

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**The receptorial responsiveness method (RRM), a tool to allow a
new insight into the A₁ adenosinergic and M₂ muscarinergic
regulatory mechanisms in eu- and hyperthyroid guinea pig atria**

BY: Dr. Krisztián Pák

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Table of contents

1	Introduction	1
1.1	Main objectives	1
1.2	Adenosine and adenosine receptors	2
1.3	Receptor reserve	3
1.4	Thyroid state	4
1.5	The significance of the interstitial adenosine level in the heart	5
1.6	Interaction between the A ₁ adenosinergic and M ₂ cholinergic systems	6
2	Materials and methods	7
2.1	Materials	7
2.2	Animals and preparations	7
2.3	Arrangement of investigations underlying the present thesis	8
2.4	Experimental groups and protocols for the Study 1	8
2.5	Experimental groups and protocols for the Study 2	9
2.6	Empirical characterization of E/c curves	10
2.7	Quantification of the E/c curve change caused by NBTI and DCF	10
2.8	Correction of adenosine's effects in the presence of NBTI or DCF	11
2.9	Data analysis	13
3	Results	15
3.1	Results of Study 1	15
3.1.1	Adenosine E/c curves of Protocols 1 and 2 before the correction	15
3.1.2	CPA E/c curves	15
3.1.3	Adenosine E/c curves of Protocols 1 and 2 after the correction	16
3.2	Results of Study 2	17
3.2.1	MC E/c curves	17
3.2.2	CPA E/c curves	18
3.2.3	Corrected MC and CPA E/c curves	19
4	Discussion	20
4.1	Interpretation of results in Study 1	20
4.2	Interpretation of results in Study 2	22
5	Summary	27
6	Acknowledgements	28

1 Introduction

1.1 Main objectives

Currently, the major challenges to health and life for the low-income countries are principally infectious diseases and the shortage of life needs. By contrast, in the high-income countries, the leading cause of death is the group of cardiovascular diseases.

Despite the rapidly evolving therapeutic strategies in this field, ischemic heart disease, the most common cardiovascular disease, has remained a major cause of fatality in the decade prior to this writing, causing 7.4 million deaths in 2012 alone, with 3 in every 10 deaths worldwide resulting from cardiovascular diseases overall in that year.

In terms of proportion of deaths caused by noncommunicable diseases (NCDs), high-income countries have the highest value – 87% of all deaths resulted from NCDs – followed by the upper-middle-income countries (81%). The proportions are lower in the lower-middle-income (57%) and low-income countries (37%).

Based on the aforementioned, a basic understanding of endogenous protective mechanisms of the heart against ischemia is a necessity for the development of new rational therapeutic strategies.

Accordingly, our first aim was to test our hypothesis, i.e. the risk for weakening of atria in response to A_1 receptor agonists might be lower in hyperthyroidism, in a model that allows characterization of the effect exerted by T_4 treatment on the A_1 receptor reserve belonging to the direct negative inotropic effect of adenosine. For this purpose, a set of special experimental protocols, based on construction of concentration-response curves, were used, followed by a unique evaluation procedure, which is an adaptation of the receptorial responsiveness method (RRM). This experimental system was designed to prevent the rapid intracellular elimination of exogenous adenosine, and then to correct for the bias caused by the consequent accumulation of endogenous adenosine.

In turn, our second goal was to develop an experimental framework suitable to gain further insight into the functional consequences of ADA inhibition, as regards the A_1 receptor-mediated direct negative inotropy, under both eu- and hyperthyroid conditions. This was important because ADA inhibition elevates the adenosine levels and thus

augments all A₁ receptor-mediated processes, therefore it is not easy to identify the efficiency enhancing action of ADA inhibition on the A₁ adenosinergic signaling, an effect that was proposed in a previous study. Another goal of the present study was to assess the alteration in the interstitial adenosine level caused by ADA inhibition by means of RRM, and then, to compare it with the change produced by nucleoside transport blockade, under both eu- and hyperthyroid conditions.

1.2 Adenosine and adenosine receptors

Adenosine is an adenine nucleoside that serves as an important regulator in the cardiovascular system. Adenosine molecules are constantly produced and eliminated in the body. Adenosine plays an important role in fundamental biochemical routes (such as energy transfer) as well as in signal transduction processes (as the endogen agonist of adenosine receptors).

Adenosine is the precursor and decomposition product of ATP (adenosine triphosphate), the most important energy transfer molecule in living tissues. Thus the level of this molecule indicates the exhaustion of the cells.

In normoxic cellular metabolism, the major portion of intracellular adenosine transforms to AMP with the help of adenosine kinase, while the remaining part is deaminated by the intracellular adenosine deaminase. Adenosine can be eliminated from the interstitial fluid in three ways: 1. breakdown by adenosine deaminase (ADA) 2. getting into the bloodstream 3. uptake into cells of certain tissues such as endothelium and myocardium.

In the heart, there are mainly equilibrative nucleoside transporter (ENT) transporters, especially ENT type 1 (ENT1), which is sensitive to inhibition with nitrobenzylthioinosine (NBTI). Therefore, the adenosine transport of cardiomyocytes is primarily determined by the difference between the intra- and extracellular adenosine concentration.

Regulatory effects of adenosine are mediated predominantly by activating cell-surface adenosine receptors. The A₁ adenosine receptor (A₁ receptor) exerts complex regulatory functions in almost all tissues, including the myocardium, in which the A₁ is the main adenosine receptor type. The myocardial A₁ receptor is involved in extensive

protective and regenerative functions. This includes negative tropic effects that limit energy consumption, and thereby contribute to the protection of the heart against ischemic-hypoxic damages.

Pharmacological activation of the A₁ adenosinergic system is a prospective, although yet modestly utilized possibility that may have preventive and therapeutic implications in numerous cardiovascular maladies, including ischemic heart disease and certain types of arrhythmias. Furthermore, the A₁ receptor stimulation as a therapy may come into play in avoiding hypoxic injury during heart transplantation, too.

The A₁ receptor mediates negative tropic effects on the heart that involve negative inotropic activity on both the atrium and ventricle. In the ventricle, A₁ receptor agonists evoke only an indirect negative inotropic effect, thereby reducing only the positive inotropic action of other agents. In contrast, A₁ receptor agonists can markedly decrease the atrial contractile force below the resting level (called direct negative inotropic effect) in most species, including guinea pigs and humans.

Accordingly, several classes of compounds that activate the A₁ receptor pathway (A₁ receptor agonists, A₁ receptor enhancers and agents elevating the endogenous adenosine levels) are under consideration or in use for a variety of indications, e.g. as antiarrhythmic, antianginal, antidiabetic and antinociceptive agents. Since these drugs, either directly or indirectly, target the same molecular object (A₁ receptor), a major safety-related challenge is to ensure the desired effect in a particular indication, while minimizing some or all other effects. In general, the direct negative inotropic effect can be considered as undesirable, because reduced atrial contractility may initiate or exacerbate a wide range of cardiovascular diseases.

