

Thyroid hormones decrease the affinity of 8-cyclopentyl-1,3-dipropylxanthine (CPX), a competitive antagonist, for the guinea pig atrial A₁ adenosine receptor

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Abstract. The aim of the present study was to investigate whether or not thyroxine (T₄) treatment affects K_B , the equilibrium dissociation constant of the antagonist-receptor complex, for the interaction between CPX, a selective and competitive orthosteric antagonist, and the guinea pig atrial A₁ adenosine receptor (A₁ receptor). The inotropic response to adenosine, a nonselective adenosine receptor agonist, or CPA, a selective A₁ receptor agonist, was investigated in the absence or presence of CPX in paced left atria isolated from 8-day solvent- or T₄-treated guinea pigs. To obtain K_B values, adenosine and CPA concentration-response curves were evaluated by Schild analysis. CPA but not adenosine obeyed the requirements of the Schild analysis to provide correct K_B values for CPX. According to the CPA concentration-response curves, affinity of CPX for the hyperthyroid guinea pig atrial A₁ receptor ($K_B = 44.16$ nM) was lower than that for the euthyroid one ($K_B = 16.63$ nM). Regarding the intense reduction in the negative inotropic effect of adenosine and CPA in hyperthyroid atria, it is reasonable to assume that the moderate decrease in affinity of the guinea pig atrial A₁ receptor is only in part responsible for the diminished A₁ receptor-mediated effect in hyperthyroidism.

Key words: A₁ adenosine receptor — 8-cyclopentyl-1,3-dipropylxanthine — Thyroid hormones — Atrium — Guinea pig

Introduction

Thyroid hormones (T₃, T₄) have a wide array of cardiovascular actions including well-known unfavorable effects (Nabbout and Robbins 2010) and others offering potential advantages in cardiology as well as cardiac surgery (Ranasinghe and Bonser 2010; Pantos et al. 2011). Some of

these actions affect the purinergic system of the heart. In the myocardium, the A₁ and A₃ adenosine receptors are primarily involved (Headrick et al. 2003, 2011; Headrick and Lasley 2009).

The A₁ adenosine receptor (A₁ receptor) is a member of the G protein-coupled receptor superfamily that comprises transmembrane receptors with extracellular orthosteric binding sites (Fredholm et al. 2001, 2011). Thyroid hormones suppress the negative inotropic effect mediated by cardiac A₁ receptors (Szentmiklós et al. 1992; Kaasik et al. 1994, 1997a; Gesztelyi et al. 2003). Although this phenomenon has not been explored yet in detail, it is established that the

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thyroid hormones influence several molecular targets that are involved in the negative inotropic action of A₁ receptors (Rubinstein and Binah 1989; Kaasik et al. 1994, 1997a,b; Bosch et al. 1999; Ojamaa et al. 2000a,b; Shenoy et al. 2001; Sunagawa et al. 2005). As adenosine exerts significant cardioprotective effects against ischemic-hypoxic events that are primarily mediated *via* A₁ receptors (Headrick et al. 2003, 2011; Linden 2005; Headrick and Lasley 2009), a decrease in A₁ receptor-mediated actions may be detrimental for the hyperthyroid heart.

Ability of a receptor to bind a ligand (affinity) plays an important role in receptor function, and beyond this, it can serve as an index of the structure of the binding site. One of the simplest and yet most reliable ways to explore prospective structural changes in a receptor is when the affinity of a selective and competitive antagonist for the given receptor is determined (Colquhoun 1998, 2007). Nevertheless, there are relatively scarce data available about ligand binding properties of the hyperthyroid A₁ receptor (either in the heart or in other organs). In the present study, therefore, we investigated whether or not thyroxine (T₄) treatment affects the affinity of CPX, a selective and competitive orthosteric antagonist (Fredholm et al. 2001), for the guinea pig atrial A₁ receptor.

To measure affinity, we aimed to determine the K_B value for CPX, i.e. the equilibrium dissociation constant of the CPX-A₁ receptor complex. For this purpose, concentration-response (E/c) curves were generated with adenosine and CPA, two A₁ receptor full agonists (Fredholm et al. 2001), in the absence and presence of CPX, and then the E/c curves were evaluated by means of the Schild method.

For the experiments, guinea pigs were used, because the A₁ receptor of this species shows the greatest similarity to the human A₁ receptor among the laboratory animals (Fredholm et al. 2001).

Similarly to our earlier studies, the inotropic effect of adenosine and CPA was detected, because the contractile force proved to be a precisely measurable and informative output for quantifying the function of the A₁ receptor together with its postreceptorial signaling (Gesztelyi et al. 2003, 2004; Karsai et al. 2006, 2007).

The large negative inotropic response mediated by the atrial A₁ receptor is attributed to the opening of G protein-coupled inwardly rectifying K⁺ (GIRK) channels (more specifically: muscarinic-operated potassium channels) that are located predominantly in the supraventricular myocardium. As this action evolves even under resting conditions, it is called as direct negative inotropic effect (Kurachi et al. 1986). In turn, A₁ receptor agonists can inhibit the effect of positive inotropic agents both in the atrium and ventricle by reducing the adenylyl cyclase activity to its resting level (Belardinelli et al. 1995). As experiments of the present study were conducted on atria

without any previous adenylyl cyclase stimulation, direct negative inotropy was investigated (nevertheless a minor indirect component cannot be excluded). If the atrium is driven at a constant frequency, negative tropic effects mediated by the A₁ receptor manifest in a pure negative inotropy. Therefore, isolated and paced guinea pig left atria were used for the present study.

Materials and Methods

Animals and preparations

The investigation conforms to the European Community guidelines for the use of experimental animals. The protocols have been approved by the institutional ethics committee (Committee of Animal Research, University of Debrecen, DE MÁB 35/2007).

