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Approximation of A_1 adenosine receptor reserve appertaining to the direct negative inotropic effect of adenosine in hyperthyroid guinea pig left atria

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Abstract. 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 cause (directly or indirectly) A_1 adenosine receptor (A_1 receptor) activation, since A_1 adenosinergic pathways initiate protective mechanisms such as ischemic preconditioning. However, 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 weakening of atria is a possible side effect of A_1 adenosinergic stimulant agents. Thus, the goal of the present investigation was to explore this receptor reserve in hyperthyroidism. Our recently developed method was used that prevents the rapid intracellular elimination of adenosine, allowing sufficient time for exogenous adenosine administered for the generation of concentration-response curves to exert its effect. Our method also allowed correction for the bias caused by the consequent endogenous adenosine accumulation. Our results demonstrate 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, the most probable adverse effect affecting the heart may be a decrease of atrial contractility in both eu- and hyperthyroid conditions.

Key words: A_1 adenosine receptor — Receptor reserve — Atrium — Heart — Inotropy — Thyroid hormones — Receptorial responsiveness method

Abbreviations: CPA, N^6-cyclopentyladenosine; NBTI, S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine; FSCPX, 8-cyclopentyl-N^3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine; RRM, receptorial responsiveness method.

Introduction

The A_1 adenosine receptor (A_1 receptor), a member of the phylogenetically ancient and ubiquitous family of adenosine receptors, exerts complex regulatory functions in almost all tissues, including the myocardium (Fredholm et al. 2001, 2011; Burnstock et al. 2010; Ijzerman et al. 2010, 2013). *Inter alia*, the A_1 receptor mediates negative tropic effects on the heart, involving 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;

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Belardinelli et al. 1995). In contrast, A_1 receptor agonists can markedly decrease the atrial contractile force below the resting level (called direct negative inotropic effect) in most species, including guinea pigs and humans (Szentmiklosi et al. 1982; Bohm et al. 1984; Marmo et al. 1986; Belardinelli et al. 1995).

The A_1 adenosinergic pathways play an essential role in ischemic preconditioning by initiating powerful protective and regenerative processes that prevent and/or remediate damages caused by ischemia and subsequent reperfusion (Headrick et al. 2003, 2011; Otani 2008). 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).

Previous studies 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).

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. 2003). 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. Therefore, the aim of the present study was to test this hypothesis in a model allowing characterization of the effect exerted by thyroxine (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 (*E/c*) 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 (for more details, see: Kiss et al. 2013).

Materials and Methods

Materials

The following chemicals were used: L-thyroxine sodium salt pentahydrate (T_4), adenosine, N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-N^3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine (FSCPX) and S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (NBTI), purchased from Sigma (St. Louis, MO, USA).

T_4 was dissolved in physiological salt solution containing 0.01% NaOH. Adenosine was dissolved at 36°C in modified Krebs-Henseleit buffer (Krebs solution) containing 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). CPA was dissolved in ethanol : water (1:4) solution (v/v). Dimethylsulfoxide (DMSO) was used as a solvent for FSCPX and NBTI. All stock solutions were adjusted to a concentration of 10 mM, except for the adenosine stock solution that was used to achieve 3 mM concentration in the bathing medium. For this purpose, 20 mM adenosine solution was freshly

prepared before each use. When appropriate, stock solutions were diluted with Krebs solution.

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 700–900 g were used. 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 (36°C; pH 7.4). Atria were paced by platinum electrodes (3 Hz, 1 ms, twice the threshold voltage) with the use of a programmable stimulator (Experimetria ST-02) and power amplifier (Experimetria PST-02). The contractile force was characterized by the amplitude of the isometric twitches, which were detected by a transducer (Experimetria SD-01) and strain gauge (Experimetria SG-01D), and recorded by a polygraph (Medicor R-61 6CH Recorder).

Experimental groups and protocols

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: S1 ($n = 5$) and T1 ($n = 5$) for Protocol 1; S2 ($n = 5$) and T2 ($n = 6$) for Protocol 2; S3-Control ($n = 7$), S3-NBTI ($n = 7$), T3-Control ($n = 8$) and T3-NBTI ($n = 9$) for Protocol 3; S4-Control ($n = 7$), S4-FSCPX ($n = 7$), T4-Control ($n = 7$) and T4-FSCPX ($n = 7$) for Protocol 4. The protocols and evaluation procedures were described previously in detail (Kiss et al. 2013), they are summarized briefly in Table 1 and below.

