SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

The role of the adenosine in the functional responses of the bronchial smooth muscle and the mononuclear cells

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1. INTRODUCTION

One of the most important aim of the experimental drug development is to find newer and newer molecules targeting special diseases' pathways to improve their therapy. In the recent decades, the adenosine receptors and their activity modification has been such a target, because it has an ubiqitous receptorial structure located on any cell membrane, being involved in so many functional and morphological disorders. Almost a century ago, when Drury and Szent-Györgyi (1929) had published their seminal work, describing in detail the cardiovascular effects of the adenosine, a cornerstone in the adenosine receptors research since that date. Many decades later new theories have appeared, like that of Robert Berne's one (1963) dealing with the adenosine induced regulation of the coronary flow. Geoffrey Burnstock was the first who created his own and actually largely accepted theory about the purine and purinergic receptors (Burnstock, 1976). The research of adenosine receptors involvement in the cardiovascular physiology and pathology has been completed by a valuable discovery on the role of adenosine in the pulmonary diseases by Barnes et al (1998). More and more evidence has been obtained to prove the important role of the adenosine mechanisms in the function of cellular elements like epithelial cells, bronchial smooth muscle cells, mononuclear cells and so on. This is a fact that modifying the adenosine receptors by multiple agonists and antagonists could induce well documented pharmacological improvements in the bronchial asthma and COPD therapy. We encounter more than 210.000 publications dealing with the adenosine or its receptors, 25.000 references with the cardiovascular effect of the adenosine and 4.700 issues with its effect on the respiratory system. All these data show the physiological, pathophysiological and clinical importance of this ubiquitous purine nucleoside. It is doubtless that any functional or morphological damage of adenosine receptors could induce serious consequences in the involved organ or system functions. Pharmacological research face huge perspectives to produce new drugs based on the adenosine receptors mechanisms involvement to cure more cardiovascular and respiratory diseases. Looking on the cardiovascular line we encounter more than 70 already finished or on going studies based on the use of different adenosine molecules (like AMISTAD I, AMISTAD II, ADVICE, ADSPECT, ADELINE, PROTECT, TEMPEST). Similar studies are also available in the asthma bronchiale or COPD therapy. The number of ongoing studies are about 40. The main purpose of my study was to provide data about the importance of the function of the adenosine receptors, about their plasticity in the bronchial system and their role in the polymorphonuclear cells involved in the inflammation.

After the recognition of the main physiological roles of the purine metabolites, Burnstock was the first to classify receptors of adenosine and its derivatives into P_1 and P_2 purinergic receptors (Burnstock, 1980). Adenosine activates the P_1 receptors, while the ATP and ADP act on the P_2 receptors. Not long ago four adenosine receptors subtypes have been defined based on structural, functional and pharmacological point of view, like A_1, A_{2A}, A_{2B} and A_3 receptors. To characterize the adenosine receptor subtypes there have been many aspects taken in view like the special pharmacological profile (the rank of order of potency of the different agonists and antagonists), their capacity binding to G-proteins, or the signal transduction mechanisms during the activation of the adenosine receptors. The International Union of Pharmacology (IUPHAR) take account of these approaches, being prescribed by the Committee on Receptor Nomenclature and Drug Classification. (Fredholm et al, 2001). All subtypes of the adenosine receptors have already been cloned. From chemical point of view these receptors are asparagine linked glycoproteins, in which the palmitoylation suitable site is in the next proximity of the terminal carboxyl molecule (excepting the A_{2A} receptor) (Fredholm and Jacobson, 2001). The receptors are parts of the cell membranes linked to different G proteins structures. As IUPHAR recently admitted based on the phylogenetic analyses, the adenosine receptors linked to G proteins belong to the I. class of the „rhodopsin subtypes" (Poulsen and Quinn, 1998).

2. AIM OF THE STUDY

The adenosine receptors of the lung play an important role to orchestrate the local physiological functions and are involved in the pathomechanism of the bronchial asthma, COPD and many other, mostly inflammatory disorders. The adenosine receptors like many other G-proteins linked receptors are strongly exposed to the receptorial regulations. A profound analysis of the adenosine receptors of the mononuclear cells involved in the regulation of the inflammatory processes of the bronchial asthma and COPD seems to be obvious.

In our experiments we tried to get answers to the following questions:

1. In what extent does the chronic caffeine intake influence the adenosine receptors reactivity in the isolated tracheal structures during the treatment? What should be the role of the epithelium in the observed modifications? (in the asthmatic process there is a damage, a denudation of the epithelium)

2. We examined the pharmacological properties of the xanthine-resistant, intracellulary located P-sites. Meanwhile we tried to clarify the xanthine-resistant A_3 receptors activation effect on the adenosine induced smooth muscle activity.
3. What is the role of the different adenosine receptor subtypes activation on the IL-1β release from the peripheral mononuclear cells?
4. What is the role of the different adenosine receptor subtypes on the arachidonic acid and its metabolites released from the peripheral monocytes?

3. MATERIALS AND METHODS

3.1. Experimental animals and treatment

Male Hartley guinea pigs weighting 440-590 g were assigned randomly to the control and caffeine-treatment groups. The animals in both groups were housed in individual cages, implementing a daily 12 hours cyclic day and night condition, with free access to food (standard guinea pig lucerne pellete) and water ad libitum. The caffeine–treated animals were provided ad libitum with drinking water that contained caffeine (600mg/l) and sucrose (20g/l), the latter added to offset the bitter taste of the caffeine. The control guinea pigs received only sucrose (20g/l) in their drinking water, similarly ad libitum. For 24 h before sacrificing, the animals in the treated group also received only sucrose instead of caffeine+sucrose, for wash-out. In a separate groups of guinea pigs (n=38), the serum caffeine levels were monitored at different times during caffeine treatment.

