated its bronchodilatory activity on 7th and 14th days. Histological analysis of specimens showed increasing signs of allergic inflammation during sensitization procedure as well as significant proinflammatory effect of pinacidil and NS 1619. Introduction of non- selective K_{ATP} agonists in clinical practice is strongly limited due to proinflammatory effect, but K_{ATP} of ASM represents a rational therapeutic target for novel drugs – tissue selective agonists of K_{ATP}.

Key words: asthma, ion channels, cough reflex, airways hyperreactivity, guinea pigs

INTRODUCTION

Asthma is an allergic inflammatory airways disease mostly characterized by representative histopathological features associated with typical clinical symptoms, e.g. reversible airway obstruction, bronchial hyperresponsiveness (BHR) and cough. From the view of the pathophysiology, asthma results from complex biological interactions between different cell types, both resident and circulating, with environmental factors such as allergens, infections and tobacco smoke [1]. In this inflammatory process T lymphocyte (TH2), a key cellular element organizing chronic inflammation, smooth muscle contraction and airway remodelling. Other immune cells such as mast cells and eosinophils also participate. Epithelial damage caused by mast cells, activated eosinophils and lymphocytes has been proposed as one of the major pathophysiological mechanisms in asthma development [2]. Mast cells and eosinophils release cationic granule proteins that have been shown to cause damage to bronchial epithelial cells cultivated *in vitro* [3] and are involved into the attraction and activation of other inflammatory cells. Allergic airways inflammation is associated

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with allergen-induced early and late asthmatic reactions, and airway hyperresponsiveness to a variety of impulses resulting in obstruction of the airways and cough [4]. There is a time association between symptoms of allergic inflammation and pathophysiological changes in airways which could contribute to more accurate diagnosis than therapy. Airway hyperresponsiveness and cough reflex sensitivity courses are importantly determined by both changes in the neurogenic and non-neurogenic control of airway smooth muscle as well as by physical alterations in the airways, such as epithelial damage, mucosal swelling and remodelling of the airway wall that is characterized by the thickening of the basement membrane, subepithelial fibrosis and increased airway smooth muscle mass [5,6].

Airways allergic inflammation is often accompanied with excessive and somewhat deleterious coughing due to a lowering of the activation threshold for initiating of this reflex. As a result, cough can be evoked by stimuli that are normally considered innocuous. Except from centrally acting antitussives, e.g. codeine (with generally known serious adverse effects), current antitussive agents provide only moderate relief for patients with such cough disorders. This, in part, reflects our limited understanding the neuroanatomical and neurophysiological mechanisms that compose the sensory cough pathway in asthma, participating certain subcellular structures [7].

The cascade of inflammatory reactions resulting functional changes, mentioned above, involves various mediators and various mediating mechanisms, e.g. ion channels. Ion channels are altered in many pathological conditions, either indirectly or directly. It is not surprising, therefore, that drugs targeting ion channels constitute important therapeutic interventions for a number of diseases, including asthma. There are many channels analyzed in airways cells, the function of which may contribute to asthma.

Potassium channels modifying membrane potential and, consequently, the activation of voltage-gated calcium ion channels, are important target proteins highly expressed on neural structures, epithelial cells and smooth airways muscles [8]. They are also known as important effectors element for several relaxant agents in the guinea pig isolated trachea, like nitric oxide and VIP [9]. Several types of potassium channels have been reported to produce smooth muscle relaxation and relieve the cough, among them are large-conductance calcium- activated (BK_{Ca}) and ATP-sensitive (K_{ATP}) potassium channels [10].

The aim of the presented study was so prove whether openers of potassium ion channels, K_{ATP} - pinacidil and BK_{Ca} – NS 1619, inhibit the cough reflex and modulate the airway smooth muscle reactivity in conditions of ovalbumine-induced airway hyperresponsiveness in guinea pigs. The experimental works also evaluated the involvement of both potassium ion channels in course of allergic airways inflammation. Knowledge based on results of presented study together with literature data of the tissue-selective expression of various SUR (sulfonylurea receptor) subunits (SUR1, SUR2A-B) and Kir6.1–2 constituting K_{ATP} channels and the different subunit compositions of BK_{Ca} channels in various tissues [11] open the possibility to find tissue-selective ion channels modulators suitable for asthma therapy.

