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

EFFECT OF THYROXINE TREATMENT ON THE ADENOSINERGIC MECHANISMS IN GUINEA PIG ATRIAL MUSCLE

Dr. Gesztelyi Rudolf

DEPARTMENT OF PHARMACOLOGY AND PHARMACOTHERAPY
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Dr. Gesztelyi Rudolf

Advisor: Dr. Szentmiklósi József

Head of the Program: Dr. Gergely Lajos

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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

ADP = adenosine-5'-diphosphate

AMP = adenosine-5'-monophosohate

ARA-A = 9-beta-D-arabinofuranoside

ATP = adenosine-5'-triphosphate

AV = atrioventricular

cAMP = 3',5'-cyclyc adenosine monophosphate

CF = coformycin

cGMP = 3',5'-cyclyc guanosine monophosphate

ClAdo = 2-chloroadenosine

CPA = N^6-cyclopentyladenosine

CPX = DPCPX

DAdo = 2'-deoxyadenosine

DCF = 2'-deoxycoformycine

DP = dipyridamole

DPCPX = 8-cyclopentyl-1,3-dipropylxanthine

E/[A] = concentration-response

EHNA = *erythro*-9-(2-hydroxy-3-nonyl)adenine

ENT1 = equilibrative and nitrobenzylthioinosine sensitive nucleoside transporter

$K_{Ach/Ado}$ = acetylcholine activated K^+ channel

L_{Ca} = L-type Ca channel

M = mol/l

MC = metacholine (acetyl- β-methylcholine)

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

NECA = 5′-(N-ethylcarboxamido)adenosine

NO = nitric oxide

NT = sarcolemmal nucleoside transport

PDE = 3',5'-cyclyc nucleoside phosphodiesterase

PDE_2 = cGMP stimulated 3',5'-cyclic nucleotide phosphodiesterase

PDE_3 = cGMP-inhibited 3',5'- cyclic nucleotide phosphodiesterase

PLB = phospholamban

8-PT = 8-phenyl-theophylline

RIA = radioimmunoassay

R-PIA = (R)-N^6-(1-methyl-2-phenylethyl)adenosine

RRM = receptorial responsiveness method

T_3 = L-triiodothyronine

T_4 = L-thyroxine (L-tetraiodothyronine)

S = solvent of L-thyroxine

SD = standard deviation

SEM = standard error of the mean

SAH = S-(5'-adenosyl)-L-homocysteine

SR = sarcoplasmic reticulum /muscle SER/

SERCA = SR Ca^{2+}-ATP-ase (SR Ca^{2+}-pump)

VERA = verapamil

INTRODUCTION

The role of adenosine (Ado) receptors (A_1, A_{2A}, A_{2B}, A_3) in both physiological and pathological conditions became more and more explicit over the past few decades. A theory emerged that endogenous biological responses evoked by adenosine basically determine the activity of all cell types. The theory of Ado receptor is getting more sophisticated, and signal transduction mechanisms fundamental of the effect of receptor activation are being elucidated. However there is relatively limited information available concerning the modulation of Ado's myocardial and vascular effects, by the metabolic and hormonal environment as well as the modulation of purinergic mechanisms by endocrine and paracrine relations. It can be supposed that Ado an endogenous substance possessing cardiovascular activity is part of a complex regulatory mechanism that allows the adaptation of myocytes and vascular smooth muscle cells to the changing environment, rather than producing an isolated effect on the target cell.

Thyroxine (T_4) and its active metabolite triiodothyronine (T_3), have diverse cardiovascular effects. The well known cardiovascular effects of hyperthyroidism are produced by the complex biochemical changes coming from the activation of triiodothyronine's nuclear receptors (including the isoforms of the gene coding the myosine heavy chains, the upregualtion of myosine Ca^{2+}-ATP-ase, influence of the expression of phospholamban etc).

It is well known that thyroxine pretreatment brings about fundamental changes in the different receptors throughout the body. A classical example of this type of regulation is seen on the level of α- and β- adrenoceptors. Hypothyroidism greatly decreases the receptor density of α- adrenoceptors parallel to the substation increase of β- adrenoceptor density. This classical theory has evolved somewhat over the past few years as some receptor subtype selectivity was identified, e.g. in T_4 treated rats decrease in the number of α_{1A} and α_{1B} receptors as well as increase in the number of α_{1D} receptors were reported similarly to the situation concerning β-adrenergic receptors where a differential regulation is seen as the β_1-receptors are upregulated while the number of β_2-receptors stay unchanged or even decrease.

In addition to all this excessive T_4 decreases the number of muscarinergic receptors and enhances the serotonin and thromboxane induced vascular contractions.

WORKING HYPOTHESIS, GOALS

There is only limited information available concerning the effect of sustained T_4 on cardiovascular receptors. There was some information that hypothyroidism enhances Ado mediated adenylyl cyclase activation in adipocytes, but scarce data is about the vascular smooth muscle cells or cardiac myocytes. During our experiments we investigated four related problems in electrically paced isolated guinea pig atrial myocardium:

1. It is well known that a the effect of thyroid hormones and Ado overlap as the proteins regulating intracellular Ca^{2+} level and Ado's effects on contractility converge. In spite of this the adenosinergic mechanisms in the pathogenesis of hyperthyroidism have not been elucidated in sufficient details. Furthermore the significance of intracellular adenosinergic mechanisms isn't recognized as these effects are masked by that of the cell surface Ado receptors. Prior investigations however indicated that under pathological conditions (e.g. hypoxia) intracellular mechanisms assume a more significant role. Our goal was to determine the possible change in the role of intracellular binding sites during sustained T_4 exposition.

