FUNCTIONAL RELAXING ADENOSINE RECEPTORS IN ISOLATED RAT TRACHEAL SMOOTH MUSCLE

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Abstract: In the presence of 10^{-7} M DPCPX (8-Cyclopentyl-1,3-dipropylxanthine, selective antagonist of A_1 receptors) adenosine induced relaxing effects on tracheal smooth muscle pre-contracted by K^+ (40 mM). The obtained results show that the relaxing effects of adenosine on isolated rat tracheal smooth muscle were associated to adenosine A_2 and A_3 receptors activation. These results were supported by the use of 10^{-5} M 8-PT (8-phenyltheophylline or 1,3-Dimethyl-8-phenylxanthine) and 10^{-6} M VUF5574 {N-(2-Methoxyphenyl)-N′-[2-(3-pyridinyl)-4-quinazo-linyl]-urea}, antagonists for adenosine A_2 and, respectively, A_3 receptors. The future challenge is related to the deeper characterization of A_2 receptor subtype (A_{2A} or A_{2B}) involved in adenosine relaxing effects and of the balance between A_2 and A_3 receptors in rat tracheal smooth muscle preparations.

INTRODUCTION

Adenosine is an important signaling molecule that is involved in several pathophysiological conditions such as asthma, inflammation, and neurodegeneration. It is known to mediate its effects through four G-protein–coupled receptors, A_1, A_2 (with subtypes A_{2A} and A_{2B}), and A_3 (the receptors are also designated as A_1AR, A_2AR, and A_3AR, respectively) in the mammalian system. These receptors have been identified in several human tissues and, because they play a role in different pathologies, are targeted for the clinical development of a variety of drugs. Much information is available regarding the function and tissue distribution of the A_1 and A_{2A} receptors; however, because of a lack of proper agonists and antagonists to study the A_{2B} and A_3 receptors, the exact role of the latter two receptors in mammalian physiology is not clear. In addition to its possible involvement in the development of rheumatoid arthritis and brain, lung, and cardiac ischemia, the A_3 receptor was shown to be overexpressed and had a proliferative effect in certain cancer tissues and cell lines. Although this receptor is known to be involved in a number of diseases, little is known about its expression and distribution in the different regions and tissue of the body. In an attempt to study the ligand binding properties of the A_3 receptor, investigators developed antagonistic pyridine analogs that showed high affinity and specificity for the receptor (Chopra, 2007).

Recently, a central role for the A_{2B} adenosine receptor in a variety of cardiovascular functions including inflammation, erectile function, coronary artery dilation, asthma and cardioprotection has been demonstrated. Despite this evidence, the low-affinity A_{2B} adenosine receptor is still poorly understood. This receptor appears to be very promiscuous in its coupling. In most tissues, it couples to G_s much like its cousin, the A_{2A} adenosine receptor, but in mast cells and now, most recently, in cardiac fibroblasts, the A_{2B} receptor also couples to G_q. Because of its low affinity, this receptor was originally thought unlikely to play any important physiological role. But the sensitivity of A_{2B} adenosine receptors can be greatly increased by interaction with protein kinase C (PKC) making this receptor, under various conditions, both an activator and a target of PKC. It has been recently documented a third coupling involving G_i. This plasticity and versatility of A_{2B} adenosine receptors position them as potential triggers of signaling in multiple signaling cascades in many physiological responses, making this a most interesting receptor indeed (Cohen *et al*., 2010).

