THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The receptorial responsiveness method (RRM), a tool to allow a new insight into the A_1 adenosinergic and M_2 muscarinergic regulatory mechanisms in eu- and hyperthyroid guinea pig atria

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DEBRECEN, 2015

"The nature of promises is that they remain immune to changing circumstances."

House of cards

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1 Abbreviations

A_1 receptor - A_1 adenosine receptor
ADA - adenosine deaminase
Ado - adenosine
ADP - adenosine diphosphate
AMP - adenosine monophosphate
ANOVA - analysis of variance
ATP - adenosine triphosphate
c - concentration
c_x - regression parameter provided by RRM for the characterization of the surplus interstitial adenosine
cAMP - cyclic adenosine monophosphate
CF - contractile force
CNP - concentrative nucleoside transporter
Co - control
COPD - Chronic Obstructive Pulmonary Disease
CPA - N^6-cyclopentyladenosine
CPX - 8-cyclopentyl-1,3-dipropylxanthine
DCF - 2'-deoxycoformycin
DMSO - dimethyl-sulfoxide
E - effect
E' - biased effect
E_x - effect produced by the surplus interstitial adenosine
EC_{50} - agonist concentration producing half-maximal effect
E/c - concentration-effect
ENT - equilibrative nucleoside transporter
ENT1 – NBTI-sensitive equilibrative nucleoside transporter
E_{max} - the maximal effect
Eq - equation
FSCPX - 8-cyclopentyl-N^3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine
M2 receptor - M2 muscarinic receptor
MC - acetyl-β-methylcholine chloride
n - Hill coefficient
NBTI - S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine
NCD - noncommunicable diseases
RRM - receptorial responsiveness method
S - solvent treatment
SEM - standard error of mean
T_4 - L-thyroxine sodium salt pentahydrate
WHO - World Health Organization

2 Introduction

2.1 Epidemiological background

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 (Fig. 1, 2).

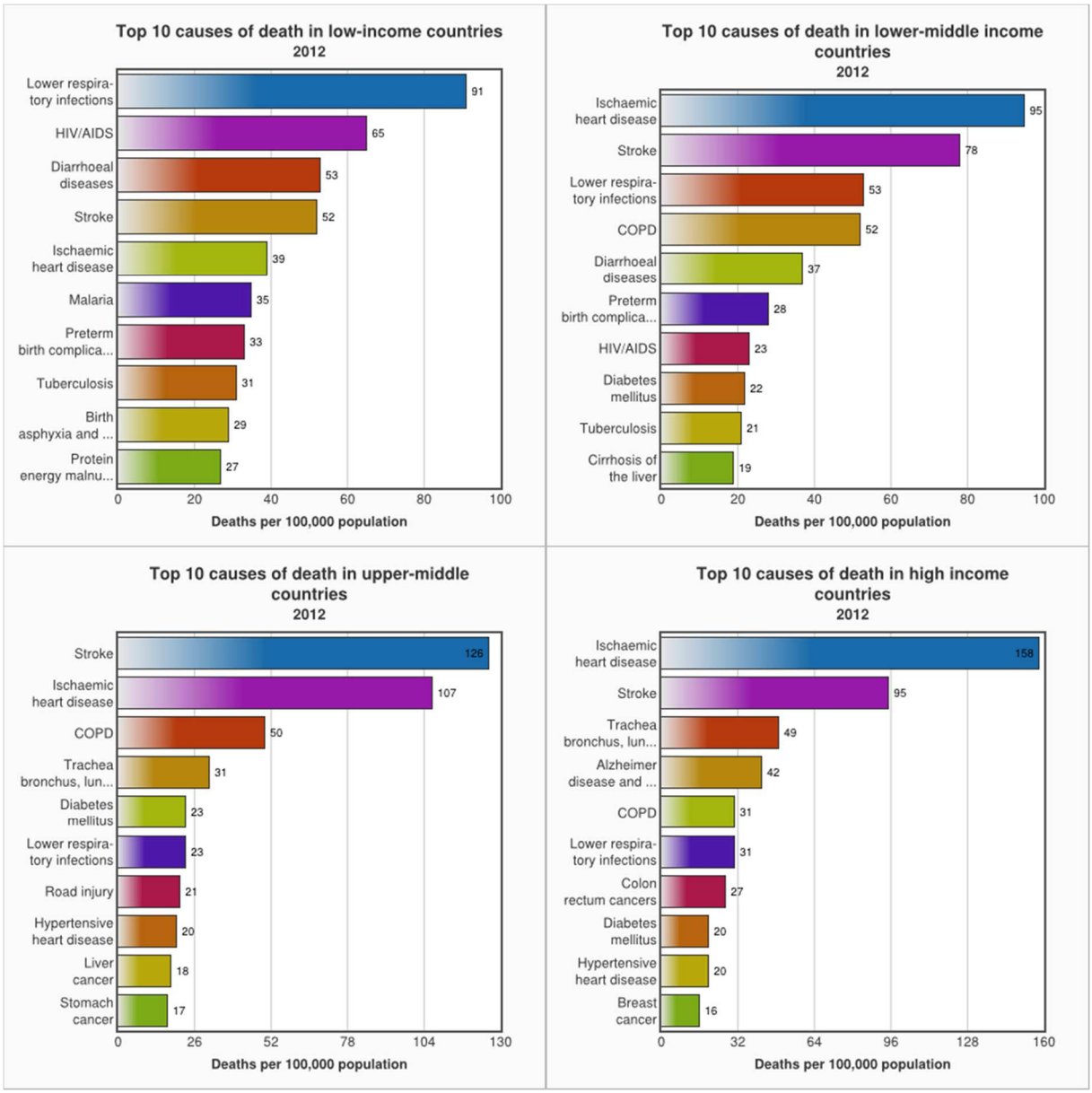


Fig. 1. Top 10 causes of death according to country income in 2012 (WHO 2014a)

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 (Fig. 2; WHO 2014b).

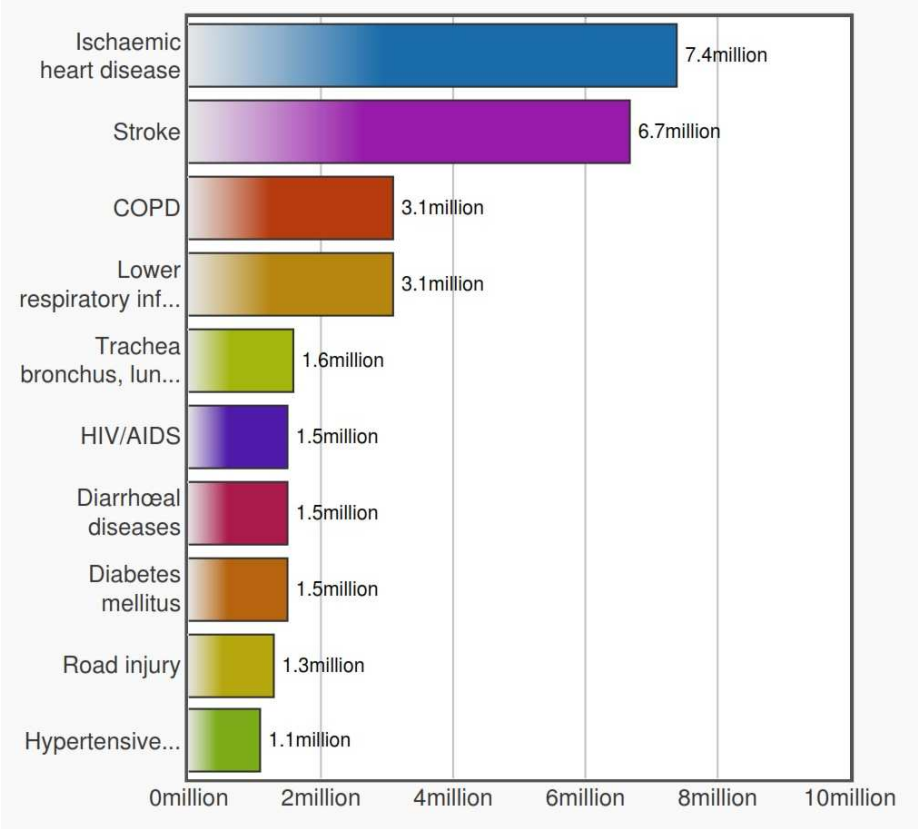


Fig. 2. The 10 leading causes of death in the world in 2012 (WHO 2014b).

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%) (Fig. 3).

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 (Perricone and Vander Heide 2014; Kleinbongard and Heusch 2015).

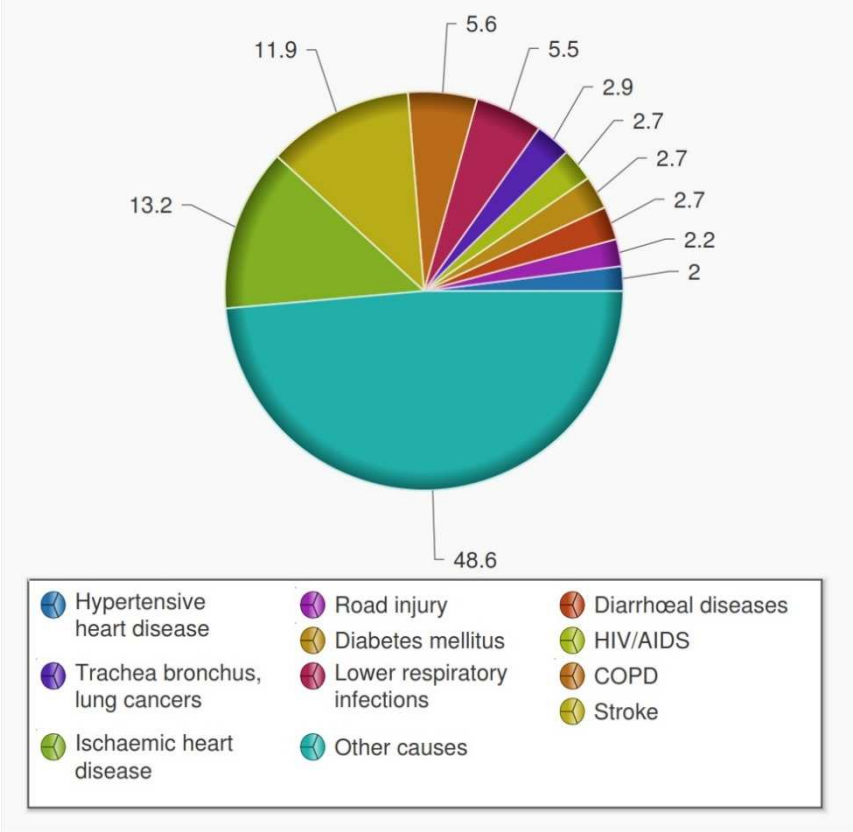


Fig. 3. The 10 leading causes of death in the world by percentage (WHO 2014b).

2.2 Adenosine and adenosine receptors

Adenosine is an adenine nucleoside that serves as an important regulator in the cardiovascular system (Fig. 4). 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) (Sato et al. 2005).

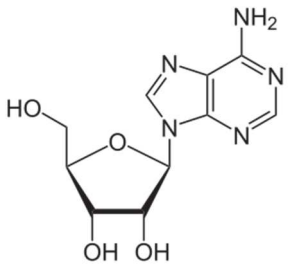


Fig. 4. The structure of adenosine

Adenosine is the precursor and decomposition product of ATP (adenosine triphosphate), the most important energy transfer molecule in living tissues, thus adenosine is a superior indicator of the energy level of the body in comparison with other nucleosides. Thus the level of this molecule indicates the exhaustion of the cells (Doenst et al. 2013).

Intracellular adenosine is generated through two primary paths. It is mainly formed via hydrolysis of 5′-AMP, and, in a lower quantity, is released from S-adenosyl-homocysteine. The pathway involving 5′-AMP, which compound is derived primarily from ATP and secondarily from cAMP, is highly regulated (being coupled to bioenergetic state), and it is the most important way in the generation of regulatory adenosine (Deussen et al. 1989; Kroll et al. 1993). Several studies have identified the intracellular 5′-AMP pool as the major source of adenosine release under both resting and ischemic/hypoxic conditions (Borst 1991; Headricket al. 1992; Darvish et al. 1996. Extracellular adenosine is generated from enzymatic degradation of interstitial ATP, ADP, AMP and cAMP. The speed limiting step is the AMP → adenosine transformation, which process is catalyzed by the ecto-5′-nucleotidase (Sommerschild and Kirkebøen, 2000, 2002; Fredholmet al. 2001, 2011).

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) (Fig. 5)
2. getting into the bloodstream
3. uptake into cells of certain tissues such as endothelium and myocardium (Sommerschild and Kirkebøen 2000, 2002; Fredholm et al. 2001).

Adenosine molecules can move across the cell membrane with active or passive transport. Active transport is carried out by concentrative nucleoside transporter (CNT) and the passive one is performed by equilibrative nucleoside transporter (ENT). In the heart, there are mainly ENT transporters, especially ENT type 1 (ENT1), which is sensitive to inhibition with NBTI (nitrobenzylthioinosine) (Conant and Jarvis, 1991, 1994). Therefore, the adenosine transport of cardiomyocytes is primarily determined by the difference between the intra- and extracellular adenosine concentration (Deussen et al. 1999; Deussen 2000a, 2000b).

Regulatory effects of adenosine are mediated predominantly by activating cell-surface adenosine receptors (Fredholm et al. 2001, 2011). There are four adenosine receptors among vertebrates, which have been denoted adenosine A_1 and A_{2A}, A_{2B} and A_3 receptors (Fredholm

et al. 2001a).

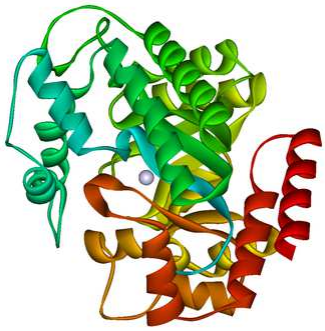


Fig. 5. The structure of adenosine deaminase with a zinc ion (bluish sphere) at center (Kinoshita et al. 2005)

All members of the adenosine receptor family are G protein-coupled receptors and each bears the characteristic motif of seven transmembrane spanning domains. The ligand biding site of these receptors faces to the interstitium. A_1 and A_3 receptors preferentially couple to G_i protein to inhibit adenylate cyclase and consequently to decrease the production of cAMP, while A_{2A} and A_{2B} subtypes stimulate the production of cAMP via coupling to G_s or G_o (Fredholm et al. 2001, 2011; Burnstock et al. 2010; Ijzerman et al. 2012). Adenosinergic signaling is a powerful endogenous tissue protective mechanism, which is a component of the ubiquitous purinergic system that has been highly conserved in vertebrate evolution (Verkhratsky and Burnstock 2014).

So, the phylogenetically ancient and ubiquitous A_1 adenosine receptor (A_1 receptor) exerts complex regulatory functions in almost all tissues, including the myocardium, in which the A_1 is the main adenosine receptor type (Fredholm et al. 2001, 2011; Burnstock et al. 2010; Ijzerman et al. 2010, 2014) (Fig. 6, 7). The myocardial A_1 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 (Headrick et al. 2011, 2013). Consistently, the A_1 adenosinergic pathways play an essential role in the phenomenon called ischemic preconditioning by initiating powerful protective and regenerative processes that prevent and remediate damage caused by ischemia and subsequent reperfusion (Headrick et al. 2003, 2011; Otani 2008).