3.2. Determination of caffeine in serum

Serum levels of caffeine were determined via HPLC as follows: 0.5 ml serum was taken, 2 ml of methanol was added, and the mixture was incubated at room temperature for 30 min. Following this, the sample was centrifugated at 100 x g for 10 min. The supernatant was removed and dried under a continuous nitrogen flow at room temperature. The dry residue was dissolved in 0.5 ml methanol, and the solution was dissolved with 0.5 ml redistilled water, filtered, and injected into CM 4000 HPLC system (Milton Roy, Riviera Beach, FL, USA) equipped with a UV 440 detector (Waters, Milford, MA, USA) at 254 nm. Samples were chromatographed on a Spherisorb C-18 (ODS2) column (5 μM,

4,6 mm x 250mm; Waters), using a mobile phase of methanol: water (50:50). The flow rate was 1 ml/min, the retention time was 4,5 min, and the temperature was 35^{o} C.

3.3. Tissue preparation

The guinea pigs were anesthetized with sodium pentobarbitone (50mg/kg, i.p.). The chest was then opened and the trachea was excised, cleaned of adhering fat and connective tissue, and opened by cutting longitudinally through the cartilage rings diametrically opposite the smooth muscle. On one of these strips, the epithelium was removed by gently rubbing the luminal surface (over both the smooth muscle and the cartilage areas) with a cotton-wrapped metal rod. Small segments of trachea were mounted into 10-ml vertical organ bath (TSZ-04; Experimetria, Budapest, Hungary) containing Krebs solution (36^{o}C) with the following composition: 118 mM NaCl, 4.7 mMKCl, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4 ,1.2 mM $MgCl_2$, 24.9 mM $NaHCO_3$ and 11.5 mM glucose, pH 7.4 gassed with a mixture of 95% O_2 and 5% CO_2. All experiments were performed in the presence of 10 μM dipyridamole to inhibit membrane purine transport and to avoid the intracellular effects of adenosine. Tissues were mounted under an initial resting tension of 10 mM and left to equilibrate for a period of 90 min before the experimental protocol was started. Tension changes were recorded by using isometric force transducers (SG-01D, Experimetria) connected to a potentiometric recorder (SP-K2V; Riken Denshi, Tokyo).

Experiments were also carried out in a coaxial bioassay system with a slight modification of the methods described by Fernandes et al. (1989), Eglen et al (1991) and Hay et al (1992). This bioassay involved an epithelium-intact or denuded guinea-pig tracheal tube (donor tissue) and an epithelium-denuded tracheal strip (sensor strip). The tracheal tube was cut longitudinally and kept in a half-open state, using a special, polyethylene mini self-retaining retractor to avoid possible mechanical friction between the tracheal tube and the assay preparation and also to allow the intensive diffusion of various substances into the lumen of the tube.

3.4. Experimental protocol

In all tracheal strip experiments, a concentration-response curve was recorded with increasing concentrations (0.1-10 μM) of methacholine. The strips were washed every 10 min for 40 min, and then they were contracted with methacholine (0.3 μM, a concentration corresponding to the approximate half-maximum response) and left until steady tone had developed. In this

precontracted condition cumulative concentration-response curves were then obtained by adding adenosine and its analogues. After a new wash-out period the tracheal strips were incubated for 50 min with the specific substances, then they were again contracted with methacholine. In this state we recorded the adenosine concentration-response curve. After this second adenosine concentration-response curve we added papaverine and we registreted the maximal relaxation rate. The effect of the adenosine and of its special analogues induced relaxation has been related in % to the maximal relaxation effect of the 100 μM of papaverine.

3.5. Preparation of human peripheral blood mononuclear cells (PBMC)

Mononuclear cell suspensions (on average 88-95% lymphocytes, 5-12% monocytes) were prapared from the peripheral blood samples of healthy human volunteers according to Boyum's method (1968). The distribution of the various subsets was detected by flow cytometry. The mean percentage of mononuclear cells was the following CD3+:69,4%, CD19+:13,5%, CD56+:9,8% and CD14+:8,3% (Coulter EPICS XL, flow cytometer, USA).

3.6. Stimulation of PBMC

Cells were cultured (5 x 10^5cells/well) in RPMI-1640 medium completed with 10% fetal calf serum of low LPS content (plus with 80 μg/ml gentamycin and 2 mM glutamine) in a humidified atmosphere with 5% CO_2 at 37^{o}C. All the samples were used in triplicates. The stimulation was achieved by the addition of 50 ng/ml of PMA and 5 μM of calcium ionophore (A23187) for 4 h.

3.7. Measurement of IL-1β release in the stimulated suspensions of PBMC

The concentrations of IL-1β were determined in the cell-free supernatants by an ELISA kit (Amersham, G. B.) and the results were given in pg/ml.