2. In close relation to this prior assumption we compared the effect of nucleoside transport (NT) blockers and adenosine deaminase (ADA) fundamental for regulating the tissue level and distribution of Ado in both eu- and hyperthyroid cardiac atria. In the well oxygenated heart NT inhibitors increase extracellular and decrease intracellular Ado levels parallel to the increase of total Ado content. Meanwhile ADA inhibitors generally increase the cytoplasmic Ado concentration in guineas pig atria, since here ADA is strictly localized to the intracellular compartment. Coming from this the comparison of the effect of NT and ADA inhibitors we wished to investigate the significance of intracellular Ado concentration in the eu- and hyperthyroid atria.

3. Inhibition of cGMP-stimulated 3',5'- cyclic nucleotide phosphodiesterases (PDE_2) by EHNA enhanced the L-type Ca^{2+} current in human, while failing to do so in rat heart. The effects of PDE_2 inhibition however were not previously investigated in guinea pig atrial myocytes, whereas the effect of PDE_2 inhibition on inotropy was not even determined on any species previously. Our goal was to work out an experimental design that allows the assessment of the inotropic effect of PDE_2 in both eu- and hyperthyroid state.

4. Currently accepted methods used for the quantification of $[Ado]_{ISF}$ provide estimations ranging from nano- to micromolar concentrations due to the diverse effects coming from Ado's metabolism and compartmentalization (Table 1.). Accordingly the measured changes in the $[Ado]_{ISF}$ also depend on the method used (Table 1.), hindering the quantitative assessment of agent producing a change in $[Ado]_{ISF}$. Out of these considerations we set out to work out a method that is suitable for the precise approximation of the changes of A_1 receptor agonists in the receptorial microenvironment, on basis of the relationship described by the Langmuir-Hill equation. In order to do this an E/A curve has to be constructed on the investigated tissue. The E/A curves constructed with Ado, however strongly depend on the functional state of the tissue's nucleoside transporters and the functional state of Ado's metabolizing enzymes since a substantial amount of Ado is metabolized by the time of reaching the cell surface receptors. Therefore we aimed to work out a method that is able to define the change in the tissue concentration of A_1 receptor agonists regardless of the hormonal state, thus the comparison of the change in $[Ado]_{ISF}$ (e.g. in response to NT blockade) may become possible between the eu- and hyperthyroid heart.

MATTERIALS AND METHODS

MATTERIALS

The following materials were used: adenosine (Ado); 2'-dezoxiadenosine (DAdo); 9-β-D-arabinofuranoside (ARA-A); N^6-cyclopentyladenosine (CPA); (R)-N^6-(1-methyl-2-phenylethyl)adenosine (R-PIA); 2-chloroadenosine (ClAdo); 5′-(N-ethylcarboxamido)adenosine (NECA); acetyl- β-methylcoline chloride (MC); (±)-verapamil (VERA); *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA); S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (NBTI); kantaridin; ciklopiazonsav; dipyridamole (DP); coformycin (CF); L-tiroxin (T_4) (Sigma, St. Louis).

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 PRETREATMENT

Male guinea pigs weighing 500-700 g were used. One group of the animals received 330 μg/kg L-thyroxine sodium salt pentahydrate (T_4) daily ip. for 8 days (in vivo T4 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 on day 9.

DETERMINATION OF T_3 AND T_4

In order verify the thyroid state blood samples obtained from the animals at the time of sacrifice were allowed to clot then centrifuged. T_3 and T_4 levels were determined from the serum with the aid of radioimmunoassay (RIA) method.

The serum T_3 and T_4 level was 3.1 (±0.4) and >280 nM; while it was <0.38 and 32.7 (±2.9) nM (significant), in the T_4 and S treated groups respectively, thus T_4 therapy was efficient.

DETERMINAITON OF ADENOSINE DESAMINASE ACTIVITY IN INTACT ATRIAL TISSUE

To assess the inhibitory effect CF confers on ADA the methods of Rogler-Brown and colleagues (1978) was adapted. Ammonium content was quantified on basis of the Kingsley és Tager (1970) method.