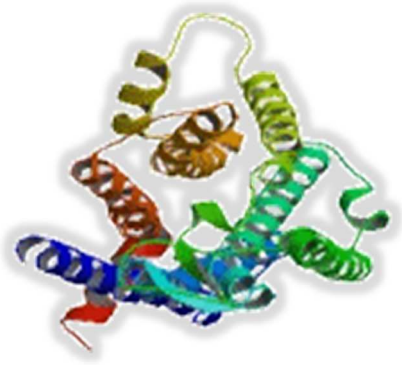


Fig. 6. The structure of A_1 adenosine receptor (USCN 2015)

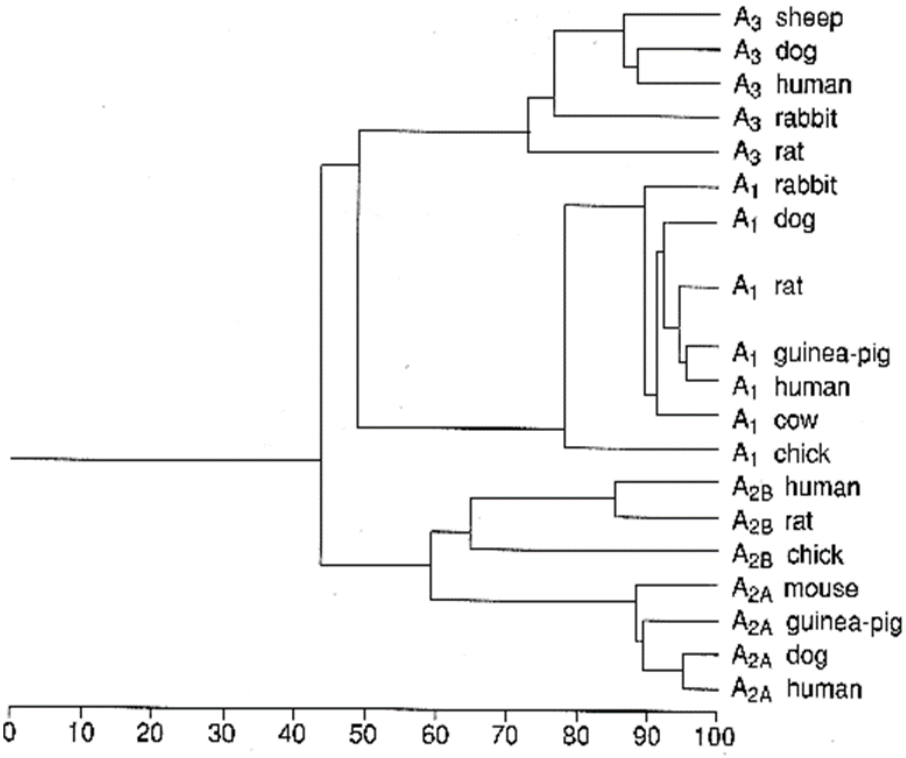


Fig. 7. Dendrogram of adenosine receptors for different laboratory and domestic animal. The scale at the bottom indicates the amino acid sequence homology in percentage. There is about 95% matching for the human and guinea-pig A_1 adenosine receptor that is the highest value for this receptor type (Fredholm et al. 2001; IJzerman et al. 2012).

Pharmacological activation of the A_1 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 (Szentmiklosi et al. 2011, 2015). Furthermore, the A_1 receptor stimulation as a

therapy may come into play in avoiding hypoxic injury during heart transplantation, too (Lim et al. 2013; Burnstock and Pelleg 2015).

As mentioned previously, the A_1 receptor mediates negative tropic effects on the heart that involve negative inotropic activity on both the atrium and ventricle (Shryock and Belardinelli 1997). In the ventricle, A_1 receptor agonists evoke only an indirect negative inotropic effect, thereby reducing only the positive inotropic action of other agents (Bohm et al. 1984, 1985; Belardinelli et al. 1995). In contrast, A_1 receptor agonists exert a significant direct negative inotropic effect on the atrium in most species, including the guinea pig and human (Szentmiklosi et al. 1982; Bohm et al. 1984; Marmo et al. 1986; Brodde et al. 1992; Belardinelli et al. 1995). This means that stimulation of A_1 receptors can decrease the atrial contractile force deeply below the resting level.

Accordingly, several classes of compounds that activate the A_1 receptor pathway (A_1 receptor agonists, A_1 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 (Elzein and Zablocki 2008; Schenone et al. 2010; Fredholm et al. 2011; Szentmiklosi et al. 2011; Albrecht-Küpper et al. 2012; Staehr et al. 2013). Since these drugs, either directly or indirectly, target the same molecular object (A_1 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 (Rossi et al. 2000; Betts 2012).

2.3 Receptor reserve

The first step of a drug-receptor interaction is the biding of the drug to the receptor, which depends on the complementarity of ‘fit’ of the two molecules. The closer the fit and the greater the number of (usually noncovalent) molecular bonds, the higher will be the affinity of the drug for the receptor. While both agonists and antagonists have affinity to the receptor, only the agonist has a special feature to intervene in some functions of the cell expressing the given receptor. This phenomenon is mediated by the agonist-receptor complex in a way that this complex bounds to some transducer elements in the cell.

According to the ternary complex model applied to G-protein coupled receptors, when an agonist binds to a free receptor, first they form an agonist/receptor/G-protein complex that possesses small ability to alter the function of the cell (because it has low affinity for a transducer that mediates the effect of the agonist) (Fig. 8). Then, this complex will switch to another conformation (isomerization step) that displays great ability to change the cell function (having high affinity for the transducer), resulting in an effective signal transduction. Classical dogma states that the ratio of the low affinity to the high affinity predicts intrinsic activity of drugs: the higher the ratio, the higher the intrinsic activity (Egan et al. 2000).

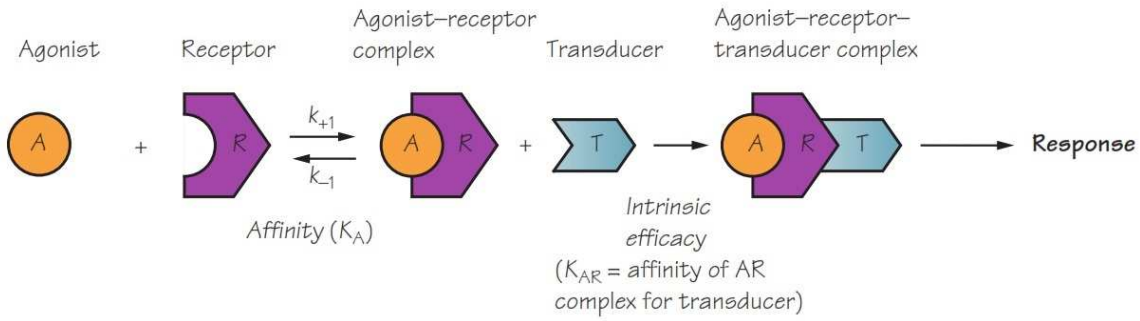


Fig. 8. The ternary complex model (Neal 2012)

The two major determinants of tissue responsiveness to a given agonist are the receptor number and the signal amplification ability of receptors related to the agonist in the given tissue. Within the traditional receptor theory, the signal amplification ability is characterized by the so-called receptor reserve. The receptor number and receptor reserve show a high positive correlation across the different tissues (Dhalla et al. 2003; Kenakin 2009).

The term 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 (Ruffolo 1982). 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) (Kenakin 1987, 2009; Dhalla et al. 2003). As receptor reserve is very sensitive to agonist's intrinsic efficacy, it is usually defined only for full (high-efficacy) agonists (Kenakin 2009; Dhalla et al. 2003). 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 (Ruffolo 1982).

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 (Dhalla et al. 2003).

Relative to the adipose and neuronal tissues, the heart has small A_1 receptor reserve for electrophysiological actions such as the inwardly rectifying potassium current, L-type calcium current, action potential duration (Dhalla et al. 2003). In contrast, previous studies carried out in our laboratory have found a considerable A_1 receptor reserve for the direct negative inotropic effect of synthetic A_1 receptor agonists (Gesztelyi et al. 2013) and adenosine (Kiss et al. 2013). 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 (Gesztelyi et al. 2013; Kiss et al. 2013).

2.4 Thyroid state

The thyroid state influences several regulatory mechanisms, including functions of the A_1 receptor (for a brief review, see: Gesztelyi et al. 2012). 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 (Szentmiklosi et al. 1992; Kaasik et al. 1994; Gesztelyi et al. 2003a). 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 (Cini et al. 2009; Nabbout and Robbins 2010). As a consequence, excess thyroid hormones increase the risk of congestive heart failure, ischemic heart disease and arrhythmias, and thereby elevate cardiovascular mortality (Franklyn and Boelaert 2012). In light of these facts, suppression of the A_1 adenosinergic system by thyroid hormones may raise concerns. Thus, it is of importance to find out possibilities to enhance the depressed function of the A_1 adenosinergic system in hyperthyroidism.

Excessively high levels of thyroid hormones place the heart under elevated stress. This phenomenon occurs due to the capacity of thyroid hormones to upregulate a wide range of metabolic processes and, thereby, to increase the oxygen and nutrient demand of the tissues. In addition, these hormones may directly evoke positive tropic effects on the heart, including positive inotropy (Pietras et al. 1972; Kiss et al. 1994), although this latter effect predominantly affects the ventricle (Kaasik et al. 1997). These factors combine to increase heart rate and cardiac output, which places an extra burden on the heart (Cini et al. 2009; Nabbout and Robbins 2010). Consequently, hyperthyroidism elevates the cardiovascular mortality by increasing the risk of congestive heart failure, supraventricular arrhythmias, embolism and ischemic heart disease (Franklyn and Boelaert 2012). Therefore, therapeutic tools, which enhance the tolerance of the heart to ischemia and subsequent reperfusion, may be particularly useful for hyperthyroid patients.

Although adenosinergic drugs seem convenient to counterbalance many of deleterious effects of hyperthyroidism, potential undesired effects of agents producing A_1 receptor activation must also be considered under hyperthyroid condition. Indeed, thyroid hormones markedly reduce the effect of A_1 receptor agonists on the contractility of atria (Szentmiklosi et al. 1992; Kaasik et al. 1994; Gesztelyi et al. 2003a). Thus, it may be hypothesized that the risk for weakening of atria in response to A_1 adenosinergic stimulant agents, which seems to be high in euthyroid condition (Gesztelyi et al. 2013; Kiss et al. 2013), might be lower in hyperthyroidism.

2.5 The significance of the interstitial adenosine level in the heart

Adenosinergic signaling is a powerful endogenous tissue-protective mechanism. In the heart, adenosine receptors also exert complex regulatory functions. The myocardial A_1

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. A basic understanding of endogenous protective mechanisms of the heart against ischemia is a necessity for the development of new rational therapeutic strategies. 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 enzymes participating in the elimination of adenosine. Adenosine deaminase (ADA), by converting adenosine into inosine, plays an important, although not pivotal role in the nucleic acid metabolism of the heart (Fredholm et al. 2001, 2011; Headrick et al. 2011). Inhibition (or deficiency) of ADA increases both the intra- and extracellular adenosine concentrations, which enables the surplus adenosine to exert its effects *via* both intra- and extracellular binding sites (Sandhu et al. 1993; Zhu et al. 1994; Hudspeth et al. 1994; Manthei et al. 1998; Peart et al. 2001, 2003; Willems et al. 2006; Abd-Elfattah et al. 2012). Consistently, the inhibition of ADA augments actions of exogenous adenosine as well (Szentmiklosi et al. 1982; Gesztelyi et al. 2003b).

In a previous study, we found that inhibition of ADA increases the signal amplification of the A_1 adenosinergic system, regarding its direct negative inotropic function in the hyperthyroid guinea pig atrium (Kemeny-Beke et al. 2007). As ADA inhibition elevates the adenosine levels and thus augments all A_1 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 A_1 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 (Deussen et al. 1999; Deussen 2000a, 2000b). In the heart, the transmembrane adenosine flux passes almost exclusively through ENT1, a nucleoside transporter type that is equilibrative and sensitive to inhibition by NBTI (Thorn and Jarvis 1996). Accordingly, in our previous studies, NBTI was found to elevate the interstitial adenosine level in the guinea pig atrium (Karsai et al. 2006; Kiss et al. 2013), which action was more pronounced in hyperthyroidism (Karsai et al. 2007; Pak et al. 2014).

As the ligand binding site of adenosine receptors is accessible from the interstitium, it is especially important to gather information about changes of the interstitial adenosine concentration. However, in the functioning heart, these data 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 (Karsai et al. 2006; Ramakers et al. 2008). Under well-defined circumstances, the receptorial responsiveness method (RRM), a method developed in our laboratory (Gesztelyi et al. 2004), may address this problem.

2.6 The receptorial responsiveness method (RRM)

RRM, although it has been only recently developed, is rooted in the classical pharmacology (Gesztelyi et al. 2004; Pak et al. 2014). 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 (Grenczer et al. 2010a, 2010b). 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 (Gesztelyi et al. 2004; Grenczer et al. 2010a, 2010b).

To unambiguously distinguish the agonists playing a role in the performance of RRM, the agonist that is to be quantified has been denominated as “biasing agonist”, while the agonist used for the construction of the E/c curve is called “test agonist”. The biasing agonist is already present in the system before taking the E/c curve, and thus it has exerted an effect when the E/c curve starts. If we ignore the biasing agonist concentration and its effect, the resulting E/c curve will not represent the real responsiveness of the receptors to the test agonist (it will underestimate this), because the biasing agonist has in part consumed the capacity of the system to respond before the generation of the E/c curve (this way, the biasing agonist “biased” the E/c curve).

As a thought experiment, let us illustrate the influence of the biasing agonist concentration (being unheeded) on the responsiveness of the receptor detected with a subsequently administered test agonist concentration (being taken into account). Let us administer a full agonist to its receptor at a concentration that evokes (practically) the maximal response. Then, let us ignore this agonist concentration and its effect by defining the evolved response as baseline value. If we administer another agonist dose to this system, (practically) no response will be detected. So, a sufficiently high concentration of a full agonist (as a biasing agonist) nullifies the responsiveness of its receptor, when detected with a subsequently administered concentration of the same agonist (or another one activating the same or a largely overlapping signaling). Of course, this is a virtual decrease of the receptor responsiveness that is produced by the ignorance of the biasing agonist and its effect.

Biasing agonist concentrations producing submaximal effects do not annul rather concentration-dependently decrease the responsiveness to a subsequently added test agonist concentration. This phenomenon can be described exactly by means of an equation (see below in the Materials and methods section). This relationship enables the estimation of a biasing agonist concentration from the extent of the decrease (bias) in the response to a test agonist concentration. If we construct E/c curves in the absence and presence of the biasing agonist concentration and compare the two curves, the determination will be more accurate and reliable. To the best, the E/c curve generated in the presence of the biasing agonist concentration should be fitted to the equation mentioned above. The fitted equation should contain the main parameters of the E/c curve constructed under the same conditions but in the absence of the biasing agonist concentration in question. These parameters can be obtained by fitting the unbiased E/c curve to the Hill equation, the simplest and most flexible receptor model (see below in the Materials and methods section). Use of these parameters is essential for RRM as they hold information about the intact (unbiased) concentration-effect relationship, against which the virtual decrease in the responsiveness can be quantified (Gesztelyi et al. 2004; Grenczer et al. 2010a, 2010b; Pak et al. 2014).

Thus, RRM is a nonlinear regression with two variables (test agonist concentration used to generate the E/c curve and the related effect value of the curve constructed in the presence of the biasing agonist) and one parameter (the biasing agonist concentration or test agonist concentration that is equieffective with the biasing agonist concentration).

Requirements of RRM (Gesztelyi et al. 2004; Grenczer et al. 2010a, 2010b):

1. The effect should be well measurable.

2. The test agonist should be stable and have the ability to penetrate.
3. The biasing agonist concentration should not change during the determination.
4. The effect evoked by the biasing agonist should be located at the medium part of the intact E/c curve (that is generated in the absence of the biasing agonist) of the test agonist.
5. The receptors should not undergo desensitization.
6. The maximal response achievable with the test agonist should be close to that achievable with the biasing agonist in the given system. In general, the biasing and test agonist should be similar with regard to their pharmacodynamic properties. If it is not possible, the efficacy of the test agonist should be lower rather than higher in comparison with the efficacy of the biasing agonist.

Fulfilment of the above-mentioned criteria in our investigations:

1. The contractile force is a robust measure that can be reliably determined.
2. Agonists used to generate E/c curves in our work (see below in the Materials and methods section) were stable and could easily penetrate the thin atrial tissue (Pavan and Ijzerman 1998).
3. The rise in the interstitial adenosine concentration that served as biasing agonist concentration in our experiments developed as a result of a dynamic equilibrium of different processes in the myocardium.
4. We generated a rise in the interstitial adenosine concentration that was sufficiently great to evoke a properly strong direct negative inotropic effect.
5. Although RRM, in principle, can be applied for each receptor, the A_1 receptor is especially suitable for this purpose, due to its slow desensitization (Mundell and Kelly 2011) relative to the duration of the measurement.
6. Agonists used to generate E/c curves were all full agonists capable of producing almost total loss of atrial contractile force, just like adenosine (the biasing agonist).