3.8. Treatment of PBMC by adenosine and receptors subtype-specific selective adenosine analogues

Various concentrations of adenosine and the selective adenosine receptor agonists were added to the stimulated suspensions of PBMC for 15 min before the stimulation by PMA+calcium ionophore. The adenosine A_{2B} receptor stimulation was achieved by using various concentrations of NECA (non-specific agonist for A_1 and A_2 receptors) in the presence of 0.3 μM concentrations of DPCPX (inhibitor of A_1 receptors) and ZM 243185 (inhibitor of A_{2A} receptors) added 15 min prior to NECA. In the experiments using specific inhibitor for A_1 receptors (DPCPX), and for A_{2A} receptors (ZM 243185) 0.1 μM concentrations of the drug were used. In the case of A_3 receptors, the antagonist MRS 1191 (10 nM) was added to the cells 15 min before the application of the selective agonist agent CPA (10 nM). The two other agonist molecules, CGS 21680 and IB-MECA, were also applied in the concentrations of 10 nM. The concentrations of the antagonists were chosen based on results from preliminary measurements. In the non-stimulated samples, aliquots of culture medium were used which contained the same amounts of dimethylsulfoxide (DMSO) as the solutions of PMA and calcium ionophore. The amounts of IL-1β released from the cells activated by the „plastic surface" (cell cultivation plates) but not stimulated by PMA and Ca^{2+} ionophore were taken into correction in every case. (The average concentration of IL-1β produced by surface activation was 12.4±2.5 pg/ml) It is noted that DMSO as solvent, in the concentration applied did not have any significant effect on IL-1β production.

3.9. Measurement of cell viability

The viability of cells treated with various drugs before the measurement of their IL-1β release was determined by trypan blue exclusion test. Only those experimental samples were evaluated in which the viability of cells was higher than 90%

3.10. Measurement of release of arachidonic acid metabolites from the non-stimulated and stimulated suspensions of PBMC

3.10.1 Measurement of AAM release in non-stimulated cells.

Cells were distributed into tissue culture plates of 24 wells (GIBCO, USA) ($0.5x10^6$ cells/well) and were incubated in RPMI medium (GIBCO, completed with 1% fetal calf serum, antibiotics and glutamine) at 37°C in sterile 5% CO_2 milieu (ASSAB, Sweden). 0.1μ Ci of ^3H arachidonate (^3H-AA) was added to the $0.5x10^6$ cells for 20 h. After labeling, the cells were washed three times in phosphate-buffered saline (PBS) and were further incubated without any stimuli for 4 h. These cells were regarded as the „non-stimulated" controls, and their cell-free supernatants were used for the ^3H-AA measurement. Since total radioactivity was measured, it could not be determined unless AA was released into the supernatant or a contamination of AA and its metabolites, therefore the results are reported as AA and its metabolites (AAM).

3.10.2 Measurement of AAM release in stimulated PBMC.

Activation of cells preincubated for 20 h with ^3H-AA was achieved by the addition of 50 ng/ml of PMA and 0.5 μM calcium ionophore (A-23187) at 37 °C for 4 h. The samples were centrifugated for 10 min (1 200 rpm) and the suspensions were added 5 ml of TRITOSOL scintillator and were counted by scintillator counter (Packard 2200CA). ^3H-AA and metabolites released into medium were determined by measurement of the total ^3H radioactivity released without separating the variouos metabolites. The values of isotope measurements were given in desintegration per minute (dpm). (Victor-Vega et al, 2002)

3.10.3 Treatment of PBMC by adenosine, CPA, CGS-216801 and IB-MECA.

Various concentrations of adenosine and the selective adenosine receptor agonists were added to the suspensions of PBMC for 15 min before non-stimulation or the stimulation with PMA plus calcium ionophore. In a series of the experiments, 10 μM EHNA (ADA inhibitor) was present in the incubation medium to inhibit the degradation of adenosine by adenosine deaminase. For selective adenosine A_{2B} receptor stimulation various concentrations of NECA (non-specific agonist for A_1 and A_2 receptors) were used in the presednce of 0,3 μM DPCPX (inhibitor of A_1 receptors)

and 0.1 μM ZM-243185 (inhibitor of A_{2A} receptors) added 15 min prior to NECA. In the study of A_3 receptors, antagonist MRS 1191 (10 nM) was added to the cells 15 min before the application of the selective agonist agent CPA (10 nM). The two other agonists, CGS-21680 and IB-MECA also were applied in a concenratation of 10 nM. The concentrations of the antagonists were chosen according to the results of preliminary measurements. In another part of our study, culture medium was completed by 0,2 U/ml adenosine deaminase to eliminate the possible contribution of endogenous adenosine. In the non-stimulated samples aliquots of culture medium were used, which contained the same amounts of dimethylsulphoxid (DMSO) as the solutions of PMA and calcium ionophore.

3.11. Statistical analysis.

As agonist-induced change in mechanical activity was expressed as a percentage reduction of the methacholine-induced precontraction. The papaverine-induced maximum effect was taken as 100% relaxation. Individual E/[A] curve data were fitted by means of the least-square iterative computer program Prism (GraphPad, San Diego, CA, USA) to a logistic function of the following form: $E = E_{max}$ [A]nH/[A]nH+[EC_{50}]nH, where E denotes the effect, E_{max} is the asymptote, [A] is the concentration of the agonist, EC_{50} is the concentration producing the half-maximum response, and nH is the midpoint slope parameter. This function is generally used and accepted for the evaluation of concentration-response curves similar to ours, and its use was further justified by our results showing a good fit to our data with correlation coefficients between 0.86 and 0.98. The concentration ratio the EC_{50} values for the adenosine-induced relaxation of solvent- and caffeine-treated tracheal tissues. The data are expressed as means ± S.E.M. The EC_{50} values are expressed throughout the text as their negative base 10 logarithms (pD_2 values). Multiple comparisons between the experimental groups were performed by one-way analysis of variance (ANOVA) with a Newman-Keuls post hoc test. The one-sample t- test was used for determining the significance of the differences of concentrations ratios from 1, whereas the paired t-test was applied in the case of appropiately matched pairs. $P < 0.05$ was taken as the level of significance.

