FUNCTIONAL RELAXING ADENOSINE RECEPTORS IN ISOLATED RAT TRACHEAL SMOOTH MUSCLE

DUMITRU IŞFAN¹, BOGDAN CRISTIAN PETRESCU², LILIANA CHELARU³, ELENA COJOCARU², ROXANA IRINA IANCU¹, BOGDAN GURZU², IRINA LUCIANA DUMITRIU², SIMONA MIHAELA SLĂTINEANU², GHEORGHE PETRESCU³, MARCEL COSTULEANU¹*

Keywords: adenosine, receptor, rat, tracheal smooth muscle.

Abstract: In the presence of 10^{-7} M DPCPX (8-Cyclopentyl-1,3-dipropylxanthine, selective antagonist of A1 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 A2 and A3 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 A2 and, respectively, A3 receptors. The future challenge is related to the deeper characterization of A2 receptor subtype (A2A or A2B) involved in adenosine relaxing effects and of the balance between A2 and A3 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, A₁, A₂ (with subtypes A₂A and A₂B), and A₃ (the receptors are also designated as A₁AR, A₂AR, and A₃AR, 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 A₁ and A₂A receptors; however, because of a lack of proper agonists and antagonists to study the A₂B and A₃ 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 A₃ 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 A₃ receptor, investigators developed antagonistic pyridine analogs that showed high affinity and specificity for the receptor (Chopra, 2007).

Recently, a central role for the A₂B 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 A₂B adenosine receptor is still poorly understood. This receptor appears to be very promiscuous in its coupling. In most tissues, it couples to Gᵢ, much like its cousin, the A₂A adenosine receptor, but in mast cells and now, most recently, in cardiac fibroblasts, the A₂B receptor also couples to Gᵢ. Because of its low affinity, this receptor was originally thought unlikely to play any important physiological role. But the sensitivity of A₂B 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 Gᵢ. This plasticity and versatility of A₂B 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, Gₐ, as the cellular target of receptor mimetic basic secretagogues that activate mast cell independently of IgE. Recently, it was demonstrated that Gₐ is the cellular target of the adenosine A₂ receptor (A₂R), 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 Gₐ fused to an importation sequence (ALL1) as a selective inhibitor of Gₐ signaling, it was shown that by coupling to Gₐ, the A₂R 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 A₂R, resulting in Gₐ-mediated signaling. Specifically, the majority of ERK1/2 signaling initiated by contact with activated T cell membranes is mediated by Gₐ, giving rise to ALL1-inhibitable cellular responses. These results unveil the physiological G-protein coupled receptor that
couples to $G_i^3$ 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 (Bâneasa 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, Iași, 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$_2$PO$_4$, 1.2; NaHCO$_3$, 25; glucose 5.5; indomethacin (Sigma) $10^{-5}$ M. The serum from the organ bath was maintained at 37°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" Iași.

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 significantly influence 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 A₁, A₂ and A₃, 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 A₁ and A₂ 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.
Dumitru Işfan et al. – Functional relaxing adenosine receptors in isolated rat tracheal smooth muscle

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 A₁, A₂ and A₃ receptor types (mean ± S.E.M, n=6). *p <0.05 statistically significant as compared to control (100%).

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 A₁, A₂A, A₂B and A₃. 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 A2B adenosine receptor antagonist will be useful as a pharmacological tool to help determine the role of the A2BAR in inflammatory and angiogenic diseases (Kalla et al., 2009). Pharmacologic evidence suggests that activation of A2B adenosine receptors results in proinflammatory effects relevant to the progression of asthma.

The study of the A3 adenosine receptor represents a rapidly growing and intense area of research in the adenosine field. Through studies utilizing selective A3AR agonists and antagonists, or A3AR 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 A3AR 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 A3 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, AT1) 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 A1 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 A2 and A3 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-quinoxalinyl]-urea}, antagonists for adenosine A2 and, respectively, A3 receptors.

The future challenge is related to the deeper characterization of A2 receptor subtype (A2A or A2B) involved in adenosine relaxing effects and of the balance between A2 and A3 receptors in rat tracheal smooth muscle preparations.

ACKNOWLEDGEMENTS

This study was partially supported by C.N.C.S.I.S. grants IDEI-PCE-1273/2007 (Project director Lect. Dr. Bogdan Gurzu) and IDEI-PCE-2670/2008 (Project director Prof. Dr. Gheorghe Petrescu).
REFERENCES


1. Department of General and Oro-Maxillo-Facial Pathology, Faculty of Dental Medicine, 2. Department of Physiology, 3. Department of Histology, Faculty of Medicine, University of Medicine and Pharmacy “Gr. T. Popa” Iași, 16, University Str., 700115, Iași, Romania.

*Author for correspondence: Prof. Dr. Marcel Costuleanu, Department of General and Oro-Maxillo-Facial Pathology, Faculty of Dental Medicine, University of Medicine and Pharmacy “Gr. T. Popa” Iași, 16, University Str., 700115, Iași, Romania; tel. +40-745-589050; fax: +40-232-211820; e-mail: mcostuleanu@yahoo.com.
Adenosine receptor antagonist potentiates the fenoterol-induced relaxation response in rat tracheal rings precontracted with CCh EC30. (A) Three cumulative concentration-response curves to fenoterol were constructed at 60-minute intervals (n = 4). (B) Control cumulative concentration-response curves to fenoterol from different tissues (n = 4â€”5). Although the cAMP efflux from isolated perfused rat lung has been mentioned by Barnard et al. (1994) and recently described in human airway epithelial cells (Huff et al., 2017), its biologic function in the airway smooth muscle has never been experimentally addressed. The current study provides reliable evidence for a functional extracellular cAMP-adenosine pathway in airway smooth muscle. Relaxation of tracheal smooth muscle independent on functional epithelium cells induced by lidocaine, bupivacaine and isomers in rats. Roberto Queiroga Lautner, gisele Zapata-sudo, Roberto T Sudo. Chemistry, Medicine. European journal of pharmacology. 2009. VIEW 3 EXCERPTS. Vasoconstriction and analgesic efficacy of locally infiltrated levobupivacaine for nasal surgery. Cellular mechanisms of airway smooth muscle relaxant effects of anesthetic agents. Michiaki Yamakage, Akiyoshi Namiki. Medicine. Journal of Anesthesia. 2003. VIEW 2 EXCERPTS. Effects of lidocaine on ratâ€™s isolated tracheal smooth muscle. Chuan-Hsiang Kao, Yueng-Hsiang Chu, Hsing-Won Wang. Medicine. European Archives of Oto-Rhino-Laryngology. 2009. VIEW 4 EXCERPTS. Tracheal tensometry also provides a functional assay to study a variety of second messenger signaling pathways that affect contraction of smooth muscle. Contraction in trachea is primarily mediated by parasympathetic, cholinergic nerves that release acetylcholine onto ASM (Figure 1). The major ASM acetylcholine receptors are muscarinic M2 and M3 which are G i/o and Gq coupled receptors, respectively 3,4,5. M3 receptors evoke contraction by coupling to Gq to activate phospholipase C, increase IP3 production and IP3-mediated. Shown is a cholinergic axon terminal innervating a tracheal smooth muscle cell.