2.7 Interaction between the A_1 adenosinergic and M_2 cholinergic systems

In the heart, the A_1 and M_2 receptors are the predominant receptor types for adenosine (Headrick et al. 2013; Ijzerman et al. 2014) and acetylcholine (Harvey 2012; Birdsall et al. 2014), respectively. In the atrium, both A_1 and M_2 receptors bind to $G_{i/0}$ 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 (Caulfield and Birdsall 1998; Fredholm et al. 2001, 2011; Harvey and Belevych 2003; Harvey 2012; Headrick et al. 2011, 2013). As a consequence, both A_1 and M_2 receptors can mediate direct negative inotropic effect (Belardinelli et al. 1995; Kurachi 1995). Thus, although *via* binding to different receptors, agonists for these receptors activate greatly overlapping signal-transduction pathways in the atrium. Further similarities between A_1 and M_2 receptors are that both of them are controlled by thyroid hormones (T_3, T_4) (Sharma and Banerjee 1977; Ishacand Pennefather 1983; Kastrup and Christensen 1984; Rubinstein and Binah 1989; El-Ani et al. 1994; Kaasik et al. 1994, 1997a, 1997b; Bosch et al. 1999; Ojamaa et al. 2000a, b; Shenoy et al. 2001; Sunagawa et al. 2005; Gesztelyi et al. 2012), and our knowledge about this regulation is incomplete and, in some areas, inconsistent yet.

3 Goals

The work presented in the present thesis can be divided into two main parts: Study 1 and Study 2.

In Study 1, the goal 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 Study 2, our major 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 A_1 receptor-mediated processes, therefore it is not easy to identify the efficiency enhancing action of ADA inhibition on the A_1 adenosinergic signaling, an effect that was proposed in a previous study (Kemeny-Beke et al. 2007).

Furthermore, an additional goal in Study 2 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.

4 Materials and methods

4.1 Materials

The following chemicals were used:

- L-thyroxine sodium salt pentahydrate (T_4)
- adenosine(non-selective adenosine receptor full agonist),
- N^6-cyclopentyladenosine (CPA) (CPA; selective A_1 adenosine receptor full agonist),
- acetyl-β-methylcholine chloride (methacholine: MC; non-selective muscarinic receptor full agonist with high affinity for the M_2 muscarinic receptor (M_2 receptor))
- 8-cyclopentyl-1,3-dipropylxanthine (CPX; selective, competitive A_1 adenosine receptor antagonist)
- 8-cyclopentyl-N^3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-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 Nipent™, which was the generous gift of Wyeth Pharmaceuticals (Collegeville, PA, USA).

Experiments were conducted in modified Krebs-Henseleit buffer (Krebs solution) that contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 24.9 mM $NaHCO_3$, 11.5 mM glucose and 0.1 mM ascorbic acid (dissolved in redistilled water). T_4 was dissolved in physiological salt solution containing 0.01% NaOH. Adenosine and MC were dissolved at 36ºC in Krebs solution. CPA was dissolved in ethanol: water (1:4) solution (v/v). CPX, FSCPX and NBTI were dissolved in dimethyl-sulfoxide (DMSO). All stock solutions were adjusted to a concentration of 10 mM, except for the adenosine stock solution, which was used to achieve 3 mM concentration in the bathing medium. For this purpose, 20 mM adenosine solution was freshly prepared before each use. DCF was dissolved in redistilled water (according to the manufacturer's instructions). Stock solutions were diluted with Krebs solution (when appropriate). The concentration of ethanol and DMSO did not

exceed 0.023% (v/v) and 0.1% (v/v), respectively, in the organ baths at any time.

4.2 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 (Fig. 9).

Fig. 9. Hartley guinea pig

A group of animals received 330 µg/kg T_4 daily (*ip.*) for 8 days (*in vivo* T_4 treatment), and the vehicle of T_4 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% O_2 and 5% CO_2 (36ºC; pH 7.4) (Fig. 10).

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 (with a displacement 200 µm/g) (Experimetria SD-01, Fig. 11) and strain gauge (Experimetria SG-01D), and recorded by a polygraph (Medicor R-61 6CH Recorder).

4.3 Arrangement of investigations underlying the present thesis

Our investigations, according to 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 A_1 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. This condition was important for the acquisition of accurate data, because the direct component of negative inotropy is very sensitive to any change in the frequency of contraction (Endoh 1999). The reliability of the raw data was essential, as opportunities inherent in RRM could be only exploited using accurate input data.

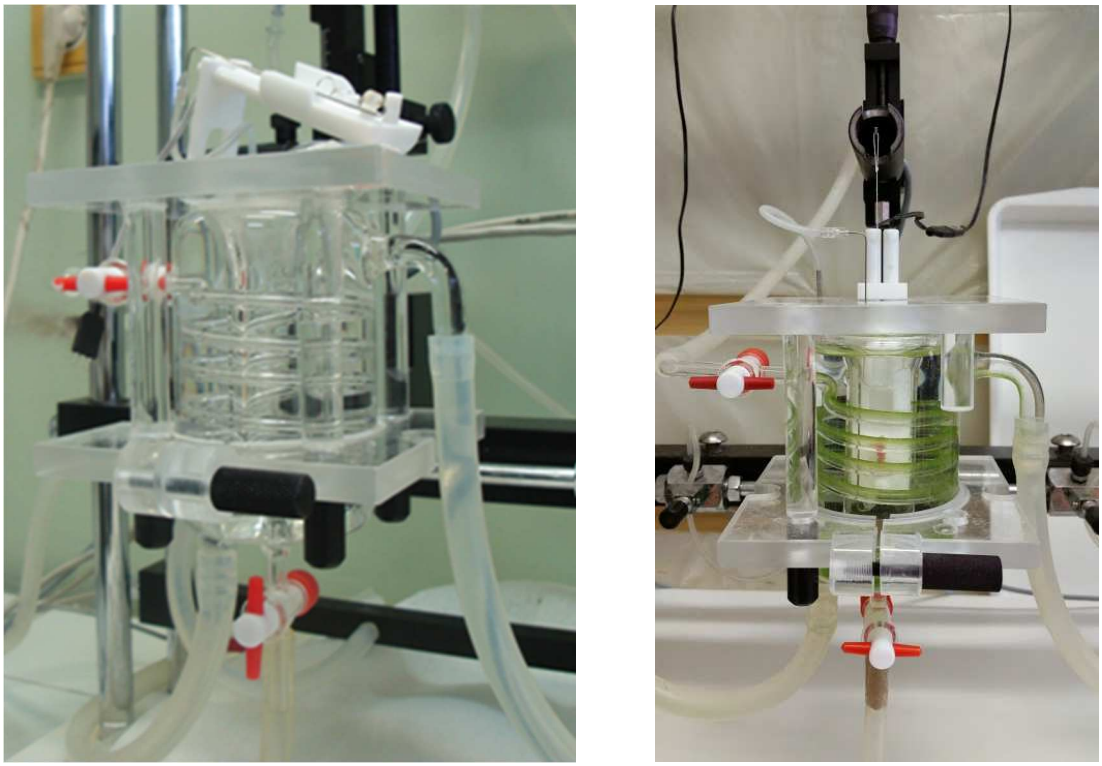


Fig. 10. Isolated-organ chamber before experiment (left panel) and in use (right panel)

As a first step, an adenosine concentration-effect (E/c) curve was constructed with **adenosine** in all protocols. Adenosine was used to assess the responsiveness of the naïve (*in vitro* untreated) A_1 receptors. Adenosine is especially suitable for this purpose because of its rapid elimination without yielding confounding byproducts (Wilbur and Marchlinski 1997).

Fig. 11. The transducer used for the detection of the amplitude of isometric twitches

If the agonist of the next E/c curve was not adenosine (rather CPA or MC), only one E/c curve followed the adenosine E/c curve, due to the slow elimination of CPA and MC from tissues relative to adenosine (Pavan and IJzerman 1998; Gesztelyi et al. 2004). Thus, only one CPA or MC E/c curve was constructed in each preparation in order to avoid the prolonged washout period that would have been necessary between two CPA or MC E/c curves.

4.4 Experimental groups and protocols for the Study 1

Both solvent- and T_4-treated atria were randomized into six-six groups (the *in vivo* solvent and T_4 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 (n = 5) and T1 (n = 5) for Protocol 1 (demonstration of the effect of FSCPX on the adenosine E/c curve)
- S2 (n = 5) and T2 (n = 6) for Protocol 2 (attempt to determine the A_1 receptor reserve for adenosine)
- S3-Control (n = 7), S3-NBTI (n = 7), T3-Control (n = 8) and T3-NBTI (n = 9) 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 (n = 7), S4-FSCPX (n = 7), T4-Control (n = 7) and T4-FSCPX (n = 7) for Protocol 4 (data collection to compute the negative inotropic effect of c_x on the FSCPX-pretreated atria).

The protocols and evaluation procedures were described previously in detail (Kiss et al. 2013), they are summarized briefly in Table 1 and Fig 12.

P	First incubation	First E/c curve
1	Krebs solution for 25 min; 100 µM adenosine for 1 min; Krebs solution for 15 min	adenosine (1 nM – 3 mM)
2	Krebs solution for 25 min; 100 µM adenosine for 1 min; Krebs solution for 15 min	adenosine (1 nM – 3 mM)
3	Krebs solution for 40 min	adenosine (1 nM – 1 mM)
4	Krebs solution for 40 min	adenosine (1 nM – 1 mM)
	Second incubation	**Second E/c curve**
1	Krebs solution for 15 min; 10 µM FSCPX for 45 min; Krebs solution for 75 min	adenosine (1 nM – 3 mM)
2	Krebs solution for 15 min; 10 µM NBTI for 15 min	adenosine (1 nM – 3 mM)
3	Krebs solution for 15 min; 10 µl DMSO (control) or 10 µM NBTI for 15 min	CPA (0.1 nM – 0.1 mM)
4	Krebs solution for 15 min; 10 µl DMSO (control) or 10 µM FSCPX for 45 min; Krebs solution for 75 min	CPA (0.1 nM – 0.1 mM)
	Third incubation	**Third E/c curve**
2	Krebs solution for 20 min; 10 µM FSCPX for 45 min; Krebs solution for 60 min; 10 µM NBTI for 15 min	adenosine (1 nM – 3 mM)

Table 1. The four protocols used for both the solvent- and T_4-treated guinea pig atria. P, number of protocol; CPA, N6-cyclopentyladenosine; NBTI, S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine; FSCPX, 8-cyclopentyl-N3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N1-propylxanthine.

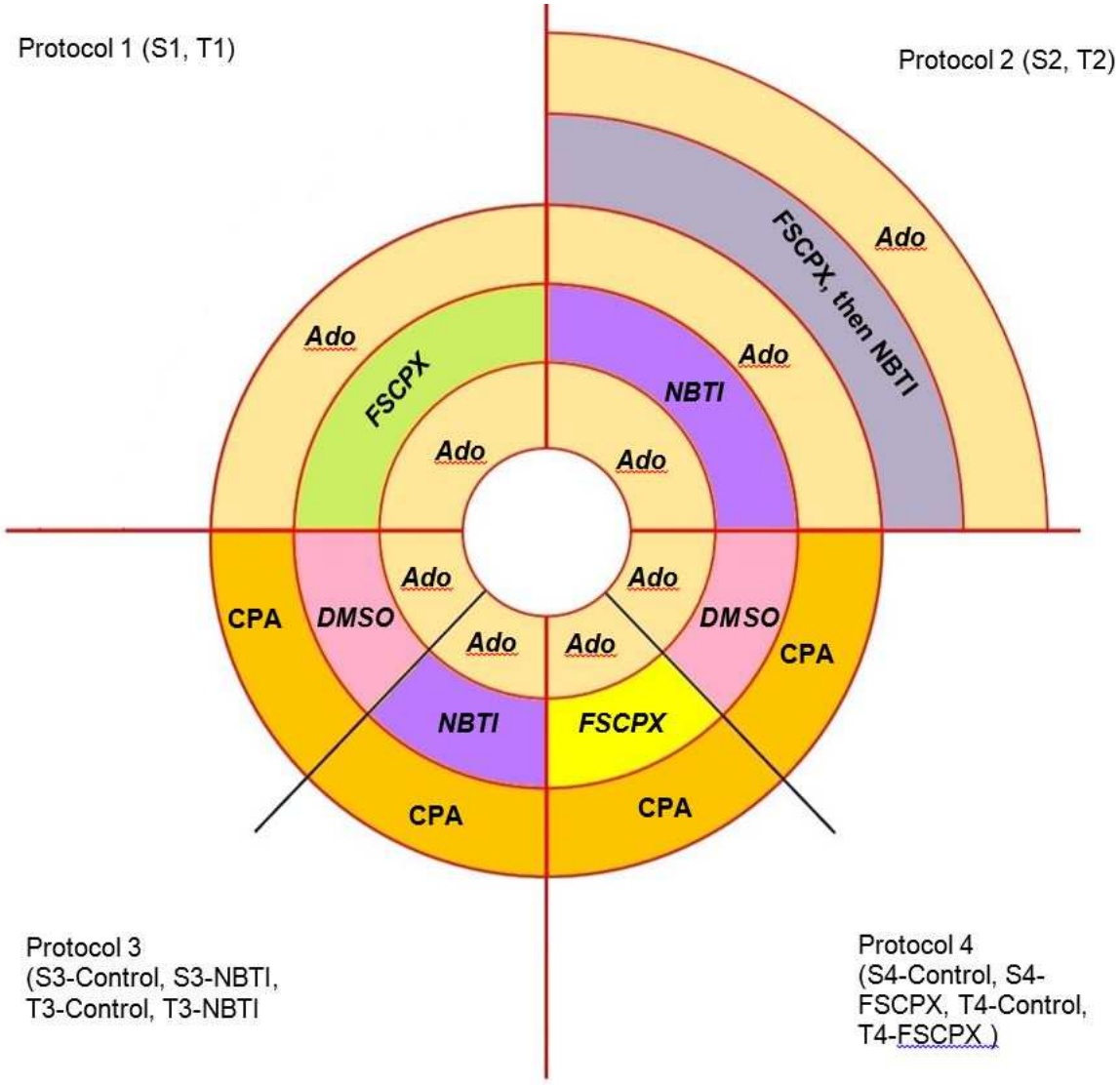


Fig.12. Conspectus of protocols and groups. The main feature of all protocols, used for the Study 1, was construction of two or three E/c curves with in vitro treatments inserted into them. The first (innermost), third and fifth annuli of the doughnut chart represent the E/c curves (showing the agonist used), while the second and fourth annuli symbolize the in vitro treatment (indicating the applied chemicals in italics). Sectors of the doughnut chart denote the particular protocols that progressed from the inside out. The names of the experimental groups, which underwent treatments specified by the protocols, are listed outside of each sector. Two groups belonging to one protocol, are defined diagrammatically above by one sector. Within this sector there are one group including solvent-treated atria and another one showing T_4-treated atria. Ado: adenosine; CPA: N^6-cyclopentyladenosine; S: solvent treatment; T: T_4 (thyroxine) treatment; Control: control; DMSO: dimethyl-sulfoxide; FSCPX: 8-cyclopentyl-N3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine; NBTI: nitrobenzylthioinosine.

4.5 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 - T_4-treated; Co - control; and abbreviations of the used chemicals):

- **S Co** (n = 16), **T Co** (n = 19)
- **S CPX** (n = 12), **T CPX** (n = 9)
- **S DMSO** (n = 6), **T DMSO** (n = 7)
- **S NBTI** (n = 6), **T NBTI** (n = 7)
- **S DCF** (n = 10), **T DCF** (n = 18)
- **S DCF CPX** (n = 8), **T DCF CPX** (n = 9)
- **S Co (CPA)** (n = 7), **T Co (CPA)** (n = 9)
- **S DCF (CPA)** (n = 7) and **T DCF (CPA)** (n = 10).