The alterations in the release of IL-1β induced by adenosine and its agonists in the suspensions of PBMC were expressed as the percent of basal IL-1β production. Individual E/[A] curve data were fitted by the means of a least-square iterative computer program to a logistic function of the form: $E = E_{max}$ [A]nH/[A]nH+[EC_{50}]nH, as in the previous form. The data were expressed as means ±S.E.M. The criterion for significance was $P < 0.05$.

The changes in the release of AAM after application of adenosine and adenosine analogues in the suspensions of PBMC were expressed as the percent of basal AAM production. The EC_{15} values (concentration inducing a 15% decrease in the basal AAM production) of the concentration-response curves was estimated by linear regression analysis. Experimental values are given as means ±S.E.M. Multiple comparison between the experimental groups were performed by one-way analysis of variance. (ANOVA) with a Newman-Keuls post hoc test. The criterion for significance was $P<0.05$ (*$P< 0.05$, **$P<0.01$, ***$P< 0.001$).

4. RESULTS AND DISCUSSION

4.1. The effect of chronic caffeine treatment on the adenosine receptors of the airways.

The main results of our experiments with chronic caffeine therapy are as follow:

(1) The continuous administration of caffeine as a non-specific adenosine-receptor antagonist led to an enhancement in the relaxation elicited by adenosine in epithelium-intact tracheal preparations from guinea pigs. The course of the sensitization process was unusual however: after an initial sensitization period (up to 2 weeks), the adenosine-induced mechanical responses and the parameters of the E/[A] curves (pD_2 and E_{max} values) returned to the control levels in week 10.

(2) The epithelium-denuded tracheal preparations of the caffeine-treated guinea pigs did not exhibit such biphasic effects: continuous, clear-cut sensitization was observed throughout the 10-week treatment period.

(3) Using the coaxial bioassay system we confirmed that these differences could be due to an adaptogenic mechanism of the tracheal epithelium, because on the epithelium-denuded tracheal strips after 6 weeks of caffein intake, the adenosine-induced sensitization remain unaltered, however using epithelial-intact donors the sensitization induced by adenosine decrease.

(4) On the epithelium-intact, caffeine treated guinea pig tracheal strips neither the cyclo-oxygenase enzyme inhibition by indomethacin and diclofenac, nor the lipoxygenase enzyme blockage with selective inhibitors like NDGA and AA861 induced any change in adenosine-induced relaxation.

(5) On the epithelium-intact tracheal strips from caffeine treated guinea pigs the adenosine induced relaxation response has been enhanced after the neuropeptides emptying from the epithelium as the effect of capsaicine.

Our results demonstrate that the continuous administration of caffeine, an adenosine antagonist, gives rise to an unusual, biphasic modulation of the adenosine-related tracheal sensitization, with an initial increase followed by a gradual loss of sensitization.

Caffeine, a widely consumed psychoactive drug, is thought to be a non-specific antagonist on adenosine receptors. The guinea pig trachea exhibit A_2 adenosine receptors (Collis and Hourani, 1993), but Losinski and Alexander (1995) have shown that isolated tracheal rings from guinea pigs exhibit a pharmacology inconsistent with either A_{2A} or A_{2B} adenosine receptors, the agonist profile of tracheal specimens is rather compatible with the presence of an A_{2B} adenosine receptor, whereas the antagonist profile is more similar to that of an A_{2A} adenosine receptor. It should be noted that some experimental data indicate the existance of xanthine-insensitive sites probably having intracellular locations (Brown and Collis, 1982). To exclude the interactions of adenosine with these putative intracellular sites, we carried out the present experiments in the presence of an inhibitor of membrane adenosine transport.

The effects of chronic caffeine treatment on the adenosine receptors are controversial. Some studies on the central nervous system suggested an upregulation of the A_1 receptors, but others indicated no upregulation after treatment with caffeine. Contrasting data have also been published on the regulation of the A_2 adenosine receptors during chronic caffeine treatment. In a number of experiments, caffeine administration was not found to be associated with an alteration in the number of A_{2A} adenosine receptors, whereas Johansson et al, (1997) reported that caffeine ingestion (1g/l) increased the number of A_{2A} receptors in the striatum of mouse brain. As far as the A_{2B} adenosine-receptors regulation is concerned, Fredholm (1982) did not observe any alterations in the number of central A_{2B} receptors after a 1-week treatment with caffeine in rats.

Relatively few studies deal with the action of long-term caffeine administration on peripheral adenosine receptors. Varani et al (1999; 2000) demonstrated that caffeine intake induces functional alterations and upregulations of human platelet A_{2A} receptors. Zhang and Wells (1990) described an increased number of A_2 adenosine receptors and A_2–receptor-related biochemical changes in rat platelets after long-term caffeine treatment, but did not observe any modification in A_1–receptor-induced lipolysis in fat cell membranes, although the number of A_1 receptors was significantly reduced. Concerning the cardiovascular implications of caffeine treatment, a 2 weeks

caffeine treatment strongly potentiated the A_1 adenosine receptor-induced suppression of the sinoatrial rate, whereas the vasodilator and reflex tachycardic responses to an A_2 -receptor agonist were unchanged.

However there is no data available on the action of chronic methylxanthine treatment on the adenosine sensitivity of the bronchial system. Such examinations may be of importance from at least three aspects:

(1) Adenosine can play an important role in the physiological functions of the respiratory system and has a significant pathogenetic role in the bronchial asthma (Fozard, 2003).

(2) The frequent consumption of caffeine as an adenosine-receptor antagonist can modify the adenosinergic regulation both in healthy and asthmatic people.

(3) Theophylline and its derivates structure and pharmacology is hardly different from the caffeine, and for long time they used them as antiasthmatic therapy. We have to emphasize that the average serum caffeine concentrations measured in our experiments can represent an excessive coffee consumption rather than a normal intake if the data from animal experiments can be extrapolated to humans, representing a normal „therapeutic level" (Fredholm et al, 1999).