1.3 Receptor reserve

The term ‘receptor reserve’ has been defined in the context of the traditional receptor theory. Receptor reserve refers to a phenomenon whereby stimulation of only a fraction of the whole receptor population apparently elicits the maximal effect, achievable in a particular tissue. The existence (and magnitude) of receptor reserve depends on the agonist (efficacy), tissue (signal amplification ability) and measured effect (pathways activated to cause signal amplification). Thus, in the most general sense, receptor reserve

is an integrative measure of the response-inducing capacity of an agonist (intrinsic efficacy) and of the signal amplification capacity of the corresponding receptor (and its downstream signaling pathways). As receptor reserve is very sensitive to agonist's intrinsic efficacy, it is usually defined only for full (high-efficacy) agonists. If receptor reserve is determined with the same (high-efficacy) agonist, it can be used as a practical measure of the signal amplification capacity of the receptor. Theoretically, signal amplification means that, on a percentage basis, the effect exceeds the receptor occupancy. In the experimental practice, the simplest index of a big signal amplification capacity (and thus, of a great receptor reserve) is the phenomenon that stimulation of even a small fraction of the whole receptor population apparently elicits the maximal effect.

As a consequence of the above-mentioned facts, high-efficacy agonists usually act on most tissues, expressing the given receptor as a full agonist. In turn, low-efficacy agonists exert significant effect only in tissues with large receptor reserve. Thus, use of low-efficacy agonists can ensure tissue selectivity in a sense that they will not evoke biologically significant effect in tissues with small (or no) receptor reserve.

Previous studies have found a considerable A_1 receptor reserve for the direct negative inotropic effect of synthetic A_1 receptor agonists and adenosine. The magnitude of the aforementioned receptor reserve was clearly demonstrated by an observation that FSCPX, a potent and irreversible A_1 receptor antagonist, was unable to significantly reduce the maximal effect of both the synthetic agonists and adenosine. This observation indicates a strong amplification of A_1 receptor stimulus, regarding the direct negative inotropy. Thus, among the possible cardiac side effects of agents that produce A_1 receptor stimulation, weakening of atria is a probable (if not the most probable) one.

1.4 Thyroid state

The thyroid state influences several regulatory mechanisms, including functions of the A_1 receptor. Among others, thyroid hormones (T_3 , T_4) markedly reduce the direct negative inotropic effect (decrease of the contractile force without prior positive inotropic stimulation exerted by another agent) of A_1 receptor agonists. Hyperthyroidism is a pathological condition that, by upregulating a wide range of metabolic processes, raises the oxygen and nutrient consumption in the tissues, and thus, increases the work of the heart. As a consequence, excess thyroid hormones increase the risk of congestive heart

failure, ischemic heart disease and arrhythmias, and thereby elevate cardiovascular mortality. In light of these facts, suppression of the A₁ adenosinergic system by thyroid hormones may raise concerns. Thus, it is of importance to find out possibilities to enhance the depressed function of the A₁ adenosinergic system in hyperthyroidism.

1.5 The significance of the interstitial adenosine level in the heart

Adenosinergic signaling is a powerful endogenous tissue-protective mechanism. The myocardial A₁ receptor is involved in extensive protective and regenerative actions. This includes negative tropic effects that limit energy consumption and thereby contribute to the protection of the heart against ischemic-hypoxic damages. The elevation of the interstitial adenosine level could trigger the adenosinergic signaling and thus initiate the cardiac protective mechanisms.

An opportunity to influence the interstitial adenosine concentration is to blunt adenosine deaminase (ADA), an enzyme that converts adenosine into inosine. Consistently, the inhibition of ADA increases both the intra- and extracellular adenosine concentrations and thereby augments actions of exogenous adenosine as well.

In a previous study, we found that inhibition of ADA increases the signal amplification of the A₁ adenosinergic system, regarding its direct negative inotropic function in the hyperthyroid guinea pig atrium. As ADA inhibition elevates the adenosine levels and thus augments all A₁ receptor-mediated processes, it is not easy to identify this particular (efficiency enhancing) action of ADA inhibition. For this purpose, a special experimental design would be needed that is suitable to distinguish the functional consequences of ADA inhibition, as regards the A₁ receptor-mediated direct negative inotropy.

Another way to manipulate the interstitial adenosine level is inhibition of the adenosine flux across the cell membrane. In the metabolically intact myocardium, adenosine mainly forms in the interstitium and is eliminated in the cell interior, therefore, the net adenosine transport is directed into the cardiomyocytes. In the heart, the transmembrane adenosine flux passes almost exclusively through ENT1. Accordingly, in our previous studies, NBTI was found to elevate the interstitial adenosine level in the guinea pig atrium, which action was more pronounced in hyperthyroidism.

However, in the functioning heart, information about changes of the interstitial adenosine concentration cannot be assessed with sufficient accuracy (at least for our purposes) by the commonly used methods, because of the rapid turnover and poor access of adenosine in the living tissues. Under well-defined circumstances, the receptorial responsiveness method (RRM), a method that has been recently developed but is rooted in the classical pharmacology, may address this problem. RRM is based on a simplified mathematical modelling of the interaction between two agonists that consume the response capacity of the same (or at least greatly overlapping) signal-transduction. This way, RRM enables the quantification of an acute increase in the concentration of an agonist *via* generating concentration-effect (E/c) curves with the same or another agonist (which latter is more stable or preferred for any other reasons) in the given tissue. As a limitation, if the two agonists are different, the surplus concentration in question can be quantified only with a surrogate parameter, i.e. the equieffective concentration of the other agonist. However, a unique feature of RRM is that - owing to its functional assay nature - it provides information about the agonist concentration in the vicinity of the specific receptors, a tissue compartment otherwise difficult to access in a working organ. Although RRM, in principle, can be applied for each receptor, the A₁ receptor is especially suitable for this purpose, due to its slow desensitization relative to the duration of the measurement.

1.6 Interaction between the A₁ adenosinergic and M₂ cholinergic systems

In the heart, the A₁ and M₂ receptors are the predominant receptor types for adenosine and acetylcholine, respectively. In the atrium, both A₁ and M₂ receptors bind to G_{i/o} proteins and, thereby, both of them can open the muscarinic-operated potassium channel and blunt the adenylyl cyclase activity with the consequent inhibition of all cAMP-dependent signaling pathways. As a consequence, both A₁ and M₂ receptors can mediate direct negative inotropic effect. Thus, although *via* binding to different receptors, agonists for these receptors activate greatly overlapping signal-transduction pathways in the atrium. Further similarities between A₁ and M₂ receptors are that both of them are controlled by thyroid hormones (T₃, T₄), and our knowledge about this regulation is incomplete and, in some areas, inconsistent yet.

2 Materials and methods

2.1 Materials

The following chemicals were used: L-thyroxine sodium salt pentahydrate (T₄); adenosine(non-selective adenosine receptor full agonist); N⁶-cyclopentyladenosine (CPA) (CPA; selective A₁ adenosine receptor full agonist); acetyl-β-methylcholine chloride (methacholine: MC; non-selective muscarinic receptor full agonist with high affinity for the M₂ muscarinic receptor (M₂ receptor)); 8-cyclopentyl-1,3-dipropylxanthine (CPX; selective, competitive A₁ adenosine receptor antagonist); 8-cyclopentyl-N³-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N¹-propylxanthine (FSCPX); S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (NBTI; selective inhibitor of nucleoside transporter type ENT1); pentostatin (2'-deoxycoformycin: DCF; selective inhibitor of adenosine deaminase) in NipentTM. Experiments were conducted in modified Krebs-Henseleit buffer (Krebs solution).

