Summary of Thesis for the degree of Doctor of Philosophy (PhD)

EFFECT OF NUCLEOSIDE TRANSPORT INHIBITION ON THE INTERSTITIAL ADENOSINE CONCENTRATION IN THE MICROENVIRONMENT OF A_1 ADENOSINE RECEPTORS IN EU- AND HYPERTHYROID GUINEA PIG ATRIA

Dénes Karsai

DEPARTMENT OF PHARMACOLOGY
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Dénes Karsai

Advisor: Dr. Árpád Tósaki

Consultant: Dr. Rudolf Gesztelyi

Head of the Program: Dr. Lajos Gergely

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ABBREVIATIONS

AC = adenylyl cyclase

A_1 receptor = A_1 adenosine receptor

ADA = adenosine deaminase

Ado = adenosine

$[Ado]_{ISF}$ = adenosine concentration of the interstitial fluid

ATP = adenosine-5'-triphosphate

cAMP = 3',5'-cyclyc adenosine monophosphate

CPA = N^6-cyclopentyladenosine

DP = dipyridamole

E/[A] = concentration-response

ENT1 = equilibrative and nitrobenzylthioinosine sensitive nucleoside transporter

M = mol/l

NBTI = S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine

NT = sarcolemmal nucleoside transport

RRM = receptorial responsiveness method

T_3 = L-3,5,3'-triiodothyronine

T_4 = L-thyroxine (L-3,5,3',5'-tetraiodothyronine)

S = solvent of L-thyroxine

INTRODUCTION

In the developed countries, cardio-vascular diseases mean the greatest challenge regarding life expectancy and the quality besides cancer. Increasing the resistance of the heart against ischemia is one of the leading research topics around the world. The influence of adenosine mediated protective mechanisms in the heart belongs to one of these topics as well.

Adenosine (Ado) composed of adenine and ribose is a precursor as well as a degradation product of the most important primer energy storing molecule, the ATP. Further significance of Ado is that it possesses regulatory functions in the whole organism at the level of cell and tissue growth, differentiation and metabolism. An acute and excessive Ado generation usually signifies a defect in ATP re-synthesis, thus it is a marker of the exhaustion of the given tissue. In accordance with this, Ado takes a part in the mediation of protective and reparative functions as well.

Effects of Ado are mainly mediated by specific cell-surface receptors, namely the A_1, A_{2A}, A_{2B} and A_3 receptors. All four are present in the human heart, and according to the most widely accepted theory, myocardial A_1, A_3, furthermore vascular A_{2A} receptors have protective functions. The binding sites of these receptors are oriented towards the extracellular space, thus the Ado concentration of the interstitial fluid ($[Ado]_{ISF}$) and the changes occurring in it have notable significance. The determinants of $[Ado]_{ISF}$ are the extra- and intracellular Ado production and elimination, and the trans-membranal nucleoside transport (NT). As the larger part of the weight of the heart is the myocardium, determination of the above-mentioned factors is of primary impact for cardiomyocytes.

In the cell membrane, two types of nucleoside transporters can be found: the equilibrative (diffusion facilitating; ENT) and the concentrative (capable of active transport; CNT) nucleoside transporter. It is generally accepted that the human cardiomyocytes contain only ENT, and within this mainly ENT1, a nitrobenzylthioinosine (NBTI) sensitive subtype of equilibrative carriers. Consequently, the direction and intensity of NT is primarily determined by the Ado concentration gradient between the two sides of the membrane. Due to the strong intracellular Ado reutilization, cardiac muscle can be characterized by a net intracellular Ado elimination and a net interstitial Ado production. In accordance with the facts mentioned above, in physiological circumstances NT is directed towards the cell interior in cardiomyocytes, therefore NT inhibition increases $[Ado]_{ISF.}$ Determination of this increase depends on the measurement of $[Ado]_{ISF}$, which has not been resolved successfully up to now.

Various methods are available to assess the $[Ado]_{ISF}$ which cannot be measured directly within an operating human heart; these are: micro dialysis, peri- or epicardial transudation, infusion and the "well" technique: „axially distributed modeling", a calculation made on basis of the difference of artery-venous Ado concentration, namely on basis of the hematocrit and coronary perfusion. The scale of $[Ado]_{ISF}$ values gained by the different measuring methods encompass three orders of magnitude (!). This variance is partly due to the different rate of damage caused to the tissues by each measuring method on one side, and on the other side it can be traced back to the difference in the tissue compartments used as samples, but it certainly shows the necessity to develop a new and reliable measuring method.