In our experiments, the continuous administration of caffeine as a non-specific adenosine-receptor antagonist led to an enhancement in the relaxation elicited by adenosine in epithelium-intact tracheal preparations from guinea pigs. The course of the sensitization process was unusual however: after an initial sensitization period (up to 3-week), the adenosine-induced mechanical responses and the parameters (pD_2 and E_{max} values) returned to the control levels. Surprisingly, the epithelium-denuded tracheal preparations of the caffeine –treated guinea pigs did not exhibit such biphasic effects: continuous, clear-cut sensitization was observed throughout the 10-week treatment period. It seemed conceivable, and was confirmed by the experiments with the coaxial bioassay system, that these differences could be due to an adaptogenic mechanism of the tracheal epithelium. The epithelium is known to be rich source of various bronchodilator and bronchoconstrictor substances, however we suppose that continuous caffeine intake can induce an adaptive response in the tracheal epithelium which will counterbalance the effect of purine responses. It has been established that the guinea pig airway epithelial cell could be a rich source for both bronchodilator (NO, PGE_2, etc.) and bronchoconstrictor agents (Barnes et al, 1998) it can express also cyclo-oxygenase and 5- lipoxygenase too. It has been reported that the granules of guinea pig and the rat

tracheal epithelium displays calcitonin gene-related peptide (CGRP) immunoreactivity, and capsaicine treatment reduces the staining of these cells for CGRP (Bertrand et al, 1993).

Besides CGRP, endothelin-like immunoreactivity has also been localized at the epithelium (Barnes et al, 1998), and endothelin release from the guinea pig tracheal epithelium has been determined. (Hay et al,1997)

As indomethacin and diclofenac (inhibitors of cyclo-oxygenase) and NDGA and AA861 (a non-selective and a selective inhibitor of lipoxygenase, respectively) did not influence the relaxation evoked by adenosine, the adenosine-induced release of bronchoconstrictor prostanoids and leukotriens could be excluded. An enhanced release of mast cell-derived constrictor agents was also unlikely, as in the presence of cromolyn (a „mast cell stabilizer") the adenosine-induced tracheal responses were also unaffected.

We would like to emphasize our results obtained using different types of capsaicine treatment. Capsaicine has been thought to deplete the neuropeptides not only from the sensory nerve fibres, but also from the airway epithelial cells (Szolcsányi, 1996, Barthó et al, 2004, Tschirhart et al, 1990). Our present findings that the depletion of neuropeptides by capsaicine pretreatment increases the adenosine-induced relaxation and that the liberation of neuropeptides in the presence of capsaicine inhibits the action of the purine nucleoside provided pharmacological proof that continuous treatment with caffeine lead to an adaptive response of the tracheal epithelial cells that counterbalances the actions of adenosine.

Moreover, we have established that during the chronic caffeine intake, the relaxant property of adenosine is strongly inhibited in the presence of inhibitors of neutral endopeptidase (phosphoramidone, thiorphan and *N*-carboxymethyl-Phe-Leu), indicating the increased involvement of bronchoconstrictor peptides of epithelial origine (eg. CGRP), although the role of non-epithelial cells can not be entirely excluded.

4.2 The role of the xanthine-resistant adenosine receptors in the guinea pigs tracheal smooth muscle

The most important results of this chapter are the following:

a. On the guinea pig tracheal strip precontracted with methacholine, the adenosine receptor activation induces a concentration-related relaxation, which has been antagonized by the non-specific A_1/A_2 adenosine-receptor antagonist 8-phenyltheophylline, but the shift of the dose-effect curves were not parallel.

b. The intracellular P-site agonist 2'-deoxyadenosine or adenine-9-beta-D-arabinofuranoside (ARA-A) induce a slight relaxation, but these effects were not influenced by the methylxantine (xanthine-resistant effect).

c. The relaxant effect of the adenosine on the trachea was significantly increased by coformycin or EHNA (an intracellulary acting, specific adenosine deaminase inhibitor).

d. There is a strong enhancement of the 2'-deoxyadenosine effect when we repeat it after inhibition of the intracellular adenosine deaminase. This potentiating effect is significant mostly in the case of the 2'-deoxyadenosine.

e. In the presence of dipyridamole, an inhibitor of the transmembrane adenosine transport, the bronchial effects of the adenosine increase, suggesting a significant functional activity of these extracellular receptors.

f. The lack of extracellular activity of two intracellular P-site agonists 2'-deoxyadenosine and ARA-A has been observed by the use of dipyridamole (a cell membrane transport inhibitor), which induced a significant antagonistic effect.

g. In the presence of specific A_3 adenosine receptor antagonist MRS 1191, the bronchial effects of the adenosine were decreased. This fact indicate that the A_3 adenosine receptors have a slight participation in the action of adenosine (non- xanthine sensitive receptors).

h. On the precontracted tracheal smooth muscle the selective A_3 adenosine receptor agonist IB-MECA induced a relaxant effect. MRS 1191 antagonized this effect, but did not modify the relaxant effect of the 2'-deoxyadenosine.

The role of the adenosine is more and more accepted in the pathogenesis of bronchial asthma (Barnes, 2003). The bronchodilator effect of methylxanthine-like adenosine receptor antagonists (theophylline, aminophylline, enprophyilline, etc.) is an obvious clinical proof for the role of the adenosine in the pathomechanism of asthma.