Empirical characterization of E/c curves

The effect was defined as a percentage decrease in the initial contractile force of atria. All *E/c* curves were fitted to the Hill equation:

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

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

Table 1. The four protocols used for both the solvent- and T_4-treated guinea pig atria

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)

P, number of protocol; CPA, N^6-cyclopentyladenosine; NBTI, S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine; FSCPX, 8-cyclopentyl-N^3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine.

The Hill equation was fitted to both individual and averaged *E/c* curve data. Empirical parameters (E_{max}, EC_{50}, n) of the individual *E/c* curves were used for statistical analysis. Empirical parameters of the averaged CPA *E/c* curves of the S3-Control, T3-Control, S4-FSCPX and T4-FSCPX groups were used for the mathematical correction (see below).

Quantification of the bias caused by NBTI in the CPA E/c curves

NBTI, which efficiently inhibit the adenosine transport across the cardiomyocyte cell membrane (Thorn and Jarvis 1996), was used to blunt the intracellular elimination of adenosine administered for the *E/c* curves. However, NBTI increases the interstitial concentration of endogenous adenosine in both euthyroid (Karsai et al. 2006) and T_4-treated guinea pig atria (Karsai et al. 2007). The surplus endogenous

adenosine biases the *E/c* curves generated in the presence of NBTI with an adenosine receptor agonist (Karsai et al. 2006). 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.

The surplus endogenous adenosine accumulated by NBTI was quantified with the equieffective CPA concentration (c_x, see below) by means of RRM (Gesztelyi et al. 2004; Grenczer et al. 2010). For this purpose, the averaged CPA *E/c* curves of the S3-NBTI and T3-NBTI groups were fitted to Eq. (2):

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

where: *E'*, 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_{max}, EC_{50}, n, the empirical parameters of the averaged CPA *E/c* curve of the S3-Control or T3-Control group; c, the concentration of CPA (administered for the *E/c* curve); c_x, the variable parameter of Eq. (2) indicating the CPA concentration that is equieffective with the surplus endogenous adenosine concentration accumulated by NBTI.

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

The effect belonging to c_x was calculated by means of the Hill equation:

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

where: E_x, the effect evoked solely by the surplus endogenous adenosine accumulated by NBTI; c_x, the CPA concentration provided by Eq. (2); E_{max}, EC_{50}, n, the empirical parameters of an appropriate CPA *E/c* curve (see the next paragraph).

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 Eq. (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 Eq. (3), respectively.

From the biased effects and corresponding E_x values, correct effects were computed by means of Eq. (4):

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

where: *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 Eq. 3).

Data analysis

Each atrium was required to meet three criteria in order to qualify for inclusion in the statistical analysis: (i) the resting contractile force had to reach 1 mN before the first *E/c* curve; (ii) the mechanical activity of the paced atrium had to be regular; (iii) the response to10 μM or 100 μM adenosine of the solvent- or T_4-treated atrium, respectively, which was obtained from the first *E/c* curve, was required to be within the mean ± 2 SD range. The mean and SD were computed using atria meeting the first two criteria. 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.

All data sets were evaluated by the normality test and passed. Two data sets were compared with the paired or unpaired *t*-test (if the equal variance test was not passed, t-test with Welch's correction was used). More than two data sets were compared using one-way ANOVA or repeated-measures one-way ANOVA followed by Tukey post-testing. Difference of means was considered significant at $p < 0.05$.

Curve fitting and statistical analysis were performed with the use of GraphPad Prism 4.03, while other calculations were made by means of Microsoft Office Excel 2013.

Results

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 ± S.E.M.) 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 ($p < 0.0001$).

Initial adenosine E/c curves

Adenosine decreased the contractile force of all atria in a concentration-dependent manner (Fig. 1). 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.

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. 2A, 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. 2B, Table 2A).