2.2 Animals and preparations

All animal use protocols were approved by the Committee of Animal Research, University of Debrecen, Hungary (3/2012/DE MÁB). Male Hartley guinea pigs weighting 600–900 g were used.

A group of animals received 330 µg/kg T₄ daily (*ip.*) for 8 days (*in vivo* T₄ treatment), and the vehicle of T₄ was administered daily (*ip.*) for 8 days to the other group (*in vivo* solvent treatment). On the ninth day, the animals were guillotined. Left atria were quickly removed and mounted at 10 mN resting tension in 10 ml vertical organ chambers (Experimetria TSZ-04) containing Krebs solution oxygenated with 95% O₂ and 5% CO₂ (36°C; pH 7.4).

Atria were paced by platinum electrodes (3 Hz, 1 ms, twice the threshold voltage), with the use of a programmable stimulator (Experimetria ST-02) and power amplifier (Experimetria PST-02). The contractile force was characterized by the amplitude of the isometric twitches, which were detected by a transducer (Experimetria SD-01) and strain

gauge (Experimetria SG-01D), and recorded by a polygraph (Medicor R-61 6CH Recorder).

2.3 Arrangement of investigations underlying the present thesis

Our investigations, according to our two major goals and the two publications used for the present thesis, were divided into two studies referred to as Study 1 and Study 2. The essence of protocols of both studies was the construction of two or three E/c curves on guinea pig atria. Between two E/c curves, *in vitro* treatments were carried out. In both studies, the direct negative inotropic function was assessed, because it is a strong, well-measurable and well-reproducible effect, mediated by the A₁ receptor located in the atrial myocardium. The isolated and paced left atria formed a greatly simplified model system, in which negative tropic effects of different agonists could manifest only in a decrease of the contractile force.

2.4 Experimental groups and protocols for the Study 1

Both solvent- and T₄-treated atria were randomized into six-six groups (the *in vivo* solvent and T₄ treatment were indicated with an S and T, respectively, in the group name). In each group, one of four protocols was carried out. Groups and protocols applied: S1 and T1 for Protocol 1 (demonstration of the effect of FSCPX on the adenosine E/c curve); S2 and T2 for Protocol 2 (attempt to determine the A₁ receptor reserve for adenosine); S3-Control, S3-NBTI, T3-Control and T3-NBTI for Protocol 3 (data collection to determine c_x, the CPA concentration that is equieffective with the surplus endogenous adenosine, accumulated interstitially in the presence of NBTI); S4-Control, S4-FSCPX, T4-Control and T4-FSCPX for Protocol 4 (data collection to compute the negative inotropic effect of c_x on the FSCPX-pretreated atria).

Protocol 1: After an incubation period in Krebs solution, a cumulative adenosine E/c curve was constructed. Subsequently, after a 15 min washout, atria were subjected to 10 μM FSCPX, a selective and irreversible A₁ receptor antagonist, for 45 min followed by a 75 min long washout with Krebs solution. Then, a cumulative adenosine E/c curve was generated.

Protocol 2: After an incubation period in Krebs solution, a cumulative adenosine E/c curve was constructed. After a 15 min washout, atria were incubated in 10 μ M NBTI, a selective nucleoside transport inhibitor, for 15 min. Then, a cumulative adenosine E/c curve was generated in the presence of 10 μ M NBTI (S2-NBTI and T2-NBTI curves). After a 20 min washout, atria were subjected to 10 μ M FSCPX for 45 min followed by a 60 min long washout with Krebs solution. Then, atria received 10 μ M NBTI and were incubated for 15 min. Afterward, a cumulative adenosine E/c curve was generated in the presence of 10 μ M NBTI.

Protocol 3: After an incubation period in Krebs solution, a cumulative adenosine E/c curve was constructed. After a 15 min washout, atria were randomized into two groups. Atria in the control groups received 10 μ l DMSO for 15 min, while atria in the NBTI groups received 10 μ M NBTI for 15 min. Then, a cumulative E/c curve was generated with CPA, a selective A₁ receptor full agonist, in the presence of 10 μ l DMSO or 10 μ M NBTI.

Protocol 4: After an incubation period in Krebs solution, a cumulative adenosine E/c curve was constructed. After a 15 min washout, atria were randomized into two groups. Atria in the control groups received 10 μ l DMSO for 45 min followed by a 75 min long washout, whereas atria in the FSCPX groups were subjected to 10 μ M FSCPX for 45 min, succeeded by a 75 min washout. Then, a cumulative CPA E/c curve was generated.

2.5 Experimental groups and protocols for the Study 2

First, all atria were allowed to equilibrate in Krebs solution for 40 min. Then, a cumulative concentration-effect (E/c) curve was constructed with adenosine.

After a washout period (Krebs solution for 15 min), atria were randomized into groups for the subsequent *in vitro* treatment. In the group names, the applied *in vivo* and *in vitro* treatments were indicated (S - solvent-treated; T - T₄-treated; Co - control; and abbreviations of the used chemicals): The *in vitro* treatment included 20 min incubation in the presence of Krebs solution alone (S Co, T Co, S Co (CPA), T Co (CPA)) or 10 μ M CPX (S CPX, T CPX) or 0.1% (v/v) DMSO alone (S DMSO, T DMSO) or 10 μ M NBTI (S NBTI, T NBTI) or 10 μ M DCF (S DCF, T DCF, S DCF (CPA), T DCF (CPA)) or 10 μ M DCF with 10 μ M CPX (S DCF CPX, T DCF CPX). Finally, a cumulative E/c curve

was generated with MC (S Co, T Co, S CPX, T CPX, S DMSO, T DMSO, S NBTI, T NBTI, S DCF, T DCF, S DCF CPX, T DCF CPX) or CPA (S Co (CPA), T Co (CPA), S DCF (CPA), T DCF (CPA)).

For the first E/c curve, adenosine was used to assess the responsiveness of the *in vitro* untreated atrial A₁ receptors. For the second E/c curve, MC or CPA (two relatively stable agonists for the M₂ or A₁ receptor, respectively) was administered in order to gather information about the effect of the different *in vitro* treatments on the M₂ and A₁ receptor responses.

2.6 Empirical characterization of E/c curves

The effect (defined as a percentage decrease in the initial contractile force), obtained from the experiments, was plotted against concentration of agonists administered. Both individual and averaged E/c curves were fitted to the Hill equation:

$$E = E_{\max} \cdot \frac{c^n}{c^n + EC_{50}^n} \quad \text{Equation 1}$$

where: c - the concentration of the agonist administered; E - the effect; E_{max} - the maximal effect; EC₅₀ - the agonist concentration producing half-maximal effect; n - the Hill coefficient.

Hill parameters (E_{max}, EC₅₀, n) of the individual E/c curves were used for the statistical analysis. Hill parameters of some averaged E/c curves were applied for the mathematical correction of some other E/c curves (see below).