In the relaxation of the bronchial smooth muscle of guinea pigs the A_2 adenosine receptors play essential role (Collis and Hourani, 1993), but Losinski and Alexander (1995) have shown that isolated tracheal rings from guinea pigs exhibit a pharmacology inconsistent with either A_{2A} or A_{2B} adenosine receptors; the agonist profile of tracheal specimen is rather compatible with the presence of an A_{2B} adenosine receptor, whereas the antagonist profile is more similar to that of an A_{2A} adenosine receptor. Many authors assume that the tracheal adenosine receptors are of atypical form, but their identification is still ongoing.

It is an interesting issue that the bronchial smooth muscle contains non-xanthine-type sites or receptors, but this represents a neglected field of research. This non-xanthine-type of ligands are

belived to act on intracellular sites (Brown and Collis, 1982; Losinski and Alexander, 1995), and A_3 adenosine receptors attached to the cell membrane.

Using pharmacological methods we proved, that in the bronchial system of the guinea pigs besides the traditionally accepted xanthine-sensitive receptors there are so called „non-xanthine type" ligand sites or „non-xanthine sensitive" A_3 adenosine receptors. We observed that dipyridamole, a membrane adenosine transport blocker significantly increases the bronchial effect of adenosine, but inhibits the effect of the intracellular P-site agonists (2'-deoxyadenosine, 9-beta-D-arabinofuranosil adenine). From this perspective, we examined the role of the intracellular xanthine-resistant site in the adenosine induced effect on the bronchial smooth muscle.

The first published non-xanthine site has been localized on the surface of the adenyl cyclase enzyme and it was described as a P-site by Londos and Wolff (1977). Among the intracellular sites the S-adenosyl-homocysteine-hydrolase (Zimmerman et al, 1980) and the so called „adenosine-binding protein"(Olsson,1992) have also been described. The pharmacological characteristics of these purine „binding-proteins" are not yet fully understood, and for this reason their involvement in the physiology and pathology needs to be clarified. It's well known that the P-site is a part of the adenyl cyclase which has an important role in the regulation of many biological processes, and its activation antagonizes the enzyme.

The adenyl cyclase itself is a hydrophob, membrane linked structure which besides of the six transmembrane domain unit contains an 35-40 kDa sized citoplasmatic component (Dessauer et al, 1999). The forskolin binding-site or P-site represent a part of this citoplasmatic domain. We have more detail on the supposed role of these P-sites in the effect of adenosine on the electrical and mechanical activity of the atrial myocardium. (Szentmiklósi et al, 1982). Marone and Casolaro (1990) provided strong evidence concerning the P-site effects in the regulation of human lymphocytes and polymorphonuclear cAMP metabolism. The P-site agonists rank order of potency accepted today is: 2',5'-dideoxyadenosine-3'- tetraphosphate> 2',5'-dideoxy-3'-ATP> 2',5'-dideoxy-3'-ADP> 2',5'-dideoxy-3'-AMP> 2'-deoxy-3'-AMP> 3'-AMP> 2'-deoxyadenosine> adenosine. In pharmacological experiments only a few P-site agonists could penetrate the cell membrane. As P-site selective agonists, we used adenosine or the ribose modified and membrane permeable adenosine analogues: 2'5'-dideoxyadenosine, 2'-deoxyadenosine, 9-beta-D-xilofuranosile adenine, 9-beta-D-arabinofuranosil adenine (Londos et al, 1980).

In our experiments, the main evidence for the physiological role of the xanthine-resistant P-sites has been provided by inhibition of the intracellular adenosine deaminase activity. This enzyme is membrane associated (Agarwal et al, 1975), and breaks the adenosine into inosine, a

pharmacologically less active purine. The coformycin is one of the most potent selective inhibitor of the adenosine deaminase. In its presence, the adenosine concentration increases in the intracellular space, and activates the intracellular binding sites. In our hands, coformycin significantly enhanced the effect of the adenosine. Our main evidence for this effect on P-site was that the effect of the specific P-site agonist 2'-deoxyadenosine effect was potentiated by 50-fold in the presence of coformycin. This conception has been strengthen by the fact that on other specific adenosine deaminase inhibitor EHNA with different chemical structure similarly potentiated the bronchial effects of the adenosine and 2'-deoxyadenosine.

For examining the further role of the xanthine-resistant structures we had the opportunity to examine the role of the extracellular A_3 adenosine receptors. We observed that the bronchodilator effect of the adenosine decreased by the A_3 adenosine receptor antagonist MRS 1191. The A_3 receptor activator IB-MECA induced a relaxant effect and was antagonised by MRS 1191.

All these results provided pharmacological evidences for the fact that besides the clinically approved methylxanthine–sensitive receptors, in the lung there are also methylxanthine-resistant intracellular sites or extracellular receptors, which induce similar effects to the A_2 adenosin receptor activation. The importance of this recognition is not only theorethical, but may represent an innovative consequences, because the inhibition of the appropriate P-site or A_3 adenosine receptor can result in a significant adjuvant effect beside of a methylxanthine based bronchodilator therapy. For the implementation of this theory we need more intensive effort because the synthesis of P-site antagonist has failed until now.

4.3 The effect of the adenosine on the interleukin-1β release from the activated human peripheral mononuclear cells

On this issue our most important results are the following:

a. In the suspensions of PBMS activated by phorbol ester (phorbol 12-miristate 13-acetate) and calcium ionophore (A23187), the action of adenosine and the subtype-specific adenosine receptor agonist, (CPA (A_1), CGS 21680 (A_{2A}) and IB-MECA (A_3) selective activators) induced a concentration-dependent inhibition of the IL-1β release.

b. On the basis of pD_2 values, the rank order of potency in the inhibition of IL-1β release was: CPA = CGS 21680>IB-MECA> adenosine> NECA (in the presence of inhibitors for A_1, A_{2A} and A_3 receptors).

c. The inhibitory effect of CPA, CGS 21680 and IB-MECA was significantly antagonized by DPCPX (an A_1 adenosine receptor antagonist), ZM 243185 (A_{2A} receptor antagonist) or MRS 1191 (A_3 receptor antagonist).