In certain cases, the classic Langmuir-Hill equation makes it possible to calculate the value of the receptor adjacent concentration of an agonist because with the help of the three parameters (E_{max} , EC_{50} , n) the equation is able to describe the relationship between the concentration of the agonist and the triggered biological response:

$$E = E_{\max} \cdot \frac{c^n}{c^n + EC_{50}^n}$$

where: c is the concentration of the agonist; E is the effect of c; E_{max} is the maximum effect that can be evoked by the given agonist (efficacy); EC_{50} is a concentration yielding half the maximum effect (reciprocal of this is potency); n is the Hill coefficient (slope).

If concentration-effect (E/[A]) date pairs are known for a given agonist and its receptor, i.e. a E/[A] curve is available; than fitting the Langmuir-Hill equation to this, the value of the three parameters can be assessed with great accuracy (this is called regression analysis). At the same time, if four from the above-mentioned five values are known, the fifth can be easily calculated in an algebraic manner using the Langmuir-Hill equation. That is to say, that change in $[Ado]_{ISF}$ could be calculated from the three parameters of the relationship between Ado and one of its cell surface receptors (E_{max} , EC_{50} , n) and from the receptorial response. However, Ado is a strongly degrading and compartmentalizing molecule within the living tissues, so it is not possible to determine exactly its level in the microenvironment of its receptor. Without this date, the E_{max} , EC_{50} and n parameters of the Ado E/[A] curve are not suitable for the above-mentioned calculation.

In principle, however, there exists a possibility to asses the changes of $[Ado]_{ISF}$ according to a recently developed method. This method makes use of the analysis of the data

received by the (E/[A]) curve with the help of curve-fitting. The estimation of concentration change made through regression analysis is based on the saturation capability of the receptor function. If in a biological system the E/[A] curve is made with an agonist where even at the beginning a $c_x \neq 0$ concentration of the given agonist is present in the system, at the time of analysis c_x must be taken into account for the designation of the starting condition and also when creating the responses of the E/[A] curve. If c_x is ignored, the results gained in such way will differ from those which could be received if the calculation was made in the correct manner. The connection between the distortion of the responses and between c_x, the element causing the distortion can be deducted from the following Langmuir-Hill equation:

$$E' = 100 - \frac{100 \cdot \left(100 - E_{\max} \cdot \frac{(c_x + c_{ad})^n}{(c_x + c_{ad})^n + EC_{50}^n} \right)}{100 - E_{\max} \cdot \frac{c_x^n}{c_x^n + EC_{50}^n}}$$

Where: c_x equals to the agonist concentration present in the system before generating the E/[A] curve; E' is the distorted response (effect calculated by ignoring c_x); c_{ad} is the concentration of the agonist calibrated during the construction of the E/[A] curve; E_{max}, EC_{50} and n are the parameters of the intact ($c_x = 0$) E/[A] curve gained by fitting the Langmuir-Hill equation, in other words the descriptors of an intact E/[A] relation.

If a system, where the intact E/[A] relationship with the given agonist is known, yields an altered response for this given agonist, than a preliminary and partial activation of the receptors (or a post-receptorial signal-transduction) can be assumed as a possible cause of it. In this case, if the parameters (E_{max}, EC_{50}, n) of the intact E/[A] relationship are used in the above equation, and after this the equation is fitted on the different points of the distorted E/[A] curve, we will receive as regression parameter (c_x) the concentration value of the agonist in the E/[A] curve, which is able to produce the same (as previously calibrated) or similar modification on the E/[A] curve as found before. C_x will provide (in an absolute way) the distorting concentration rate if the distortion is caused by the presence of a certain concentration of the agonist used for making the E/[A] curve.

If the agent causing the distortion is not the same as the agonist of the E/[A] curve, then c_x is the concentration rate of the agonist of the E/[A] curve, which is equally effective as the distorting concentration rate from the point of view of the studied effect. In this case c_x is to be considered as indicative as well, although it is only a relative index of the distorting

concentration. The relationships described above will be referred to as “concentration-determining” equation henceforth.

Because of the above described inaccuracy of the E_{max} , EC_{50} and n parameters of the Ado E/[A] curve, unfortunately the concentration-determining equation is not suited to determine $[Ado]_{ISF}$ modification with an absolute index. In principle however, it provides a possibility to characterize $[Ado]_{ISF}$ alterations with the help of a relative index, if fitted on an E/[A] curve of an agonist where the parameters (E_{max} , EC_{50} , n) describing the relationship between the agonist and the receptor is known. Such an Ado receptor agonist is the A_1 receptor selective N^6-cyclopentyladenosine (CPA) which is degraded and compartmentalized with a negligible rate in the asanguineous myocardium. Processing data of the CPA E/[A] curves with or without the presence of NT inhibitors with the help of the concentration-determining equation seems suited to characterize the alteration caused by NT inhibitors in $[Ado]_{ISF}$.