Male Hartley guinea pigs weighing 500–700 g were used. One group of animals received 330 µg/kg L-thyroxine sodium salt pentahydrate (T₄) daily (*i.p.*) for 8 days (*in vivo* T₄ treatment), and the vehicle of T₄ was administered daily (*i.p.*) for 8 days to another group (*in vivo* solvent treatment). The animals were guillotined on the ninth day. Left atria were quickly removed, rinsed and mounted under 10 mN of resting tension in 10 ml vertical organ baths (TSZ-04, Experimetria, Budapest) containing Krebs solution (composition in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, NaH₂PO₄ 1, MgCl₂ 1.2, NaHCO₃ 24.9, glucose 11.5 and ascorbic acid 0.1 in redistilled water), which was gassed with 95% O₂ and 5% CO₂ (pH 7.4; 36°C). Atria were electrically paced by platinum electrodes (3 Hz, 1 ms, twice the threshold voltage) with a programmable stimulator (PST-02, Experimetria, Budapest). The amplitude of the isometric twitches was measured as contractile force and recorded by a transducer (SD-01, Experimetria, Budapest) and polygraph (BR-61, Medicor, Budapest).

Materials

The following drugs were used: adenosine, 8-cyclopentyl-1,3-dipropylxanthine (CPX), N⁶-cyclopentyladenosine (CPA), and L-thyroxine sodium salt pentahydrate (T₄) from Sigma (St. Louis, MO, USA).

A 10 mM solution of adenosine, CPX and CPA was prepared using Krebs solution (at 36°C), dimethyl-sulfoxide (DMSO) and a solution of ethanol-water (1/4; v/v), respectively. For the E/c curves, adenosine and CPA were further diluted with Krebs solution. The concentration of ethanol and DMSO did not exceed 0.23% and 0.1%, respectively, by volume in the organ baths at any time. T₄ was dissolved in physiological salt solution containing 0.01% NaOH.

Experimental groups

The solvent- (S, $n = 33$) and T₄-treated (T, $n = 32$) atria underwent one of two protocols. Protocol 1 involved two groups: S1 ($n = 9$) and T1 ($n = 9$). Protocol 2 included six groups: S2-control ($n = 10$), S2-1 μM CPX ($n = 7$), S2-10 μM CPX ($n = 7$), T2-control ($n = 9$), T2-1 μM CPX ($n = 7$), T2-10 μM CPX ($n = 7$). The accompanying number reflects the protocol used.

Protocol 1

Atria in the groups S1 and T1 were allowed to equilibrate in Krebs solution for 30 min, and then 100 μM adenosine was administered for 2 min to exercise the preparations. Following a 15 min long wash-out period, a cumulative E/c curve was generated using adenosine. After a 15 min long wash-out, atria were incubated in the presence of 0.1 μM CPX for 20 min, and then (without wash-out) another cumulative E/c curve was constructed with adenosine. Following a 15 min long wash-out, atria were incubated in the presence of 1 μM CPX for 20 min, and then a third cumulative E/c curve was generated with adenosine. After a 15 min long wash-out, atria were incubated in the presence of 10 μM CPX for 20 min, afterward a fourth cumulative E/c curve was generated with adenosine.

Protocol 2

Atria were allowed to equilibrate in Krebs solution for 45 min. Afterward, a cumulative E/c curve was constructed with adenosine to assess the responsiveness of A₁ receptors. After a 15 min long wash-out period, the following *in vitro* treatments were performed: in the groups S2-control and T2-control, atria were incubated in Krebs solution for 20 min; in the groups S2-1 μM CPX and T2-1 μM CPX, atria were subjected to 1 μM of CPX for 20 min; and in the groups S2-10 μM CPX and T2-10 μM CPX, atria received 10 μM of CPX for 20 min. Finally, a cumulative E/c curve was generated with CPA in all groups.

Inotropic response to adenosine, CPA and CPX

The negative inotropic response to adenosine and CPA, both A₁ receptor agonists, was computed from the smallest contractile force developed in the presence of the different adenosine and CPA concentrations. In response to adenosine, the contractile force reached a minimum and right after it started to increase. In the case of CPA, the contractile force achieved a minimum and maintained that until the administration of the next CPA dose. The effect of adenosine and CPA was quantified by the percentage decrease of the initial contractile force of atria.

After addition of CPX, an A₁ receptor antagonist, the equilibrium was indicated by the stabilization of the contractile force.

Curve fitting

Hill equation: The first adenosine E/c curves in both protocols and the control CPA E/c curves of protocol 2 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 agonist; E , the effect of agonist at c ; $E_{\max}$, the maximal effect; EC_{50} , the agonist concentration producing half-maximal effect (median effective concentration, midpoint location); n , the Hill coefficient (midpoint slope factor).

For the statistical analysis, individual E/c curves were fitted to the Hill equation to obtain parameters $E_{\max}$, $\log EC_{50}$ and n , which were then compared. For the sake of illustration, the Hill equation was also used to fit the averaged adenosine E/c curves of protocol 2.

Schild equation: To obtain A_2 (pA_2) and K_B (pK_B) values, the corresponding E/c curves of both protocols were globally fitted to an advanced version of the Schild equation according to Motulsky and Christopoulos (2004):

$$E = E_{\max} \cdot \frac{c^n}{c^n + \left(EC_{50}^* \cdot \left(1 + \left(\frac{B}{A_2} \right)^S \right) \right)^n} \quad (2)$$

where: c , E , $E_{\max}$ and n – see the notation for Eq. 1; EC_{50}^* , the median effective concentration in the absence of antagonist (the median effective concentration of the control E/c curve); B , the concentration of antagonist (being zero for the control E/c curve); A_2 , the antagonist concentration causing two-fold rightward shift of the E/c curve (i.e. the antagonist concentration producing $EC_{50} = 2EC_{50}^*$); S , the Schild coefficient (Schild slope factor) (for more details see: Appendix).

The Schild analysis was performed by globally fitting the Schild equation, i.e. the corresponding E/c curves were simultaneously fitted to the Schild equation with shared $E_{\max}$, EC_{50}^* , n , A_2 and S values (Motulsky and Christopoulos 2004). Corresponding curves herein meant an E/c curve family that consisted of E/c curves generated: 1) on atria receiving the same *in vivo* treatment (solvent or T₄), 2) with the same agonist (adenosine or CPA), and 3) in the presence of different concentrations (including zero) of CPX. To improve the reliability of the results, the E/c curves (belonging to the same group and generated with the same agonist in

the presence of the same antagonist concentration) were averaged before fitting to the Schild equation. The Schild equation was fitted with both variable and fixed ($S = 1$) Schild coefficients, and then the fit of these two models were compared with an F test. If the Schild equation with $S = 1$ showed a better fit, A_2 was considered to equal K_B ($pA_2 = pK_B$), if not, then A_2 did not give K_B ($pA_2 \neq pK_B$).