Mast cells are key players in mediating and amplifying allergic and inflammatory reactions. Some time ago there was identified the G-protein, G_{i3}, as the cellular target of receptor mimetic basic secretagogues that activate mast cell independently of IgE. Recently, it was demonstrated that G_{i3} is the cellular target of the adenosine A_3 receptor (A_3R), a G-protein coupled receptor involved in inflammation and the pathophysiology of asthma. By using a cell permeable peptide comprising the C-terminal end of $G_{\alpha i3}$ fused to an importation sequence (ALL1) as a selective inhibitor of G_{i3} signaling, it was shown that by coupling to G_{i3}, the A_3R stimulates multiple signaling pathways in human mast cells, leading to upregulation of cytokines, chemokines, and growth factors. It was further shown that after contact with activated T cell membranes, endogenous adenosine binds to and activates the A_3R, resulting in G_{i3}-mediated signaling. Specifically, the majority of ERK1/2 signaling initiated by contact with activated T cell membranes is mediated by G_{i3}, giving rise to ALL1-inhibitable cellular responses. These results unveil the physiological G-protein coupled receptor that

couples to G_{i3} and establish the important role played by this G-protein in inflammatory conditions that involve adenosine-activated mast cells (Baram *et al*., 2010).

Thus, the aim of the present study was to characterize the functional relaxing adenosine receptors in isolated rat tracheal smooth muscle preparations.

MATERIALS AND METHODS

To obtain the tracheal rings were used adult, male, Wistar rats (Băneasa source), weighing 180-200 g, bred in identical laboratory conditions. Briefly, thoracic trachea was quickly removed, dissected of connective tissue and cut into rings 2 mm long. Epithelium of the rings was removed by light rubbing with a smooth surface (rings of trachea were randomly selected to undergo histological examination to confirm the successful removal of the epithelium). The rings were then fitted with two hooks in organ bath and mechanical activity recorded using isometric force transducers and writers with pen potentiometric (writer OH-827, Radelkis, Budapest, Hungary, and K 201, Karl Zeiss Jena, Germany) or with a data acquisition program online on computer (Data 4U, Optimex, Iaşi, Romania) using a data acquisition card, DAS-1601. Organ baths of 2 ml each contained serum Krebs-Henseleit (pH=7.4) with the following composition (mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgSO_4$, 1.6; KH_2O_4, 1.2; $NaHCO_3$, 25; glucose 5.5; indomethacin (Sigma) 10^{-5} M. The serum from the organ bath was maintained at 37^{o}C and continuously bubbled with a gas mixture consisting of 95% O_2 and 5% CO_2. Resting tension was maintained at 2.5 g and the preparations were left to balance for 2 hours before beginning the experiment. During that time, serum was changed every 15 minutes.

Values of tonic contractions of tracheal smooth muscle induced by 40 mM K^+ (depolarization) were taken as reference (100%). Contractions were considered reproducible if the difference between the maximum of two consecutive contractions was less than 10%. Preparations without reproducible contractions were discarded. Adenosine, in doses ranging between 10^{-10} and 10^{-3} M, was cumulatively administered in the plateau (20 minutes after reaching and maintaining top level) of depolarization (40 mM K^+)-induced contractions in the isolated rings of rat trachea. The antagonists of adenosine receptors were added 15 minutes before adenosine administrations.

The statistical significance of test results was highlighted using the Variance One-Way ANOVA (possibly complemented by Bonferroni test) and Student t-test and the results were expressed as mean ± S.E.M (n = 6). Value of $p<0.05$ was always considered as being statistically significant.

Present studies were carried out in accordance with the "Guide for Care and Use of Animal Experiments" of U.S. National Institutes of Health (NIH), published by the U.S. National Academy in 1996, and approved by the Ethics Committee of the University of Medicine and Pharmacy "Gr T. Popa" Iaşi.

Drugs used. DPCPX (8-Cyclopentyl-1,3-dipropylxanthine), 8-PT (8-phenyltheophylline or 1,3-Dimethyl-8-phenylxanthine), VUF5574 {N-(2-Methoxyphenyl)-N′-[2-(3-pyridinyl)-4-quinazolinyl]-urea}, and dilazep dihydrochloride {N,N′-bis(3-[3,4,5-Trimethoxybenzoyloxy]propyl)homopiperazine dihydrochloride} were all obtained from *Sigma-Aldrich Co*. All other compounds used were of analytical grade.