2.7 Quantification of the E/c curve change caused by NBTI and DCF

The surplus interstitial adenosine, accumulated over the basal level in response to the inhibition of nucleoside transport or ADA, biased the shape of the E/c curves constructed in the presence of NBTI or DCF. This bias affects both concentration and

effect values of the E/c curves, because the biased effect values are plotted against concentration values of the administered (exogenous) agonist, while the concentration of the surplus endogenous adenosine is neglected. As DCF was found previously to influence the signaling efficiency of atrial A_1 adenosinergic system, the biased E/c curves, generated with the A_1 receptor agonist CPA in the presence of DCF, were excluded from the quantification. The bias of E/c curves, constructed with CPA or MC, was quantified with the use of RRM, by fitting the averaged data of the biased E/c curves to the following equation:

$$E' = 100 - \frac{100 \cdot \left(100 - E_{\max} \cdot \frac{(c_x + c)^n}{(c_x + c)^n + EC_{50}^n} \right)}{100 - E_{\max} \cdot \frac{c_x^n}{c_x^n + EC_{50}^n}} \quad \text{Equation 2}$$

where: E' *in Study 1*: the effect value of the averaged CPA E/c curve of the S3-NBTI or T3-NBTI group that is considered to be biased; E' *in Study 2*: the averaged effect value of the biased MC E/c curve of the group S NBTI, T NBTI, S DCF or T DCF; E_{\max} , EC_{50} , n *in Study 1*: the empirical parameters of the averaged CPA E/c curve of the S3-Control or T3-Control group; E_{\max} , EC_{50} , n , *in Study 2*: Hill parameters of the corresponding control-type averaged MC E/c curve of the group S DMSO, T DMSO, S Co or T Co, respectively; c *in Study 1*: the concentration of CPA (administered for the E/c curve); c *in Study 2*: the concentration of MC administered for the E/c curve; c_x *in Study 1*: the variable parameter of equation (2) indicating the CPA concentration that is equieffective with the surplus endogenous adenosine concentration accumulated by NBTI; c_x *in Study 2*: the variable parameter of equation (2) denoting the MC concentration equieffective with the surplus interstitial adenosine produced by NBTI or DCF.

2.8 Correction of adenosine's effects in the presence of NBTI or DCF

The correction procedure was performed as described previously First, an effect belonging to c_x was calculated by means of the Hill equation (equation 3):

$$E_x = E_{\max} \cdot \frac{c_x^n}{c_x^n + EC_{50}^n} \quad \text{Equation 3}$$

where: *in Study 1*: E_x , the effect evoked solely by the surplus endogenous adenosine accumulated by NBTI; c_x , the CPA concentration provided by equation (2); E_{\max} , EC_{50} , n , the empirical parameters of an appropriate CPA E/c curve (see the next paragraph); *in Study 2*: E_x , the effect evoked solely by the surplus interstitial adenosine produced by NBTI or DCF; c_x , the MC concentration conveyed by the equation 2 (belonging to the averaged MC E/c curve of the group S NBTI, T NBTI, S DCF or T DCF); E_{\max} , EC_{50} , n , Hill parameters of the corresponding control-type E/c curve (i.e. the averaged MC E/c curve of the group S DMSO, T DMSO, S Co or T Co, respectively).

When E_x was computed for the averaged S2-NBTI or T2-NBTI curve, empirical parameters of the averaged CPA E/c curve of the S3-Control or T3-Control group were substituted into equation (3), respectively. When E_x was calculated for the averaged S2-FSCPX+NBTI or T2-FSCPX+NBTI curve, empirical parameters of the averaged CPA E/c curve of the S4-FSCPX or T4-FSCPX group were written into equation (3), respectively.

Then, from effect values of a biased E/c curve (E') and the corresponding E_x , corrected effect values were computed with the use of the following equation (equation 4).

$$E = 100 - \frac{(100 - E') \cdot (100 - E_x)}{100} \quad \text{Equation 4}$$

where: *in Study 1* - E , the correct (unbiased) effect (belonging to the averaged S2-NBTI, T2-NBTI, S2-FSCPX+NBTI or T2-FSCPX+NBTI curve); E' , the biased effect (related to the foregoing curves); E_x , the effect of the surplus endogenous adenosine produced by NBTI (see equation 3); *in Study 2*: E , the corrected effect; E' - the biased effect; E_x , the effect of the extra interstitial adenosine produced by NBTI or DCF (belonging to the averaged MC E/c curve of the group S NBTI, T NBTI, S DCF or T DCF).

In order to correct effect values of MC E/c curves of groups S NBTI, T NBTI, S DCF and T DCF, the averaged biased effects of these E/c curves (as E') and the corresponding E_x values were substituted into the equation 4. For the correction of CPA

E/c curves of groups S DCF (CPA) and T DCF (CPA), averaged biased effects of these E/c curves were substituted into the equation 4 along with E_x values belonging to the averaged MC E/c curves of groups S DCF and T DCF, respectively. The reason to do this was that the amount and effect of the surplus interstitial adenosine, produced by DCF, did not depend on the nature of the agonist, used for a subsequent E/c curve. All corrected effects were plotted *versus* the MC and CPA concentrations administered for the given E/c curve.

2.9 Data analysis

Each atrium was required to meet three criteria in order to qualify for inclusion in the statistical analysis: 1) the initial contractile force had to reach 1 mN before the first E/c curve; 2) the mechanical activity of the paced atrium had to be regular; 3) the response to 10 μ M or 100 μ M adenosine of the solvent- or T₄-treated atrium, respectively, was required to be within a mean \pm 2 SD range. The mean and SD were computed using atria meeting the first two criteria (separately for the solvent- and T₄-treated population). All experimental outcomes conforming to these three criteria were subjected to statistical workup.

According to the recommendation of Motulsky and Christopoulos (2004), agonist concentration, EC_{50} and c_x in the equations, used for curve fitting, were expressed as common logarithms. Statistical significance for the difference of means (or medians) was defined at $p < 0.05$. Curve fitting and statistical analysis were performed with the use of GraphPad Prism 6.05, while other calculations were made by means of Microsoft Office Excel 2013.

In Study 1: All data sets were evaluated by the normality test and passed. Two data sets were compared with the paired or unpaired *t*-test (if the equal variance test was not passed, *t*-test with Welch's correction was used). More than two data sets were compared using one-way ANOVA or repeated-measures one-way ANOVA (with Geisser-Greenhouse correction), followed by Tukey post-testing.

In Study 2: Hill parameters of the pooled adenosine E/c curves (solvent-treated atria *vs.* T₄-treated ones) and raw E/c data of selected E/c curve pairs were compared with unpaired Student *t*-test or *t*-test with Welch's correction (if equal variance test was not

passed) or Mann-Whitney U-test (if either equal variance test or normality test were not passed). Hill parameters of adenosine E/c curves of the different groups were compared (separately for the solvent and T₄ treatment) by one-way ANOVA (using Geisser-Greenhouse correction) with Tukey post-testing, or by Kruskal-Wallis test with Dunn's post-testing (if the normality test was not passed). Hill parameters of the MC and CPA E/c curves were compared using two-way ANOVA with Sidak post-testing (as all data sets passed the normality test).