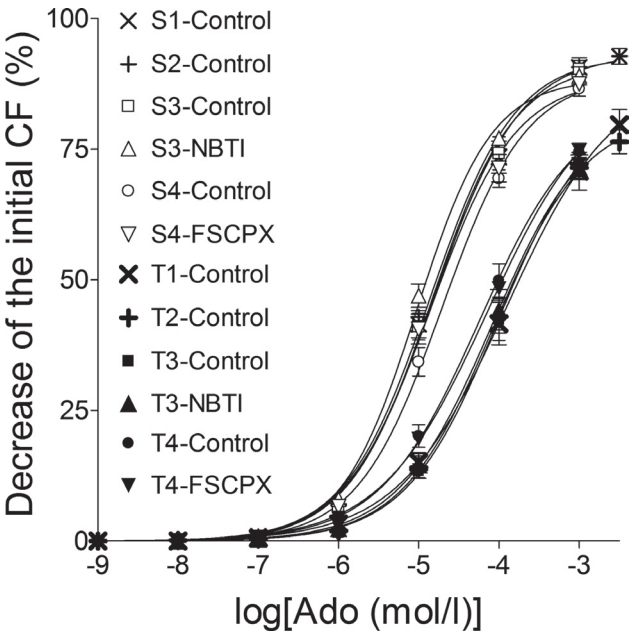

Figure 1. The direct negative inotropic effect of adenosine (Ado) in solvent- (open symbols) and T_4-treated (filled symbols) guinea pig left atria divided into six-six groups. In groups S1, T1, S2 and T2, the first adenosine *E/c* curve is shown (Control curves), while in the other groups, the first 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 (Eq. 1). CF, contractile force.

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. 2, Table 2A).

CPA E/c curves

CPA also reduced the contractile force of all atria in a concentration-dependent manner (Fig. 3). 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. 3A, Table 2B). The T_4-treated atria produced outcomes similar to the solvent-treated ones (Fig. 3B). 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. 3, 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 Eq. (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 (logc_x = –7.231 with –7.334 and –7.129 95% confidence limits).

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. 4). For this reason, the most useful data