MATERIAL AND METHODS

All experiments were approved by the local Ethics Committee of the Jessenius Faculty of Medicine in accordance with the revised Declaration of Helsinki of 1983 and follow the EU criteria of experimental animals wellfare. Animals used in the studies - adult male TRIK strain guinea pigs, weighing 150-350 g – were obtained from Department of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Slovakia and breeding facility Velaz, Prague, Czech Republic- were housed in approved animal holding facility.

Design of the study

The animals in total number of 128 were used in experiments after one-week adapting period and after adaptation of guinea pigs to experimental condition. During several days,

animals were daily placed into the bodyplethysmograph box to achieve 60 min time interval of undisturbed breathing. The guinea pigs were divided into 16 groups, each consisting of 8 animals. The airways hyperresponsiveness was induced by ovalbumine in 12 groups of guinea pigs as it is shown in table 1. The rest 4 groups of animals were unsensitized. Presented study was performed in following 4 partial experimental procedures:

1. Experiments with unsensitized guinea pigs;
2. Experimental works with animals on 7th day of sensitization;
3. Experimental works with animals on 14th day of sensitization;
4. Experimental works with animals on 21st day of sensitization.

During each step, 4 groups of guinea pigs consisting of 8 animals were used. Two experimental groups of animals received K_{ATP} opener pinacidil in the dose of 1 mg.kg^{-1} b.w. by subcutaneous route (s.c.) or were exposed to an aerosol of BK_{Ca} agonist NS 1619 (200 μM) for 10 min interval. The third and fourth groups were positive controls. Animals received codeine or salbutamol in airways reactivity tests as intraperitoneally administered dose of 10 mg.kg^{-1}. The doses of all used chemicals were selected according to previous experiments [12, 13 and 14].

Chemicals

The modulators of potassium ion channels, citric acid and salbutamol were obtained from Sigma-Aldrich (Lambda life, Slovakia). Codeine (codeinium dihydrogenphosphoricum) was purchased from Lachema (Czech Republic). Pinacidil and NS 1619 were dissolved in 10 % DMSO, salbutamol and codeine in water for injection.

Antigen-induced airway hyperresponsiveness

Sensitization of animals by the antigen ovalbumin, which causes airway reactivity changes on immunological basis, was performed during 21 days. The method of experimental asthma model in guinea pigs was described by Franova et al. [13]. The allergen (ovalbumin in c= 10^{-5} mol.l^{-1}) adsorbed on Al $(OH)_3$ was administered on the 1st day of sensitization intraperitoneally (0.5 ml) and subcutaneously (0.5 ml), and on the 3rd day intraperitoneally (1 ml). Inhalation of allergen during 30-60 s time interval was performed at 9th, 12th, 15th, 18th and 20th days using double chamber whole bodyplethysmograph box for small laboratory animals (HSE type 855, Hugo Sachs Elektronik, Germany) and ovalbumin aerosol generated by jet nebulizer (PARI jet nebuliser, Paul Ritzau, Pari-Werk GmbH, Germany, output 5 l.s^{-1}, particles mass median diameter 1.2 μm) delivered to head chamber. All tests with sensitized animals were accomplished 24 h after last allergen provocation.

Cough reflex assessment

The method of chemically-induced cough was used for assessing the cough reflex [14]. We used citric acid aerosol in concentration of 0.3 M in saline for cough provocation. The followed two methods for detection of cough were used to distinguish the cough efforts from sneezing and movements:

– The changes of the expiratory airflow interrupting the basic respiratory pattern during cough effort were measured by pneumotachograph connected to the head chamber of bodyplethysmograph.
– The typical cough reflex movements and sounds were recognized by trained observer.

The inhalation of citric acid in double chamber plethysmograph lasted 3 min. During this time the number of the cough efforts was evaluated on the basis of sudden enhancement of expiratory flow accompanied by a typical cough movement and sound and counted. The cough response was measured prior to any agents administration (baseline measurement; N value in graphs) and then after their application in confirmed time intervals (30, 60, 120 and 300 min).

Minimal time difference between two measurements was two hours to prevent cough receptors adaptation on that kind of irritation [14, 15].

Evaluation of in vivo airway reactivity

In vivo airway smooth muscle reactivity was evaluated using double chambers body-plethysmograph box for laboratory animals consisting of head and body chambers. The specific airway resistance values calculated by Pennock [16] and their changes were regarded as indicator of *in vivo* airways reactivity. The specific airway resistance is proportional to phase difference between nasal and thoracic respiratory airflow. This non-invasive plethysmograph technique is commonly used for evaluation of bronchoactive substances effect [17].