Among the Ado receptors of the heart muscle type A_1 is the one occurring in the biggest concentrations, and its occurrence is exclusive on the atrium muscle of guinea pigs. In the labor exerting muscle sells of the atrium A_1 receptors mediate strong and direct negative trop effects (which can develop without previous raise of the cAMP-level), and among these negative inotropy can be measured with the greatest ease. We have performed our experiments on left atriums of guinea pigs provoked on stable frequency. Thanks to this the trop effects of the A_1 receptor function are clearly manifested in the alteration of the contraction force, thus during the whole experiment this outgoing changeable was registered as effect.

It is well known that the tri- and tetraiodotyronin (T_3 , T_4) hormones of the thyroid gland have a significant effect on the heart, this effect is rather adverse in the presence of excess T_3 , T_4 hormones putting strain on the heart. In accordance with this it has been shown that in the case of hyperthyroidism the effect of the A_1 receptor agonists diminishes in the heart. According to recent data excess T_3 and T_4 levels have a cardio protective effect on the short run, and this effect is essentially similar to the protective effect of ischemia preconditioning. In this latter the A_1 receptors play an important role. This fact modulates the picture and inspires researchers to study adenosinergic mechanisms on a hyperthyroid heart.

AIMS

The protective and regenerative effects of Ado within the heart are mediated mainly by A_1 receptors and to a smaller extent by A_3 receptors. Because the binding site of these receptors is oriented towards the extracellular space, baseline $[Ado]_{ISF}$ and the changes occurring in it have a great significance in respect of cardio protective mechanisms. The fact that the assessed basal $[Ado]_{ISF}$ values encompass three orders of magnitude is a result of the dependence on the applied measuring instruments, indicating the necessity of developing a new and reliable method. In accordance with this we have examined whether the classical Langmuir-Hill equation and the "concentration-determining" equation deducted from it could be used to characterize changes occurring in $[Ado]_{ISF.}$ Because NT inhibition strongly increases $[Ado]_{ISF}$ on metabolically intact heart muscle, we have set it as our primary goal to determine this effect.

It is well know that thyroid hormones (T_3 , T_4) influence at various points the signal-transduction mediated by the A_1 receptor originated by a decreased response of the A_1 subtype agonists of an hyper-thyroid heart. According to our best knowledge no data are available upon the effect of thyroid hormones on myocardial $[Ado]_{ISF}$ and its changes under NT inhibition, although this information could lead to a more exact expedition of the effect of T_3 and T_4 hormones on the heart. Our secondary aim was to study the influence of hyperthyroidism on the effect of NT blockade on $[Ado]_{ISF}$. This provided an opportunity to use our recently tested method (RRM), a procedure based on fitting of a "concentration-determining" equation in order to solve a practical problem.

MATERIALS AND METHODS

MATERIALS

The following materials were used: adenosine (Ado); N^6-cyclopentyladenosine (CPA); dipyridamole (DP); S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (NBTI); adenosine deaminase (ADA); L-tiroxin (T_4).

EXPERIEMENTAL ANIMALS

Our experiments were performed on the isolated left atria of male guinea pigs weighing 500-700 g. The housing, pretreatment and processing of animals was according to the European Community guidelines and in agreement with the Ethical Codex of the Committee of Experimental Animal Research (University of Debrecen).

THYROXINE TREATMENT

For the experiments aiming to compare the eu- and the hyperthyroid state, the animals were randomly divided into two groups: the T_4 treated and the solvent (S) treated group.

One group of the animals received 330 μg/kg L-thyroxine sodium salt pentahydrate (T_4) daily ip. for 8 days (*in vivo* T_4 treatment), while the vehicle of T_4 (S) was administered daily ip. for 8 days to another group (*in vivo* solvent treatment). The animals were sacrificed on the ninth day by one firm blow on the head. The thyroid state of the animals was confirmed by the bodyweight and rectal temperature.

TISSUE PREPARATIONS

After opening the thorax of sacrificed guinea pigs, hearts were removed and placed into an oxygenated Krebs solution at room temperature. The left atrium was mounted in a 10 cm3 organ chamber filled with Krebs solution (TSZ-04, Experimetria, Budapest) /figure 3/. The Krebs solution was oxygenated with 95 % O_2 and 5% CO_2 in order to maintain the pH at

7.4 . Atria were fixed to an isometric transducer (SG-01 D, Experimetria, Budapest) under a tension of 10 nM.

Atria were stimulated by a programmable electrical stimulator (ST-02, Experimetria, Budapest) through platinum electrodes. Stimulation was performed at a frequency of 3 Hz with 1 ms impulse width and under 150 % threshold tension (approximately 1 V) the electrical signs were registered with a 6 channel polygraph (BR-61, Medicor, Budapest).