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 µM CPX** (S CPX, T CPX) or **0.1% (v/v) DMSO alone** (S DMSO, T DMSO) or **10 µM NBTI** (S NBTI, T NBTI) or **10 µM DCF** (S DCF, T DCF, S DCF (CPA), T DCF (CPA)) or **10 µM DCF with 10 µM 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)) (Fig. 13).

For the first E/c curve, adenosine was used to assess the responsiveness of the *in vitro* untreated atrial A_1 receptors. For the second E/c curve, MC or CPA (two relatively stable agonists for the M_2 or A_1 receptor, respectively) was administered in order to gather information about the effect of the different *in vitro* treatments on the M_2 and A_1 receptor responses. In the atrium, signaling pathways of these two receptors are almost the same, concerning the direct negative inotropic effect (Belardinelli et al. 1995; Harvey and Belevych 2003).

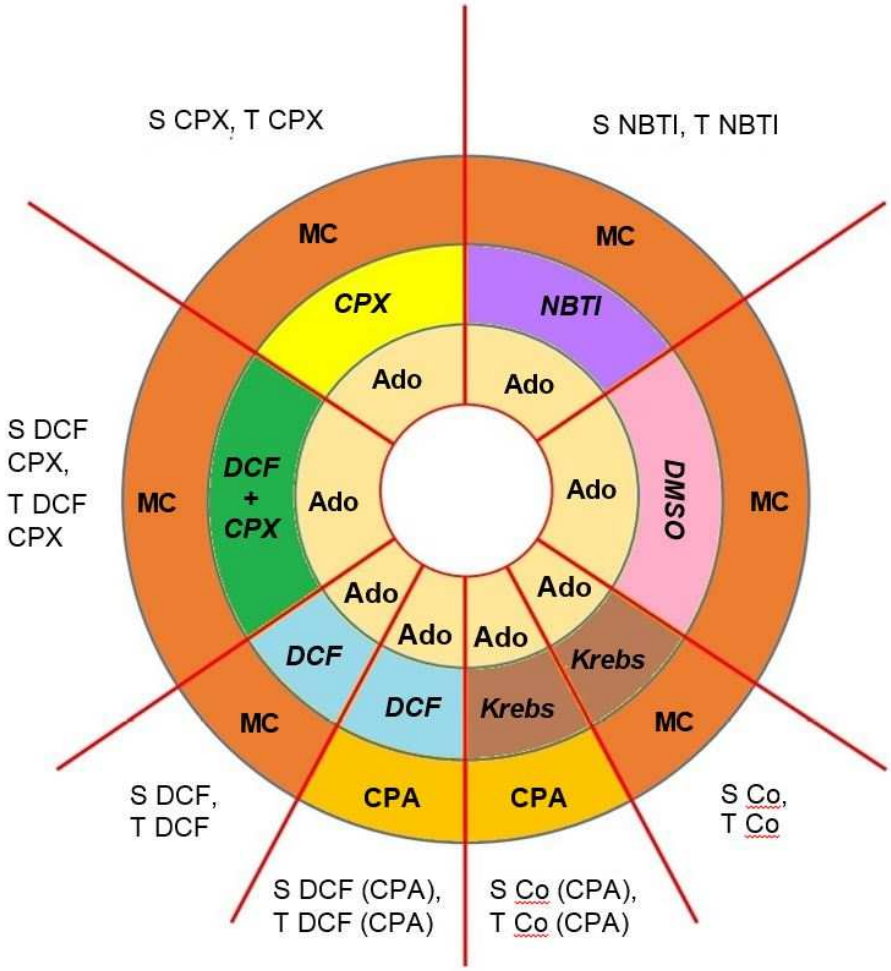


Fig. 13. Conspectus of protocols and groups in Study 2. The pivot of Study 2 protocols was the construction of two E/c curves with an in vitro treatment inserted into them. The inner and outer annuli of the doughnut chart represent the E/c curves (showing the agonist used), and the medium annulus symbolizes the in vitro treatment (indicating the applied chemicals in italics). Sectors of the pie chart denote the particular protocols that progressed from the inside out. The names of the experimental groups, which underwent the protocols, are listed outside of the sectors. Two groups belong to one protocol (one sector): one group including solvent-treated atria and another one involving T_4-treated atria. Ado: adenosine; CPA: N6-cyclopentyladenosine; MC: methacholine; S: solvent treatment; T: T4 (thyroxine) treatment; Co: control; Krebs: Krebs solution; CPX: 8-cyclopentyl-1,3-dipropylxanthine; DMSO: dimethyl-sulfoxide; NBTI: nitrobenzylthioinosine; DCF: 2'-deoxycoformycin.

According to the major goal of this study, the ADA inhibitor DCF was administered during the *in vitro* treatment to investigate the effects of ADA inhibition on the atrial A_1 adenosinergic system (which has a significant overlap with the M_2 muscarinergic machinery). The A_1 receptor antagonist CPX was added solely (to check its effect alone) and together with

DCF (to explore the contribution of A_1 receptors, activated by endogenous adenosine evading the deamination by ADA, to the effect of DCF on the MC E/c curve). Blockade of the inward adenosine transport, elicited by NBTI, was applied to generate data about the increase in concentration of interstitial adenosine in this particular experimental setup (using MC), and thereby, to enable the comparison between ADA inhibition (by DCF) and nucleoside transport blockade (by NBTI) regarding their effect on the interstitial adenosine level. DMSO treatment served as control for the NBTI treatment.

4.6 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 (Hill 1910):

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

where: c - the concentration of the agonist administered; E - the effect; E_{max} - the maximal effect; EC_{50} - the agonist concentration producing half-maximal effect; n - the Hill coefficient.

Hill parameters (E_{max}, EC_{50}, 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).

4.7 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 (Kemeny-Beke et al. 2007), 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 (Gesztelyi et al. 2004; Grenczer et al. 2010a), by fitting the averaged data of the biased E/c curves to the following equation:

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

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.

4.8 Correction of effect values of adenosine E/c curves generated in the presence of NBTI or DCF

The correction procedure was performed as described previously (Kiss et al. 2013). First, an effect belonging to c_x was calculated by means of the Hill equation (equation 3):

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

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).

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

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.

4.9 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 to10 μM or 100 μM adenosine of the solvent- or T_4-treated atrium, respectively, was required to be within a mean ± 2 SD range. The mean and SD were computed using atria meeting the first two criteria (separately for the solvent- and T_4-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. Data presented are expressed as mean ± SEM or value with lower and upper 95% confidence interval limits. Statistical significance for the difference of means (or medians) was assigned into one of four categories: $p > 0.05$ (not significant), $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). 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 D'Agostino-Pearson omnibus 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.* T_4-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 D'Agostino-Pearson omnibus normality test were not passed). Hill parameters of adenosine E/c curves of the different groups were compared (separately for the solvent and T_4 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).

5 Results

5.1 Results of Study 1

5.1.1 Thyroid status

The initial body weight of T_4-treated guinea pigs did not differ significantly from that of the solvent-treated ones. By the ninth day, the body weight (mean ± SEM) of the solvent-treated animals changed from 827 ± 19 g to 835 ± 20 g (non-significant), while that of the T_4-treated guinea pigs decreased from 836 ± 18 g to 641 ± 13 g ($p < 0.0001$).

5.1.2 Initial adenosine E/c curves

Adenosine decreased the contractile force of all atria in a concentration-dependent manner (Fig. 14). Empirical parameters of the first adenosine *E/c* curves did not differ significantly when compared the same *in vivo*-treated experimental groups with one another (i.e. the solvent- and T_4-treated atria formed two homogenous populations with regard to the response to adenosine). The T_4 treatment decreased E_{max} from 90.65 ± 0.62% to 85.37 ± 0.94% ($p < 0.0001$), increased logEC_{50} from –4.82 ± 0.03 to –4.08 ± 0.04 ($p < 0.0001$), and decreased n from 0.86 ± 0.02 to 0.74 ± 0.02 ($p < 0.0001$) when compared the pooled data of the solvent-treated atria ($n = 38$) to those of the T_4-treated atria ($n = 42$), respectively.

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

In the solvent-treated atria, consistent with our previously reported findings (Kiss et al. 2013), 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 (Fig. 15A, Table 2A). 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 (Fig. 15B, Table 2A).

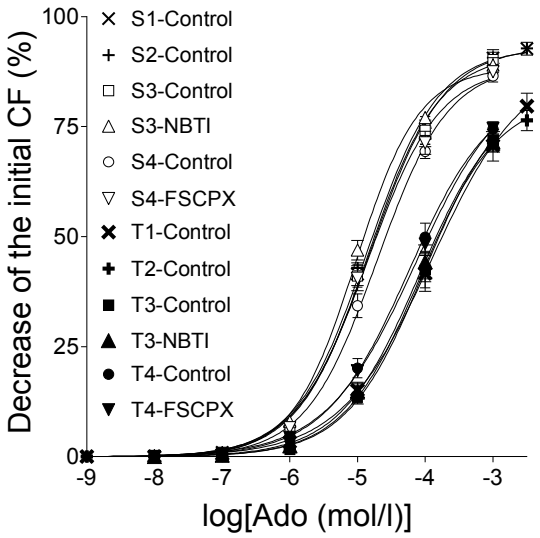


Fig. 14. The direct negative inotropic effect of adenosine (Ado) in solvent- (open symbols) and T_4-treated (filled symbols) guinea pig left atria divided into six-six groups. In groups S1, T1, S2 and T2, the first adenosine E/c curve is shown (Control curves), while in the other groups, the first and only adenosine E/c curve is indicated. The terms NBTI and FSCPX in the group names refer to a subsequent (and not the current) in vitro treatment. The symbols denote the responses to adenosine averaged within the groups (± SEM), and the curves illustrate the fitted Hill equation (equation 1). CF, contractile force.

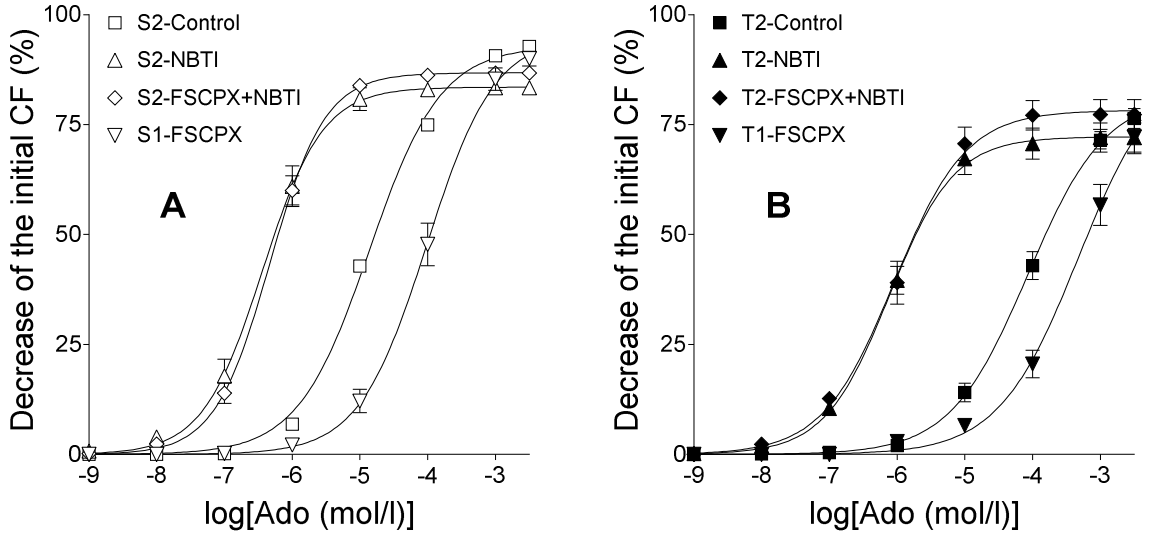


Fig.15. The direct negative inotropic effect of adenosine (Ado) before and after an FSCPX pretreatment, and in the absence and presence of NBTI (alone and in combination), in in vivo solvent- (A) and T_4-treated (B) guinea pig left atria. The curve names refer to the in vivo treatment (S or T), applied protocol (1 or 2) and in vitro treatment (Control, NBTI, FSCPX or FSCPX+NBTI). For simplicity, the S1-Control and T1-Control curves are omitted. The symbols represent the responses to adenosine averaged within the groups (± SEM), and the curves illustrate the fitted Hill equation (equation 1). CF, contractile force.

A

Curves	E_{max} (%)	$logEC_{50}$	EC_{50} (μM)	n
S1-Control	93.47 ± 1.18	–4.8 ± 0.06	15.85	0.82 ± 0.05
S1-FSCPX	93.65 ± 1.01 (ns)	–4.03 ± 0.1 (***)	93.33	0.96 ± 0.09 (ns)
S2-Control	92.9 ± 1 (ns)	–4.86 ± 0.03 (ns)	13.8	0.83 ± 0.04 (ns)
S2-NBTI	83.57 ± 1.5 (***)	–6.43 ± 0.1 (***)	0.37	1.02 ± 0.08 (ns)
S2-FSCPX+NBTI	86.76 ± 1.03 (**; ≠)	–6.33 ± 0.06 (***; ns)	0.47	1.1 ± 0.08 (ns; ns)
T1-Control	88.04 ± 1.09	–3.92 ± 0.12	120.23	0.68 ± 0.03
T1-FSCPX	88.96 ± 1.01 (ns)	–3.32 ± 0.12 (**)	478.63	0.77 ± 0.05 (ns)
T2-Control	81.95 ± 2.91 (ns)	–4.07 ± 0.09 (ns)	85.11	0.78 ± 0.03 (ns)
T2-NBTI	72.3 ± 3.42 (ns)	–6.1 ± 0.05 (***)	0.79	0.92 ± 0.04 (ns)
T2-FSCPX+NBTI	78.26 ± 3.29 (ns; ns)	–6.03 ± 0.08 (***; ns)	0.93	0.85 ± 0.02 (ns; ns)

B

Groups	E_{max} (%)	$logEC_{50}$	EC_{50} (nM)	n
S3-Control	93.07 ± 1.67	–7.57 ± 0.08	26.92	0.99 ± 0.03
S3-NBTI	83.55 ± 2.09 (***)	–7.22 ± 0.11 (*)	60.26	0.78 ± 0.08 (*)
S4-Control	90.26 ± 1 (ns)	–7.61 ± 0.04 (ns)	24.55	0.92 ± 0.03 (ns)
S4-FSCPX	91.63 ± 0.85 (ns)	–6.86 ± 0.1 (***)	138.04	0.85 ± 0.04 (ns)
T3-Control	83.08 ± 1.29	–7.28 ± 0.04	52.48	0.8 ± 0.04
T3-NBTI	69.02 ± 2.54 (***)	–6.72 ± 0.14 (***)	190.55	0.78 ± 0.05 (ns)
T4-Control	81.62 ± 2.03 (ns)	–7.29 ± 0.08 (ns)	51.29	0.8 ± 0.05 (ns)
T4-FSCPX	84.28 ± 0.98 (ns)	–6.77 ± 0.09 (**)	169.82	0.77 ± 0.01 (ns)

Table 2. The influence of FSCPX-pretreatment and NBTI (alone or together) on the direct negative inotropic effect of adenosine (panel A) or CPA (panel B) on left atria, isolated from solvent- or T_4-treated guinea pigs. E_{max}, $logEC_{50}$ and n (mean ± SEM) are best-fit values of the Hill equation (equation 1) fitted to the individual E/c curves. EC_{50} (mean) is the antilog of $logEC_{50}$. The level of statistical significance is indicated: ns, not significant; one, two or three marks (* or ≠), $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively. Panel A: the adenosine E/c curve generated after FSCPX pretreatment (S1-FSCPX, T1-FSCPX) or in the presence of NBTI (S2-NBTI, T2-NBTI) or both (S2-FSCPX+NBTI, T2-FSCPX+NBTI) vs. the corresponding (i.e. the same in vivo treatment and protocol) control adenosine E/c curve (S1-Control, S2-Control, T1-Control, T2-Control); ≠ the adenosine E/c curve influenced by both inhibitors (S2-FSCPX+NBTI, T2-FSCPX+NBTI) vs. the same in vivo-treated adenosine E/c curve constructed in the presence of NBTI (S2-NBTI, T2-NBTI). The corresponding control adenosine E/c curves were also compared (S1-Control vs. S2-Control; T1-Control vs. T2-

Control). Panel B: the CPA E/c curve generated in the presence of NBTI (S3-NBTI, T3-NBTI) or after FSCPX pretreatment (S4-FSCPX, T4-FSCPX) vs. the corresponding (the same in vivo treatment and protocol) control CPA E/c curve (S3-Control, S4-Control, T3-Control, T4-Control). The corresponding control CPA E/c curves were also compared (S3-Control vs. S4-Control; T3-Control vs. T4-Control). CPA, N^6-cyclopentyladenosine; NBTI, S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine; FSCPX, 8-cyclopentyl-N3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine.