The specific airway resistance was measured consecutively after the citric acid exposure and cough response registration during 1 min interval. Their intensity prior to administration of solvent, codeine and both potassium ion channels openers was considered as baseline (N value in graphs). The next values were measured 30, 60, 120 and 300 min time intervals. Between the cough response recording and measurements of airways specific resistance was an interval of minimum 5 min. During intervals, fresh air was insufflating into the nasal chamber.

Histological evaluation

Specimen processing:

For histological analysis we used both, tracheal and pulmonary tissue samples of each animal. All specimens were fixed in 10% formaldehyde solution at least for 24 hours. The size of specimens (10x6 mm) enabled their total processing. After fixation the samples were dehydrated in alcohol-xylen mixtures and embedded into paraffin blocks. Tissue sections (4-6 m thick) were cut by slide microtome and consecutively stained by basic haematoxylin-eosin stain. For the assessment of inflammatory cells, especially of mast cells, slides stained with Giemsa staining were used.

Histological analysis:

All microscopic slides were assessed by two independent observers. Cases, where the discrepancy between observers result was noticed, were repeatedly assessed by both observers together by dualhead microscope. In every specimen the evaluation was focussed on basic signs of allergic inflammation- presence of inflammatory infiltrate and the grade of blood perfusion (normal/hyperaemia). These parameters were semiquantitatively assessed by 4-level grading system (0- absence of cellular infiltrate / normal blood supply, 1 – slight increase of cells / slight increase in blood perfusion, 2 – moderate increase of cells / moderate vascular hyperaemia, 3- distinct cellular infiltrate / distinct vascular hyperaemia). The histological findings corresponded with grade 0 and 1 were regarded as negative and both, grade 2 and 3, were evaluated as positive.

Statistical analysis

All obtained data are shown as means ± SEM. For statistical analysis Student t-test was used. A $p<0.05$ was considered statistically significant.

RESULTS

The influence of ovalbumine sensitization on the number of coughs and antitussive effect of potassium ion channels agonists

The cough response on the citric acid irritation is significantly changed by allergen sensitization. As it is shown in the figure 1B and 1C, number of the citric acid - induced coughs recorded on 7^{th} and 14^{th} days of sensitization was significantly higher than number of the coughs registered in unsensitized group (Fig. 1A). The cough response measured on 21^{st} day of sensitization was unambiguously attenuated in comparison with previous recordings (Fig. 1D).

Previously, it was shown that administration of K_{ATP} as well as BK_{Ca} ion channels openers resulted in significant decline of citric acid - induced coughs [14]. Despite of the increased cough response on 7th and 14th days and decreased on 21st day of ovalbumine sensitization, measurements showed persistent cough suppressive effect of selective K_{ATP} opener pinacidil. Cough suppressive effect of pinacidil was almost similar to codeine, most active antitussive agent used in clinical practice, the agonist of central opioid receptors. Unlike pinacidil, the antitussive activity of BK_{Ca} ion channels agonist, agent NS 1619 remained only on 7th day of sensitization. Whereas duration of significant cough suppression after one inhalatory applied dose of NS 1619 was 2 h in unsensitized guinea pigs and ovalbumine - sensitized animals tested on 7th day, number of citric acid - induced coughs was significantly suppressed by NS 1619 only in first measured time interval (30 min after administration of drug) on 14th and 21st day of sensitization (Fig.1).