PROTOCOLS

After starting the stimulation, every atrium was incubated in a Krebs solution for 50 minutes, during which period the contractility parameters had a chance to stabilize. During the incubation (here and on other occasions as well) the content of the organ tubs was changed every 15 to 20 minutes (washing). Atria were classified randomly in groups and subgroups during pre-incubation. During the experiments the isometric contractions of the atria were registered and the amplitudes of these contractions were assessed as contraction force.

Ado E/[A] curves

First in all experimental groups and subgroups, a cumulative E/[A] curve was generated with Ado. Anomalous atria not meeting the inclusion criteria were excluded with the help of the Ado E/[A] curves.

In vitro treatment

Following the construction of the Ado E/[A] curve, atria were subjected to different compounds for 50 minutes (*in vitro* treatment), these compounds defined the groups and subgroups in addition to the *in vivo* treatment.

In our first experimental set, *in vivo* untreated animals were used. Within this set the following groups were formed after the *in vitro* treatment: (1.) Krebs solution: **control group**; (2.) 10 μM DP: **DP group**; (3.) 10 μM NBTI: **NBTI group**.

In our second experimental set the *in vivo* treatment resulted in the formation of two groups (S and T_4 treated groups respectively), and these were divided following the *in vitro* treatment into 3 subgroups respectively: (1.) Krebs solution: **S control subgroup**; (2.) 10 μM NBTI: **S & NBTI subgroup**; (3.) 2 U/ml adenosine deaminase (ADA) + 10 μM NBTI: **S & ADA+NBTI subgroup**; (4.) Krebs solution: **T_4 control subgroup**; (5.) 10 μM NBTI: **T_4 & NBTI subgroup**; (6.) 2 U/ml ADA + 10 μM NBTI: **T_4 & ADA+NBTI subgroup**.

CPA E/[A] curves

Following the *in vitro* treatment a cumulative E/[A] was constructed for each group and subgroup with CPA, which is a highly selective A_1 receptor agonist.

Because CPA is not a substrate of the Ado degrading and reutilizing enzymes present in the myocardium, its concentration scarcely diminished during our experiment (approximately 20-40 minutes) in the bloodless atrium. Because of its slow elimination, CPA can be hardly removed from the atrium. For this reason, we did not perform self controlling experiments during our research, rather we created a distinct control group or control subgroups.

DATA ANALYSIS

Evaluation of E/[A] curves

The percentage decline of the initial contraction force was regarded as the concentration effect of a given agonist. Langmuir-Hill equation was fitted on the points of the E/[A] curve. Responsiveness of each preparations for the given agonist was characterized by the E_{max} , EC_{50} and n parameters of their E/[A] curve.

Concentration estimation with the help of the Langmuir-Hill equation (classical method)

In the Langmuir-Hill equation the decline of the contraction force in the presence of DP and NBTI respectively was used as effect (repose value in percentages). As E_{max} , EC_{50} and n parameters we have used the equivalent E_{max} , EC_{50} and n values of the averaged Ado E/[A] curves made for the *in vivo* treated (or *in vivo* not treated) atria. The c values received in this manner indicate Ado concentrations which were administered into the organ bath and induced the same inotrop effect as 10 μM DP or NBTI. This method used for the assessment of $[Ado]_{ISF}$ change will be here referred to as classic method.

Concentration estimation by means of the Receptorial Responsiveness Method (RRM)

The relative index (c_x) characterizing the changes occurring in $[Ado]_{ISF}$ as a result of NT inhibition was received fitting the concentration-determining equation on the data of the CPA E/[A] curve in the DP and NBTI treated groups. The equation contains the parameters (E_{max} , EC_{50} , n) of the averaged control CPA E/[A] curves (for groups receiving the same *in vivo* treatment) defining the intact relationship between the CPA and the A_1 receptors. The

relative index received in this manner indicates thus a CPA concentration that induces the same inotrop effect as 10 µmol/l DP or NBTI. This method will be hereafter referred to as Receptorial Responsiveness Method, in short the RRM. Fitting was made to both individual and averaged CPA E/[A] curve data.

For the sake of comparing the values received by using the classical method with those gained by RRM assessment, CPA concentrations found by RRM assessment were converted into Ado concentration values with the help of the Langmuir-Hill equation. First the negative inotrop effects (E_x) pertaining to the c_x values were calculated with the help of the Langmuir-Hill equation for which E_{max} , EC_{50} and n parameters of the averaged CPA E/[A] curbs (for the same *in vivo* treated groups) were used. After this the Ado concentrations pertaining to the E_x –s were calculated with the help of the Langmuir-Hill equation for which E_{max} , EC_{50} and n parameters of the averaged Ado E/[A] curbs (for the same *in vivo* treated groups) were used. Because we have used the parameters of Ado E/[A] curves, the received concentrations – similarly to the classic method – indicate concentrations in the organ bath that have the same inotrop effect as 10 µM DP or NBTI.