Form of the fitted equations: To obtain the correct confidence intervals and a better fit, concentrations of agonists (c) and some parameters to be fitted (EC_{50} , EC_{50}^* , A_2) are expressed as a decimal logarithm; furthermore the effect against the decimal logarithm of concentration was plotted (Motulsky and Christopoulos 2004).

Statistical analysis

Each atrium had to meet three criteria to be included in the statistical analysis: 1) the resting contractile force had to reach 1 mN before the first E/c curve; 2) the mechanical activity of the paced atrium had to be regular; 3) the response to 10 or 100 μ M adenosine (concentrations closest to EC_{50} values of adenosine E/c curves for solvent- or T_4 -treated atria, respectively) had to be within the mean ± 2 standard deviations (S.D.) range. The mean and S.D. values were determined separately for the solvent- and T_4 -treated atria obeying the first two criteria. All data meeting the above-mentioned three criteria were processed.

Two data sets passing the normality test as well as the equal variance test were compared by paired or unpaired t -test. If only the normality test was passed, Welch's test was used. More than two data sets were compared with one-way ANOVA followed by Tukey post-testing (because all data

sets passed both the normality and equal variance tests). Values of $p < 0.05$ were considered to be significant. Data are presented as mean $\pm$ S.E.M..

GraphPad Prism version 4.03 for Windows was used for the statistical analysis and curve fitting. Calculations were done with the help of Microsoft Office Excel 2003.

Results

Thyroid state

The initial body weight of the T_4 -treated guinea pigs did not differ significantly from that of the solvent-treated ones. By the ninth day, body weight (mean $\pm$ S.E.M.) of the solvent-treated guinea pigs changed from 854 ± 21 g to 861 ± 23 g (not significant), while body weight of the T_4 -treated animals decreased from 887 ± 19 g to 687 ± 14 g ($p < 0.0001$).

Adenosine E/c curves of protocol 1

Adenosine evoked a concentration-dependent reduction in the atrial contractile force (Fig. 1).

The control E/c curves of protocol 1 were compared to each other by means of their best-fit values obtained from the fitting of the empirical Hill equation (Eq. 1). The effect of T_4 treatment manifested in a nonsignificant decrease of $E_{\max}$ ($89.74 \pm 1.23\%$ vs. $82.94 \pm 2.93\%$), a significant increase of $\log EC_{50}$ (-4.72 ± 0.05 vs. -3.88 ± 0.1 ; $p < 0.0001$), and a nonsignificant decrease of n (0.81 ± 0.04 vs. 0.7 ± 0.04), when control E/c curves of the S1 group were compared to control E/c curves of the T1 group, respectively.

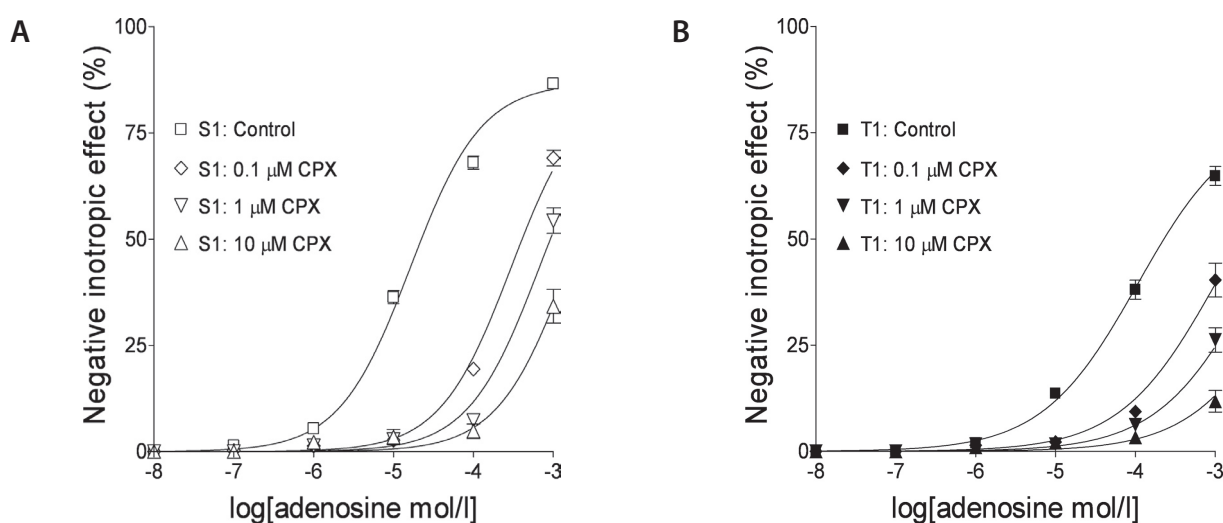


Figure 1. The effect of adenosine on the contractile force of solvent- (A) and T_4 -treated (B) guinea pig left atria in the presence of 0 (control), 0.1, 1 and 10 μ M CPX (according to the protocol 1). The symbols represent the averaged adenosine E/c curve data ($\pm$ S.E.M.) of the groups indicated. The lines show the globally fitted Schild equation (Eq. 2) with variable Schild slope.

CPX concentration-dependently inhibited the effect of adenosine in both the solvent- and T₄-treated atria (Fig. 1).

Adenosine E/c curves of protocol 2

Adenosine concentration-dependently reduced the atrial contractile force (Fig. 2).

The adenosine E/c curves of protocol 2 were also compared to each other using the regression parameters of the fitted Hill equation (Eq. 1). The T₄ treatment caused a nonsignificant decrease in $E_{\max}$ ($86.51 \pm 1.38\%$ vs. $80.98 \pm 3.15\%$), a significant increase in $\log EC_{50}$ (-5.06 ± 0.05 vs. -3.88 ± 0.1 ; $p < 0.0001$) and a significant decrease in n (0.88 ± 0.04 vs. 0.7 ± 0.04 ; $p = 0.0044$), when adenosine E/c curves of all S2 groups were compared to adenosine E/c curves of all T2 groups, respectively.