DETERMINATION OF THE CONTRACTILE FORCE IN ELECTRICALL PACED ATRIAL MYOCARDIUM

Guinea pigs were killed by a blow on the head after ether narcosis. Following the opening of the thorax the hearts were quickly removed, and placed into oxygenated room temperature Krebs solution. Left atria were isolated and set up in 10 ml vertical organ baths (TSZ-04, Experimetria, Budapest) containing regular Krebs solution of (mmol*l^{-1}): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; NaH_2PO_4 1.0; $MgCl_2$ 1.3; $NaHCO_3$ 24.9 and glucose 11.5; oxygenated with the mixture of 95% O_2 and 5% of CO_2, and kept at 37 ^oC. During the 60 minute equilibration period the initial tension was adjusted to get maximum contractions. The preparations were incubated for 60 minutes in normal Krebs solution allowing the equilibration of contractile parameters. The drugs were administered in this steady state. The stimulation was carried out by platinum electrodes of a programmable electro stimulator (ST-02 Experimetria, Budapest). The basal stimulation frequency was 3 Hz. The stimulation was performed out by rectangular impulses of 1 ms at supramaximal voltage (twice the threshold voltage). Isometric contractions were measured by means of a transducer (SD-01 Experimetria, Budapest) converting the mechanical signals for the double ray oscilloscope (EMG 1555, Type TR-4657, EMG Budapest EO 213, RFT Dresden), recorded by a 6 channel polygraph (BR-61, Medicor Budapest). The dose response curves were carried out according to the cumulative method of Van Rossum (1963). Throughout the investigations the isometric contractions were recorded. The amplitude of these contractions were used to quantify the contractile force.

IN VIVO ELECTROPHYSIOLOGICAL INVESTIGATIONS IN ANAESTHETIZED ANIMALS

Guinea pigs were fixed on an electrically isolated investigational board following pentobarbital narcosis (30 mg/kg i.p.). Following subcutaneous needle electrodes were implanted to the extremities of the animals. The standard ECG leads were recorded by a three channel ECG device.

PROTOCOLS

The initial E/[A] curves

The preparations were incubated for 60 minutes after the initiation of electrical pacing in normal Krebs solution allowing the equilibration of contractile parameters. The bathing solution was changed in every 15-20 minutes (washing). During this time the atria were randomly assigned to the in-vitro treatment group defined by the protocol (in vitro treatment).

After this stabilization period, a cumulative E/[A] curve was constructed with Ado in all groups. The steady state effect was denoted by the evolution of sustained lowest contractile force seen after the administration of the agent. (Figure 3.).

The first E/[A] curve may have had 2 distinct fates regarding further manipulations. If the second E/[A] curve was also constructed with Ado then the first curve served as a control, while if a different agent was used following incubation (e.g. CPA, metacholine (MC) or verapamil (VERA) than the Ado E/[A] curves were only used to assess the samples with respect to the inclusion criteria used for data analysis. In these cases a distinct subgroup provided the control data of the *in vitro* treatment.

In vitro treatment and further E/[A] curves

After the construction of the first E/[A] curve a 50 minutes long incubation was performed in the presence of the predefined concentrations of the investigative agent(s) (*in vitro* treatment). Next, a cumulative E/[A] curve was generated. In some protocols this was followed by a further washout-incubation- E/[A] curve generation cycle. During data analysis the contractile force prior to the construction of the second E/[A] as well as the parameters of the second and third E/[A] curves were used.

DATA ANALYSIS

Contractile force

The intact contractile force of a preparation was defined as the initial contractile force seen upon constructing the first E/[A] curve. The change of contractile force produced by the in vitro treatment was expressed as the percentage decrease of the initial force and it was used to characterize the inotropic effect of the preparation.

Concentration-response curves

The negative inotropic responses to Ado and CPA were expressed as the percentage decrease of contractile force

$$E = \frac{F_0 - F_c}{F_0} \cdot 100\%$$

where: E, the response (effect); F_0, the resting contractile force (the contractile force in equilibrium before the administration of the first agonist dose); F_c the contractile force produced by the given [A] agonist concentration.

The individual E/[A] curve data were characterized by fitting the Langmuir-Hill equation (based on the law of mass action)

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

where: c, the concentration of the agonist; E, the effect at c; E_{max}, the maximal effect (maximum efficacy, asymptote); EC_{50}, the agonist concentration producing half maximal effect (midpoint location); n_H , the Hill coefficient (midpoint slope). The data are expressed as mean ± standard error of the mean (SEM).

The responsiveness to specific agonists were characterized by the following E/[A] curve parameters: E_{max}, EC_{50} and n_H. These parameters were used latter for statistical analysis as well as for concentration estimation (see Results).

Statistical analysis

Statistical analysis: Three criteria had to be met by each atrium in order to be included in examination: (1) The resting contractile force had to reach 1 mN before the first E/[A] curve. (2) The mechanical activity of the paced atrium had to be regular. (3) After fitting the Langmuir-Hill equation to the individual Ado E/[A] curve data (for atria meeting the first two criteria) to determine the individual Emax, EC50 and nH parameters, the response to Ado dose closest to the EC50 (10 μmol/l) had to be within the mean ± 2 standard deviations (S.D.) range. All atria that met the three inclusion criteria were processed.

If all data sets passed the normality test and the equal variance test, means were compared by one-way ANOVA with Newman-Keuls post-testing. If any data set failed, then Kruskal-Wallis test with Dunn's post-testing was performed. Ado concentrations computed by the two procedures were compared by using two-way ANOVA, as well. Values of $P<0.05$ were considered to be significant.