Statistical analysis

For the comparison of the average values of data sets (groups) yielding normal distribution and homogenous variations in the case of two groups we have conducted a two sided, bi-patterned unmatched Student test. For groups of more than two participants we have made an ANOVA test with a Newman-Keuls post-test. If the variations differed for two groups of normal distribution we have used the Welch-adjusted test. For the comparison of two groups not showing normal distribution the Mann-Whitney U test was used, for the rest the Kruskal-Wallis test was used completed with a Dunn post-test.

In the case of the E/[A] curves the E_{max} , $\log EC_{50}$ and n values were compared. Expressing EC_{50} in logarithm was necessary to have data of normal distribution which could be tested by a parametric statistic probe (in case they met all criteria mentioned above).

For the calculated Ado and CPA concentrations two types of averages were made: (1.) we have taken the mathematical average of the individual concentrations; (2.) first we have calculated the mathematical average of the basic data and we have calculated the concentration from it. For the statistical analysis we have compared the average of the individual concentrations, whereas the values of the averaged basic data were used as illustrations because a preliminary averaging is able to compensate for biological variables and eventual disturbing effects of measurement inaccuracies.

We have compared with a two-way ANOVA test the Ado concentration values received by use of the classical method and those gained with RRM.

Values of $p < 0.05$ were considered to be significant.

RESULTS

INOTROPIC RESPONSE TO ADENOSINE

In accordance with earlier observations, Ado concentration-dependently decreased the contractile force of both eu- and hyperthyroid atria.

The responses to Ado observed between the *in vivo* untreated groups and between the same *in vivo* treated subgroups did not differ significantly, indicating a proper randomization of atria.

Treatment with T_4 significantly reduced the inotropic response to Ado.

INOTROPIC RESPONSE TO CPA

Similarly to Ado, CPA concentration-dependently reduced the contractile force of both eu-, and hyperthyroid atria treated with S and T_4. The effect of CPA depended on its concentration.

Treatment with T_4 significantly decreased the inotropic response to CPA. On *in vivo* untreated atria, both DP and NBTI significantly and to a similar degree reduced response to CPA.

NBTI reduced the response to CPA more significantly in atria treated with T_4 than in those treated with S. ADA considerably reduced the effects of NBTI on atria treated with S as well as T_4.

EFFECTS OF NUCLEOSIDE TRANSPORT INHIBITION ON THE ADENOSINE LEVEL AT A_1 RECEPTORS

We used two methods for the description of the changes of $[Ado]_{ISF}$ in response to *in vitro* treatment: the classic method and the RRM. The first was only applied in the *in vivo* untreated atria in order to be able to compare the results with the findings received from RRM. RRM was also used for the atria treated *in vivo* with S and T_4.

In the case of the classic method, we calculated Ado concentrations from the inotropic effect of the *in vitro* treatment. These Ado concentrations were supposed to be equi-effective to those administered in the organ bath. In the course of the RRM two different E/[A] curves

were taken with the CPA: one was the base curve, used for comparison, and the one the difference of which from the base curve was described with the CPA concentration (c_x). It was performed in the following day: the parameters of the base CPA E/[A] curve (E_{max} , EC_{50} , n) were entered into the equation used for the definition of the concentration. It was then compared to the data of the distorted CPA E/[A] curve. The following comparisons were carried out:

(1.) the parameters of the average CPA E/[A] curve of the base group were compared with **a)** the data of the average CPA E/[A] curve of the DP and NBTI groups respectively, and **b)** and the data of the individual CPA E/[A] curves of the DP and NBTI groups (Table Ø);

(2.) the parameters of the average CPA E/[A] curve of the S control group were compared with **a)** the data of the average CPA E/[A] curve of the S & NBTI and S & ADA+NBTI sub-groups, and **b)** the data of the individual CPA E/[A] curves of the S & NBTI and S & ADA+NBTI sub-groups (Table S);

(3.) the parameters of the average CPA E/[A] curve of the T_4 control sub-group were compared with **a)** the parameters of the average CPA E/[A] curve of the T_4 & NBTI and T_4 & ADA+NBTI sub-groups, and **b)** with the parameters of the individual CPA E/[A] curves of the T_4 & NBTI and T_4 & ADA+NBTI sub-groups (Table T_4).