Regarding the $E_{\max}$, $\log EC_{50}$ and n parameters belonging to the distinct groups, no significant difference was detected among groups receiving the same *in vivo* treatment (in agreement with the fact that adenosine E/c curves of protocol 2 were constructed prior to the CPX treatment). Thus, both the solvent- and T₄-treated atria formed a homogenous population regarding susceptibility to adenosine (Fig. 2).

CPA E/c curves of protocol 2

Similarly to adenosine, CPA concentration-dependently decreased the atrial contractile force (Fig. 3).

Similarly to adenosine, the CPA E/c curves of protocol 2 were compared to each other using best-fit values of the fit-

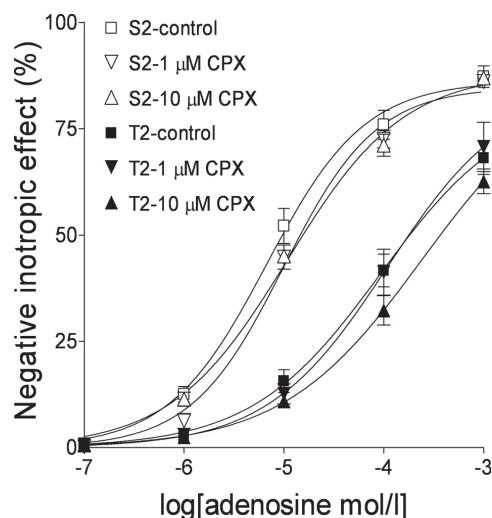


Figure 2. The effect of adenosine on the contractile force of solvent- (open symbols) and T₄-treated (closed symbols) guinea pig left atria in the absence of CPX (according to the protocol 2). The symbols represent the averaged adenosine E/c curve data ($\pm$ S.E.M.) of the groups shown. The names of groups refer to a subsequent and not the current *in vitro* treatment. The lines denote the fitted Hill equation (Eq. 1).

ted Hill equation (Eq. 1). Comparing the S2-control group to the T2-control group, T₄ treatment reduced the effect of CPA that was indicated by a nonsignificant decrease in $E_{\max}$ ($88 \pm 2.02\%$ vs. $81.64 \pm 2.72\%$), a significant increase in $\log EC_{50}$ (-7.36 ± 0.06 vs. -6.9 ± 0.1 ; $p = 0.001$) and a significant decrease in n (0.98 ± 0.04 vs. 0.74 ± 0.05 ; $p = 0.0016$), respectively.

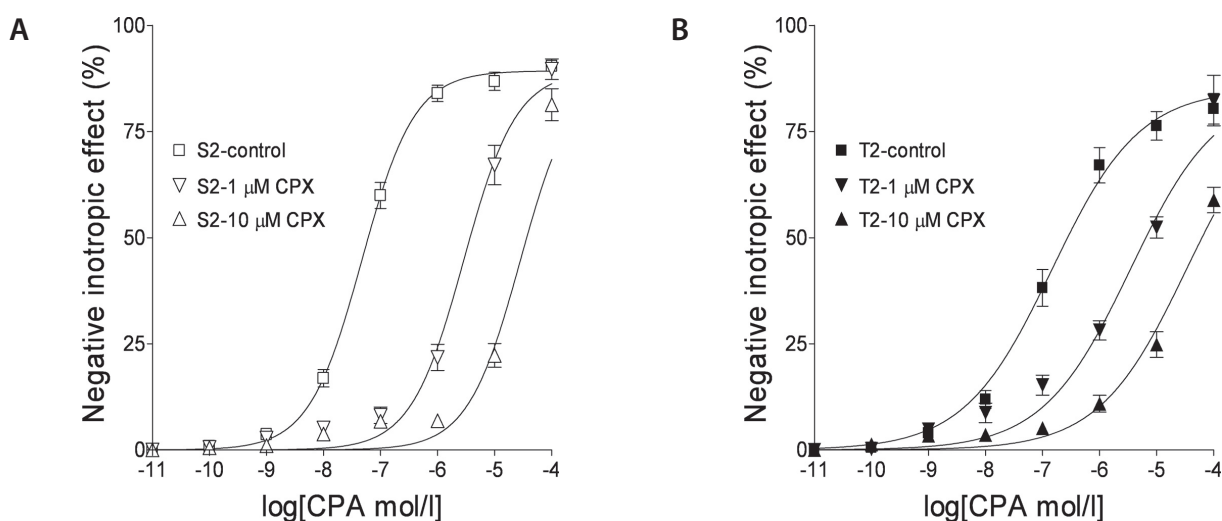


Figure 3. The effect of CPA on the contractile force of solvent- (A) and T₄-treated (B) guinea pig left atria in the presence of 0 (control), 1 and 10 μ M CPX (according to the protocol 2). The symbols represent the averaged CPA E/c curve data ($\pm$ S.E.M.) of the groups indicated. The lines denote the globally fitted Schild equation (Eq. 2) with a Schild slope fixed at unity.

CPX concentration-dependently antagonized the effect of CPA as well, in the case of both the solvent- and T₄-treated atria (Fig. 3).

pA₂ and pK_B values of CPX provided by adenosine and CPA E/c curves

When fitting the corresponding adenosine E/c curves, fit of the model was significantly better if the Schild slope was variable rather than unity, in the case of both solvent- and T₄-treated atria. Consequently, pA₂ (and not pK_B) values could only be determined for CPX (Table 1). The pA₂ related to the solvent-treated atria was considerably greater than the pA₂ assessed for the T₄-treated atria, and the 95% confidence intervals of these parameters did not overlap (Table 1). This indicates that CPX (used in the same concentration) can exert a stronger inhibition on the direct negative inotropic effect of adenosine under euthyroid conditions than in hyperthyroidism.

In contrast, when fit of the Schild equation to the CPA E/c curve families were evaluated, the model with a Schild slope fixed at unity was significantly superior to that with variable Schild slope in both the solvent- and T₄-treated atria. Thus, the use of CPA enabled to determine pK_B values. The pK_B related to the solvent-treated atria was greater

than that belonging to the T₄-treated ones (95% confidence intervals did not overlap), indicating that CPX had lower affinity for the hyperthyroid guinea pig atrial A₁ receptor than for the euthyroid one (Table 1). As pK_B values characterize purely the interaction between CPX and the atrial A₁ receptors (independently from properties of the given agonist and the particular effect measured), the observed difference between those strongly suggest a difference in the orthosteric binding sites of the eu- and hyperthyroid A₁ receptors.