3 Results

3.1 Results of Study 1

3.1.1 Adenosine E/c curves of Protocols 1 and 2 before the correction

In the solvent-treated atria, consistent with our previously reported findings, pretreatment with FSCPX (selective and irreversible A_1 receptor antagonist) was observed to significantly shift the adenosine E/c curve to the right, whereas NBTI (selective nucleoside transport inhibitor) significantly displaced the adenosine E/c curve to the left and significantly decreased its E_{\max} , as compared with the corresponding control curves. The T_4 -treated atria responded to FSCPX pretreatment and NBTI the same way, although decrease in E_{\max} caused by NBTI did not reach the level of statistical significance.

In comparison with the corresponding curves generated in the presence of NBTI, the FSCPX pretreatment along with NBTI paradoxically increased E_{\max} (without affecting the other two empirical parameters) in both the solvent- and T_4 -treated atria. As a consequence, E_{\max} values of the FSCPX+NBTI curves are located between E_{\max} values of the corresponding control and NBTI curves, but differences are only statistically significant in the solvent-treated atria.

3.1.2 CPA E/c curves

CPA also reduced the contractile force of all atria in a concentration-dependent manner. In the solvent-treated atria, in agreement with our earlier results, NBTI significantly decreased E_{\max} (as well as Hill coefficient) and increased $\log EC_{50}$, while FSCPX pretreatment significantly increased $\log EC_{50}$, imitating the action of a competitive, rather than irreversible A_1 receptor antagonist. The T_4 -treated atria produced outcomes similar to the solvent-treated ones. The two main differences were that NBTI induced a more pronounced depression, whereas FSCPX pretreatment produced a smaller dextral displacement in the hyperthyroid CPA E/c curve, as compared to the corresponding control curves.

3.1.3 Adenosine E/c curves of Protocols 1 and 2 after the correction

As the interstitial adenosine levels in the microenvironment of A₁ receptors were unknown, only the effect values of the NBTI and FSCPX+NBTI curves could be corrected, which procedure was based on the equivalence of adenosine and CPA in their negative inotropic effect. Thus, for lack of a better option, the corrected effect values were plotted *versus* the concentration of exogenous adenosine in the bathing medium. For this reason, the most useful data, conveyed by these transformed E/c curves, are the corrected effect values belonging to the highest concentration (because after the saturation of the transformed E/c curves, the exact value of the surplus endogenous adenosine concentration caused by NBTI becomes irrelevant). The maximal corrected effect values uniquely represent the maximal negative inotropic responses, achievable with adenosine under the specified conditions in the guinea pig atrium. This is due to two facts. On one hand, NBTI enabled full saturation for the adenosine E/c curves *via* reducing the adenosine transport into the cell interior, the main site for adenosine elimination. On the other hand, the correction by means of RRM eliminated the bias caused by the endogenous adenosine accumulated by NBTI.

Negligible differences were found between the maximal effect values of the averaged S2-Control curve and the corrected S2-NBTI curve. Thus, NBTI was unable to enhance the maximum of the direct negative inotropic response to adenosine in the euthyroid atrium. In contrast, the maximal effect value of the corrected T2-NBTI curve considerably exceeded that of the T2-Control curve. This fact shows that NBTI significantly enhanced the maximum of the direct negative inotropic effect of adenosine in the hyperthyroid atrium.

The major features of the corrected curves representing the euthyroid status (S2-NBTI and S2-FSCPX+NBTI) were the same as those observed in our earlier study: they changed places with each other, as compared to the original curves, and their final parts got close to each other, indicating a great A₁ receptor reserve for the direct negative inotropic effect of adenosine. These characteristics also apply to the corrected hyperthyroid curves (T2-NBTI and T2-FSCPX+NBTI), indicating that T₄ treatment did not significantly influence the aforementioned A₁ receptor reserve. However, while the corrected S2-FSCPX+NBTI curve ran (a bit) below the S2-Control curve at the two highest adenosine concentrations, the final part of the corrected T2-FSCPX+NBTI curve ran considerably above the T2-Control curve (similar to the corrected T2-NBTI curve).

Experiments underlying the results of Study 1 were carried out by the author of this thesis (approximately 75%) and his supervisor (about 25%). Statistical analysis was made by the supervisor, while interpretation of the outcome was also a result of collaboration (author: 40%; supervisor: 60%)

3.2 Results of Study 2

3.2.1 MC E/c curves

Response to MC: MC also decreased the contractile force of atria in a concentration-dependent manner (direct negative inotropic effect).

Effect of CPX and DMSO on the response to MC: The control-type groups (Co, CPX, DMSO) receiving the same *in vivo* treatment did not differ significantly from one another, when either the responses to the different MC concentrations or the Hill parameters of the MC E/c curves (data not shown) were compared. This observation indicates that DMSO, vehicle of CPX and NBTI, and CPX did not influence significantly the response to MC.

Effect of T₄ on the response to MC: Based on the comparison of groups T Co and T DMSO to their solvent-treated counterparts (S Co and S DMSO), the T₄ treatment moderately suppressed the response to MC, which was only significant at higher MC concentrations. In line with this, the T₄ treatment caused a moderate diminution in E_{max} (significant) and n (on the border of statistical significance), while the increase of logEC₅₀ did not reach the significance threshold.

Modification of the response to MC by NBTI: In both the solvent- and T₄-treated groups, NBTI significantly reduced the response to MC, according to the conventionally plotted (and thereby biased) E/c curves. This manifested in a significant decrease of E_{max} and in a minor increase of logEC₅₀ with a practically unchanged. The effect of NBTI was more intense in the group T NBTI than in the group S NBTI.

Effect of NBTI on the interstitial adenosine level: Based on the depression of the conventionally plotted MC E/c curves generated in the presence of NBTI, the surplus interstitial adenosine was found to be equieffective with 101.2 nM and 151.1 nM MC in the solvent- and T₄-treated atria, respectively. It means that nucleoside transport blockade produces a greater interstitial adenosine accumulation in the T₄-treated atria than in the

solvent-treated ones, consistent with our earlier studies, in which CPA served as an agonist for the E/c curves.

Modification of the response to MC by DCF: DCF decreased the response to MC in both the solvent- and T₄-treated atria, according to the conventionally plotted (biased) E/c curves. This bias was rather symbolic under euthyroid conditions, while it was well-marked in hyperthyroidism. In turn, CPX abolished this effect of DCF on the MC E/c curves in both thyroid states. These observations indicate that DCF exerted its effect on the response to MC the same way as NBTI did, i.e. *via* elevating the interstitial adenosine level. It should be noted that the effect of DCF on the response to MC was statistically significant only in the T₄-treated atria (except for the response at 10 nM MC in the group S DCF, but it was considered irrelevant). Consistently, the decrease of E_{max} was only significant in the group T DCF.

Effect of DCF on the interstitial adenosine level: Fitting the equation 2 to MC E/c data of groups S DCF and T DCF, the surplus interstitial adenosine proved equieffective with 28.05 nM and 44.36 nM MC in the solvent- and T₄-treated atria, respectively. This outcome is similar to that seen in response to NBTI, i.e. DCF appears to produce a greater interstitial adenosine accumulation in the T₄-treated atria than in the solvent-treated ones. However, DCF increased the interstitial adenosine level to a smaller extent than NBTI did, in both the solvent- and T₄-treated groups.