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 (Fig. 15, Table 2A).

5.1.4 CPA E/c curves

CPA also reduced the contractile force of all atria in a concentration-dependent manner (Fig. 16). The T_4 treatment decreased E_{max} from 91.57 ± 0.98% to 82.31 ± 1.21% ($p< 0.0001$), increased logEC_{50} from –7.59 ± 0.04 to –7.29 ± 0.05 ($p< 0.0001$), and decreased n from 0.93 ± 0.02 to 0.8 ± 0.03 ($p = 0.0029$) when comparing the pooled data of the S3-Control and S4-Control groups ($n = 15$) to those of the T3-Control and T4-Control groups ($n= 14$), respectively.

In the solvent-treated atria, in agreement with our earlier results (Kiss et al. 2013), NBTI significantly decreased E_{max} (as well as Hill coefficient) and increased logEC_{50}, while FSCPX pretreatment significantly increased logEC_{50}, imitating the action of a competitive, rather than irreversible A_1 receptor antagonist (Fig. 17A, Table 2B). The T_4-treated atria produced outcomes similar to the solvent-treated ones (Fig. 16B). 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 (Fig. 16, Table 2B).

Interstitial accumulation of surplus endogenous adenosine, caused by NBTI in the solvent-treated atria, was found to be equieffective with 38.19 nM CPA (the best-fit value provided by equation (2) was logc_x = –7.418 with a 95% confidence interval from –7.537 to –

7.3). In the T_4-treated atria, the equieffective CPA concentration was 58.75 nM ($\log c_x$ = –7.231 with –7.334 and –7.129 95% confidence limits).

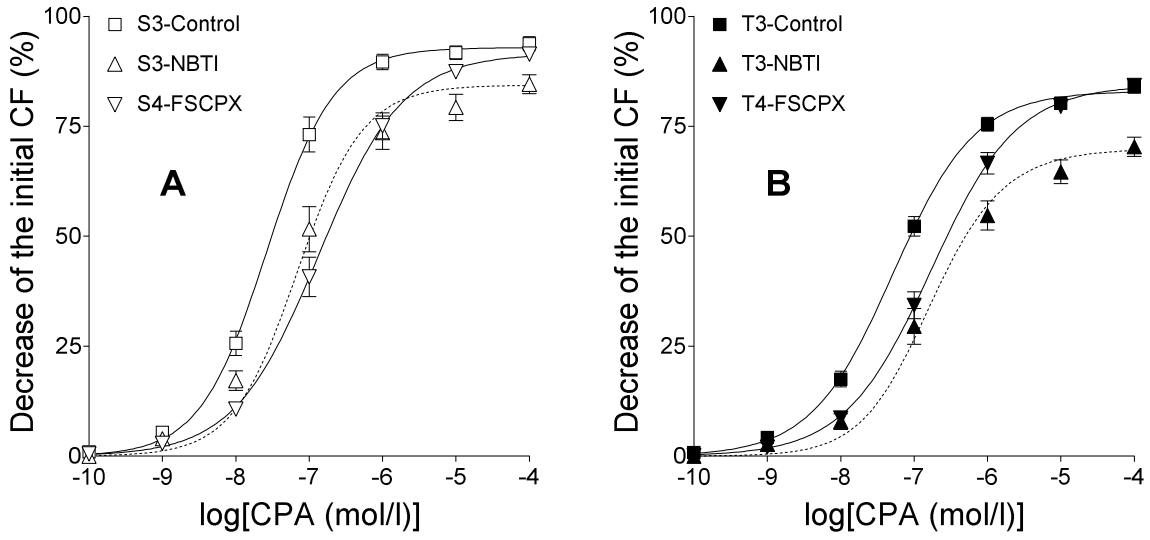


Fig.16. The direct negative inotropic effect of CPA with or without an FSCPX pretreatment, furthermore in the presence and absence of NBTI, in in vivo solvent- (A) and T_4-treated (B) guinea pig left atria. The group names refer to the in vivo treatment (S or T), applied protocol (3 or 4) and in vitro treatment (Control, NBTI or FSCPX). For simplicity, the S4-Control and T4-Control groups are omitted. The symbols indicate the responses to CPA averaged within the groups (± SEM). The continuous curves represent the fitted Hill equation (equation 1), while the dotted curve illustrates the fitted RRM model (equation 2). CF, contractile force.

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

As the interstitial adenosine levels in the microenvironment of A_1 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 (Fig. 17). 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.

The corrected effect values (and effect values of the control curves considered to be inherently correct) appertaining to 3 mM adenosine, the highest adenosine concentration used, were as follows: 92.77% (S2-Control curve), 92.45% (S2-NBTI curve), 90.11% (S2-FSCPX+NBTI curve), 76.4% (T2-Control curve), 85.47% (T2-NBTI curve) and 82.42% (T2-FSCPX+NBTI curve). The initial corrected effect values (at zero exogenous adenosine concentration) were as follows: 54.59% (S2-NBTI curve), 25.46% (S2-FSCPX+NBTI curve), 48.05% (T2-NBTI curve) and 26.44% (T2-FSCPX+NBTI curve) (Fig. 17).

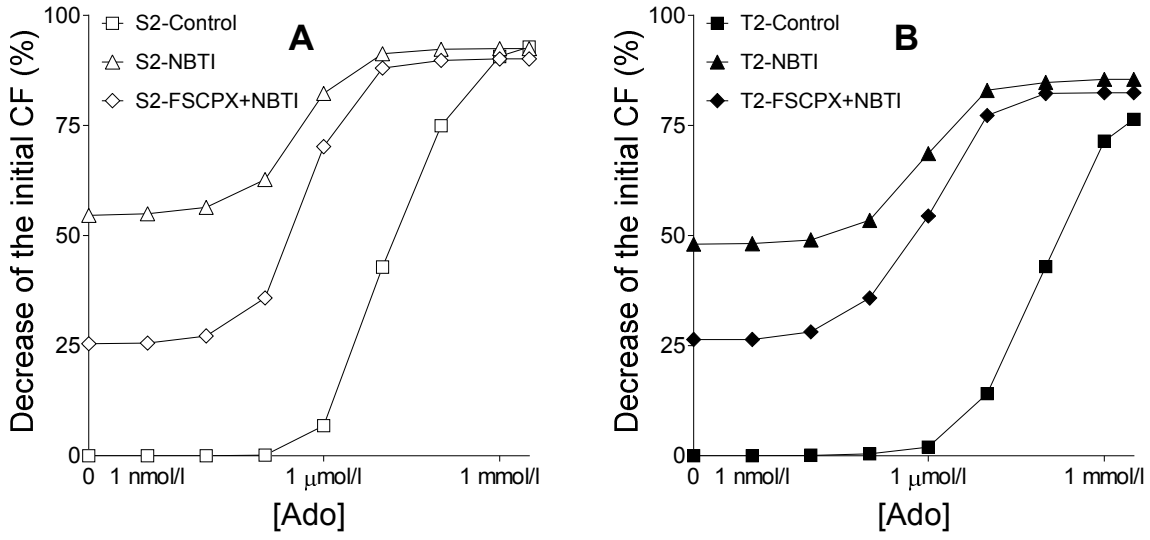


Fig.17. The corrected effect values of adenosine E/c curves generated in the presence of NBTI plotted versus the exogenous adenosine (Ado) concentrations developed in the bathing medium, together with the original S2-Control and T2-Control curves. The solvent- and T_4-treated atria are represented on panel A and B, respectively. The curve names refer to the in vivo treatment (S or T), applied protocol (2) and in vitro treatment (Control, NBTI or FSCPX+NBTI). The correction was made with the use of regression parameters of the averaged CPA E/c curves of Protocols 3 and 4. The symbols represent the responses to adenosine averaged within the groups. CF, contractile force.

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 (Fig. 17A). (In our previous study (Kiss et al. 2013), the maximal effect value of the corrected euthyroid NBTI curve was marginally greater than that of the euthyroid control curve, which result fits the theoretical expectations better than the present one. This discrepancy, noted in the present investigation, may reflect a minor uncertainty in the raw experimental data.) 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 (Fig. 17B).

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 (Kiss et al. 2013): they changed places with each other, as compared to the original curves, and their final parts got close to each other, indicating a great A_1 receptor reserve for the direct negative inotropic effect of adenosine (Fig. 17A). These characteristics also apply to the corrected hyperthyroid curves (T2-NBTI and T2-FSCPX+NBTI), indicating that T_4 treatment did not significantly influence the aforementioned A_1 receptor reserve. However, while the corrected S2-FSCPX+NBTI curve ran (a bit) below the S2-Control curve at the two highest adenosine concentrations (Fig. 17A), the final part of the corrected T2-FSCPX+NBTI curve ran considerably above the T2-Control curve (similar to the corrected T2-NBTI curve) (Fig. 17B).

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%)

5.2 Results of Study 2

5.2.1 Thyroid status

No difference was detected between the initial body weight of the solvent-treated guinea pigs and the T_4-treated ones. By the ninth day, body weight of the solvent- and T_4-treated guinea pigs altered from 712 ± 11 g to 723 ± 11 g, and from 719 ± 12 g to 538 ± 11 g ($p < 0.001$), respectively. The ratio of left atrial weight to body weight in the solvent- and T_4-treated group was 0.089 ± 0.009 mg/g and 0.132 ± 0.009 mg/g ($p < 0.001$), respectively.

5.2.2 Initial contractile forces in Study 2

In Study 2, we analyzed and interpreted the raw contractile forces, in addition to the evaluation of adenosinergic responses, consistent with the advanced complexity of Study 2.

The initial contractile forces, measured prior to the *in vitro* treatment (before the first E/c curve), did not differ significantly, either between the pooled solvent- and T_4-treated atria (9.3 ± 0.35 mN and 8.67 ± 0.4 mN, respectively) or among the different groups (Fig. 18).

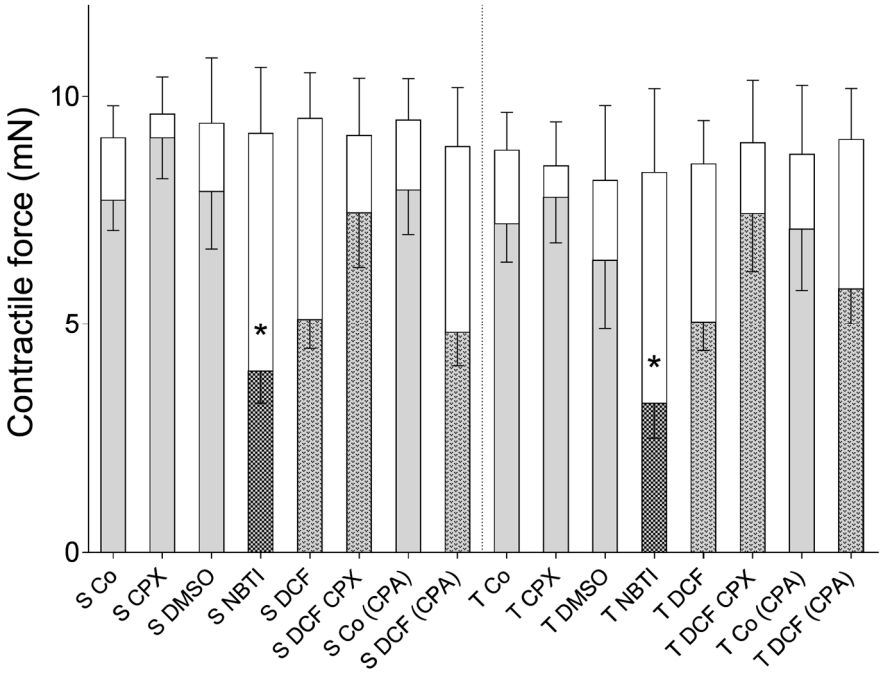


Fig. 18. The contractile force of guinea pig left atria in the different groups before and after the in vitro treatment. The open columns show the contractile forces of atria measured before the in vitro treatment (mean + SEM). The shorter filled columns in front of the open ones denote the contractile forces determined at the end of the in vitro treatment (mean – SEM). The homogeneous light gray filling means an in vitro treatment containing neither NBTI nor DCF, while light gray filling with darker or less dark pattern shows an in vitro treatment containing NBTI or DCF, respectively. Group names are indicated below the columns. Comparing the contractile forces of the different in vitro treated groups (filled columns), significant difference was found only in the case of NBTI: S Co vs. S NBTI; S CPX vs. S NBTI; T Co vs. T NBTI; and T CPX vs. T NBTI (*).

During the *in vitro* treatment, CPX non-significantly moderated the small decay in the contractile force over time, as compared to the corresponding controls. In contrast, both NBTI

and DCF decreased the atrial contractile forces, although this effect was significant only in the case of NBTI ($p < 0.05$). The co-treatment with DCF and CPX produced an outcome similar to the corresponding controls, thus DCF and CPX appeared to mutually cancel out each other's effects. Contractile forces of the different T_4-treated groups did not differ significantly from their solvent-treated counterparts (Fig. 18).

5.2.3 Adenosine E/c curves

Response to adenosine: Adenosine concentration dependently reduced the contractile force of atria (Fig. 19). As no previous intervention evoking positive inotropic effect was done, the response to adenosine was considered as direct negative inotropic effect that is typical for the atrial (but not ventricular) myocardium (Belardinelli et al. 1995; Kurachi 1995).

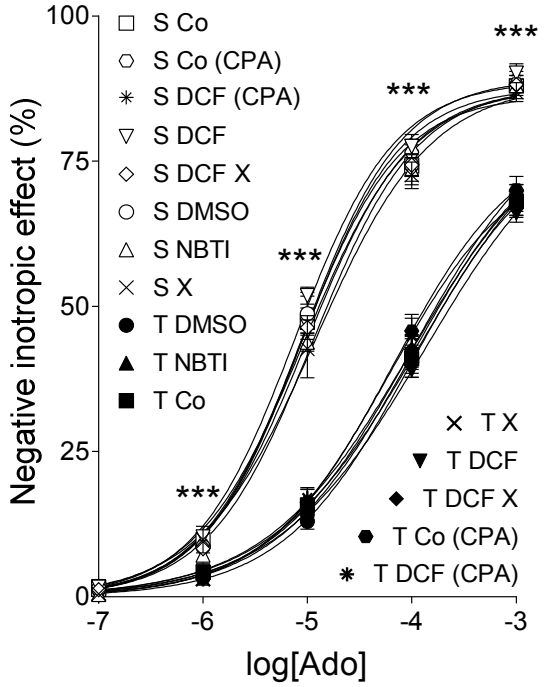


Fig. 19. The direct negative inotropic effect of adenosine (Ado) in solvent- (open/thin symbols) or thyroxine- (T_4) treated (filled/thick symbols) guinea pig left atria divided into eight-eight groups (before any in vitro treatment). In the group names, abbreviations refer to a previous in vivo treatment (S: solvent treatment; T: T_4 treatment) and to a subsequent (and not the current) in vitro treatment (Co: control; DCF: 2'-deoxycoformycin; X: CPX, i.e. 8-cyclopentyl-1,3-dipropylxanthine; DMSO: dimethyl-sulfoxide; NBTI: S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine), and to a certain agonist used for a next (and not the present) concentration-effect curve (CPA: N6-cyclopentyladenosine). The axis x shows the common

logarithm of molar concentration of adenosine, and the axis y denotes the effect as a percentage decrease of the initial contractile force. The symbols indicate the responses to adenosine averaged within the groups (± SEM). The curves illustrate the fitted Hill equation (equation 1). The responses to adenosine differed significantly between groups S Co vs. T Co (*).