Discussion

To the best of our knowledge, this is the first study showing that the affinity of CPX, a selective and competitive A₁ receptor antagonist, is lower for the hyperthyroid A₁ receptor than for the euthyroid one in the guinea pig atrial myocardium.

Determining the affinity of an agonist for its receptor is fraught with theoretical pitfalls, especially when a G protein-coupled receptor is investigated and, in addition, a functional assay is performed (Colquhoun 1998, 2007). Thus, in the present study, we assessed affinity of CPX, a well-known selective and competitive orthosteric A₁ receptor antagonist (Fredholm et al. 2001, 2011), for A₁ receptors located in eu- and hyperthyroid guinea pig atria. Since the A₁ receptor-mediated direct negative inotropic action (characteristic of the atrium, see: Belardinelli et al. 1995) was measured as an effect, our findings apply particularly to the A₁ receptor situated in atrial cardiomyocytes.

As an agonist, adenosine and CPA were employed. Adenosine is the physiological agonist of all adenosine receptor types and intracellular adenosine binding sites including the so-called P-site (Tesmer 2000; Fredholm et al. 2001, 2011; Peart et al. 2003). Adenosine quickly metabolizes in the living tissues (Wilbur and Marchlinski 1997; Pavan and IJzerman 1998). In contrast, CPA is a synthetic adenosine analogue that selectively stimulates the A₁ receptor (Fredholm et al. 2001, 2011) and is slowly eliminated under our experimental conditions, i.e. in the asanguineous guinea pig atrium (Gesztelyi et al. 2004). Due to the difference in their rate of elimination, adenosine enabled a self-controlled arrangement (E/c curves in the absence and presence of antagonist were subsequently generated in the same atria), while for experiments with CPA, separate control and antagonist-treated groups were created.

E/c curve data representing the interaction of CPX with adenosine or CPA on the atrial A₁ receptors were evaluated by performing a Schild analysis according to Motulsky and Christopoulos (2004). It is widely accepted that the Schild analysis is an exact and reliable method to characterize the binding site of a receptor. The Schild method provides a physico-chemically meaningful constant, K_B (the equi-

Table 1. The pA₂ and pK_B values for CPX, a selective and competitive A₁ receptor antagonist, determined in solvent- and T₄-treated guinea pig left atria with the use of adenosine, a nonselective adenosine receptor agonist, or of CPA, a selective A₁ receptor agonist

	Adenosine		CPA	
	S	T	S	T
pA ₂	10.37	8.84	7.96	7.2
95% CI	9.8 – 10.93	8.35 – 9.33	7.61 – 8.3	6.86 – 7.54
Schild slope	0.37	0.5	0.92	1.09
pK _B	7.77	7.66	7.78	7.36
95% CI	7.64 – 7.9	7.53 – 7.79	7.69 – 7.87	7.2 – 7.51
K _B (nM)	–	–	16.63	44.16

The pA₂ and pK_B values were yielded by the globally fitted Schild equation with variable (upper row) or fixed (at unity) Schild slope (lower row). Values of the preferable model are shown in bold, while those being to reject are in italics. S, solvent treatment; T, T₄ treatment; CPA, N⁶-cyclopentyladenosine (a selective A₁ adenosine receptor full agonist); CPX, 8-cyclopentyl-1,3-dipropylxanthine (an A₁ adenosine receptor competitive antagonist); A₂, the CPX concentration reduplicating EC₅₀ of the control E/c curve; pA₂, the negative decimal logarithm of A₂; K_B, the equilibrium dissociation constant of the CPX-A₁ adenosine receptor complex; pK_B, the negative decimal logarithm of K_B; 95% CI, 95% confidence interval for the fitted pA₂ or pK_B (indicating precision of the curve fitting).

librium dissociation constant of the antagonist-receptor complex), even for a functional assay (Motulsky and Christopoulos 2004; Colquhoun 2007; Giraldo et al. 2007; Wyllie and Chen 2007; Kenakin 2009). In addition, the results obtained by the Schild method do not depend on the signaling of the receptor investigated (Colquhoun 2007). Thus, the Schild method is suitable for comparing the affinity of A₁ receptors in different thyroid states, despite the fact that thyroid hormones affect several signaling pathways involved in the negative inotropic action mediated by the atrial A₁ receptor (Rubinstein and Binah 1989; Kaasik et al. 1994, 1997a, b; Bosch et al. 1999; Ojamaa et al. 2000a, b; Shenoy et al. 2001; Sunagawa et al. 2005).

The corresponding E/c curves of the present study were globally fitted to an advanced form of the Schild equation (Eq. 2). Global fitting possesses three advantages over the original method described by Arunlakshana and Schild (1959): 1) The simultaneous fitting with certain parameters being shared among more than one E/c curve minimizes the effect of errors in the fitted E/c curve data. 2) Using the global fitting method, Schild analysis can be performed even when E/c curves representing the effect of antagonist are not saturated. As unsaturated E/c curves are inappropriate for individual curve fitting to obtain reliable EC₅₀ values, global fitting is the only option in such cases. 3) The global fitting, in addition to preserving the Schild coefficient as an internal check, quantifies the contrast between the fit with variable and fixed Schild slope by allowing of a model comparison with an F test.

When evaluating the corresponding adenosine E/c curves of the present study, fit of the Schild model containing variable Schild slope was the better one, so the obtained pA₂ parameters cannot be considered pK_B values (Table 1). In contrast, in the case of the corresponding CPA E/c curves, constraining the Schild slope at unity led to a better fit than allowing it to vary. Consequently, the obtained parameters are pK_B values that purely characterize the interaction between CPA and the A₁ receptor (Table 1). This difference between results provided by the adenosine and CPA E/c curves may stem from the difference between receptor (and/or other binding site) specificity of adenosine and CPA: the former one also acts on intracellular binding sites including the P-site on adenylyl cyclase (Tesmer et al. 2000), whereas the latter one is a selective A₁ receptor agonist (Fredholm et al. 2001, 2011). This assumption is supported by the previous observation of Collis et al. (1989), who showed that, in the presence of EHNA, an adenosine deaminase inhibitor, and of dipyridamole, a nucleoside transport blocker capable of inhibiting the adenosine intake into the cardiomyocytes (Karsai et al. 2006), the Schild analysis did provide a Schild slope not differing significantly from unity, when investigated the effect of CPX on adenosine E/c curves generated in guinea pig atria (Table 2). Since hyperthyroidism does not

change direction of the transmembrane adenosine flux in the guinea pig atrium (Karsai et al. 2007), intracellular actions of the exogenous adenosine could influence the effect of CPX in the T₄-treated atria of the present study as well.