3.2.2 CPA E/c curves

Response to CPA: CPA also reduced the atrial contractile force in a concentration-dependent manner (direct negative inotropic effect).

Effect of T₄ on the response to CPA: Comparing groups T Co (CPA) and S Co (CPA), the T₄ treatment considerably decreased the response to CPA that was significant from medium to high concentrations. In agreement with this, T₄ treatment significantly reduced both E_{max} and n, and increased logEC₅₀. Thus, T₄ induced a greater depression of the E/c curve for CPA than for MC.

Modification of the response to CPA by DCF: In contrast to that seen with MC, DCF augmented the response to CPA in both the solvent- and T₄-treated atria, according to the conventionally plotted (biased) E/c curves. While this effect of DCF was minor in the solvent-treated atria, it was significant in the T₄-treated ones in the lower and medium concentration ranges. In agreement with this, DCF significantly decreased logEC₅₀ of E/c

curves of T₄-treated but not solvent-treated atria. This outcome is consistent with our previous finding that inhibition of ADA enhances the efficiency of the direct negative inotropic function mediated by the A₁ receptor in the hyperthyroid guinea pig atrium.

3.2.3 Corrected MC and CPA E/c curves

The corrected E/c curves have two points of interest, the starting and final ones. The starting point shows E_x, the effect belonging to c_x, while the last point reflects the maximal response of the given system to the agonist in question. As c_x values have been addressed previously, herein the emphasis is on the final point of the corrected E/c curves, specifically on its position relative to the last point of the corresponding control E/c curve (this latter considered to be *a priori* correct).

NBTI with MC: The corrected MC E/c curves (generated in the presence of NBTI) ended somewhat below their control curves. Thus, if we consider the observed small difference between maximal values of the corrected and control E/c curves to be an error, it can be concluded that NBTI does not affect the efficiency of the M₂ muscarinergic signaling, irrespectively of the thyroid state.

DCF with MC: The corrected MC E/c curves (constructed in the presence of DCF) ran to the maximum of their control curves. Conclusions to be drawn are the same as those with NBTI, i.e. DCF does not influence the signal amplification of the atrial M₂ muscarinergic mechanisms, regardless of the T₄ treatment.

DCF with CPA: Effect values of the corrected CPA E/c curves (generated in the presence of DCF) exceeded their control effect values at each concentration, even at the highest one. This finding indicates that DCF augments the efficiency of the A₁ adenosinergic system regarding its direct negative inotropic function in the guinea pig atrium. This phenomenon was visibly more pronounced in the T₄-treated atria, in agreement with our previous finding. However, the present result denotes that DCF can exert its efficiency enhancing effect even in the euthyroid state.

Experiments for Study 2 were performed by the author (≈50%) and Tamás Erdei, a TDK (Students' Research Society) student (≈50%). The statistical workup was made by the supervisor. Experimental data were interpreted by the author (≈40%) and the supervisor (≈60%).

4 Discussion

4.1 Interpretation of results in Study 1

To the best of our knowledge, our Study 1 is the **first** to show that T₄ treatment does not substantially affect the A₁ receptor reserve appertaining to the direct negative inotropic effect of adenosine in the guinea pig atrium. **Secondly**, results of the present research revealed that reduction of intracellular adenosine elimination with the use of NBTI considerably augments the maximal response to adenosine in the hyperthyroid but not euthyroid atrium.

In two previous studies, we observed substantial receptor reserve for negative inotropy by use of stable synthetic agonists and adenosine, the degradable physiological agonist. This outcome led to a hypothesis that agents producing A₁ receptor activation, even those with low efficacy, may significantly weaken the mechanical activity of atria.

Hyperthyroidism is a pathological condition that modifies numerous elements of the A₁ adenosinergic signaling pathways. As a consequence, thyroid hormones reduce the effect of A₁ receptor agonists on atrial contractility, although the underlying mechanisms are not fully clarified yet. Thus, it might be expected that thyroid hormones affect, presumably reduce, the great atrial A₁ receptor reserve belonging to the direct negative inotropic effect. The aim of the present study was to test this possibility.

The present investigation revealed that T₄ treatment did not substantially influence the A₁ receptor reserve appertaining to the direct negative inotropic effect of adenosine, although it significantly suppressed the direct negative inotropic response to both adenosine and CPA. This result suggests that administration of agents causing A₁ receptor stimulation, irrespective of their indication of use, presents a similar risk in eu- and hyperthyroid hearts for weakening of atria. Thus, when an A₁ receptor agonist is administered in increasing concentrations to the whole body, this effect can be expected foremost among the A₁ receptor-mediated adverse cardiac effects in both eu- and hyperthyroid conditions. The major finding of the present study, i.e. unchangingness of A₁ receptor reserve for the direct negative inotropic effect of adenosine may be surprising, with regard to the observation that a given A₁ receptor agonist concentration decreases the contractile force to a lesser extent in the hyperthyroid atrium than in the euthyroid one.

The significance of this finding is that weakening of atria worsens the booster pump function and thereby decreases the ventricular filling. Additionally, the decreased atrial pumping capacity increases the risk for atrial thrombus formation. For these reasons, it is important to consider that atrial contractility may decrease during the use of agents that cause A₁ receptor stimulation even in hyperthyroid patients. It should be noted that these detrimental consequences may differ in extent in different individuals and arise more frequently with the coexistence of certain conditions, such as worsened ventricular filling for other reasons (mitral stenosis, restrictive or hypertrophic cardiomyopathy and pericarditis) and procoagulant states.

After the correction of adenosine *E/c* curves constructed in the presence of NBTI, it has also been established that nucleoside transport blockade produces a greater increase in the maximal response to adenosine in the hyperthyroid atria than in the euthyroid ones. Thus, although the direct negative inotropy evoked by adenosine is suppressed in hyperthyroidism, there is a greater possibility of it, increasing in hyperthyroidism than in euthyroid condition. This observation corroborates previous observations that the nucleoside transport capacity was increased in the hyperthyroid rat ventricle, and the inward adenosine transport was enhanced in the hyperthyroid guinea pig atrium, as compared to their euthyroid controls. The increased inward adenosine transport is likely to contribute to the suppressed response to adenosine in hyperthyroidism, because it removes adenosine faster from the interstitium and thus from the microenvironment of binding sites of A₁ receptors.

A limitation of Study 1 is that conclusions were drawn from experiments performed on guinea pigs. The extrapolation of our results to humans is based on the similarity of guinea pigs and humans, with regard to the atrial A₁ receptor and its downstream signaling pathways.

In summary, the present investigation has revealed that, although the A₁ receptor-mediated direct negative inotropic effect is suppressed in hyperthyroidism, the signal amplification capacity belonging to this effect seems to be similarly great in both eu- and hyperthyroid states. This finding suggests that if an A₁ receptor agonist, even a partial one, is administered for any indication, a decrease of atrial contractility will be a probable side effect under both eu- and hyperthyroid conditions. It is possible (but not inevitable) that this adverse effect occurs even at A₁ receptor agonist (or enhancer) concentrations that are necessary to evoke a desired effect anywhere in the body. In addition, the present study has demonstrated that nucleoside transport blockade considerably augments the

maximum of the direct negative inotropic effect of adenosine in the hyperthyroid but not euthyroid guinea pig atrium.