Comparison before the in vitro treatment: Responses to the different adenosine concentrations (Fig. 19), as well as Hill parameters of the adenosine E/c curves (data not shown), did not differ significantly when compared the same *in vivo* treated groups with one another. Thus, the solvent- and T_4-treated atria formed two homogenous populations regarding the response to adenosine.

Effect of T_4 on the response to adenosine: The responses to adenosine at the different concentrations (except the starting 0.1 μM) showed a significant decrease when comparing the pooled T_4-treated atria to the pooled solvent-treated ones (($p < 0.001$; data not presented). Consistently, the T_4 treatment diminished E_{max} from 88.27 ± 0.65 % to 84.53 ± 0.84 % ($p < 0.01$), increased $logEC_{50}$ from –5 ± 0.03 to –3.96 ± 0.03 ($p < 0.001$), and decreased n from 0.9 ± 0.03 to 0.66 ± 0.01 ($p < 0.001$).

5.2.4 MC E/c curves

Response to MC: MC also decreased the contractile force of atria in a concentration-dependent manner (direct negative inotropic effect) (Fig. 20-23).

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 (Fig. 20) 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. Nevertheless, minor difference can be shown between groups S Co *vs.* S DMSO and T Co *vs.* T DMSO (Fig. 20B). Based on prior experiences of our team with DMSO, this cannot be ascribed to the effect of DMSO, rather to the fact that investigations using NBTI (including their controls) were performed after experiments using CPX and DCF (and their controls). Seasonal differences can affect the atrial inotropic response (Kemeny-Beke et al. 2007).

Effect of T_4 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 T_4 treatment moderately suppressed the response to MC, which was only significant at higher MC concentrations (Fig. 20). In line with this, the T_4 treatment caused a moderate diminution in E_{max} (significant) and n (on the border of statistical significance), while the increase of $logEC_{50}$ did not reach the significance threshold (Table 3).

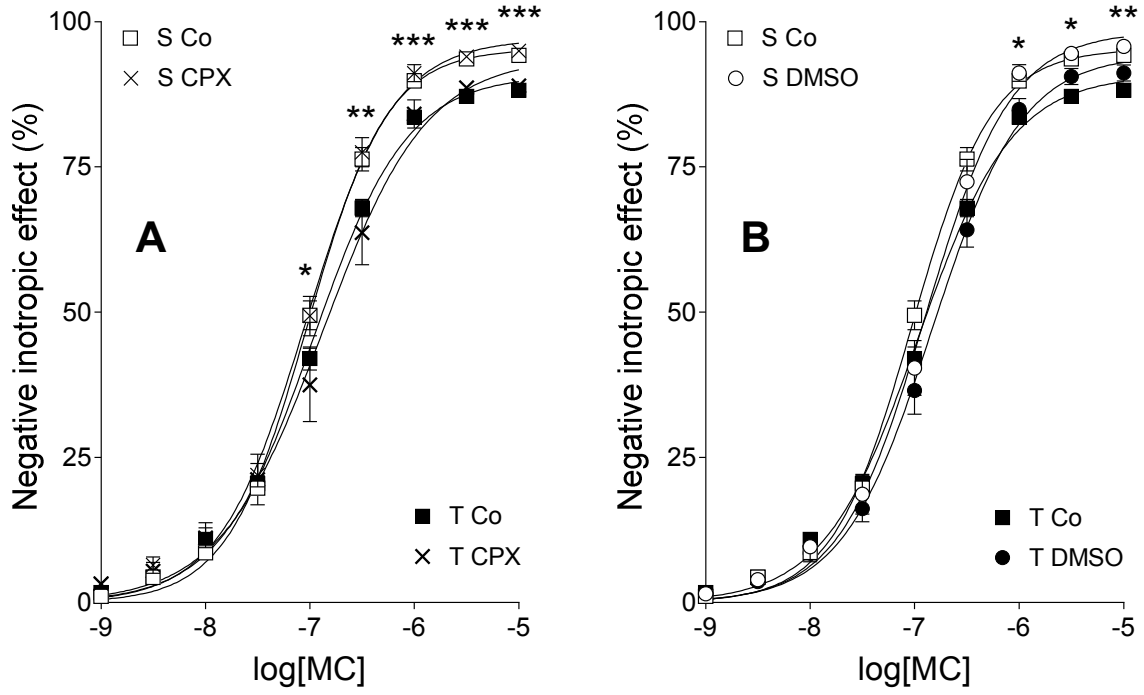


Fig. 20. The direct negative inotropic effect of MC in the presence of CPX or DMSO (alone) or in the absence of both of them (Co), in solvent- (open/thin symbols) or T_4-treated (filled/thick symbols) guinea pig left atria. The axis x shows the common logarithm of molar concentration of MC, while the axis y indicates the effect as a percentage decrease of the initial contractile force of atria. The symbols represent the responses to MC averaged within the groups (± SEM), and the curves illustrate the fitted Hill equation (equation 1). The responses to MC differed significantly between groups S Co vs. T Co and S DMSO vs. T DMSO (*).

Modification of the response to MC by NBTI: In both the solvent- and T_4-treated groups, NBTI significantly reduced the response to MC, according to the conventionally plotted (and thereby biased) E/c curves (Fig. 21). This manifested in a significant decrease of E_{max} and in a minor increase of $logEC_{50}$ with a practically unchanged n (Table 3). The effect of NBTI was more intense in the group T NBTI than in the group S NBTI (Fig. 20, Table 3).

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 (Fig. 21), the surplus interstitial adenosine was found to be equieffective with 101.2 nM and 151.1 nM MC in the solvent- and T_4-treated atria, respectively (Table 4A). It means that nucleoside transport blockade produces a greater interstitial adenosine accumulation in the T_4-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 (Karsai et al. 2007; Pak et al. 2014).

	E_{max}	$logEC_{50}$	n
MC curves			
S Co	95.5 ± 0.41		1.18 ± 0.03
S DMSO	97.52 ± 0.81		
S NBTI	90.23 ± 0.75 ##		
T Co	90.77 ± 0.79 **		1 ± 0.03 *
T DCF	86.23 ± 1.43 ≠		
T DMSO	94.1 ± 1.44 *		
T NBTI	82.85 ± 2.11 ##		
CPA curves			
S Co (CPA)	90.9 ± 1.59	-7.86 ± 0.08	1.06 ± 0.05
T Co (CPA)	84.04 ± 1.01 **	-7.5 ± 0.06 **	0.7 ± 0.04 ***
T DCF (CPA)		-7.77 ± 0.08 ≠	

Table 3. The Hill parameters exhibiting statistically significant change in response to the different in vivo (*) or in vitro (≠) treatments. E_{max}, $logEC_{50}$ and n (mean ± SEM) are best-fit values of the Hill equation (equation 1), fitted to the individual E/c curves generated with MC or CPA. Significant differences were found between groups S Co vs. T Co, S DMSO vs. T DMSO and S Co (CPA) vs. T Co (CPA) (*); furthermore S DMSO vs. S NBTI, T DMSO vs. T NBTI, T Co vs. T DCF and T Co (CPA) vs. T DCF (CPA) (≠).

Modification of the response to MC by DCF: DCF decreased the response to MC in both the solvent- and T_4-treated atria, according to the conventionally plotted (biased) E/c

curves. This bias was rather symbolic under euthyroid conditions (Fig. 22A), while it was well-marked in hyperthyroidism (Fig. 22B). In turn, CPX abolished this effect of DCF on the MC E/c curves in both thyroid states (Fig. 22). 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 T_4-treated atria (except for the response at 10 nM MC in the group S DCF, but it was considered irrelevant) (Fig. 22). Consistently, the decrease of E_{max} was only significant in the group T DCF (Table 3).

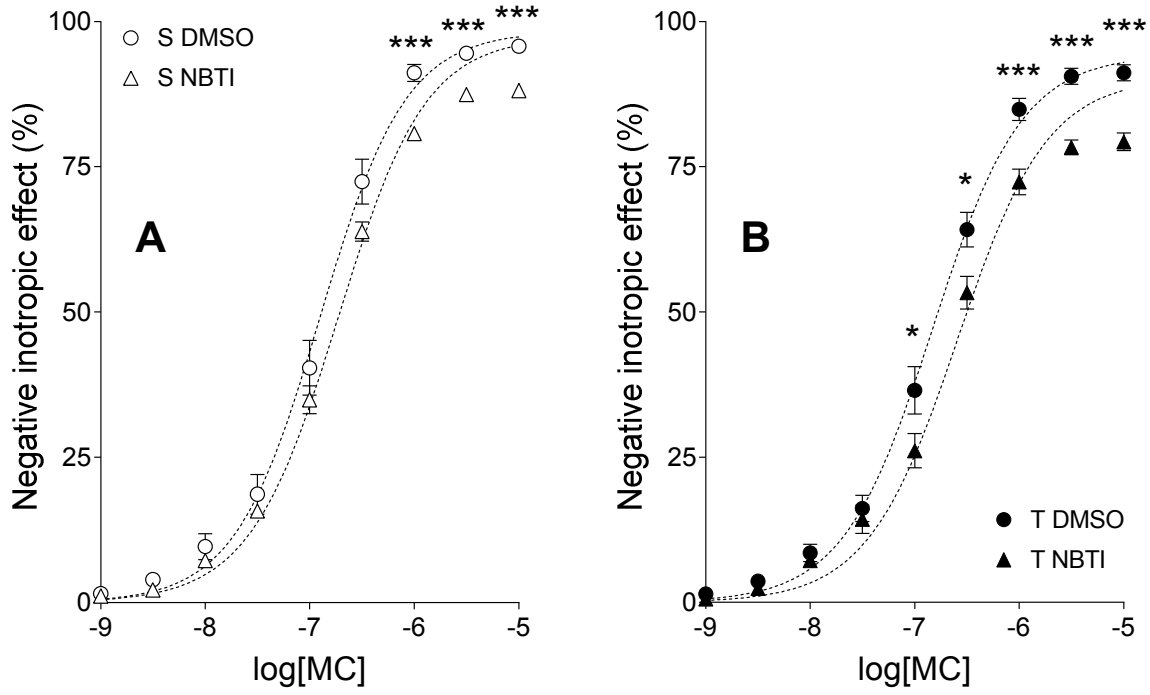


Fig. 21. The direct negative inotropic effect of MC in the presence of DMSO (alone) or NBTI, in solvent- (open symbols in panel A) or T_4-treated (filled symbols in panel B) guinea pig left atria. The axis x shows the common logarithm of molar concentration of MC, and the axis y indicates the effect as a percentage decrease of the initial contractile force of atria. The symbols denote the responses to MC averaged within the groups (± SEM), and the dotted curves illustrate the fitted RRM model (equation 2). The responses to MC differed significantly between groups S DMSO vs. S NBTI and T DMSO vs. T NBTI (*).

Effect of DCF on the interstitial adenosine level: Fitting the equation 2 to MC E/c data of groups S DCF and T DCF (Fig. 22), the surplus interstitial adenosine proved equieffective with 28.05 nM and 44.36 nM MC in the solvent- and T_4-treated atria, respectively (Table 4B).

This outcome is similar to that seen in response to NBTI (Table 4A), namely DCF appears to produce a greater interstitial adenosine accumulation in the T_4-treated atria than in the solvent-treated ones (Table 4B). However, due to reasons specified in the Discussion section, this latter finding should be treated with caution. Nevertheless, DCF increased the interstitial adenosine level to a smaller extent than NBTI did, in both the solvent- and T_4-treated groups (Table 4).

A	**S DMSO**	**S NBTI**	**T DMSO**	**T NBTI**
$\log c_x$	-12.62	-6.99	-9.07	-6.82
95%CI from	-18000	-7.1	-17.62	-6.93
to	17975	-6.88	-0.53	-6.72
c_x(nmol/l)	0.00024	**101.2**	0.84	**151.1**

B	**S Co**	**S DCF**	**T Co**	**T DCF**
$\log c_x$	-13.98	-7.55	-53.25	-7.35
95%CI from	-91603	-7.73	ambig-	-7.45
to	91576	-7.37	uous	-7.26
c_x(nmol/l)	0.00001	**28.05**	0	**44.36**

Table 4. The c_x values obtained with RRM characterizing the concentration of the surplus interstitial adenosine produced by NBTI or DCF in solvent- (S) or T_4-treated (T) guinea pig left atria. The $\log c_x$ is the best-fit value of equation 2 fitted to the averaged E/c curves generated with MC in the presence of DMSO or NBTI or DCF or in the absence of all of them (Co). Precision of the fit was characterized with the 95% confidence interval (95% CI) for the best-fit value. Fitting of the control-type groups (S DMSO, T DMSO, S Co, T Co) to the equation 2 served only for verification, the expected value of c_x was zero. In turn, fitting of the groups S NBTI, T NBTI, S DCF and T DCF was expected to provide a c_x value (shown in bold) that indicates the MC concentration equieffective with the extra interstitial adenosine produced by NBTI or DCF.

5.2.5 CPA E/c curves

Response to CPA: CPA also reduced the atrial contractile force in a concentration-dependent manner (direct negative inotropic effect) (Fig. 24).

Effect of T_4 on the response to CPA: Comparing groups T Co (CPA) and S Co (CPA),

the T_4 treatment considerably decreased the response to CPA that was significant from medium to high concentrations (Fig. 24). In agreement with this, T_4 treatment significantly reduced both E_{max} and n, and increased $logEC_{50}$ (Table 3). Thus, T_4 induced a greater depression of the E/c curve for CPA than for MC (Fig. 20, 24, Table 3).

Modification of the response to CPA by DCF: In contrast to that seen with MC (Fig. 22), DCF augmented the response to CPA in both the solvent- and T_4-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 T_4-treated ones in the lower and medium concentration ranges (Fig. 24). In agreement with this, DCF significantly decreased $logEC_{50}$ of E/c curves of T_4-treated but not solvent-treated atria (Table 3). This outcome is consistent with our previous finding that inhibition of ADA enhances the efficiency of the direct negative inotropic function mediated by the A_1 receptor in the hyperthyroid guinea pig atrium (Kemeny-Beke et al. 2007).

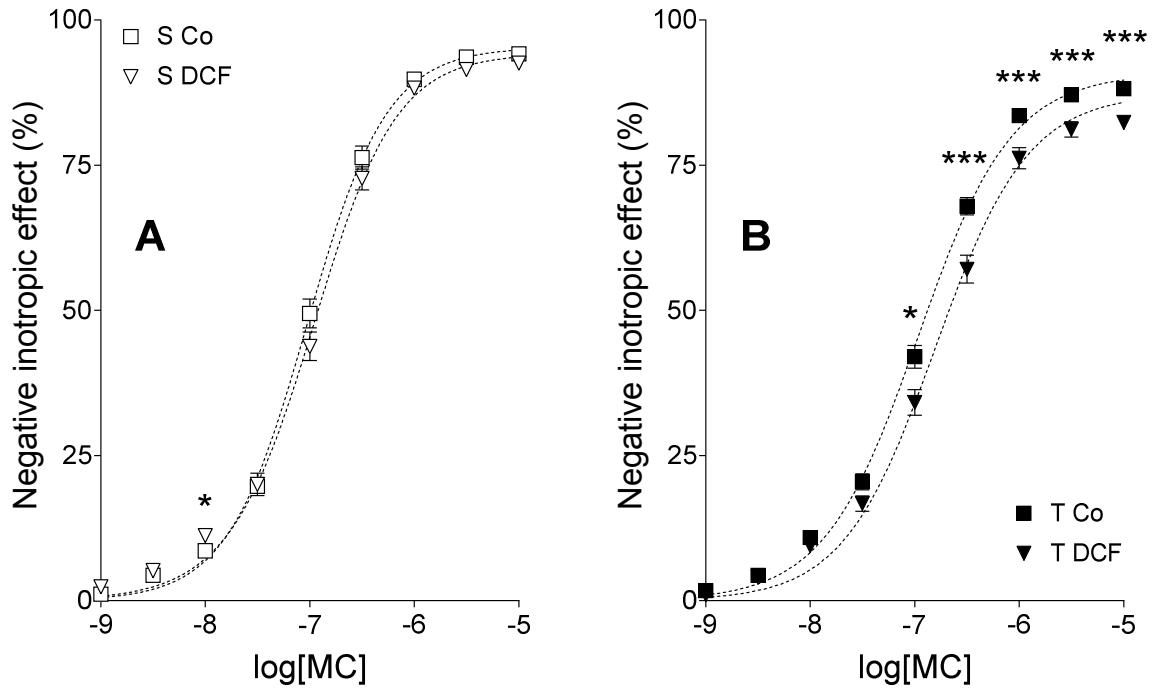


Fig. 22. The direct negative inotropic effect of MC in the absence or presence of DCF, in solvent- (open symbols in panel A) or T_4-treated (filled symbols in panel B) guinea pig left atria. The axis x denotes the common logarithm of molar concentration of MC, and the axis y indicates the effect as a percentage decrease of the initial contractile force of atria. The symbols show the responses to MC averaged within the groups (± SEM), and the dotted curves illustrate the fitted RRM model (equation 2). The responses to MC differed significantly between groups S Co vs. S DCF and T Co vs. T DCF (*).