The means of the computed CPA and Ado concentrations were generated two ways: by averaging the individual values (for statistical analysis) and by averaging the input data before the concentration estimation (in order to counteract the biasing effect of the biological diversity and measurement errors). The individual Ado concentrations were compared both including and excluding outliers. Values out of the mean ± 2 S.D. interval were considered to be outlier in each data set.

For statistical analysis and curve fitting, GraphPad Prism version 4.03 for Windows was used.

RESULTS AND CONCLUSIONS

The major conclusions of the experiments presented in my thesis:

A./ Chang in the activation of extra- and intracellular binding sites in T_4 treated guinea pig atria

Extracellular adenosine receptor agonists

1. The thypreotoxic state of experimental animal treated with T_4 for 8 days could be characterized with the following changes: weight loss, increase of the rectal temperature, heart rate, marked elevation of serum T_4 and T_3 concentration, and increase of the ratio of left atria to body weight.
2. Ado (0,01 μM - 1mM) concentration-dependently reduced the atrial contractile force. No significant differences were detected among the same in vivo treated subgroups, thus the solvent as well as the T4 treated group proved to be homogenous. The pD_2 value of the S and T_4 treated atria was 4,94 ± 0,09 (n = 26) and 4,08 ± 0,08 respectively ($p < 0,01$), and the maximal effect was also significantly decreased by T_4 treatment.
3. CPA also induced a concentration-dependent decrease in the atrial contractile force (Fig. 2).
4. Effect of T4 treatment: The hyperthyroid atria showed smaller response to CPA and R-PIA (a specific agonist of the A_1 adenosine receptor) than the same in vitro treated euthyroid counterparts, as the pD_2 values for CPA and R-PIA in the S and T_4 treated atria were 7.74 ± 0.09 and 7.14 ± 0.08, as well as 6.75 ± 0.11 and 6.25 ± 0.19 respectively. The decrease of efficacy was statistically significant ($p < 0.01$) as well as the decrease in the A pD_2 value.
5. These values were as follows for the less specific A_1 receptor agonist ClAdo (small A_2 receptor agonist effect) the pD_2 value was 7.17 ± 0.16 and 6.29 ± 0.16 in the solvent and T_4 treated atria ($p < 0.01$). And again the E_{max} values also decreased.
6. A smaller shift in the E/[A] curves was seen in response to the NECA that activates both A_1 and A_2 receptors upon the comparison of eu- and hyperthyroid specimens. The pD_2 value was 7.72 ± 0.07, and 7.26 ± 0.18 in the S and T_4 treated atria ($p < 0.05$). E_{max} vas also significantly decreased in this case as well.

Intracellular P-site agonists

1. We used Dado and ARA-A as P-site agonists throughout our experiments. These agents met the criteria for P-site activity in our experimental design: (a) their effect was not influenced by methylxantines (8-PT), (b) the inotropic effect increased after the activation of adenylyl cyclase by forskolin, (c) blockade of transsarcolemmal purinergic transport by NBTI significantly decreased their effect.
2. DAdo and ARA-A evoked concentration dependent but restricted negative inotrtopic response that was even smaller in the T_4 treated atria. The -logEC_{25} was 3.05 ± 0.12 and 2.60 ± 0.09 for the S and T_4 treated atria respectively. Similarly the ARA-A effect was also less remarkable in the T_4 treated group.
3. The effect of Ado (extracellular Ado receptor and P-site agonist) was only minimally potantiate by CF in solvent treated atria as the efficacy of Ado doubled on basis of the pD_2 values. A significant enhancement in efficacy was seen in the T_4 treated atria in the presence of Ado as a 10 fold increase in sensitivity was seen.
4. In order to delineate extracellular Ado effects we conducted experiments in the presence of 10 μM 8-PT. Under these conditions a 7 fold increase in efficacy was seen in the control atria (this was only 2 fold in atria in the absence of 8-PT). After T_4 treatment efficacy increased by 16 fold and 8 fold in the presence and absence of 8-PT respectively.
5. DAdo moderately decreased the contractile force of the electronically paced solvent treated atria while after inhibiting ADA by CF this effect increased 24 fold. The T_4 treated atria showed a much more pronounced increase in efficacy as after incubating with 8 μM CF a 765 fold increase was seen (!).
6. We saw similar tendencies with ARA-A the other P-site agonist as well. Here efficacy increase by 3 and 12 fold in the solvent and T_4 treated atria respectively on basis to the EC_{25} values computed.
7. The characteristics of enhancement was similar when another ADA inhibitor (EHNA 10 μM) was used instead of CF-

Conclusions

On basis of the experiments presented in the dissertation it may be concluded that T_4 treatment significantly decreases the response to Ado as reflected by the pD_2 and E_{max} values. A similar phenomenon is seen in case of NT and synthetic Ado analogues that are not the substrate of ADA (CPA, ClAdo, R-PIA, NECA), and also have efficacy in the extracellular binding sites of Ado receptor. Since the guinea pig atria expresses A_1 receptors, on basis of the results we may suppose that the effects at least in part are manifested at the level of this receptor.