4.2 Interpretation of results in Study 2

The **first** finding of Study 2 is that, in the guinea pig atrium, ADA inhibition (but not nucleoside transport blockade) enhances the efficiency of the direct negative inotropic function of the A₁ adenosine (but not M₂ muscarinic) receptor. This finding suggests that inhibition of ADA affects the atrial A₁ adenosinergic system in a part other than the joint signaling pathways of the A₁ and M₂ receptors. **Secondly**, ADA inhibition enhances the A₁ adenosinergic direct negative inotropy even in the euthyroid state, although to a less extent than in hyperthyroidism. This outcome implies that ADA inhibition can partially reset the A₁ receptor-mediated direct negative inotropy suppressed by thyroid hormones. **Thirdly**, ADA inhibition produces a smaller rise in the interstitial adenosine concentration than nucleoside transport blockade does. **Fourthly**, our results demonstrate that T₄ treatment suppresses the direct negative inotropic function of the M₂ receptor in a guinea pig model as well. Nevertheless, this reduction in the M₂ muscarinergic function in response to thyroid hormones is quite small relative to the decrease in the A₁ adenosinergic one.

Previously we found that ADA inhibition elicited by DCF potentiated the direct negative inotropic effect of CPA, a selective A₁ receptor agonist, in the hyperthyroid guinea pig atrium. Since CPA is not a substrate for ADA, this counterintuitive result was attributed to that ADA inhibition increases the signal amplification of the atrial A₁ receptor and/or its downstream signaling pathways under hyperthyroid conditions. We assumed that this efficiency enhancing effect of ADA inhibition may be associated with the intracellular adenosine accumulation rather than the interstitial one (which latter is otherwise responsible for the stimulation of the cell-surface A₁ receptors, a known trigger of several beneficial effects of ADA inhibition. This assumption is supported by the fact that blockade of the physiologically inward nucleoside transport, which also increases the interstitial adenosine concentration but decreases the intracellular one, does not enhance the efficiency of the atrial A₁ adenosinergic system under either euthyroid or hyperthyroid conditions.

The major difficulty to investigate this phenomenon is the fact that ADA

inhibition, besides enhancing the efficiency of A₁ receptor function, elevates the tissue adenosine content, which leads to A₁ receptor activation that also augments the A₁ receptor-mediated functions (a well-known effect). In the experimental setup used for the above-mentioned study, this problem occurred in a form that the decrease of the contractile force (evoked by the surplus interstitial adenosine caused by ADA inhibition) interfered with the efficiency enhancing effect of ADA inhibition on the A₁ receptor-mediated direct negative inotropy. To clarify this case, we provide a brief explanation. When DCF was administered, the interstitial adenosine level increased and the surplus adenosine exerted a direct negative inotropic effect on the atria. So, this condition served as baseline for the further manipulations, i.e. administration of CPA to generate an E/c curve. As the surplus interstitial adenosine had already consumed a part of the response capacity of the A₁ adenosinergic system, a biased (smaller than expected) response to CPA was detected. Thus, the two actions of ADA inhibition (mentioned at the top of this paragraph) worked against each other in our earlier experimental setup.

In the present study we aimed to separate these two actions of ADA inhibition in order to gain a deeper understanding of the influence of ADA inhibition on the regulation of contractility of the atrium. To address this challenge, we repeated our previous experiments with the replacement of CPA with MC, a muscarinic receptor agonist with high affinity for the M₂ receptor.

The key concept of the experimental design used for the present study is as follows: If the A₁ receptor and ADA are inhibited simultaneously, the direct negative inotropic effect of the surplus interstitial adenosine produced by ADA inhibition can be prevented (with the consequent preservation of the response capacity of the A₁ adenosinergic system). However, the major signaling pathways, underlying the direct negative inotropy, remain accessible from the M₂ receptor. In this setup, if the molecular target, the change of which is responsible for the enhanced efficiency of the direct negative inotropic function of the A₁ receptor under ADA inhibition, is located in the joint part of the postreceptorial signaling of A₁ and M₂ receptors, an enhanced response to MC is expected relative to the naïve state (lacking A₁ receptor antagonist and ADA inhibitor). If this is the case, we succeed in narrowing the circle of possible mechanisms of action for the efficiency enhancing effect of ADA inhibition. If not, besides narrowing the circle of possible action mechanisms (i.e. the molecular target in question is out of the shared part of signaling of the A₁ and M₂ receptors), we have the opportunity to quantify the concentration of the surplus interstitial adenosine, produced by ADA inhibition. This is

because a prerequisite of the quantification is the fixedness of signal amplification properties of the M₂ muscarinergic system throughout the investigation.

Our results show that, in the presence of CPX, ADA inhibition afforded by DCF was unable to influence the response to MC. Thus, we have concluded that ADA inhibition does not affect the shared part of postreceptorial signaling of A₁ and M₂ receptors. Therefore, we could quantify the effect of ADA inhibition on the interstitial adenosine concentration, and then compare it with the similar action of nucleoside transport blockade (by NBTI).

The response to MC during inhibition of the nucleoside transport or ADA (without A₁ receptor blockade) showed a decrease relative to the naïve state. This phenomenon was due to the fact that the surplus interstitial adenosine, by activating the A₁ adenosinergic machinery, biased the effect mediated by the M₂ receptor. Namely, because of the overlapping signaling pathways, when a fraction of the response capacity of the A₁ adenosinergic system was depleted, the responsiveness of the M₂ muscarinergic system also decreased. The magnitude of the change (“bias”) of the E/c curve is characteristic of the magnitude of the biasing effect.

To estimate the surplus interstitial adenosine from the bias of MC E/c curves, RRM presented itself. The motif of RRM is the interchangeability of agonists evoking the same kind of effect, irrespective of what sort of receptor they bind to. In the present case, RRM has quantified the extra interstitial adenosine with a surrogate parameter, i.e. the equieffective MC concentration (c_x).

The c_x values of the present study show that NBTI substantially elevated the interstitial adenosine level and this effect was greater in the hyperthyroid guinea pig atrium than in the euthyroid one. This finding corroborates our previous results about the effect of NBTI, in which c_x values were obtained as equieffective CPA concentrations.

The c_x values of the present study also indicate that DCF, similarly to NBTI, increased the interstitial adenosine level in both the eu- and hyperthyroid guinea pig atria. Expressing this action in numbers, NBTI produced an about 3.5-fold greater c_x than DCF did, irrespectively of the thyroid state. This denotes that the nucleoside transport blockade has greater influence on the interstitial adenosine level than ADA inhibition (under our *ex vivo* conditions ensuring well-oxygenated bathing medium). However, nucleoside transport inhibitors increases the interstitial adenosine level only in the metabolically intact myocardium. In hypoxia, nucleoside transport blockers can decrease the interstitial

adenosine level by inhibiting the adenosine release from the cells. Thus, to elevate the interstitial adenosine concentration, ADA inhibition appears to be an intervention more reliable than nucleoside transport blockade. It should be noted that ADA inhibitors have a wide range of actions throughout the body that forms the basis for several side effects. However, these side effects are less problematic if ADA inhibitors are applied in an isolated organ rather than the whole body, e.g. in a heart to be transplanted. ADA inhibitors have been found to reduce hypoxic injury during cardiac surgery.