The present study is the first to describe the direct inhibition of IL-1β release by adenosine and various subtype-selective adenosine receptor agonists in human mononuclear cells activated by phorbol ester and Ca^{2+} ionophore. For testing the involvement of the various receptors of adenosine, selective agonists were applied, CPA for A_1, CGS 21680 for A_{2A} and IB-MECA for A_3 receptors. In order to determine the specific effects of the adenosine A_{2B} receptors, the A_1/A_2 receptor specific NECA was used in the presence of DPCPX and ZM 243185, as selective antagonists of A_1 and A_{2A} receptors to produce an A_{2B} specific response. For the exclusion of the potential A_3 type effect of NECA, the selective inhibitor of A_3 receptors MR 1191 was used. The specificity of changes related to the A_1, A_2 and A_3 receptors was also tested in the presence of their selective antagonists.

The results of the current study clearly show that the inhibitory effect of adenosine on the release of IL-1β from activated monocytes can also be mediated by A_1, A_{2A} and A_3 receptors, while the effect of A_{2B} receptor is negligible. The pharmacological analyses of the concentration-response curves for adenosine and its receptor specific agonists demonstrates that the Hill coefficient (n_H) for adenosine versus specific adenosine receptor activators is different. The slopes of the concentration-response curves for the adenosine receptor selective agonists do not differ from the unity (1). On the other hand, the slope of adenosine is aproximately 3, suggesting a strong positive correlation between multiple receptors. In the case of A_1 and A_3 receptors the inhibition of IL-1β release from the mononuclear cells can be mediated by adenyl cyclase independent pathways, whereas the involvement of A_2 receptors may have connections with adenyl cyclase and PKC enzymes (Németh et al, 2003; Majumdar and Agarwal, 2003). The clearly dose dependent inhibition of IL-1β release via A_2 receptors is likely due to some PKC dependent phosphorylation (Palmer and Stiles, 1999). Otherwise, a specific cross-talk exist between adenosine and the receptors of various proinflammatory cytokines including IL-1β. These cytokines can up-regulate the expression of A_{2A} receptors, at least in PC12 cells (Trincavelli et al, 2002). The slight inhibition of IL-1β release mediated by A_{2B} receptors is most likely a consequence of the relatively low affinity of these receptors to adenosine.

In the current study IL-1β was primarily produced by the monocytes in the suspension of peripheral mononuclear cells (PBMC) consisting of both monocytes and lymphocytes. The advantage of unfractionated PBMC versus purified monocytes was that the purification procedure

could have resulted in some functional damage in the cells. (Sipka et. al, 2001). Therefore, the unfractionated non-damaged cell preparation was used for these studies.

The physiological and clinical importance of the present findings is that the inhibitory effect of adenosine and of its receptor specific analogues on the release of IL-1β has been observed in activated human peripheral mononuclear cells. Specifically, A_{2A} receptors may serve as potential anti-inflammatory agents (Gomez and Sikovsky, 2003). It was noteworthy that previously we reported that the average concentration of adenosine in the sera of healthy volunteers was about 40 nM, whereas the level of adenosine reached a concentration of 0,75 μM in patients with septic shock (Sipka et al, 1995). According to Martin et al, (2000) the adenosine concentrations of circulating blood can be 4 -10 μM in patients suffering from sepsis. In addition, it has been documented that adenosine concentrations can reach as high as 100 μM in some inflammatory regions. (Cronstein et al, 1994). According to our present results, adenosine is able to inhibit the release of IL-1 from the activated mononuclear cells at lower concentrations than previously mentioned. As IL-1 is one of the most effective natural inducer of the hyperthermic reactions, one of the main physiologic roles of adenosine may be the prevention of hyperpyrexia by the inhibition of IL-1 release from the activated monocytes, lymphocytes and macrophages. In addition, it may moderate the hyperactivation of inflammatory cells in general. The capability of adenosine to regulate the actual level of IL-1 in certain part of the organism can play an important role in its action on processes related to innate immunity. (Haskó and Cronstein, 2004).

In summary, an inhibition of IL-1β release by adenosine and selective adenosine receptor agonists from activated human peripheral mononuclear cells in vitro has been demonstrated. Furthermore, aspects of the physiological and clinical importance of the inhibition of IL-1β release caused by adenosine have been proposed. The observation that A_1, A_3, but especially A_{2A} receptor activation can reduce the release of IL-1β from the activated mononuclear cells provides additional data to better understand the mechanism(s) of the anti-inflammatory effects of this endogenous purine nucleoside.

4.4. The effect of the adenosine on the arachidonic acid release from the activated human mononuclear cells.

The main results in this issue are the following:

a. The adenosine and specific adenosine agonists CPA (A_1) and CGS 21680 (A_{2A}) induce a concentration-dependent inhibition of arachidonic acid release from the phorbol ester

(phorbol 12-miristate 13-acetate) and Ca^{2+}-ionophor (A23187) activated human peripheral mononuclear cells, whereas the A_{2B} and A_3 receptor agonists had no significant effect.

b. The rank order of potency of the agonists on the arachidonic acid release was: CGS 21681 = CPA> adenosine> NECA (in the presence of ZM 24185 as A_{2A} antagonist and DPCPX as A_1 adenosine receptor antagonist) = IB-MECA. The inhibitory effect of the adenosine was effective only above the 100 μM concentration, whereas the adenosine receptor agonists effect was already from the 0,1 μM concentration of the adenosine.