Ado in addition to binding to extracellular A_1 receptors it also activates the intracellular P site. Therefore we investigated whether T_4 treatment differentially modulates the alteration of Ado response. It is known the CF and EHNA are a specific and non-specific blockers of the intracellular ADA respectively. Since the guinea pig atria does not possess extracellular ADA, the inhibition of intracellular ADA primarily significantly increases the intracellular Ado concentration. We showed that Ado effect was greatly enhanced after ADA inhibition in the T_4 treated animals thus it may suggest that T_4 treatment may enhance the ADA activity, parallel to the decrease in the effect of the intracellular component while the number and affinity of P sites also increase. This phenomenon is only revealed after the inhibition of ADA. This decrease in inotropy was so remarkable that the phenomenon known in classical pharmacology as "Umkehr"-effect was seen, thus inversely the contractility increased. In the presence of Coformycin a dramatic enhancement of P site activation was seen in T_4 treated atria, thus it is supposed that in addition to enhanced ADA activity, the P site is also upregulated.

We saw an interaction between extra- and intracellular P site effects as the increase of efficacy was only 2 fold in the S treated atria after ADA inhibition by CF while this became 8 fold after 8-PT treatment. We presume a negative cooperation between extracellular Ado receptors and intracellular P site.

We do not know what the role of P site is in hyperthyroid state. It should be highlighted that the P site effects decrease in the ADA is left to function while they dramatically increase upon ADA inhibition, thus under pathological conditions (hypoxia) when intracellular ADA activity greatly decreases this may assume great significance.

B./ Changes seen following nucleoside transport blockade and adenosine desaminase inhibition in hyperthyreoid atria

1. NT blocker DP (3 μM) and NBTI (1 μM), as well as the ADA inhibitor EHNA (10 μM) and CF (8 μM) both potantiate the effect of Ado in the S and T_4 treated atria. NT inhibitors significantly decreased pD_2 (leftward shift of the E/[A] curve), while not influencing the E_{max} value. On the contrary ADA inhibitors significantly decreased pD_2 whiel greatly increased E_{max} values.
2. The potency ratio obtained by comparing the EC_{50} values of the Ado E/[A] curves (regarding the state seen prior and following the first Ado E/[A] curve construction) didn't differ significantly between the eu- and hyperthyreoid specimens. On the contrary the potency ratio of hyperthyreoid atria was significantly higher in the than that of the euthyroid.
3. Chantaridine (0.5 μM) a phophoprotein phosphatase (PP1 and PP2A type) produced a small decrease of Ado's effect that only became statistically significant in the presence of EHNA. The inhibitory effect of cantharidine seen in the presence of EHNA was much more remarkable in the T_4 treated atria than in the S treated ones.
4. Cyclopyazonic acid inhibitory of SERCA (20 μM) didn't influence the rune of the Ado E/[A] curve nor in the presence neither in the absence of EHNA.

Conclusions

The major findings of these experiments were as follows: (i) specific parameters of E/[A] curves for adenosine-induced depression of mechanical activity (pD_2 and E_{max}) in guinea pig atrial myocardium were significantly reduced after an 8-day thyroxine treatment, (ii) in thyroxine-treated atria, membrane purine transport inhibitors (dipyridamole, NBTI) induced similar leftward shifts in E/[A] curves for adenosine in both euthyroid and hyperthyroid atrial myocardium without influencing the depressed maximum responses, (iii) the leftward displacement induced by inhibitors of intracellularly located ADA (EHNA, coformycin) was more prominent in myocardium obtained from thyroxine-treated guinea pigs than in those prepared from solvent-treated animals. In addition, inhibitors of ADA induced a complete reversal of the maximum adenosine actions, (iv) inhibition by cantharidin of serine/threonine protein phosphatases type 1 (PP_1) and type 2A (PP_{2A}) differently affected the

adenosine-induced reduction of force development in myocardial tissues depending on the absence or presence of ADA inhibitors. When ADA inhibitor was not applied, cantharidin did not alter the responses evoked by the purine nucleoside in any of the treatment groups. On the other hand, after the inhibition of ADA, cantharidin was capable of attenuating the purine-induced responses in the mechanical activity. The inhibition of the adenosine action was significantly stronger in the hyperthyroid atria. (v) Pharmacological inhibition of SERCA by cyclopiazonic acid did not alter the adenosine-induced cardiac responses in either euthyroid or hyperthyroid tissues.

The difference may be explained by the fact that while NT blockers increase $[Ado]_{ISF}$ while decreasing the intracellular Ado level the inhibition of ADA directly increases the level of intracellular Ado. This points to the possibility that primarily elevating the cytosolic Ado concentration by ADA inhibition and the enhancement of $[Ado]_{ISF}$ by NT inhibition differently influence the adenosinergic mechanisms. This proposition is supported by the fact that the environment altered by excessive T_4 altered the effect of ADA inhibitors. A further support is that chantaridine barely inhibited the negative inotropic effect of Ado (partially mediated by phosphatases), while if ADA was functionally eliminated the inhibition became explicit especially in the hyperthyroid state. Since cyclopiazonic acid the inhibitor of SERCA didn't alter the inotropic response to Ado the SERCA-PLB system probably fails to assume a role in the guinea pig lef atria as a molecular effectory, as well as in the adenosinergic responses mediated by ADA inhibitors.