Although there were no statistically significant differences between the results obtained from the examinations carried out in the two different ways in the case of the *in vivo* untreated atria, the difference was considerably larger in the DP group (Table Ø: Average 1 values). There was, however, a considerable difference in the data of the Ado concentration of the different *in vitro* treated groups examined with the classic method ($p<0.01$) (Table Ø: Average 1 values). The source of variation, according to ANOVA, is the difference between the two NT inhibitors ($p=0.034$) and not any difference between the methods ($p=0.88$).

In the case of the *in vivo* treated atria, the average concentration of c_x was 2,5 times higher in the T_4 & NBTI sub-groups than the average c_x concentration in the S & NBTI sub-groups ($p<0.05$) (Tables S and T_4: Average 1 values). The c_x concentrations determined from the averaged CPA E/[A] curves were in accordance with these values: the c_x values found in the T_4 & NBTI sub-groups were 1,5 times higher than the values of c_x determined in the S & NBTI sub-group (Tables S and T_4: values in the Average 2 line). If ADA was present together with NBTI, the values of c_x received were considerably lower (Tables S and T_4: Average 1 and Average 2 values), although it was statistically significant only in the case of the atria treated with T_4 ($p<0.05$) (Tables S and T_4: Average 1 values).

Ø	**DP**		**NBTI**	
	Average 1	Average 2	Average 1	Average 2
RRM [CPA] (nM)	16.2 ± 3.7	16.1	25.3 ± 9.2	20.4
RRM [Ado] (µM)	32.3 ± 12.5	25.5	58 ± 28.6	36.9
C [Ado] (µM)	9.8 ± 3.7	7.5	87.6 ± 31.8 **	40

S	**NBTI**		**ADA+NBTI**	
	Average 1	Average 2	Average 1	Average 2
RRM [CPA] (nM)	18.8 ± 3	18.5	7.9 ± 1.9	7.8

T_4	**NBTI**		**ADA+NBTI**	
	Average 1	Average 2	Average 1	Average 2
RRM [CPA] (nM)	46.5 ± 13.7 †	37	7.4 ± 2.3 ≠	6.6

Tables Ø, S and T_4. CPA concentrations characterizing the changes in $[Ado]_{ISF}$ near the A_1 receptor determined by RRM (c_x); Ado concentrations calculated from c_x by the Langmuir-Hill equation; and the Ado concentrations calculated by the traditional method. The *in vivo* treatment of the groups and sub-groups concerned are indicated in the upper left corners of the tables (Ø, S, T_4), whereas the *in vitro* treatment was entered into the upper lines of the tables.

Lines starting with "RRM [CPA] (nM)" contain CPA concentrations (c_x) equi-effective with the changes of $[Ado]_{ISF}$ in nM, whereas the Ado levels (in µM) calculated from these with the Langmuir-Hill equation are entered into the lines starting in "RRM [Ado]". The line with "C [Ado] (µM)" shows Ado concentrations in µM determined with the classic method. Average 1: the arithmetical mean average of the specified individual values, ± SEM. Average 2: the values determined from the preliminarily averaged initial data.

The statistical levels of significance apply to the following comparisons: the NBTI and DP groups within the same concentration estimation method (*); the S and T_4 treated sub-group within the same *in vitro* treatment (†); and the NBTI and ADA+NBTI subgroups within the same *in vivo* treatment (≠). One symbol: $p<0.05$; two symbols: $p<0.01$.

DISCUSSION

A characteristic of the A_1 receptors, the most investigated Ado receptor of the heart, is that they tend to desensitize even in the presence of a large dose of agonist. It suggests that the even a lasting change in the concentration of agonists available to the Ado receptors is able to influence the functions of the Ado receptors, including the receptors belonging to the A_1 type. It opens up a perspective in the research into the effects of adenosinergic agonists and sensitizers, and the tissue distribution of Ado.

Providing interstitium Ado has a great significance in the protection against the ischaemia of the cardiac muscles. Interstitial Ado primarily offers a protection through stimulating the Ado receptors on the cell-surface, but the exact mechanisms in detail are yet unclear, due to measurement limitations and partly contradictory results. The scale of the values obtained about cardiac $[Ado]_{ISF}$ by the currently used methods of estimating cover three ranges and are largely influenced by the measuring methods. All this indicates that it is necessary to develop now methods.

It has been well-known that inhibition of NT increases the effects of both endogenous and exogenous Ado in the well-oxygenated cardiac muscle. As the results of the direct $[Ado]_{ISF}$ measurement largely depend on the methods applied, it is difficult to evaluate the effects of NT inhibitors, particularly in the microenvironment of A_1 receptors, a tissue compartment difficult to assess in the functioning heart.