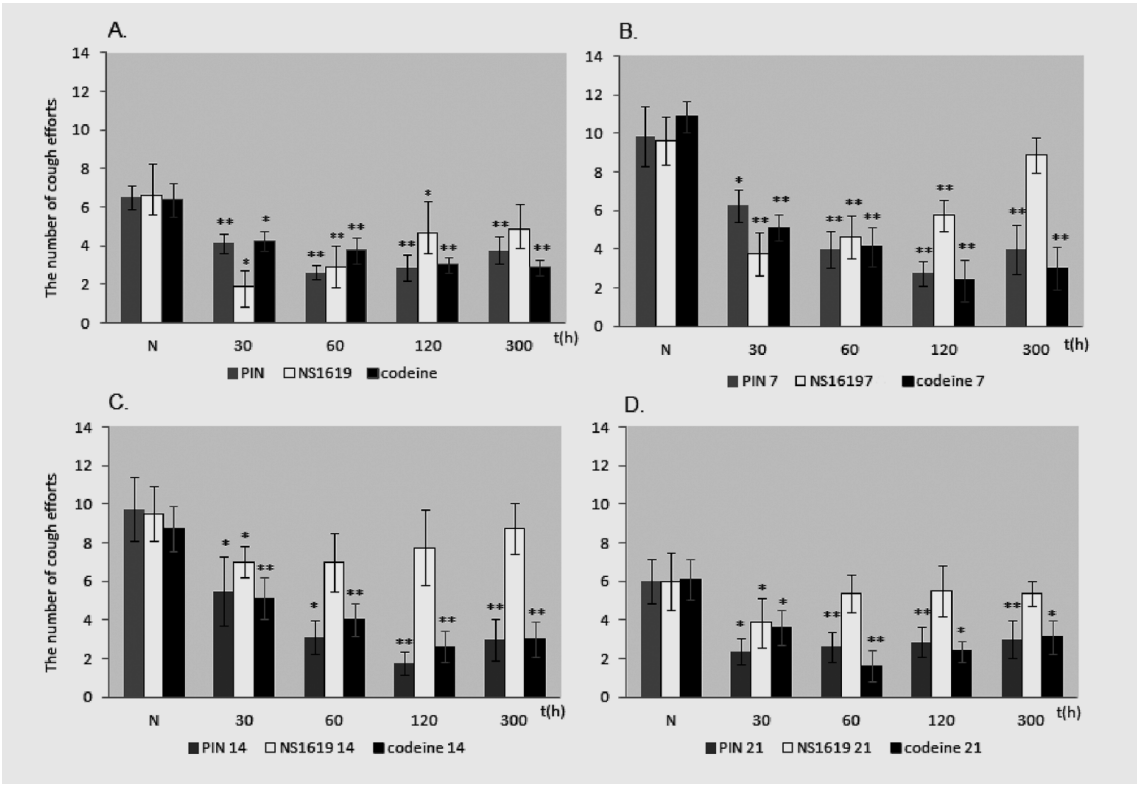

Fig. 1. The number of cough efforts (expressed as average ± S.E.M.) on administration of pinacidil (PIN) and NS 1619 compared to commercially used drug codeine registered in unsensitized guinea pigs (A) and on 7th (B), 14th (C) and 21st (D) days of sensitization procedure. N represents baseline value before any agent administration. *p < 0.05; ** p < 0.01.

The influence of ovalbumine sensitization on both, the airways smooth muscle reactivity and relaxing effect of potassium ion channels agonists (in vivo conditions)

Sensitization by ovalbumine gradually increased airways smooth muscle contraction on bronchoprovoking agent citric acid. According to figure 2, the values of specific airways resistance known as very sensitive predictor of airways smooth muscle reactivity were progressively increased during process of ovalbumine sensitization, even though significantly higher values in comparison with unsensitized controls were recorded on 21st day of exposure to allergen.

It had been demonstrated earlier that both potassium ion channels agonists decreased specific airway resistance in conscious guinea pigs [14]. Pinacidil administered to unsensitized animals suppressed values of R.V during whole experiment. Furthermore, only shortened duration of bronchodilatory effect was observed in process of sensitization by ovalbumine (Fig. 2A). In comparison by positive control agent tested on 21st day of sensitization procedure, bronchodilatory drug salbutamol, we followed more significant airway smooth muscle (ASM) relaxation on pinacidil (Fig. 2B). In unsensitized guinea pigs, NS 1619 significantly reduced R.V values compared to baseline measurement (N) in 30 and 60 min time interval. Sensitization by ovalbumine attenuated bronchodilatory activity on 7th and 14th day. Moreover, values of specific airways resistance measured on 21st day of sensitization had not shown any significant decline (Fig. 3).