These results correlate well with the biochemical changes seen in hyperthyroidism as described in the scientific literature, thus provide further insight to the signal transduction mechanisms of Ado.

C./ Alteration of the PDE_2 inhibitory effect in hyperthyroidism

1. The PDE_2 inhibitory effect of EHNA (the inhibitor of both ADA and cGMP stimulated phosphodiesterases (PDE_2)) may be more specifically investigated in it is described by its influence on the ADA-resistant CPA E/[A] curve.
2. T_4 treatment doesn't influence the contractile force in guinea pig atria.
3. EHNA more significantly potentiated the negative inotropic effect of CPA in T_4 treated atria than in the S treated ones.

Conclusions:

The positive inotropic effect of PDE_2 inhibition is hard to assess in the euthyroid state, since EHNA the most frequently used selective PDE_2 inhibitor (among PDE inhibitors), is also a strong inhibitor of ADA; thus the elevated tissue adenosine level decreases the cAMP level to the extent that the increase of cAMP due to PDE_2 inhibition is not revealed. T_4 treatment enhanced the positive inotropic effect of PDE_2 inhibition, a synergism possibly mediated by the effects of thyroid hormones and EHNA on the L-type Ca^{2+} channels. When evaluating the effect of EHNA however inhibition of ADA must also be accounted for that may take part in the enhancement of the response to CPA *via* the previously discussed mechanism as ADA inhibition in hyperthyreoid atria may more expensively enhance the adenosinergic signal transduction as compared to the euthyroid state.

D./ A new method for the concentration estimation of A_1 receptor agonists

By using mathematical tools, we were able to characterize the following phenomenon: We suppose that the model contained either the test agent or another one using the same signal-transduction pathway ("surplus agent") in excess of zero or a conventionally ignored baseline level ("surplus concentration") before and during the construction of the E/[A] curve. Plotting this E/[A] curve, the test agent appears to be less efficient than it would be in the absence of the surplus concentration. The cause of this apparently decreased efficiency is the surplus agent, which, by occupying a part of the binding sites, partially consumes the ability to respond of the specific receptors even before generating the E/[A] curve with the test agent. Based on the law of mass action, the decrease in the response to the test agent correlates with the surplus concentration, therefore it is possible to obtain an index of the surplus concentration by fitting an appropriate equation) to the E/[A] curve generated in the presence of the surplus concentration. This equation must contain the Emax, EC_{50} and n_H parameters of the E/[A] curve constructed with the test agent in the absence of the surplus concentration (otherwise upon the same conditions), to define the real relationship between the concentration of the test agent and the evoked response. The index produced is either an estimate of the surplus concentration (when the surplus and the test agent are the same) or a relative value (if the surplus and the test agent are different). The relative index denotes the

concentration of the test agent that is equieffective with the surplus concentration. This method was denoted the receptor responsiveness method (RRM).

Conclusions:

The major advantage of the RRM is that it enables the replacement of an unstable agent (or one that is difficult to determine due to any other cause) with a stable and well-determinable "test" agent sharing the same signal-transduction pathway during the concentration estimation utilizing the law of mass action. In our first attempt to address a question by the RRM, we found a considerable rise in $[Ado]_{ISF}$ equivalent to 16.1 or 20.4 nmol/l CPA in the presence of 10 μmol/l DP or NBTI, respectively (see: Mean 2 values in Table 3). Due to the multi-layer preparations used, these CPA concentrations generally characterize the elevation in $[Ado]_{ISF}$ at the cardiomyocyte A1 receptors of isolated guinea pig atrium.Our results show an interstitial Ado accumulation upon ENT1 blockade in all atria, which was more extensive in the hyperthyroid samples (2-3 times greater with regards to the CPA equivalents of the surplus $[Ado]_{ISF}$), suggesting an enhanced Ado influx via ENT1 in hyperthyroidism. It is concluded that hyperthyroidism does not alter the prevailing direction of the Ado transport, moreover intensifies the Ado influx in the guinea pig atrium.

GENERAL CONCLUSIONS

Upon reaching a milestone in the scientific work, one must summarize the results in a way that these are placed into the general context of scientific knowledge on the topic, while further investigative goals, visions also should be voiced in order to move on with the science.

On basis of the results presented in this current dissertation it could be said that there are remarkable links between the thyroid state and the receptorial adenosine effects. T_4 clearly decreases receptorial A_1 response, and under special circumstances (ADA inhibition) it enhances intracellular P-site mechanisms.

It is well known that hyperthyroidism increases the density and sensitivity of β-adrenoceptors, leading to well circumscribed clinical manifestations. At the same time it is also known that Ado and the extracellular Ado analogues as well as P site activators also decrease the myocardial effects of endogenous agents that utilize the AC pathway (epinephrine, histamine, norepinephrine etc.). Upon the comparison of these effects with our results the assumption seems to stand that sustained presence of T_4 initiates a process in myocytes that may alter catecholamine effects.