RESULTS AND DISCUSSIONS

In the presence of 10^{-7} M DPCPX (8-Cyclopentyl-1,3-dipropylxanthine) and 10^{-5} M 8-PT (8-phenyltheophylline or 1,3-Dimethyl-8-phenylxanthine), with full antagonistic effects against A_1 and A_2 receptors types (figure no. 1), adenosine induced relaxing effects on tracheal smooth muscle contraction induced by K^+ (40 mM). These relaxing effects of adenosine were statistically significant. By blocking the adenosine receptor type A_2, relaxing effects of adenosine in this case remain to be mediated by activating only the A_3 adenosine receptor or intracellular effects, unspecific.

In the presence of 10^{-7} M DPCPX, 10^{-5} M 8-PT and 10^{-6} M VUF5574 {N-(2-Methoxyphenyl)-N′-[2-(3-pyridinyl)-4-quinazolinyl]-urea} (the latter being a potent, selective and competitive antagonist at the A_3 receptor level) adenosine does not induce any significant effect of relaxation or contraction when it is administered in the plateau of tracheal smooth muscle contraction induced by K^+ (40 mM) (figure no. 2). These effects demonstrate that the relaxing effects of adenosine are due to the activation of the receptors of type A_2 and A_3.

Furthermore, dilazep (10^{-5} M), a specific blocker of nucleoside transporters at the membrane level, did not statistically significant influenced the relaxing effects of adenosine on tracheal smooth muscle contraction induced by K^+ (40 mM), as compared with adenosine

administration without any pretreatment (data not shown). We thus excluded the mechanisms of action of adenosine receptors of type A_1, A_2 and A_3, adenosine intracellular intervention, but also possible intracellular actions (related to surface adenosine receptors).

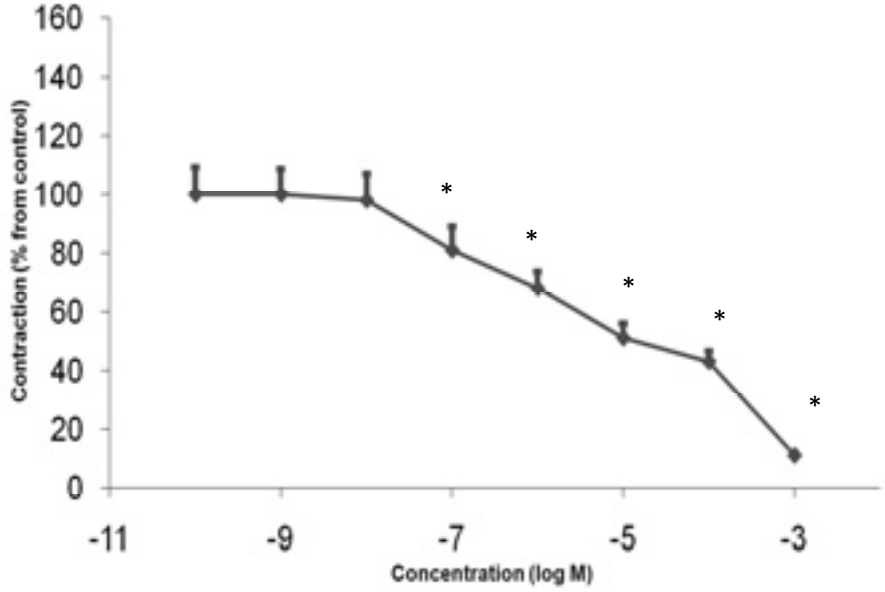

Figure no. 1: Effects of adenosine on tracheal smooth muscle contraction induced by K^+ (40 mM) in the presence of 10^{-7} M DPCPX and 10^{-5} M 8-PT, with full antagonistic effects against A_1 and A_2 receptor types (mean ± S.E.M, n=6). *p <0.05 statistically significant as compared to control (100%).