In the course of experiments using animals without *in vivo* treatment, the changes of $[Ado]_{ISF}$ elicited by DP and NBTI, inhibitors of NT, were estimated by two methods: the classic method and the RRM. The latter was used for the first time. The measuring process with the classic method was carried out in order to provide a foundation for comparison with the findings obtained with the RRM.

In the experiments carried out on *in vivo* untreated guinea pigs, both the DP and NBTI significantly reduced the responses of the left atria to CPA. A common feature of the non-nucleoside DP and the nucleoside-analogous NBTI is that they both effectively inhibit ENT1-type membranal Ado-carriers. As in a metabolically intact heart Ado streams from the interstitium towards the inside of the cells, the effects of DP and NBTI on the CPA may be attributed to the increase of $[Ado]_{ISF}$ triggered by the inhibition of NT. The increased amount of interstitial Ado generates negative inotropic effect through the stimulation of myocardial

A_1 receptors, thus consumes a certain part of the negative inotropic capacity of A_1 receptors. The CPA E/[A] curve constructed following this process will be therefore depressed.

On the basis of the results provided by RRM, 10 µM DP or NBTI increased $[Ado]_{ISF}$ in the isolated guinea pig left atrium. The increase in $[Ado]_{ISF}$ in the microenvironment of A_1 receptors was equi-effective with 16.1 nM and 20.4 nM CPA. The c_x values are unique in that the changes in the level of degradable Ado generated by NT inhibition were characterized at the A_1 receptors, which compartment is very difficult to access by any other method although it is essential for the adaptation of the heart to hypoxia.

As the values of c_x received by RRM are CPA concentrations, they are directly comparable neither with data in the literature nor with Ado concentrations estimated by the classic method. For this reason, by using the parameters of Ado E/[A] curves, the16.1 nM and 20.4 nM CPA were converted into 25.5 µM and 36.9 µM Ado concentrations, respectively. For reasons explained in the introduction, these Ado levels do not indicate an increase in $[Ado]_{ISF,}$ but exogenous Ado concentrations in the organ bath. These Ado levels are able to generate the increase of $[Ado]_{ISF}$ in the direct environment of the A_1 receptors in the tissue of the atrium. As Ado is rapidly eliminated from the cardiac tissue, there may be a considerable difference between the concentrations of Ado in the tank and those in the direct environment of the receptors (as the $[Ado]_{ISF}$ in the environment of A_1 receptors is the product of a dynamic balance of diffusion, transport and elimination). Consequently, a conversion of CPA levels into Ado concentration values carries the same bias into the results as the classic method does. In this way, although it is not possible to compare our data to those in the literature, it is possible to compare Ado levels obtained by the two methods (classic *vs.* RRM).

An analysis of the Ado concentrations generated by the increase of $[Ado]_{ISF}$ as a result of NT suggest that the variety in the results is probably caused by the variety of NT blockers (DP vs. NBTI) and not by the difference between the two analytical methods (classic vs. RRM). It offers the conclusion that the application of CPA in the course of RRM did not considerably distort the description of changes in $[Ado]_{ISF}$. It underpins our initial hypothesis. It is to be noted that in the classic method we did not use anything apart from the negative inotropic effect of the NT inhibitors, parameters of intact Ado E/[A] curves and the Langmuir-Hill equation.

Our experiments with *in vivo* untreated animals offered the following conclusions: (1.) In the case of using different agonists, the RRM produces relative indices, but a larger relative index always suggests the higher equi-effective concentration of the agonist to be specified. In

order to obtain reliable data, it is worth to use relative indices in the case of agents difficult to determine. Instead, it is better to use relative indices. Furthermore, (2.) NBTI is more suitable for studying the effects of NT inhibition than the less selective DP.

In the *in vivo* treated animals, we compared the changes in $[Ado]_{ISF}$ elicited by NT inhibition between eu- and hyperthyroid atria. As carried out in the course of our preliminary experiments, we described the changes in $[Ado]_{ISF}$ with the help of RRM, based upon the inotropic response to CPA. We used selective ENT1 inhibitor NBTI as an NT inhibitor.

The NBTI reduced the response to CPA in all atria. As the NBTI is a selective ENT1 inhibitor, we ascribed the drop in the effects of CPA, at least in part, to the increased amount of interstitial Ado. It suggests hyperthyroidism does not change the direction of NT observed in the euthyroid guinea pig atrium. It appears to confirm our assumption that accumulated extracellular Ado is responsible for the negative inotropic effect of NBTI. According to this, ADA blunted the effect of NBTI on the response to CPA. The values of c_x, determined by RRM, corresponded to the findings described above.