The pK_B of CPX (provided by CPA E/c curves) was moderately smaller for the hyperthyroid atria than for the euthyroid ones (with separate 95% confidence intervals). In agreement with this, pA₂ (obtained from adenosine E/c curves) was considerably smaller in the hyperthyroid atria as compared to the euthyroid ones (Table 1). So, results of the present study show that affinity of the orthosteric binding site of the atrial A₁ receptor decreases in response to thyroid hormones.

There are relatively few data available about the change in affinity of the cardiac A₁ receptor in hyperthyroidism. Most of *K* values (estimating the equilibrium dissociation constant of CPX and the A₁ receptor) refer to euthyroid conditions. *K* values of others obtained from the guinea pig atrium are in good agreement with our euthyroid data (Table 2). After T₃ or T₄ treatment, no significant change in *K* values was found in the rat ventricle (moreover, affinity tended to increase rather than decrease in response to thyroid hormones). In the hyperthyroid rat atrium, however, a slight decrease in A₁ receptor affinity for tritiated CPX was reported. This change was quite small (smaller than that observed in the present study, see Table 1 and 2), and due to this fact it was opined to be insignificant by the authors. Interestingly, lack of thyroid hormones was also reported to reduce the A₁ receptor affinity in the rat kidney (Franco et al. 2004; Table 2).

T₄ treatment produced a substantial reduction in the negative inotropic effect of A₁ receptor activation (Figs. 2, 3). This phenomenon cannot be exclusively explained by the moderately decreased A₁ receptor affinity explored in the present study, so (an)other mechanism(s) should be supposed. Thyroid hormones increased the A₁ receptor number in both the rat atrium (Kaasik et al. 1994) and ventricle (El-Ani et al. 1994), with unchanged concentration of the relevant G protein subtypes (El-Ani et al. 1994; Kaasik et al. 1994). Starting from this, the suppressed negative inotropic response cannot be ascribed to a change in the A₁ receptor or G protein density in hyperthyroidism.

In the atrium, the A₁ receptor mediated negative inotropy involves two major signaling pathways: opening of the GIRK channel, and inhibition of the activated adenylyl cyclase. The adenylyl cyclase inhibition blunts the cAMP dependent protein kinase A and activates phosphoprotein phosphatases. The resulting attenuated phosphorylation enhances the activity of phospholamban and decreases the L-type Ca²⁺ current. The dephosphorylated phospholamban inhibits more efficiently the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) that, together with the repressed L-type Ca²⁺ current, leads to the weakening of the contractile force (for a brief review, see: Gesztelyi et al. 2003).

Table 2. K values estimating the equilibrium dissociation constant for the CPX (or [^3H]CPX)- A_1 receptor complex, obtained by different methods and from different samples

K (nM)	pK	Thyroid status	Preparation	Reference
$K_B = 8.1$	8.09	eu	guinea pig atrium	Tawfik-Schlieper et al. 1989
$K_d = 1.3$	8.89			
$K_B = 12.3$	7.91	eu	guinea pig atrium	Collis et al. 1989
$K_B = 9.6$	8.02	eu	guinea pig atrium	Wilken et al. 1990
$K_i = 2$	8.7	eu	recombinant human A ₁ receptor	Robeva et al. 1996
$K_i = 0.12$	9.92	eu	rat kidney cortex	Franco et al. 2004
$K_i = 0.82$	9.09	<i>hypo</i>		
$K_i = 0.74$	9.1	eu	rat kidney medulla (outer part)	
$K_i = 0.84$	9.08	<i>hypo</i>		
$K_d = 0.13$	9.89	eu	rat ventricle	El-Ani et al. 1994
$K_d = 0.11$	9.96	hyper		
$K_d = 0.46$	9.34	eu	rat atrium	Balas et al. 2002
$K_d = 0.53$	9.28	hyper		
$K_d = 0.29$	9.54	eu	rat ventricle	
$K_d = 0.27$	9.57	hyper		

K_B values were yielded by functional assays (see: Table 1). K_d and K_i values were provided by radioligand binding assays, in which the antagonist was the radioligand ([^3H]CPX) and the competitor (CPX), respectively. pK , the negative decimal logarithm of K ; CPX, 8-cyclopentyl-1,3-dipropylxanthine; [^3H]CPX, tritiated CPX; eu, euthyroid; *hypo*, hypothyroid (in italics); **hyper**, hyperthyroid (in bold).

The GIRK current, thought to be pivotal for the A_1 receptor mediated direct negative inotropic action (Kurachi et al. 1986; Belardinelli et al. 1995), was found by most authors to be unchanged in the hyperthyroid rat atrium (Sunagawa et al. 2005) as well as in the rabbit atrium and ventricle (Shimoti and Banno 1993). Accordingly, it was observed that hypothyroidism left the GIRK current unchanged in the guinea pig ventricle (Bosch et al. 1999). On the other hand, Sakaguchi et al. (1996) found that acute administration of T_3 enhanced the GIRK current in the guinea pig ventricle (that was ascribed to a nongenomic effect of T_3).

In the hyperthyroid rat ventricle, the activity of adenylyl cyclase was found to be repressed (Ojamaa et al. 2000b), although the whole adenylyl cyclase cascade showed an activation when compared to the euthyroid state (Watanabe et al. 2005). Nevertheless, it has been unequivocally established that thyroid hormones increase the level of SERCA and decrease the amount and efficiency of phospholamban both in the atrium and ventricle (Kaasik et al. 1997a; Ojamaa et al. 2000a; Shenoy et al. 2001). However, since the phospholamban level in the atrium is already low and it further decreases in hyperthyroidism (Shenoy et al. 2001), the phospholamban as a target of thyroid hormones appears to be less significant concerning results of the present study.