(1) It is known on basis of the scientific literature that excessive T_4 elevates tissue Ado concentration by speeding up the metabolism (our current RRM investigations also support this). This increase in Ado concentration possibly moderates the β-adrenergic effects of catecholamines. (2) Our data show that the presence of T_4 decreases the response to Ado, thus it may be very well assumed that that this enhances the β-adrenergic effects of catecholamines, as the effect of endogenous Ado is decreased. This was T_4 upregulates β-adrenoceptors and also sensitizes these receptors. Based on our experiments we propose that this sensitization is in part due to the decreased responsiveness to Ado (3) According to data published in international journals P site agonist also have inherent catecholamine inhibitory effects, thus we think that P site upregulation will decrease the catecholamine effect if the pronounced effect of thyroid hormones will lead to a mismatch between oxygen delivery and utilization. Since previous results of our workgroup showed that hypoxia substantially decreases ADA activity, this may lead to the restriction of β-adrenergic stimulation *via* the activation of the intracellular binding sites (AC inhibition) resultant of increased intracellular Ado levels.

If our assumption is correct than this adenosinergic mechanism would provide appropriate basis for the myocardial effects of T_4 hormones, especially for the analysis of myocardial effects mediated by the β-adrenoceptors.

The investigation of this working hypothesis will determine our future research goals, as we set out to provide more and more insight into this interesting and yet not completely clarifies topic with the use of modern molecular biology techniques.

IN EXTENSO PUBLICATIONS IN SUPPORT OF THE THEESIS

Gesztelyi R, Zsuga J, Bajza Á, Cseppentő Á, Szabó JZ, Szentmiklósi AJ (2003) Special sensitization pattern in adenosine-induced myocardial responses after thyroxine treatment. *J Pharmacol Sci* 91 (4): 295-304 (IF: 1.419)

Gesztelyi R, Zsuga J, Hajdú P, Szabó JZs, Cseppentő Á, Szentmiklósi AJ (2003) Positive inotropic effect of the inhibition of cyclic GMP-stimulated 3',5'-cyclic nucleotide phosphodiesterase (PDE_2) on guinea pig left atria in eu- and hyperthyroidism. *Gen Physiol Biophys* 22: 501-513 (IF: 0.794)

Gesztelyi R, Zsuga J, Juhász B, Dér P, Vecsernyés M, Szentmiklósi AJ (2004) Concentration estimation via curve fitting: quantification of negative inotropic agents by using a simple mathematical method in guinea pig atria. *Bull Math Biol* 66 (5): 1439-1453 (IF: 1.485)

CITABLE ABSTRACTS IN SUPPORT OF THE THEESIS

Cseppentő Á, Ujfalusi A, Zsuga J, Gesztelyi R, Kovács P, Szabó JZ, Szentmiklósi AJ (1999) The effect of chronic thyroxine treatment on cardiovascular adenosine receptors. *Fundam Clin Pharm* 13(Suppl 1): PT54 (IF: 0.81)

Gesztelyi R, Zsuga J, Bajza Á, Cseppentő Á, Szabó JZ, Szentmiklósi AJ (2002) Modified responsiveness of atrial myocardium to inhibitors of membrane purine transport and adenosine deaminase after chronic thyroxine treatment. *Acta Physiol Hung* 89(1-3): 171

OTHER *IN EXTENSO* PUBLICATIONS:

Gesztelyi R, Fekete I, Kellermann M, Csiba L, Bereczki D (1999) Screening for depression among post-stroke outpatients in Eastern Hungary. *J Geriatr Psych Neur* 12: 194-199 (IF: 0.881)

Kellermann M, Fekete I, Gesztelyi R, Kollár J, Sikula J, Csiba L, Bereczki D (1999) Screening for depressive symptoms in the acute phase of stroke. *Gen Hosp Psychiat* 21: 116-121 (IF: 1.4)

Körtvély Á, Szigeti G, Gesztelyi R, Zsuga J, Bányász T, Magyar J, Szigligeti P, Kovács L, Jednákovits A, Szentmiklósi AJ, Nánási PP (2000) Cardiovascular effects of BRX-005: comparison to bimoclomol. *Life Sci* 67: 1783-1789 (IF: 1.808)

Kellermann M, Gesztelyi R, Kovács A, Bereczki D (2001) Post-stroke depresszió – irodalmi áttekintés. *Psychiat Hung* 16 (4): 407-417

Zsuga J, Pórszász R, Gesztelyi R, Németh J, Szolcsányi J, Szilvássy Z, Paragh G, Kovács P (2003) A hepatikus inzulin szenzitizáció mechanizmus szenzoros nitrerg jellege éber nyulakon. *Metabolizmus* I. évolyam 1. szám 57-59

Rubovszky B, Szentmiklósi AJ, Márián T, Cseppentő Á, Gesztelyi R, Székely A, Fórizs F, Gáspár R, Krasznai Z (2003) Comparative pharmacological studies on the A2 adenosine receptor agonist 5'-n-ethyl-carboxamidoadenosine (NECA) and its F^{19} isotope labelled derivative (FNECA). *J Pharmacol Sci* 93 (3): 356-363 (IF: 1.419)

Zsuga J, Gesztelyi R, Török J, Kéki S, Bereczki D (2005) Asymmetric dimethylarginine: A molecule responsible for the coexistence of insulin resistance and atherosclerosis via dual nitric oxide synthase inhibition. *Med Hypotheses* 65 (6): 1091-1098 (IF: 0.607)