Although theophylline is the most used medication for asthma maintenance in the world, its mechanisms of action are not fully understood. In addition, theophylline presents a high toxicity potential. Therefore, the discovery of new methylxanthine derivatives with potent effects in treating asthma and reduced toxic effects is furthermore dictated by medical practice. Xanthine derivatives inability to antagonize some effects of adenosine, important mediator involved in the pathogenesis of asthma at respiratory tract level, requires rigorous characterization of adenosine receptor subtypes met in the airways (Dellabianca *et al.*, 2009).

Theophylline has an anti-inflammatory action that may account for its clinical effectiveness in the reduction of inflammatory cells in the airways. Dendritic cells (DCs) are professional antigen-presenting cells, capable of priming naïve T cells, and play key roles in the activation of immune responses in asthma. Some studies aimed to investigate the effects of theophylline on human monocyte differentiation into DCs and whether this involved antagonism

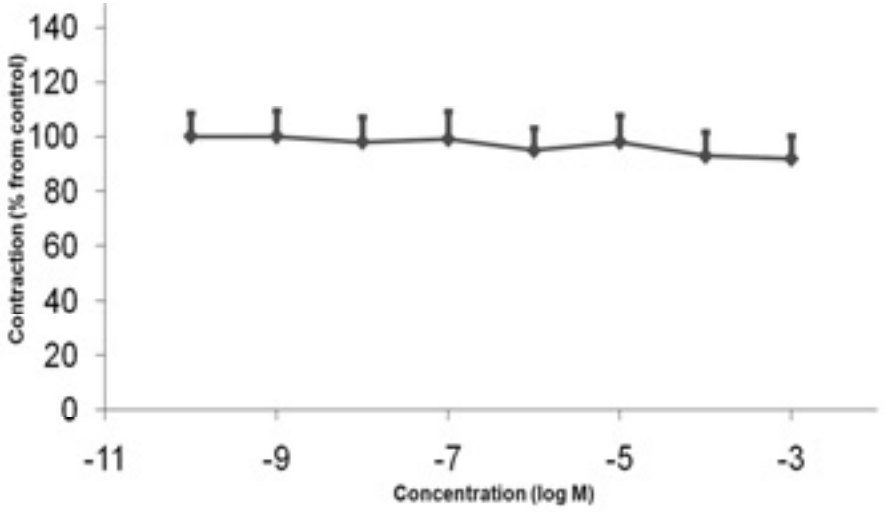

Figure no. 2: Effects of adenosine on tracheal smooth muscle contraction induced by K^+ (40 mM) in the presence of 10^{-7} M DPCPX, 10^{-5} M 8-PT and 10^{-6} M VUF5574, with full antagonistic effects against A_1, A_2 and A_3 receptor types (mean ± S.E.M, n=6). *p <0.05 statistically significant as compared to control (100%).

of adenosine receptors. Peripheral human blood monocytes were cultured in the presence of granulocyte/macrophage-colony stimulating factor and IL-4 to induce DC differentiation. The cells were incubated with theophylline, KF17837 (a selective A_{2a} receptor antagonist) and enprofylline (A_{2b} receptor antagonist) and co-incubated with selective adenosine A_1 and A_{2a} receptor agonists, a phosphodiesterase inhibitor (rolipram) and adenosine deaminase (ADA) to determine their effects on DC differentiation. In addition, depletion of adenosine receptors by small interfering RNA (siRNA) was also examined. Monocytes differentiated into myeloid DCs in the culture system. The number of DCs was remarkably reduced by 60-70% when theophylline was administered at a therapeutic concentration. This effect was concentration-dependently exacerbated, was partly mediated by cellular apoptosis and was effectively reversed by the addition of the A_1 agonists [2-chloro-N(6)-cyclopentyladenosine, N(6)-cyclohexyladenosine, and N-ethylcarboxamidoadenosine (NECA)] or the A_{2a} agonist (CGS-21680, NECA). The depletion of the adenosine A_1 receptor by siRNA and addition of ADA remarkably reduced DC differentiation. Meanwhile, both enprofylline and rolipram had little effect. These findings suggest that the adenosine A_1 (and possibly coordinated with A_{2a}) receptors contribute to DC differentiation and survival. These findings provide further evidence that theophylline has an anti-inflammatory action in bronchial asthma (Yasui *et al*., 2009).