Increased L-type Ca^{2+} current was measured in the guinea pig ventricle (Rubinstein and Binah 1989) and human atrium (Kreuzberg et al. 2000) in hyperthyroidism. However, in the hyperthyroid rat atrium, a decreased L-type Ca^{2+} current was found (Sunagawa et al. 2005), while in the hyperthyroid rabbit atrium and ventricle, no change in the L-type Ca^{2+} current was detected (Shimoni and Banno 1993). These conflicting results may be explained by assuming a complex, time-dependent effect for thyroid hormones, a hypothesis corroborated by the finding that, in the rat ventricle, the L-type calcium current was measured to be increased on 4th day of T_3 treatment, whereas it decreased on 8th day of T_3 treatment as compared to the euthyroid level (Watanabe et al. 2005).

Furthermore, it seems probable that enhanced activity of adenosine-handling carriers and enzymes in the hyperthyroid heart (Smolenski et al. 1995) contributes to the diminished negative inotropic effect of adenosine. In agreement with this, the effect of CPA, an A_1 receptor agonist much more resistant to catabolic enzymes than adenosine (Pavan and IJzerman 1998; Gesztelyi et al. 2004), showed a smaller decrease under hyperthyroid conditions than that of adenosine (see Fig. 2 and 3). However, the dextral displacement of the CPA E/c curve in response to T_4 is still too large in

light of the moderate decrease in affinity of the hyperthyroid A₁ receptor. Taking all together, further investigations are needed to explore all details in the mechanism of action for thyroid hormones in the heart.

Beyond its inotropic action, the A₁ receptor has numerous functions in the heart (e.g. negative chronotropic and dromotropic effect) and in almost all mammalian tissues (such as initiation of adaptive and regenerative processes) (Fredholm et al. 2001, 2011; Headrick et al. 2003, 2011; Headrick and Lasley 2009). As the orthosteric binding site of the A₁ receptor is identical throughout the body, the reduction in its affinity in hyperthyroidism may theoretically affect every biological process that is influenced by A₁ receptors (e.g. it may contribute to the enhanced susceptibility to cardiac arrhythmias).

Conclusion

The major finding of the present study is that affinity of CPX for the hyperthyroid guinea pig atrial A₁ receptor is moderately lower than that for the euthyroid one. This decrease in affinity suggests a modification in the orthosteric binding site of the hyperthyroid A₁ receptor, and can contribute to the diminution of the A₁ receptor mediated responses in hyperthyroidism. With regard to the intense reduction in the negative inotropic effect of adenosine and CPA in the hyperthyroid atria, it is reasonable to assume that the moderate decrease in affinity of the guinea pig atrial A₁ receptor is only in part responsible for the diminished negative inotropic effect of A₁ receptor agonists under hyperthyroid conditions. On the other hand, the reduction in affinity of the A₁ receptor to its orthosteric ligands may theoretically affect every A₁ receptor mediated biological process in hyperthyroidism. In addition, CPX proved to be a competitive antagonist not only for the euthyroid but hyperthyroid guinea pig atrial A₁ receptor.

Acknowledgements. This study was supported by grants of Hungarian Scientific Research Fund Programs (OTKA-K 72315; TAMOP 4.2.2-08/1-2008-0007), furthermore by the Sectoral Operational Programme Human Resources Development, financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/89/1.5/S/60782.

Appendix

The term Schild equation refers to a family of equations that contain the relationship between two equieffective concentrations of an agonist acting in the absence and presence of an antagonist concentration. These formulae were progressively developed by several researchers (Gaddum 1937; Schild 1947; Arunlakshana and Schild 1959; Waud et

al. 1978). The simplest form of the Schild equation does not contain any slope factor:

$$\frac{EC_{50}}{EC_{50}^*} - 1 = \frac{B}{K} \quad (3)$$

where: EC_{50} and EC_{50}^* , the agonist concentration producing half-maximal effect in the presence and absence of antagonist, respectively; B , the antagonist concentration (when antagonist is present); K , a constant characterizing the given antagonist and system.

If the antagonism is purely competitive, K equals with K_B , the equilibrium dissociation constant of the antagonist-receptor complex. The EC_{50}/EC_{50}^* quotient is traditionally called dose ratio (any two equieffective agonist concentrations can be written into the Eq. 3 as dose ratio, but the most useful values are the corresponding median effective agonist concentrations).

For some analyses, Gaddum as well as Schild raised B to a power, which has since been named Schild coefficient (S):

$$\frac{EC_{50}}{EC_{50}^*} - 1 = \frac{(B)^S}{K} \quad (4)$$

In another form of the Schild equation, the dose ratio is also raised to a power (which is different from that of B). The original consideration to introduce exponents into the Schild equation was the same as it was for the Hill equation: to allow for the molecularity of the binding reaction, i.e. number of ligands required to bind to one receptor in order to exert an effect or to prevent it (Clark 1937; Gaddum 1937). However, it has been observed that the experimentally obtained Schild coefficient does not have such an exact physico-chemical meaning (Colquhoun 2007), similarly to the Hill coefficient (Katz 1978; Gesztelyi et al. 2011). Later, Waud proposed to raise the whole B/K quotient to the Schild coefficient (Waud et al. 1978). In this case, K equals with A_2 , the antagonist concentration reduplicating the median effective agonist concentration:

$$\frac{EC_{50}}{EC_{50}^*} - 1 = \left(\frac{B}{A_2} \right)^S \quad (5)$$

Similarly to the Eq. 3 and 4, if the antagonist is purely competitive, A_2 equals with K_B .

In addition, Waud combined the Schild and Hill equations by expressing EC_{50} from the Eq. 4 (Waud 1975, 1976) or 5 (Waud et al. 1978) and writing it into the Eq. 1. This latter form of the Schild equation is identical with the equation 2 used in the present study (see: Materials and methods). The Eq. 2 can be directly fitted to E/c curve data, moreover it has been reported to provide more reliable estimates of A_2 via minimizing the correlation

between S and A₂ (Lazareno and Birdsall 1993; Motulsky and Christopoulos 2004).