Zsuga J, Gesztelyi R, Bereczki D (közlés alatt) Aszimmetrikus dimetilarginin: egy új rizikófaktor az ateroszklerózis és inzulinrezisztencia kialakulásában. *Metabolizmus*

OTHER CITABLE ABSTRACTS

Bereczki D, Gesztelyi R, Kellermann M, Fekete I, Csiba L (1996) A post stroke depressziós zavarok elõfordulása: prospektív epidemiológiai vizsgálat. *Agyérbetegségek* 1996(3): 13

Bereczki D, Gesztelyi R, Kellermann M, Fekete I, Csiba L (1996) Post stroke depressive disorders in the acute and chronic phases of stroke: a prospective study. *Eur J Neurol* 3(Suppl 5): 51

Szerafin T, Leny A, Szentgyörgyi L, Gesztelyi R, Szentmiklósi AJ, Péterffy Á (2003) Effect of hydrostatic and pulsatory distension on free flow, vasoreactivity and endothelium of the radial artery grafts (Abstract). *Coronary Artery Dis* (IF: 1.303)

CONFERENCE PARTICIPATION

Kellermann M, Gesztelyi R (1996) Depresszív hangulatzavar a stroke acut szakaszában: prospektív vizsgálat cerebrovascularis osztályon. A Debreceni Orvostudományi Egyetem Tudományos Diáktalálkozója Ph.D. TDK

Gesztelyi R, Kellermann M (1996) A post-stroke depresszió rehabilitációs szakában: prospektív vizsgálat cerebrovascularis szakrendelésen. A Debreceni Orvostudományi Egyetem Tudományos Diáktalálkozója Ph.D. TDK

Gesztelyi R, Bereczki D, Fekete I, Kellermann M, Csiba L (1996) A post-stroke depresszió a stroke rehabilitációs szakaszában: prospektiv vizsgálat cerebrovascularis szakrendelésen. Fiatal Neurológusok Fóruma, Miskolc

Kellermann M, Bereczki D, Fekete I, Gesztelyi R, Csiba L (1996) Post-stroke depresszív zavar: prospektív epidemiologiai vizsgálat a stroke acut szakaszában cerebrovascularis osztályon. Fiatal Neurológusok Fóruma, Miskolc

Kellermann M, Bereczki D, Gesztelyi R, Fekete I (1998) A post stroke depresszió előfordulása: prospektív epidemiológiai vizsgálat. A Magyar Pszichiátriai Társaság IV. Nemzeti Kongresszusa

Gesztelyi R, Zsuga J (2000) Thyroxin kezelés hatása a myocardiális A1-, valamint a vascularis A_1- és A_{2B} típusú adenosine receptorokon mediálódó válaszokra. DEOEC ÁOK Ph.D. és TDK Tudományos Diáktalálkozója

Gesztelyi R (2001) A kardiovaszkuláris adenosine receptorválaszok időfüggő módosulása thyroxin kezelés és azt követő „wash-out" periódus során. DEOEC ÁOK Ph.D. és TDK Tudományos Diáktalálkozója

Gesztelyi R, Zsuga J, Bajza Á, Cseppentő Á, Varga A, Szabó JZ, Szentmiklósi AJ (2001) Extra- és intracelluláris adenosine receptorválaszok időfüggő módosulása tiroxin kezelés során tengerimalac bal pitvari myocardiumon. MÉT LXVI. Vándorgyűlése, Szeged

Gesztelyi R, Zsuga J, Cseppentő Á, Bajza Á, Szabó JZ, Szentmiklósi AJ (2003) Foszfoprotein foszfatáz gátlók hatása az adenosine kardiodepresszív hatására kísérletes hipertireózisban. MÉT LXVII. Vándorgyűlése, Pécs

Gesztelyi R, Zsuga J, Cseppentő Á, Vecsernyés M, Szentmiklósi AJ (2004) Az A_1 típusú adenosine receptor-válasz változásának felhasználása adenosine analógok interstitiális koncentrációjának meghatározására. MÉT LXVIII. Vándorgyűlése, Debrecen

Gesztelyi R, Karsai D, Zsuga J, Szentmiklósi AJ, Tósaki Á (2004) A nukleozid transzport gátlásának hatása az interszticiális adenosine szintjére eu- és hipertiroid tengerimalac bal pitvaron. Magyar Kísérletes és Klinikai Farmakológiai Társaság VI. Kongresszusa, Magyar Klinikai Farmakológusok VI. Továbbképző Kongresszusa, Debrecen

Gesztelyi R, Karsai D, Zsuga J, Vecsernyés M, Tósaki Á (2005) An old-new approach for estimating the change in interstitial adenosine concentration in guinea pig left atria. A Magyar Experimentális Farmakológia Tavaszi Szimpóziuma, Budapest

Zsuga J, Gesztelyi R, Karsai D, Szentmiklósi AJ, Tósaki Á (2005) Implementation of concentration-response curves for concentration estimation: the extension of the scope of the Langmuir–Hill equation. A Magyar Experimentális Farmakológia Tavaszi Szimpóziuma, Budapest