The adenosine level in normal human plasma have been reported to be 100–120 nM (Sipka et al, 1995). The level of the adenosine in a patients with septic shock reached a concentration of 750 nM. (Cronstein et al, 1994). The local adenosine concentration can be as high as 100 μM in some inflammatory sites. According to our findings, the physiological 100–120 nM concentration of adenosine may not have a significant inhibitory effect on the release of arachidonic acid from the activated monocytes stimulated by the PKC signal transduction system or via the increased intracellular Ca^{2+} level. In special clinical situations, however, when arachidonic acid producing cells are accumulated at certain inflammatory sites, adenosine can be released at much higher concentrations, which may induce significant local reduction of arachidonic acid release.

This inhibition appears to be mainly via the A_1 and A_2 receptors according to the reported findings, whereas the role of A_{2B} and A_3 receptors seems to be negligible in this process. The actual level of AA is mainly dependent on the activity and interaction of three intracellular enzymes: cytosolic (Ca^{2+} dependent) PLA_2 ($cPLA_2$, type IV), Ca^{2+} independent PLA_2 ($iPLA_2$, type V) and diacylglycerol (DAG) lipase (Wu et al, 2004). It should be emphasized, however, that extracellular IL-1 produced by monocytes is one of the „best physiological" activator of AA production and release, (Chang et al, 1986) mainly via the activation of $cPLA_2$ (Morri et al, 1994). We suggest that the decreased release of AA found in the activated monocytes by adenosine and the A_1 and A_{2A} specific agonists is correlated with a reduced production of IL-1 in the cells stimulated by PMA and Ca^{2+}-ionophore (Sipka et al, 2005). However, some amount of secretable PLA_2 (type II.A,14kD) can appear from the interaction of activated monocytes surrounded by activated platelets (Sipka et al, 1994). It can not be excluded that this enzyme also can produce some amount of extracellular AA. Nevertheless, we think that the monocytes may require the permanent presence of IL-1 in their surrounding to produce (Liu and Young, 2004). It is possible that the activity of $cPLA_2$ in the monocytes is directly correlated with the extracellular concentration of IL-1 stimulating the release of AA. It can be one of the biological roles of endogenous adenosine, that in elevated

concentrations, it can suppress (mainly indirectly by the inhibition of IL-1 production) the release of AA from the monocytes via A_2 and A_1 receptors (Sipka et al, 2005). The A_2 receptor mediated (cPLA2 dependent) suppression of AA release by increased extracellular adenosine level was observed also in neutrophils (Németh et al, 2003). To our best knowledge, this is the firts report to describe the inhibitory action of adenosine on the AA release of activated human monocytes. It should be emphasized that this mechanism is most likely not operational at physiological concentrations of adenosine. In pathological condition, e.g. in inflammatory tissues, where the monocytes are in high numbers and the concentration of adenosine is also elevated, the activation of A_1 and A_{2A} receptors can effectively inhibit the arachidonic acid metabolites (AAM) release from monocytes. According to the present data, it can be concluded that the potent antiinflammatory action of adenosine directed to the monocytes, may be due, at least in part, to inhibition of the production of IL-1, thereby decreasing the release of AAM.

5. CONCLUSIONS

Our experiments have provided a series of evidences that the long term caffeine intake produce pronounced modifications in the adenosine receptor function and sensitivity of the bronchial system. It looks quite unequivocal that these modifications induced by the long term caffeine intake appear at the level of the epithelial cells too, which influence the adenosine receptors sensitivity, and bypassing this interactions, appears an obvious up-regulation at the level of the adenosine receptors. Our data suggest that in this process located in the epithelial cells or their direct environment the neuropeptides might have a significant role. Because the worldwide consumption of coffee and caffeine-containing beverages, as long as we extrapolated our results to the caffeine consuming population we may expected the same adaptive modulations. Taking account of that the endogenous adenosine play a fundamental role in the lung physiologic functions, the previously mentioned changes in the normal regulations should induce still unpredictable health effects. Because the results of our experiments are originals, it may be worth to complete the research in a close collaboration with the clinicians.

As regards the role of the xanthine-insensitive adenosine receptors and sites, we concluded that adenosine, mainly in higher concentrations could activate and implement biologic effects, modifying the bronchial tone. The fact is that these effects are stronger when the adenosine deaminase activity is inhibited. Because there are a number of drugs which inhibit this intracellular enzyme, and as our previous results data show, the hypoxic conditions reduce significantly the

potency of adenosine deaminase, in pulmonary hypoxia, we should expect the increase of activity of these xanthine-insensitive sites. It's a conceivable situation that in asthma and COPD these modulations could be increased. This phenomenon might be a role in the explanation of the pathophysiologic pathways and the development of future drugs for pulmonary diseases.

The results from the study of the mononuclear cells role in the inflammatory processes enhance our better understanding of the pathomechanisms of pulmonary inflammatory diseases. The fact is that the IL-1β and the arachidonic acid play a role in the inflammatory processes and the adenosine receptors, in a receptor subtype dependent way inhibit the release of inflammatory mediators allowing the hypothesis, that the adenosine has a concentration-dependent anti-inflammatory role. The importance of the results is mainly pathophysiological because relatively high concentration of adenosine is necessary to induce these effects.

The final conclusion is that extrapolating the experimental results of our studies we obtained significant results concerning not only the pathophysiology but the future implementation in the innovative drug research as well.

6. Key words:

Adenosine, Adenosine receptors, Caffeine, Tracheal smooth muscle, IL-1β, arachidonic acid, inflammation, bronchiale asthma

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