Because of unaltered signal amplification properties of the M₂ muscarinergic system under ADA inhibition, c_x values obtained from MC E/c curves could be used to correct the conventionally plotted MC and CPA E/c curves generated in the presence of NBTI and DCF (without CPX) for the change produced by the surplus interstitial adenosine. As the exact concentration of extra adenosine at the A₁ receptors remained unknown, the biased effect values were only corrected, and then they were plotted against the concentration of the agonist administered for the E/c curve. Therefore, the two most useful points of the corrected E/c curves are those at zero and at the highest concentration. The starting point shows the effect evoked by the extra interstitial adenosine alone (E_x), while the final one represents the maximal response of the given system to the given agonist (owing to the fact that well-saturated E/c curves were corrected).

The most important feature of the corrected MC E/c curves is that all of them end practically *ibidem* as their controls (considered to be inherently correct). This behavior of the curves confirms that the efficiency of the M₂ muscarinergic control on atrial contractility did not change in response to the inhibition of either nucleoside transport or ADA.

In contrast, the corrected CPA E/c curves exceed their controls at the highest CPA concentration that behavior is especially conspicuous in the hyperthyroid atria. It can be concluded that ADA inhibition increases the efficiency of the A₁ adenosinergic direct negative inotropic function, even in the euthyroid state. Nevertheless, consistent with our previous observation, this efficiency enhancing effect of ADA inhibition is stronger in hyperthyroidism. Based on the comparison of the present results obtained using NBTI with those applying DCF, the efficiency enhancing effect of ADA inhibition may be speculated to be induced by a rise in the intracellular rather than interstitial adenosine level.

Our present results denote that ADA inhibition readjusts the T₄-induced

suppression in the capacity of the A₁ receptor-mediated direct negative inotropic function, an adenosinergic protective (energy consumption limiting) effect. The impact of this finding stems from the fact that excess thyroid hormones place an extra burden on the heart and increase the risk of ischemic heart disease, supraventricular arrhythmias and congestive heart failure. Thus, the enhancement of endogenous protective ability of the heart by means of ADA inhibition seems to be an especially promising possibility in hyperthyroidism.

In summary, we have found that inhibition of ADA increases the signal amplification of the A₁ adenosinergic system as regards the direct negative inotropic effect in the euthyroid and, *a fortiori*, hyperthyroid guinea pig atrium. This outcome indicates that ADA inhibition, besides producing an increase in the interstitial adenosine level with a consequent stimulation of the A₁ receptor, intensifies the A₁ adenosinergic direct negative inotropic function in another way (forasmuch the extra adenosine can evoke a stronger effect if using a more efficacious signaling). Thus, our results propose a new, thyroid hormone-sensitive mechanism of action of ADA inhibition that may have practical significance in improving ischemic tolerance of the heart. Of course, this practical impact depends on whether this phenomenon affects other A₁ receptor-mediated protective functions as well, and whether it extends to the whole heart. It is especially interesting that this action of ADA inhibition is stronger in hyperthyroidism, a condition that places an extra burden on the heart with a simultaneous reduction of some A₁ receptor functions. In addition, it has been concluded that the site of the efficiency enhancing action of ADA inhibition is not located in the joint part of signaling pathways of A₁ and M₂ receptors. Furthermore, it has been found that ADA inhibition can produce a smaller rise in the interstitial adenosine concentration than nucleoside transport blockade can, in both eu- and hyperthyroid atria.

5 Summary

Hyperthyroidism elevates cardiovascular mortality by several mechanisms, including increased risk of ischemic heart disease. Therefore, therapeutic strategies, which enhance tolerance of heart to ischemia-reperfusion injury, may be particularly useful for hyperthyroid patients. One promising cardioprotective approach is use of agents that activate (directly or indirectly) the A₁ adenosine receptor (A₁ receptor), because A₁ adenosinergic pathways are involved in protective mechanisms such as ischemic preconditioning. These agents stimulating the A₁ adenosinergic system can be A₁ receptor agonists, furthermore they can act *via* elevating the tissue adenosine content, e.g. by inhibiting the adenosine deaminase (ADA), an enzyme that eliminates adenosine.

However, application of exogenous A₁ receptor agonists, even being partial, implies the risk of undesirable side effects. Indeed, previously we found great A₁ receptor reserve for the direct negative inotropic effect of adenosine in isolated guinea pig atria. This phenomenon suggests that A₁ adenosinergic stimulant agents may reduce the mechanical activity of atria *in vivo* as well. Due to the modulating effects of thyroid hormones in the heart, hyperthyroidism might be speculated to affect this possible side effect of A₁ receptor agonists. During the first set of our investigations, however, we have found that thyroxine treatment does not substantially affect the A₁ receptor reserve for the direct negative inotropic effect of adenosine. Consequently, if an agent causing A₁ receptor activation is administered for any indication, a decrease of atrial contractility should be considered as an adverse effect in both eu- and hyperthyroid conditions. In addition, this finding also suggests that cardioprotective potential of A₁ adenosinergic mechanisms is mainly preserved in hyperthyroidism, at least at the level of A₁ receptor and its signaling.

During the second set of our investigations, we have found that ADA inhibition (but not nucleoside transport blockade) increased the signal amplification of the A₁ adenosinergic (but not M₂ muscarinergic) system. This action of ADA inhibition developed in both thyroid states, but it was greater in hyperthyroidism. Nevertheless, ADA inhibition produced a smaller rise in the interstitial adenosine concentration than nucleoside transport blockade did, in both thyroid states. These results indicate that ADA inhibition, besides increasing the interstitial adenosine level, intensifies the atrial A₁ adenosinergic function in another (thyroid hormone-sensitive) way, suggesting a new mechanism of action of ADA inhibition.

6 Acknowledgements

This research was supported by the University of Debrecen and by the Hungarian Scientific Research Fund (OTKA-K 104017), furthermore by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 ‘National Excellence Program’ (providing personal support to Arpad Tosaki and Rudolf Gesztelyi), and of TÁMOP 4.2.2.A-11/1/KONV-2012-0045 (providing personal support to Balazs Varga, Bela Juhasz and David Haines).



Registry number: DEENK/150/2015.PL
Subject: Ph.D. List of Publications

Candidate: Krisztián Pák
Neptun ID: B9W7R6
Doctoral School: Doctoral School of Pharmaceutical Sciences
MTMT ID: 10037912

List of publications related to the dissertation

1. **Pák, K.**, Zsuga, J., Képes, Z., Erdei, T., Varga, B., Juhász, B., Szentmiklósi, J.A., Gesztelyi, R.:
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IF:0.852

Total IF of journals (all publications): 7,122

Total IF of journals (publications related to the dissertation): 3,644

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

30 July, 2015