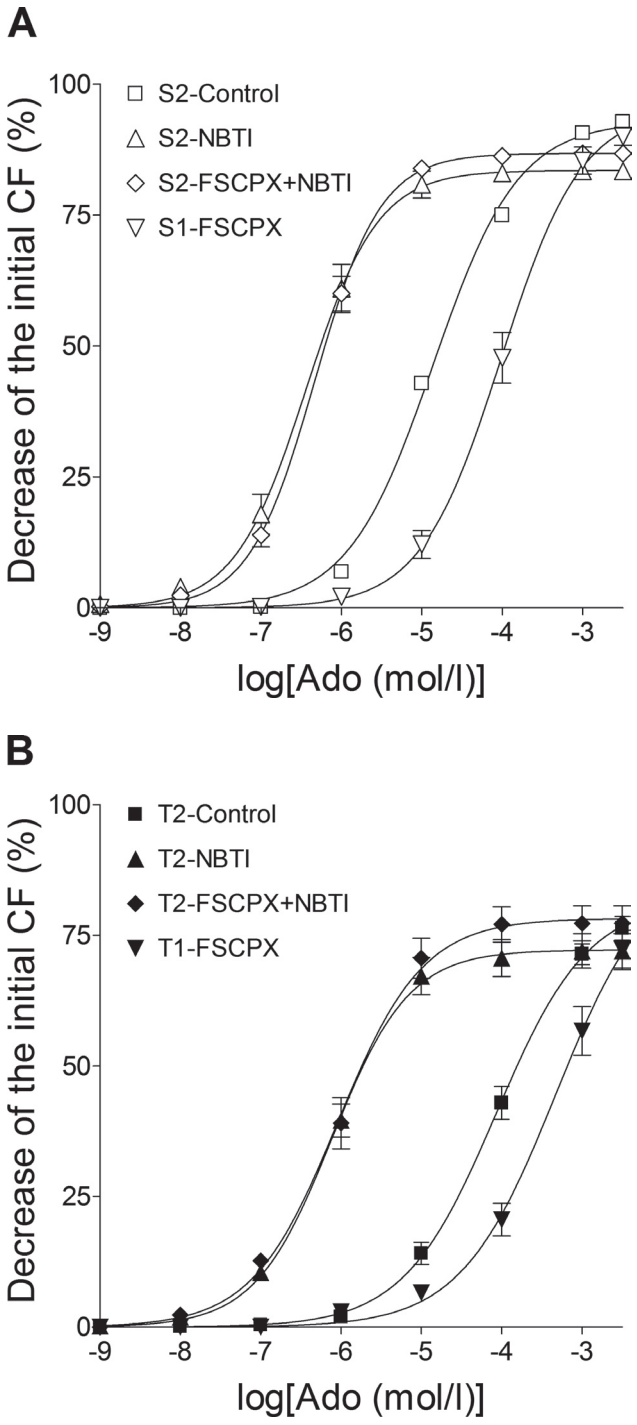

Figure 2. The direct negative inotropic effect of adenosine (Ado) before and after an FSCPX pretreatment, and in the absence and presence of NBTI (alone and in combination), in *in vivo* solvent- (**A**) and T_4-treated (**B**) guinea pig left atria. The curve names refer to the *in vivo* treatment (S 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 (Eq. 1). CF, contractile force.

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 concentrations 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. 4).

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. 4A). (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. 4B).

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. 4A). 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

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

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)

E_{max}, logEC_{50} and n (mean ± SEM) are best-fit values of the Hill equation (Eq. 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 inhibitor (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-N^3-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N^1-propylxanthine.

curve at the two highest adenosine concentrations (Fig. 4A), 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. 4B).

Discussion

To the best of our knowledge, this is the first study showing 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. In addition, 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.

The term receptor reserve has been defined in the context of the traditional receptor theory (Ruffolo 1982). In the most general sense, receptor reserve is an integrative measure of the response-inducing capacity of an agonist (in some receptor models it is termed 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