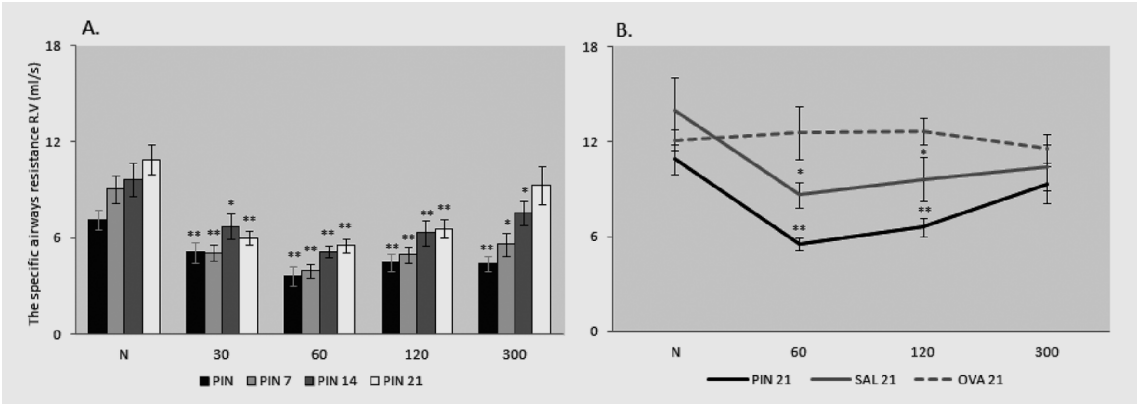

Fig. 2. The changes of specific airways resistance values (R.V) registered on pinacidil administration in unsensitized and sensitized guinea pigs (on 7th, 14th and 21st days; A). The pinacidil effect compared to salbutamol efficacy on 21st day of sensitization procedure.

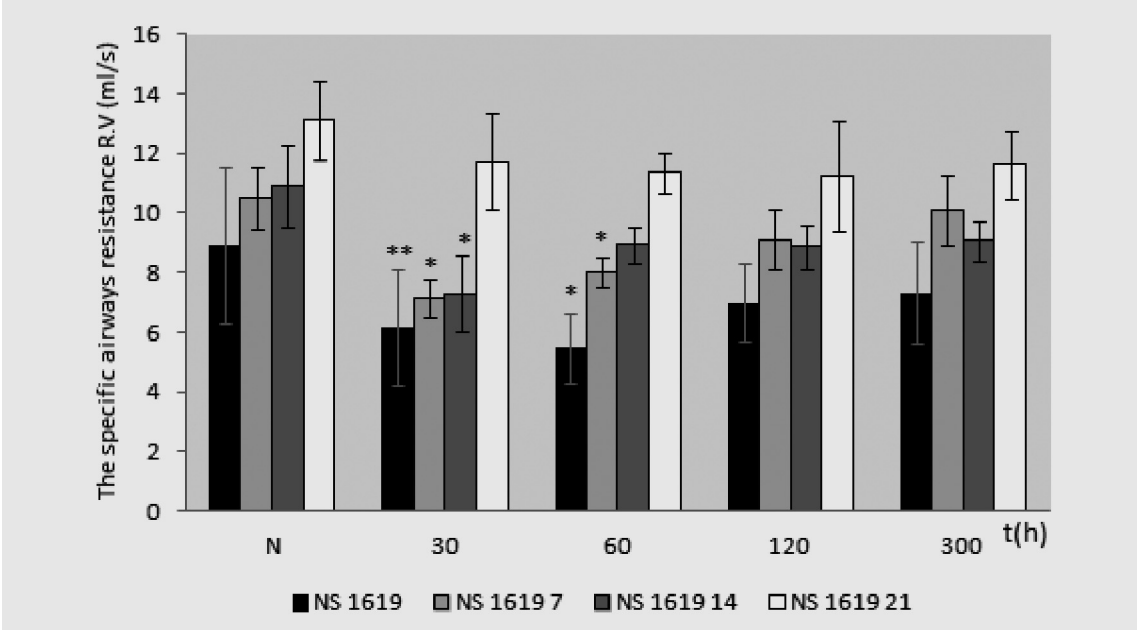

Fig. 3. The values of specific airways resistance values (R.V) calculated according to Pennock influenced by NS 1619 administration in unsensitized and sensitized guinea pigs (on 7th, 14th and 21st days).

The results of histological analysis

Histological analysis of pulmonary and tracheal specimens investigated on 7th, 14th and 21st days of ovalbumine sensitization showed increased signs of allergic inflammation. Characteristic cellular pattern of allergic inflammation in tracheal and pulmonary tissues was observed on 14th and 21st days of allergen sensitization (Tab. 1). Except for that, the next important facts were noticed after single applied doses: pinacidil and NS 1619 markedly increased mast cells infiltration both, in trachea and lungs, and increased pulmonary eosinophilia. All these findings pointed on involvement of both potassium ion channels types in development of allergic inflammation.