Before the interpretation of the values of c_x, it is necessary to consider that hyperthyroidism changes the activity of several of the enzymes and transporters of Ado. As in this case we did not use data from the Ado E/[A] curve for the RRM, and CPA is not influenced by the enzymes and transporters of the bloodless tissue of the atrium, the CPA E/[A] curves reflected the functional status of the A_1 receptors. In a hyperthyroid status the very function of the A_1 receptor declines. In did not, however, distorted the results of a comparison of the eu- and hyperthyroid states by RRM, because we compared the NBTI and ADA+NBTI treated sub-groups to the same *in vivo* treated controls. The thyroid hormone-dependent functions of the A_1 receptor are included in the E_{max}, EC_{50} and n parameters of the CPA E/[A] curves of control atria. These parameters define the base status in the equation used for determining the concentration.

The CPA equivalent of the extra $[Ado]_{ISF,}$ caused by the NT inhibition was twice as high in the case of hyperthyroid atria (37 nM), as in the euthyroid ones (18.5 nM). It suggests that (in the case of no inhibition) the intensity of Ado intake through ENT1 increases as a result of T_4 treatment in the well-oxygenated guinea pig atrium. Increased NT may contribute to the reduced effect of Ado and well-transported Ado-analogues in a hyperthyroid heart, by reducing the chance of Ado binding to cell-surface Ado receptors.

An increased influx of Ado through ENT1 may, theoretically, be caused by the following changes in hyperthyroidism: (1.) higher activity of extracellular enzymes producing

Ado; (2.) higher activity of intracellular enzymes eliminating (degrading/reutilizing) Ado; (3.) a larger participation of ENT1 in the entire NT. Whatever is the exact mechanism, in our findings thyroid hormones do not reverse the inward driving force of Ado. This inward driving force may, in theory, be reinforced by concentrative carriers generating a transport opposite in direction to that of ENT1, but we found no such data in the related literature. What is more, in the generally view, there is exclusively equilibrative nucleoside carrier, and within that, ENT1 is found on the cardiomyocytes. It suggests that the preserved or increased Ado in a hyperthyroid heart, the inward driving force is supposedly generated by the preserved or increased net interstitial Ado generation.

Our experiments, carried out on left atria isolated from *in vivo* S and T_4 treated animals, suggest the following: (1.) In hyperthyroid myocardium the inhibition of ENT1 causes a higher accumulation of interstitial Ado than under euthyroid circumstances. (2.) When examining the background to this, we were led to the conclusion that T_4 did not reverse the net direction of NT. On the contrary: in the cardiomyocytes of hyperthyroid atria (with no inhibition present) inward transport of Ado through ENT1 increased. (3.) RRM provided evidence that in the course of NT blockade, A_1 receptors are stimulated in both eu- and hyperthyroid atria, and this stimulation is larger in the hyperthyroid preparations. As A_1 receptors and the processes they initiate are important elements of the protection of the heart against ischaemic and hypoxic damages in many species (including guinea pigs as well as humans), NT inhibitors are likely to play a major role in the further investigations of the hyperthyroid heart.

SUMMARY

In our studies, two methods were used to characterize and compare the change in $[Ado]_{ISF}$ elicited by NT blockade: an algebraic calculation based on the relationship expressed in the Langmuir-Hill equation, and a regression analysis by fitting a concentration determining equation derived from the Langmuir-Hill equation („receptorial responsiveness method": RRM). On the basis of our results, the RRM enables the characterization of a change in concentration of an agonist or a modulator in the microenvironment of its receptor, when the Langmuir-Hill equation cannot be used due to degradability of the given agent. The impact of this method stems from the fact that concentration of degradable agents is difficult to assess in the compartment containing the receptors of the agents. By means of the RRM, changes in concentration of degradable agents practically immeasurable directly can be characterized by concentrations of stable agents, which have signal-transduction similar to degradable agents in question.

With the help of the index provided by the RRM, changes in concentration of degradable agents become comparable even between tissues with different enzyme and transporter activities. It is well-known that several enzyme and carrier activities are modified in the hyperthyroid heart. Since these alterations hardly affect elimination of CPA, a relatively stable compound in asanguineous atrial tissue, the change in $[Ado]_{ISF}$ accompanying NT blockade can be reliably characterized by the equieffective concentration of CPA computed by the RRM (c_x). Our results show that NT inhibition causes an increase in c_x approximately 2-2.5 fold greater in hyperthyroid atria than in euthyroid ones, indicating a greater increase in $[Ado]_{ISF}$ elicited by NT blockade in hyperthyroid atria. It can be concluded that hyperthyroidism does not alter the physiological direction of NT; moreover it does enhance NT directed to the cell interior as compared to the euthyroid condition.

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