Growing evidence emphasizes that the purine nucleoside adenosine plays an active role as local regulator in airway inflammation and pulmonary diseases. The notion that increased adenosine concentrations are associated with lung inflammation indicates the importance of this signaling pathway, which involves the activation of a family of cell surface G-protein coupled receptor subtypes named as A_1, A_{2A}, A_{2B} and A_3. Recently, important progress has been made to better clarify the role of these receptors in a variety of inflammatory airway disorders including

asthma. As a consequence, new molecules with high affinity and high selectivity for the human adenosine receptors subtypes designed to control the airway inflammatory component of asthma have been launched and are currently tested in clinical trials as anti-asthma treatments. With the availability of these molecules for testing in humans, the role of adenosine receptors in asthma can now be validated (Caruso *et al*., 2009).

A selective, high-affinity A_{2B} adenosine receptor antagonist will be useful as a pharmacological tool to help determine the role of the A_{2B}AR in inflammatory and angiogenic diseases (Kalla *et al*., 2009). Pharmacologic evidence suggests that activation of A_{2B} adenosine receptors results in proinflammatory effects relevant to the progression of asthma.

The study of the A_3 adenosine receptor represents a rapidly growing and intense area of research in the adenosine field. Through studies utilizing selective A_3AR agonists and antagonists, or A_3AR knockout mice, it is now clear that this receptor plays a critical role in the modulation of ischemic diseases as well as in inflammatory and autoimmune pathologies. The discussions principally address the use of A_3AR agonists and antagonists in the treatment of brain and heart ischemia, asthma, sepsis and glaucoma (Borea *et al*., 2009).

Adenosine plays a significant role in regulation of airways tone and reactivity by multiple and incompletely known mechanisms, including the release of endogenously active peptides from mast cells via activation of the A_3 receptors. Our previous results suggested that releasing of enzymes from activated mast cells could activate the intrapulmonary renin angiotensin system (RAS). Thus, we investigated the involvement of angiotensin II (Ang II) in adenosine-induced bronchoconstriction in an experimental model of allergic asthma. In bronchial rings from ovalbumin (OVA) sensitized rats, after *in vitro* challenge, adenosine induced small contractile effects which became significant after indomethacin pre-treatment. On the other hand, adenosine pre-treatment amplified bronchoconstriction induced by the OVA challenge and reduced bronchial relaxation of acetylcholine pre-contracted bronchial rings by cumulative doses of terbutaline. All these effects were significantly lower in rats treated with losartan (a blocker of specific angiotensin type 1 receptors, AT_1) in the last two weeks of sensitization protocol (50 mg/kg/day). Our data confirmed that adenosine-induced bronchial hyperreactivity could be partially a result of RAS activation in abnormal conditions as antigen sensitization and challenge (Cojocaru *et al*., 2009).

CONCLUSIONS

In the presence of 10^{-7} M DPCPX (8-Cyclopentyl-1,3-dipropylxanthine, selective antagonist of A_1 receptors) adenosine induced relaxing effects on tracheal smooth muscle pre-contracted by K^+ (40 mM). The obtained results show that the relaxing effects of adenosine on isolated rat tracheal smooth muscle were associated to adenosine A_2 and A_3 receptors activation. These results were supported by the use of 10^{-5} M 8-PT (8-phenyltheophylline or 1,3-Dimethyl-8-phenylxanthine) and 10^{-6} M VUF5574 {N-(2-Methoxyphenyl)-N′-[2-(3-pyridinyl)-4-quinazolinyl]-urea}, antagonists for adenosine A_2 and, respectively, A_3 receptors.

The future challenge is related to the deeper characterization of A_2 receptor subtype (A_{2A} or A_{2B}) involved in adenosine relaxing effects and of the balance between A_2 and A_3 receptors in rat tracheal smooth muscle preparations.

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