5.2.6 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).

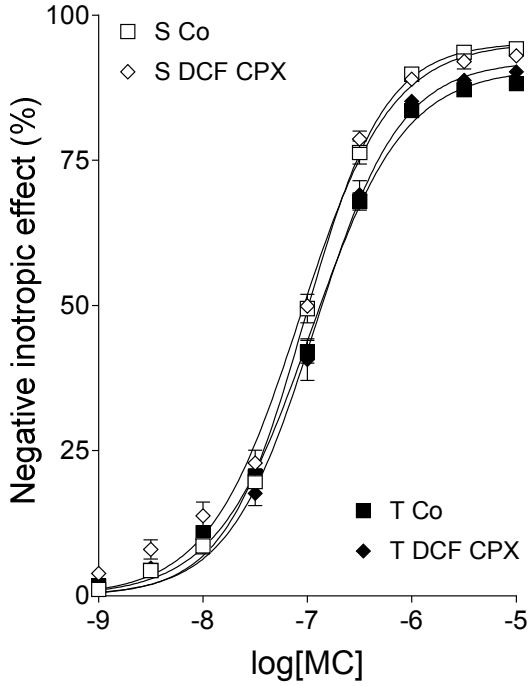


Fig. 23. The direct negative inotropic effect of MC in the absence or presence of DCF added together with CPX, in solvent- (open symbols) or T_4-treated (filled symbols) guinea pig left atria. The axis x denotes the common logarithm of molar concentration of MC, and the axis y indicates the effect as a percentage decrease of the initial contractile force of atria. The symbols show the responses to MC averaged within the groups (± SEM), and the curves illustrate the fitted Hill equation (equation 1).

NBTI with MC: The corrected MC E/c curves (generated in the presence of NBTI) ended somewhat below their control curves (Fig. 25A, 25B). 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 M_2 muscarinergic signaling, irrespectively of the thyroid state (Fig. 25A, 25B).

DCF with MC: The corrected MC E/c curves (constructed in the presence of DCF) ran to the maximum of their control curves (Fig. 25C, 25D). Conclusions to be drawn are the same as those with NBTI, i.e. DCF does not influence the signal amplification of the atrial M_2 muscarinergic mechanisms, regardless of the T_4 treatment (Fig. 25C, 25D).

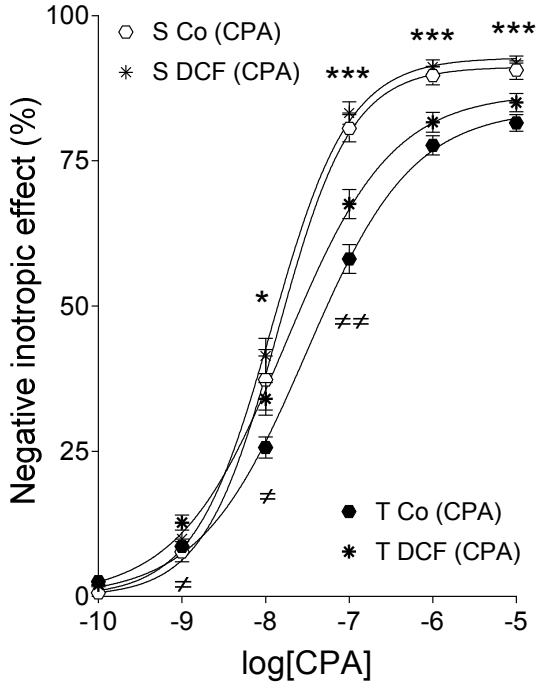
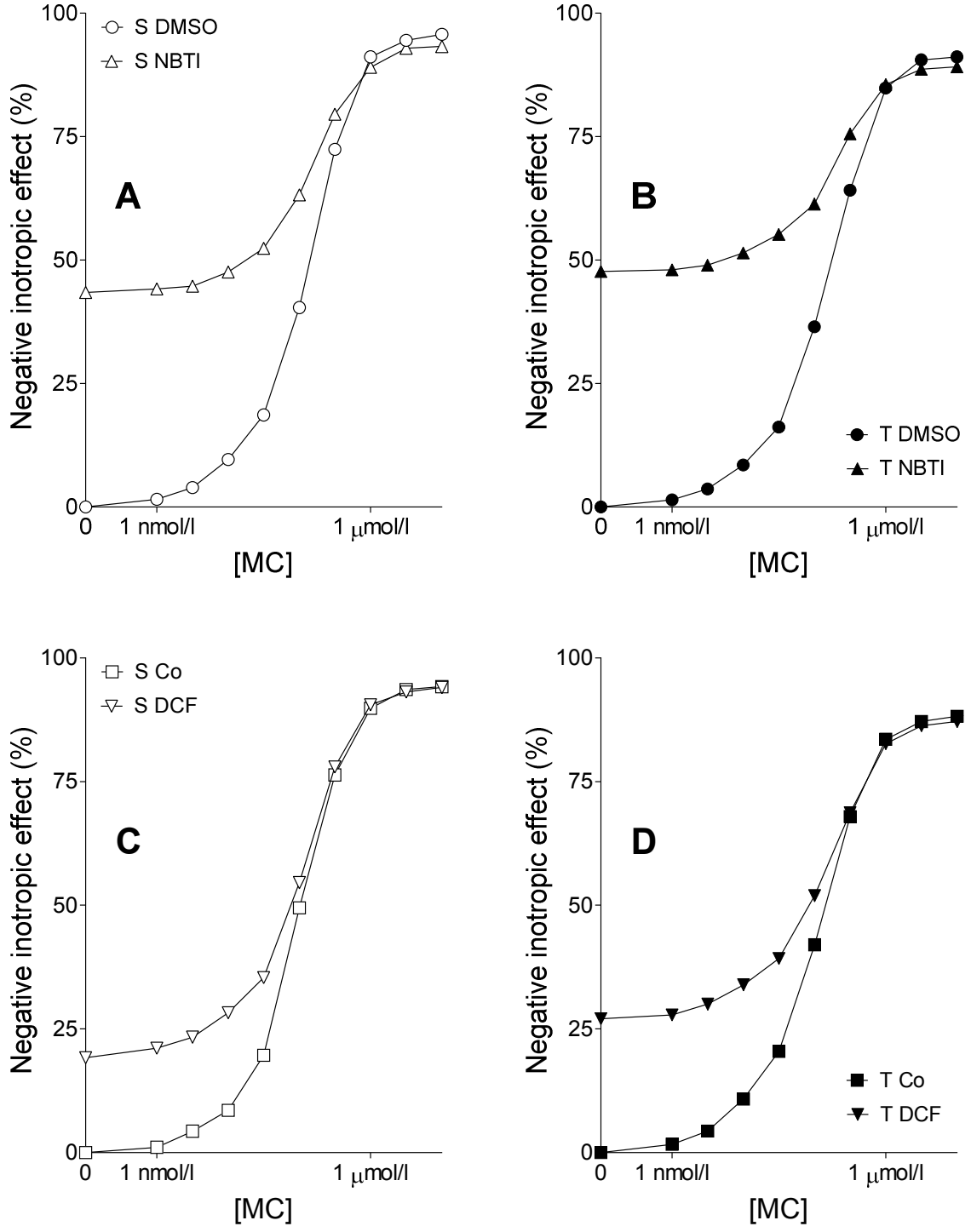


Fig. 24. The direct negative inotropic effect of CPA in the absence or presence of DCF, in solvent- (open/thin symbols) or T_4-treated (filled/thick symbols) guinea pig left atria. The axis x shows the common logarithm of molar concentration of CPA, while the axis y indicates the effect as a percentage decrease of the initial contractile force of atria. The symbols denote the responses to CPA averaged within the groups (± SEM), and the curves represent the fitted Hill equation (equation 1). The responses to CPA differed significantly between groups S Co (CPA) vs. T Co (CPA) (*) and T Co (CPA) vs. T DCF (CPA) (≠).

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 (Fig. 25E, 25F). This finding indicates that DCF augments the efficiency of the A_1 adenosinergic system regarding its direct negative inotropic function in the guinea pig atrium. This phenomenon was visibly more pronounced in the T_4-treated atria (Fig. 25E, 25F), in agreement with our previous finding (Kemeny-Beke et al. 2007). 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%).



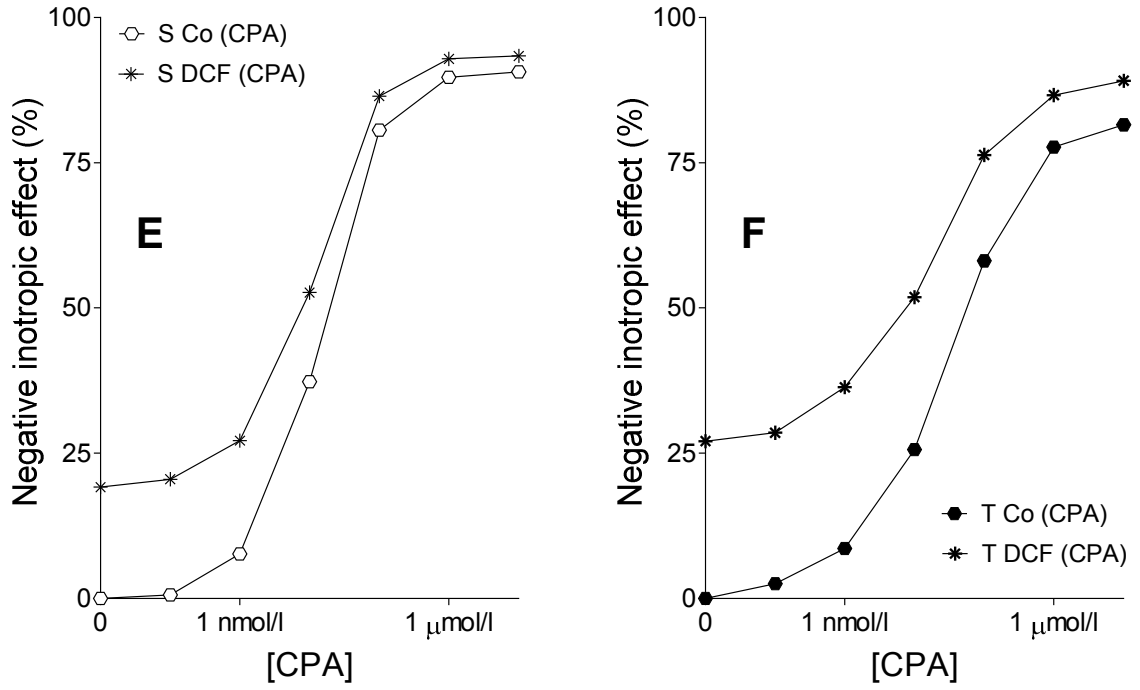


Fig. 25. The corrected direct negative inotropic effect values of concentration-effect curves generated in the presence of NBTI or DCF, plotted versus concentration of the administered MC or CPA, together with the corresponding control concentration-effect curves (in their original form).The correction was made for the direct negative inotropic effect of the surplus interstitial adenosine produced by NBTI or DCF in a manner that this effect value has been incorporated into effect data depicted herein. The axis x indicates the agonist concentration on a base-10 logarithmic scale, and the axis y shows the effect as a percentage decrease in the initial contractile force of atria. The symbols (open/thin: solvent treatment; filled/thick: T_4 treatment) represent the responses to the given agonist averaged within the groups (controls) and the corrected effects computed from the raw effects averaged within the groups.

6 Discussion

The leading cause of death in most countries is the group of cardiovascular diseases in high-income countries. Ischemic heart disease has remained the top major killer during the past decade (WHO 2014a, 2014b). A basic understanding of endogenous protective mechanisms of the heart against ischemia is a necessity for the development of new rational therapeutic strategies. One powerful endogenous protective mechanism in the living tissues is the adenosinergic signaling (Burnstock and Pelleg 2015).

There is a number of humoral factors that affects cardiac adenosinergic system. The homeostatic mechanisms, in which these hormones participate, may become dysregulated. Hyperthyroidism is a relatively frequent example of these regulation disturbances. Our investigations, underlying the Study 1 of this thesis, aimed to explore the influence of excess thyroid hormones on the A_1 receptor reserve for the direct negative inotropic effect.

There are several experimental and therapeutical procedures to increase the basal interstitial adenosine level in the heart that are thought to prevent and/or cure damages caused by ischemia and subsequent reperfusion. A possibility among these procedures is to inhibit ADA, a catabolic enzyme in adenosine homeostasis. Based on previous experiments of our work team, it has emerged that ADA inhibition exerts regulatory effect on the myocardial A_1 receptor function, besides to elevate the cardiac adenosine concentration. The major goal of our investigations, carried out in the frame of the Study 2 for this thesis, was to address this important issue. In addition, we aimed to compare the effects of inhibition of ADA and nucleoside transport on the interstitial adenosine concentration.

6.1 Interpretation of results in Study 1

To the best of our knowledge, our Study 1 is the **first** to show that T_4 treatment does not substantially affect the A_1 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.

Information regarding the magnitude of a particular receptor reserve may be used to predict the behavior of an agonist in a tissue. If receptor reserve in a tissue is small enough, low-efficacy agonists cannot evoke biologically significant effects, while high-efficacy agonists are able to do so. Accordingly, relative tissue selectivity can be achieved by means of partial agonists, i.e. they will only act on tissues possessing large receptor reserve. However, it should be noted that receptor reserve depends not only on the agonist and tissue, but also on the effect measured (Kenakin 1987, 2009; Dhalla et al. 2003).

Prior to our two recent investigations (Gesztelyi et al. 2013; Kiss et al. 2013), the A_1 receptor reserve had not been determined for the direct negative inotropic effect, which is characteristic of the atrium in most species, including guinea pigs and humans (Szentmiklosi et al. 1982; Bohm et al. 1984; Marmo et al. 1986; Belardinelli et al. 1995). The magnitude of this receptor reserve may predict sensitivity of atrial mechanical activity to A_1 receptor stimulation. The value of such a predictor may be appreciated by considering that the A_1 receptor is a therapeutic target in many tissues (Elzein and Zablocki 2008; Schenone et al. 2010; Fredholm et al. 2011; Szentmiklosi et al. 2011; Albrecht-Küpper et al. 2012; Staehr et al. 2013).

In two previous studies, we observed substantial receptor reserve for negative inotropy by use of stable synthetic agonists (Gesztelyi et al. 2013) and adenosine, the degradable physiological agonist (Kiss et al. 2013). This outcome led to a hypothesis that agents producing A_1 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 A_1 adenosinergic signaling pathways. As a consequence, thyroid hormones reduce the effect of A_1 receptor agonists on atrial contractility (Szentmiklosi et al. 1992; Kaasik et al. 1994; Gesztelyi et al. 2003a; Fig. 15, 17), although the underlying mechanisms are not fully clarified yet (for more details, see: Gesztelyi et al. 2012). Thus, it might be expected that thyroid hormones affect, presumably reduce, the great atrial A_1 receptor reserve belonging to the direct negative inotropic effect. The aim of the present study was to test this possibility.