Table 1: The histological analysis of tracheal and pulmonary sections during the sensitization procedure. The changes of cellular infiltration induced by single dose of potassium channels agonists pinacidil and NS 1619.
OVA 7 – histological analysis of sections on 7th day of ovalbumine sensitization.
OVA 14 – histological analysis of sections on 14th day of ovalbumine sensitization.
OVA 21 – histological analysis of sections on 21st day of ovalbumine sensitization.

	TRACHEA				LUNGS			
Group	Mast cells		Eosinophils		Mast cells		Eosinophils	
	negative	positive	negative	positive	negative	positive	negative	positive
OVA 7	4	2	2	4	4	2	1	5
OVA 14	0	6	2	4	2	4	0	6
OVA 21	4	4	1	7	4	4	2	6
Pinacidil	0	8	0	8	1	7	1	7
NS1619	2	6	2	6	3	5	0	8

DISCUSSION

Asthma is a typical chronic inflammatory disease in which various resident and migrated cells and cell- derived molecules play a role. Cellular elements of allergic inflammation-eosinophils, mast cells and CD^{4+} Th^2 cells, are source of IgE and cytokines, including IL-4, IL-5, IL-8, IL-10 and IL-13. Cytokine individual role in inflammatory process, e.g. further eosinophils activation or accented angiogenesis is well documented [18, 19]. Histological investigation of tracheal and pulmonary section of ovalbumine-sensitized animals in these studies showed typical cellular pattern consisting of eosinophils and mast cells on 14th and 21st days.

In presented experiments, we used citric acid to induce both, cough reflex and bronchoconstriction. This agent is known as potent tussigen as well as bronchoconstrictor. Mechanism of citric acid action is accompanied with direct action of protons on sensory nerves through the activation or sensitization of the capsaicin-operated VR1 (vaniloid receptor 1). The subsequent release of tachykinins seems to be the major mechanism of bronchoconstriction induced by citric acid [20]. Tachykinins are also known as important mediators of inflammation. Studies using various animal models of inflammation showed that wide scale of inflammatory mediators sensitize airway afferent nerve endings, thereby lowering their threshold for activation [7]. Stimuli that are typically subthreshold for activat-

ing sensory fibres in the normal airways readily activate the same fibre subtypes in diseased airways. The inflammatory processes that occur during many airway diseases coincide with changes in the excitability of airway sensory nerves and it is associated with recruitment of afferent nerve subtypes that may not be active under normal conditions. Released tachykinins had been shown to have potent effects on the further development of allergic inflammation, specifically on ASM tone, airways secretions and bronchial circulation and on inflammatory and immune cells [21]. This suggests that airways inflammation is associated with increased responsiveness to both, tussive and bronchoprovoking stimuli. In our experiments we recorded significant increase in cough response on citric acid irritation on 7th day of ovalbumine sensitized guinea pigs although microscopic changes were not significant. Enhanced cough response was followed on 14th day, but not on 21st day of sensitization procedure. Unlike cough reflex changes, we observed gradual increase of ASM contractility expressed as raised values of R.V corresponded with week of sensitization. This supported Mokry et al. [12] pointed on enhanced cough response and ASM reactivity on 14th day of sensitization. It was declared that developing allergic reaction is represented by physical alterations in the airways, such as epithelial damage, mucosal swelling and remodelling of the airway wall, including numeral, morphological and physiological transformation of ASM cells [6]. The possible explanation of decreased cough response on 21st day of sensitization could be a damage to cough receptors by inflammatory changes. However, the assessment of such changes was out of our scope.