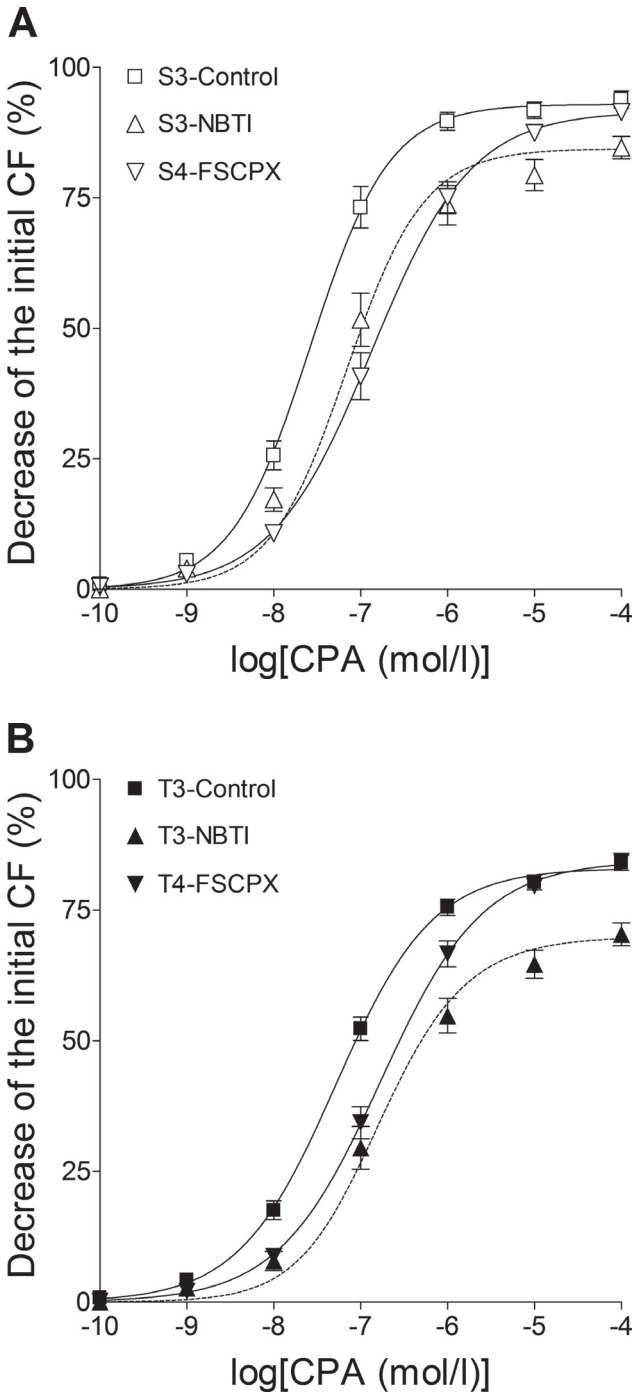

Figure 3. The direct negative inotropic effect of CPA with or without an FSCPX pretreatment, furthermore in the presence and absence of NBTI, in *in vivo* solvent- (**A**) and T_4-treated (**B**) guinea pig left atria. The group names refer to the *in vivo* treatment (S or T), applied protocol (3 or 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 (Eq. 1), while the dotted curve illustrates the fitted RRM model (Eq. 2). CF, contractile force.

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).

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. 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. 2003; Fig. 1, 3), 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

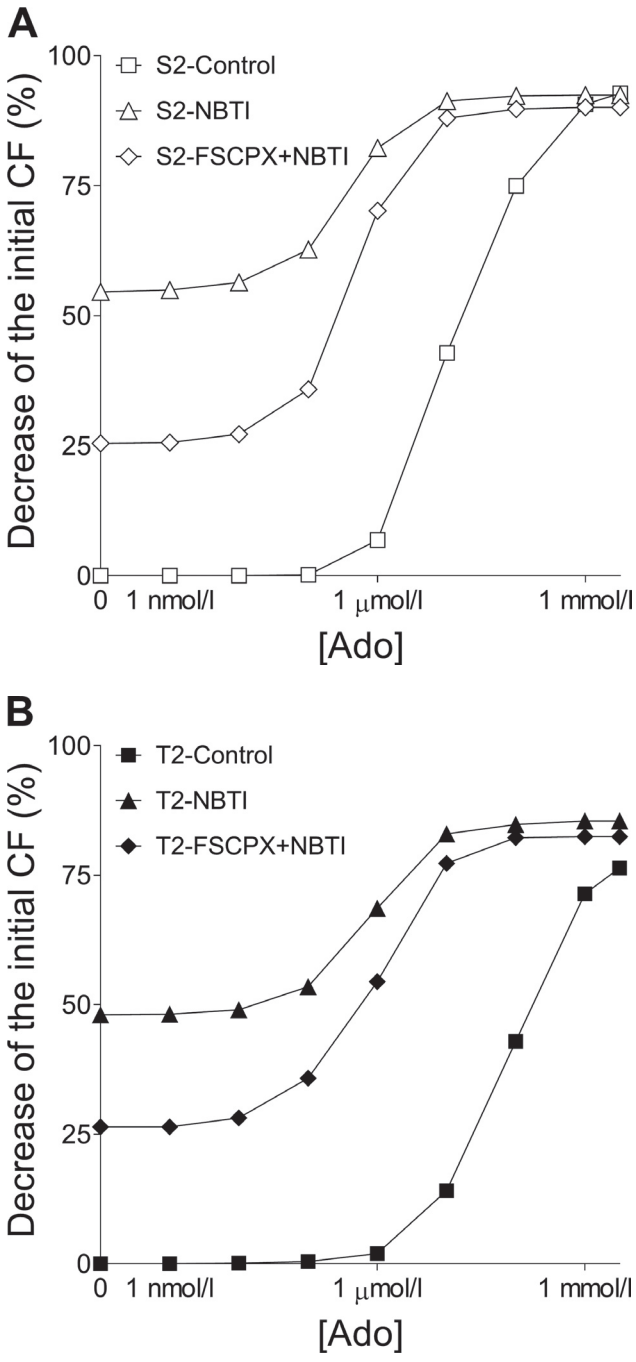

Figure 4. The corrected effect values of adenosine *E/c* curves generated in the presence of NBTI plotted *versus* the exogenous adenosine (Ado) concentrations developed in the bathing medium, together with the original S2-Control and T2-Control curves. The solvent- and T_4-treated atria are represented 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.

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. 4), although it significantly suppressed the direct negative inotropic response to both adenosine and CPA (Fig. 1, 3). 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. 4). Thus, although the direct negative inotropy evoked by adenosine is suppressed in hyperthyroidism (Szentmiklosi et al. 1992; Gesztelyi et al. 2003; Fig. 1, 3), 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 present study is its qualitative nature. This is due to the fact that the interstitial adenosine concentration that 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. 2013).

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.

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