Because A_1 receptor enhancers and agents that elevate the endogenous adenosine levels are also in development or approved for clinical use in addition to synthetic A_1 receptor agonists (Elzein and Zablocki 2008; Fredholm et al. 2011; Szentmiklosi et al. 2011), adenosine was selected as an agonist for determining the A_1 receptor reserve in the present study. Under our *ex vivo* experimental conditions (used in the present study as well), only

stable synthetic agonists proved to be suitable for the exact quantification of receptor reserve (Gesztelyi et al. 2013). In the case of adenosine, the failure of determination was attributed to adenosine's very short half-life under physiological conditions. Notwithstanding, after a mathematical correction using RRM, adenosine *E/c* curves could be transformed suitable for a qualitative determination of receptor reserve (as described previously: Kiss et al. 2013).

The present investigation revealed that T_4 treatment did not substantially influence the A_1 receptor reserve appertaining to the direct negative inotropic effect of adenosine (Fig. 18), although it significantly suppressed the direct negative inotropic response to both adenosine and CPA (Fig. 15, 17). This result suggests that administration of agents causing A_1 receptor stimulation, irrespective of their indication of use, presents a similar risk in eu- and hyperthyroid hearts for weakening of atria. Thus, when an A_1 receptor agonist is administered in increasing concentrations to the whole body, this effect can be expected foremost among the A_1 receptor-mediated adverse cardiac effects in both eu- and hyperthyroid conditions. The major finding of the present study, i.e. unchangingness of A_1 receptor reserve for the direct negative inotropic effect of adenosine may be surprising, with regard to the observation that a given A_1 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 (Rossi et al. 2000). Additionally, the decreased atrial pumping capacity increases the risk for atrial thrombus formation (Betts 2012). For these reasons, it is important to consider that atrial contractility may decrease during the use of agents that cause A_1 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, pericarditis) and procoagulant states (Rossi et al. 2000; Betts 2012).

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 (Fig. 18). Thus, although the direct negative inotropy evoked by adenosine is suppressed in hyperthyroidism (Szentmiklosi et al. 1992; Gesztelyi et al. 2003a; Fig. 15, 17), 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 (Smolenski et al. 1995), and the inward adenosine transport was enhanced in the hyperthyroid guinea pig atrium (Karsai et al. 2007), 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 A_1 receptors (Karsai et al. 2007).

A limitation of the Study 1 is its qualitative nature. This is due to the fact that the interstitial adenosine concentration, which would have been necessary for a quantitative assessment, cannot be measured with accuracy sufficient for our purpose (Bassingthwaighte 1992; Karsai et al. 2006; Ramakers et al. 2008). The present investigation has nevertheless yielded evidence about the similarly great signal amplification capacity appertaining to the direct negative inotropic effect mediated by the atrial A_1 receptor in eu- and hyperthyroid conditions. Another limitation 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 A_1 receptor and its downstream signaling pathways (Fredholm et al. 2001, 2011; Ijzerman et al. 2014).

In summary, the present investigation has revealed that, although the A_1 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 A_1 receptor agonist, even a partial one, is administered for any indication, the most probable side effect affecting the heart will be a decrease of atrial contractility under both eu- and hyperthyroid conditions. It is possible (but not inevitable) that this adverse effect occurs even at A_1 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.

6.2 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 A_1 adenosine (but not M_2 muscarinic) receptor. This finding suggests that inhibition of ADA affects the atrial A_1 adenosinergic system in a part other than the joint signaling pathways of the A_1 and M_2 receptors. **Secondly**, ADA inhibition enhances the A_1 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 A_1 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 T_4 treatment suppresses the direct negative inotropic function of the M_2 receptor in a guinea pig model as well. Nevertheless, this reduction in the M_2 muscarinergic function in response to thyroid hormones is quite small relative to the decrease in the A_1 adenosinergic one.

Previously we found that ADA inhibition elicited by DCF potentiated the direct negative inotropic effect of CPA, a selective A_1 receptor agonist, in the hyperthyroid guinea pig atrium (Kemeny-Beke et al. 2007). Since CPA is not a substrate for ADA (Pavan and Ijzerman 1998), this counterintuitive result was attributed to that ADA inhibition increases the signal amplification of the atrial A_1 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 A_1 receptors, a known trigger of several beneficial effects of ADA inhibition: Zhu et al. 1994; Peart et al. 2001; Willems et al. 2006; Szentmiklosi et al. 2011). 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 (Deussen 2000a, 2000b), does not enhance the efficiency of the atrial A_1 adenosinergic system under either euthyroid or hyperthyroid conditions (Karsai et al. 2006, 2007).

The major difficulty to investigate this phenomenon is the fact that ADA inhibition, besides enhancing the efficiency of A_1 receptor function (an effect first described by Kemeny-Beke et al. in 2007), elevates the tissue adenosine content, which leads to A_1 receptor activation that also augments the A_1 receptor-mediated functions (a well-known effect). In the experimental setup used for the above-mentioned study (Kemeny-Beke et al. 2007), 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 A_1 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 A_1 adenosinergic system, a biased (smaller than expected) response to CPA was detected (for more details about this phenomenon, see: Gesztelyi et al. 2004; Grenczer et al. 2010a). Thus, the two actions of ADA inhibition (mentioned at the top of this paragraph) worked against each other in our earlier experimental setup (Kemeny-Beke et al. 2007).

In the Study 2, 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 M_2 receptor.

The key concept of the experimental design used for the present study is as follows: If the A_1 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 A_1 adenosinergic system). However, the major signaling pathways, underlying the direct negative inotropy, remain accessible from the M_2 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 A_1 receptor under ADA inhibition, is located in the joint part of the postreceptorial signaling of A_1 and M_2 receptors, an enhanced response to MC is expected relative to the naïve state (lacking A_1 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 A_1 and M_2 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 M_2 muscarinergic system throughout the investigation.

Results of a preliminary study, carried out in our laboratory using a simple protocol and small sample size, suggested that ADA inhibition might enhance the response to MC (in addition to CPA) (Grenczer et al. 2007). During the present investigation we have expanded

our protocol with the use of CPX, a selective A_1 receptor antagonist, and applied sufficiently large sample size. Our current results show that, in the presence of CPX, ADA inhibition afforded by DCF was unable to influence the response to MC (Fig. 23).Thus, we have every reason to conclude that ADA inhibition does not affect the shared part of postreceptorial signaling of A_1 and M_2 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 A_1 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 A_1 adenosinergic machinery, biased the effect mediated by the M_2 receptor. Namely, because of the overlapping signaling pathways, when a fraction of the response capacity of the A_1 adenosinergic system was depleted, the responsiveness of the M_2 muscarinergic system also decreased (Gesztelyi et al. 2004). The magnitude of the change ("bias") of the E/c curve is characteristic of the magnitude of the biasing effect (Grenczer et al. 2010a).

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 (Gesztelyi et al. 2004; Grenczer et al. 2010a, 2010b; Pak et al. 2014). 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 (Table 3). This finding corroborates our previous results about the effect of NBTI, in which c_x values were obtained as equieffective CPA concentrations (Karsai et al. 2006, 2007; Kiss et al. 2013; Pak et al. 2014).

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 (Table 3). 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 (Deussen et al. 1999; Deussen 2000a, 2000b). In hypoxia, nucleoside transport blockers can decrease the interstitial adenosine level by inhibiting the adenosine release from the cells (Görge et al. 1998; Schreieck and Richardt 1999). 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 (Bazl et al. 2012). 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 (Zhu et al. 1994; Hudspeth et al. 1994; Abd-Elfattah et al. 2013).

Because of unaltered signal amplification properties of the M_2 muscarinergic system under ADA inhibition (Fig. 23, 25C, 25D), 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 A_1 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 M_2 muscarinergic control on atrial contractility did not change in response to the inhibition of either nucleoside transport or ADA (Fig. 25A-D).

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 (Fig. 25E, 25F). It can be concluded that ADA inhibition increases the efficiency of the A_1 adenosinergic direct negative inotropic function, even in the euthyroid state. Nevertheless, consistent with our previous observation (Kemeny-Beke et al. 2007), 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 T_4-induced suppression (Szentmiklosi et al. 1992; Kaasik et al. 1994) in the capacity of the A_1 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 (Cini et al. 2009; Nabbout and Robbins 2010) and increase the risk of ischemic heart disease, supraventricular arrhythmias and congestive heart failure (Franklyn and Boelaert 2012). Thus, the enhancement of endogenous protective ability of the heart by means of ADA inhibition seems to be an especially promising possibility in hyperthyroidism.

However, an important (and until now overlooked) factor should be also taken into account concerning the quantification of extra interstitial adenosine and the correction for the resulting E/c curve bias: the change in the signal amplification of A_1 adenosinergic system in response to ADA inhibition. This phenomenon could affect the assessment of c_x values under ADA inhibition, namely it probably caused an overestimation of c_x values (Fig. 25C, 25D, Table 4B). For explanation, the following should be thought over. If the A_1 adenosinergic machinery is more sensitive (because of ADA inhibition), a given quantity of adenosine evokes a greater effect (E_x) and thereby causes a larger change in the MC E/c curve. From this larger change, a greater c_x can be estimated by means of RRM (with the use of Hill parameters of the corresponding control MC E/c curve; see: equation 2). (Thus, c_x values determined under ADA inhibition are MC concentrations that are equieffective with the extra interstitial adenosine if it activates an A_1 adenosinergic system amplified by ADA inhibition.)

The overestimation of c_x values for ADA inhibition has two important consequences concerning our results. First, as the efficiency enhancing effect of ADA inhibition is stronger in the hyperthyroid atria, no conclusion is worth being drawn from the greater c_x in the presence of DCF in hyperthyroidism (Table 4B). Second, this phenomenon also affects the comparison of c_x values reflecting the effect of NBTI with those of DCF. However, the former c_x values are even so greater than the latter (although overestimated) c_x values (Table 3), therefore the conclusion drawn previously (i.e. NBTI is superior to DCF in increasing the interstitial adenosine level) has remained true (moreover, it has become even truer).

At the same time, it can be stated that the corrected E/c curves generated in the presence of DCF are even so appropriate. This is due to that although c_x values determined under ADA inhibition are overestimated, they were used to correct E/c curves constructed under ADA inhibition. During the correction, c_x values were converted into biasing effects

(E_x) by means of the same Hill parameters, as were used to obtain these c_x values. So, in the course of the correction, we got correct E_x values and these effects were applied for the further calculations. Thus, conclusions drawn from the corrected E/c curves are all valid.

In addition, to the best of our knowledge, this study is the first to report about the suppressing effect of T_4 treatment on the inotropic control exerted by the atrial M_2 muscarinic system in a guinea pig model. On the other hand, while thyroid hormones remarkably decreased E_{max} and potency of the direct negative inotropic action of muscarinic agonists (MC and carbachol) in the rat left atrium (Ishac and Pennefather 1983), we found this effect to be quite small in the guinea pig left atrium, manifested in a moderate decrease in E_{max} and a minor reduction in the Hill coefficient (Fig. 18, Table 3).

The major limitation of the Study 2 is the pure functional approach applied in it. This was necessary to unequivocally detect the nature and extent of the influence of ADA inhibition on the contractility of the working supraventricular myocardium. Of course, this does not substitute subsequent molecular assays that are essential to exploit all possibilities coming from our results.

In summary, we have found that inhibition of ADA increases the signal amplification of the A_1 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 A_1 receptor, intensifies the A_1 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 A_1 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 A_1 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 A_1 and M_2 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.

7 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 A_1 adenosine receptor (A_1 receptor), because A_1 adenosinergic pathways are involved in protective mechanisms such as ischemic preconditioning. These agents stimulating the A_1 adenosinergic system can be A_1 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 A_1 receptor agonists, even being partial, implies the risk of undesirable side effects. Indeed, previously we found great A_1 receptor reserve for the direct negative inotropic effect of adenosine in isolated guinea pig atria. This phenomenon suggests that A_1 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 A_1 receptor agonists. During the first set of our investigations, however, we have found that thyroxine treatment does not substantially affect the A_1 receptor reserve for the direct negative inotropic effect of adenosine. Consequently, if an agent causing A_1 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 A_1 adenosinergic mechanisms is mainly preserved in hyperthyroidism, at least at the level of A_1 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 A_1 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 A_1 adenosinergic function in another (thyroid hormone-sensitive) way, suggesting a new mechanism of action of ADA inhibition.

8 Összefoglalás

A hyperthyreosis több mechanizmus révén is növeli a kardiovaszkuláris mortalitást, beleértve az iszkémiás szívbetegség magasabb rizikóját is. Ezzel összhangban azok a terápiás stratégiák, amelyek fokozzák a szív iszkémia-reperfúzióval szembeni védekezőképességét, különösen hasznosak lehetnek a hyperthyreoid betegek számára. Az egyik ilyen ígéretes lehetőség az A_1 adenozin receptor (direkt vagy indirekt módon való) aktiválása, mivel az A_1 adenozinerg útvonalak is részt vesznek az iszkémiás prekondícionálás jelenségének kialakításában. Ezek az A_1 adenozinerg stimuláló anyagok lehetnek A_1 receptor agonisták, továbbá a szöveti adenozin-szintet növelő anyagok, mint pl. az adenozin eliminációjában szereplő adenozin dezamináz enzim (ADA) gátlószerei.

Az exogén A_1 receptor agonisták alkalmazása azonban, még ha parciálisak is, mellékhatások megjelenését vonja maga után. Egy korábbi vizsgálatunkban nagy A_1 receptor rezervet találtunk a negatív inotrópiára nézve izolált tengerimalac bal pitvaron. Ez arra utal, hogy az A_1 receptorok stimulációja csökkenteti a pitvarok mechanikai aktivitását in vivo is. A pajzsmirigyhormonok szívre gyakorolt hatása miatt a hyperthyreosis befolyásolhatja az A_1 receptor agonisták lehetséges mellékhatásait is. Első vizsgálatunk során azt találtuk, hogy a tiroxin kezelés nem befolyásolja lényegesen az A_1 receptor rezervet az adenozinnal kiváltott direkt negatív inotróp hatás tekintetében. Következésképpen egy A_1 adenozinerg stimuláns, bármilyen indikációban alkalmazzák is, feltehetően ugyanolyan mértékben képes csökkenteni a pitvari kontrakciós erőt hyperthyreoid állapotban, mint euthyreoidban. Eredményeink egyben azt is sugallják, hogy az A_1 adenozinerg rendszer kardioprotektív működése is megtartott hyperthyreoid állapotban, legalábbis az A_1 receptor és szignalizációja szintjén.

A második vizsgálatunkban azt találtuk, hogy az ADA gátlás (szemben a nukleozid transzport blokáddal) növelte az A_1 adenozinerg rendszer jelerősítését (míg az M_2 muszkarinerg rendszerét nem). Az ADA gátlás ezen hatása mind eu-, mind hyperthyreoid állapotban kifejlődött, de erősebbnek mutatkozott hyperthyreosisban. Mindazonáltal az ADA gátlása kisebb mértékű interstitialis adenozin-szint növekedést okozott, mint a nukleozid transzport gátlása, mindkét thyreoid állapotban. Eredményeink alapján az ADA gátlás az interstitialis adenozin-szint emelése mellett egy másik (pajzsmirigyhormon-érzékeny) mechanizmuson keresztül fokozza a pitvari A_1 adenozinerg működést, ezzel az ADA gátlás egy új hatásmechanizmusának lehetőségét sugallva.

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

Candidate: Krisztián Pák
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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.: The effect of adenosine deaminase inhibition on the A1 adenosinergic and M2 muscarinergic control of contractility in eu- and hyperthyroid guinea pig atria.
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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

DEBRECENI EGYETEM KENÉZY ÉLETTUDOMÁNYI EGYETEMI ÉS NEMZETI KÖNYVTÁRA

10 Key words

Heart; Atrium; Guinea pig; A_1 adenosine receptor; M_2 muscarinic receptor; Receptor reserve; Inotropy; Receptorial responsiveness method; RRM; Adenosine deaminase inhibition; Thyroid hormones

11 Kulcsszavak

Szív; pitvar; tengerimalac; A_1 adenozin receptor; M_2 muszkarinos receptor; receptor rezerv; inotrópia; receptorial responsiveness method; RRM; adenozin dezamináz gátlás; pajzsmirigyhormonok

12 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).