Potassium channels participated on the relaxation of ASM by hyperpolarizing the membrane potential and, thereby, preventing the activation of voltage-gated Ca^{2+} channels (VGCC). Electrophysiological and molecular approaches have facilitated the identification of both, Ca^{2+} activated K^+ channels (BK_{Ca}) and ATP-sensitive K^+ channels (K_{ATP}) potassium channels in ASM. Previously, we pointed on ability of K_{ATP} and BK_{Ca} openers to inhibit ASM contraction both, *in vivo* and *in vitro* condition [14]. These data are in correlation with El-Hashim et al. [22]. The allergic inflammation of the airways distinctly decreased relaxing activity of NS 1619, agonist of BK_{Ca}. Unlike NS 1619, K_{ATP} opener pinacidil maintained property to relax ASM cells according to recorded values of R.V. It could be assumed that K_{ATP} ion channels retained their defence property despite the developing allergic airways inflammation, while the role of BK_{Ca} progressively changed. Seibold et al. [23] showed that loss of function of the VGCC and BK_{Ca} has been associated with asthma severity in African Americans. Furthermore, Shepherd et al. [24] declared changes in "contractile" BK_{Ca} and "proliferative" intermediate conductance potassium channels (IK_{Ca}) expression in human ASM cells exposed to TGF-β (transforming growth factor-β), a regulatory process that is more pronounced in asthmatics. We can presume that gradual diminution and BK_{Ca} loss of cough suppressive and bronchodilatory functions on 21st day of sensitization could be the result of phenotypic modulation of ASM cells accompanied by changes in calcium–sensitive potassium channel expression. According to Bardou et al. [25], potassium ion channels could participate in complex inflammatory responses in the airways by regulating immune cell functions. As in many other cells, ion channels in immune cells mainly aim to control cytosolic Ca^{2+} signals, which in turn will regulate short and long term cellular responses [26]. Particularly relevant is the Ca^{2+} entry mechanism (the calcium release activated current, CRAC) triggered by the activation of antigen receptors, phospholipase - C/inositol trisphosphate (IP3) pathway and the subsequent depletion of endoplasmic reticulum Ca^{2+} stores. This event, named store-operated Ca^{2+} entry, relies on two recently discovered elements, the Ca^{2+} sensor STIM (stromal interaction molecule) that communicates to the plasma membrane Ca^{2+} channel Orai the need to replenish the intracellular store. CRAC current predominantly mediates inflammatory response of mast cells and eosinophils [27]. The function of many other ion channels in immune cells is principally to regulate CRAC current by modulating the driving force for calcium entry through Orai. Potassium channel activation favouring Ca^{2+} entry via channels other than VGCC while potassium channel inhibition prevents it. Inflammation could regulate potassium channel function and expres-

sion. It has been reported that inflammation stimulates K_{ATP} channels [28] and decreases BK_{Ca} open probability [29] in smooth muscle cells. Likewise Ramstorm et al. [30] also reported that proinflammatory cytokine tumour necrosis factor alpha (TNF) exerts a complex action on potassium channels by upregulating of K_{ATP} and downregulating others, including BK_{Ca}. We followed corresponding data - constant antitussive and bronchodilating activity of K_{ATP} agonist pinacidil despite lowered threshold of cough reflex and increased ASM contractility during the development of experimental allergic inflammation and gradually diminished cough suppression and relaxation of ASM on administration of BK_{Ca} agonist NS 1619. We suppose that the decreased open probability of BK_{Ca} in inflammatory conditions could be possibly the reason of loss of antitussive and bronchodilatory functions on 21st day of sensitization. Presented studies also supported Ghanshani et al. [31] experiments followed that BK_{Ca} has also been involved in mast cells IgE mediated histamine release and regulate T cell activation and proliferation. Histological investigation of tracheal and pulmonary section revealed increase of mast cells infiltration and pulmonary eosinophilia on single dose of potassium ion channels agonists, pinacidil and NS 1619. These findings pointed on involvement of both potassium ion channels in development and progression of allergic inflammation.

It can be summarized that the presented experiments confirmed the specific role of plasmalemmal potassium ion channels in the pathophysiology of disorders accompanied by allergic airways inflammation. K_{ATP} ion channels retained their defence property despite the developing allergic airways inflammation, while the role of BK_{Ca} progressively changed. However, introduction of non- selective K_{ATP} agonists in clinical practice is strongly limited by their proinflammatory effects. Accordingly, K_{ATP} of ASM represents a more rational therapeutic target for novel antiasthmatic drugs – tissue selective agonists of K_{ATP}.

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Acknowledgement: This work was supported partly by grant VEGA 1/0020/11, VEGA 1/0062/11 and Center of Experimental and Clinical Respirology II co-financed from EU sources.

Received: Oct, 26, 2011
Accepted: Nov, 30, 2011