References

- Arunlakshana O., Schild H. O. (1959): Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.* **14**, 48–58
<http://dx.doi.org/10.1111/j.1476-5381.1959.tb00928.x>
- Balas N., Arad M., Rabinowitz B., Shainberg A. (2002): Modulation of cardiac A₁-adenosine receptors in rats following treatment with agents affecting heart rate. *Mol. Cell. Biochem.* **231**, 107–116
<http://dx.doi.org/10.1023/A:1014412814735>
- Belardinelli L., Shryock J. C., Song Y., Wang D., Srinivas M. (1995): Ionic basis of the electrophysiological actions of adenosine on cardiomyocytes. *FASEB J.* **9**, 359–365
- Bosch R. F., Wang Z., Li G. R., Nattel S. (1999): Electrophysiological mechanisms by which hypothyroidism delays repolarization in guinea pig hearts. *Am. J. Physiol.* **277**, H211–220
- Clark A. J. (1937): *General Pharmacology*. Vol. 4. Springer, Berlin
<http://dx.doi.org/10.1007/978-3-642-91463-8>
- Collis M. G., Stogdall S. M., Martin F. M. (1989): Apparent affinity of 1,3-dipropyl-8-cyclopentylxanthine for adenosine A₁ and A₂ receptors in isolated tissues from guinea-pigs. *Br. J. Pharmacol.* **97**, 1274–1278
<http://dx.doi.org/10.1111/j.1476-5381.1989.tb12589.x>
- Colquhoun D. (1998): Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br. J. Pharmacol.* **125**, 924–947
<http://dx.doi.org/10.1038/sj.bjp.0702164>
- Colquhoun D. (2007): Why the Schild method is better than Schild realised. *Trends Pharmacol. Sci.* **28**, 608–614
<http://dx.doi.org/10.1016/j.tips.2007.09.011>
- El-Ani D., Jacobson K. A., Shainberg A. (1994): Characterization of adenosine receptors in intact cultured heart cells. *Biochem. Pharmacol.* **48**, 727–735
[http://dx.doi.org/10.1016/0006-2952\(94\)90050-7](http://dx.doi.org/10.1016/0006-2952(94)90050-7)
- Franco M., Galicia O., Quintana A., Martínez F. (2004): Experimental hypothyroidism modifies specific binding of A₁ and A_{2A} analogues to adenosine receptors in the rat kidney. *Br. J. Pharmacol.* **142**, 461–468
<http://dx.doi.org/10.1038/sj.bjp.0705799>
- Fredholm B. B., IJzerman A. P., Jacobson K. A., Klotz K. N., Linden J. (2001): International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **53**, 527–552
- Fredholm B. B., IJzerman A. P., Jacobson K. A., Linden J., Müller C. E. (2011): International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors – an update. *Pharmacol. Rev.* **63**, 1–34
<http://dx.doi.org/10.1124/pr.110.003285>
- Gaddum J. H. (1937): The quantitative effects of antagonistic drugs. *J. Physiol. (London)* **89** (Suppl.), P7–9
- Gesztelyi R., Zsuga J., Hajdu P., Szabo J. Z., Cseppento A., Szentmiklósi A. J. (2003): Positive inotropic effect of the inhibition of cyclic GMP-stimulated 3',5'-cyclic nucleotide phosphodiesterase (PDE2) on guinea pig left atria in eu- and hyperthyroidism. *Gen. Physiol. Biophys.* **22**, 501–513
- Gesztelyi R., Zsuga J., Juhász B., Dér P., Vecsernyés M., Szentmiklósi A. J. (2004): Concentration estimation via curve fitting: quantification of negative inotropic agents by using a simple mathematical method in guinea pig atria. *Bull. Math. Biol.* **66**, 1439–1453
<http://dx.doi.org/10.1016/j.bulm.2004.03.001>
- Gesztelyi R., Zsuga J., Kemeny-Beke A., Varga B., Juhász B., Tosaki A. (2011): The Hill equation and the origin of quantitative pharmacology. *Arch. Hist. Exact Sci.* **66**, 427–438
<http://dx.doi.org/10.1007/s00407-012-0098-5>
- Giraldo J., Serra J., Roche D., Rovira X. (2007): Assessing receptor affinity for inverse agonists: Schild and Cheng-Prusoff methods revisited. *Curr. Drug Targets.* **8**, 197–202
<http://dx.doi.org/10.2174/138945007779315687>
- Headrick J. P., Hack B., Ashton K. J. (2003): Acute adenosinergic cardioprotection in ischemic-reperfused hearts. *Am. J. Physiol.* **285**, H1797–1818
- Headrick J. P., Lasley R. D. (2009): Adenosine receptors and reperfusion injury of the heart. *Handb. Exp. Pharmacol.* **193**, 189–214
http://dx.doi.org/10.1007/978-3-540-89615-9_7
- Headrick J. P., Peart J. N., Reichelt M. E., Haseler L. J. (2011): Adenosine and its receptors in the heart: regulation, retaliation and adaptation. *Biochim. Biophys. Acta.* **1808**, 1413–1428
<http://dx.doi.org/10.1016/j.bbame.2010.11.016>
- Kaasik A., Seppet E. K., Ohisalo J. J. (1994): Enhanced negative inotropic effect of an adenosine A₁-receptor agonist in rat left atria in hypothyroidism. *J. Mol. Cell. Cardiol.* **26**, 509–517
<http://dx.doi.org/10.1006/jmcc.1994.1061>
- Kaasik A., Minajeva A., Paju K., Eimre M., Seppet E. K. (1997a): Thyroid hormones differentially affect sarcoplasmic reticulum function in rat atria and ventricles. *Mol. Cell. Biochem.* **176**, 119–126
<http://dx.doi.org/10.1023/A:1006887231150>
- Kaasik A., Paju K., Vetter R., Seppet E. K. (1997b): Thyroid hormones increase the contractility but suppress the effects of beta-adrenergic agonist by decreasing phospholamban expression in rat atria. *Cardiovasc. Res.* **35**, 106–112
[http://dx.doi.org/10.1016/S0008-6363\(97\)00069-2](http://dx.doi.org/10.1016/S0008-6363(97)00069-2)
- Karsai D., Zsuga J., Juhász B., Dér P., Szentmiklósi A. J., Tósaki A., Gesztelyi R. (2006): Effect of nucleoside transport blockade on the interstitial adenosine level characterized by a novel method in guinea pig atria. *J. Cardiovasc. Pharmacol.* **47**, 103–109
<http://dx.doi.org/10.1097/01.fjc.0000196239.51018.a0>
- Karsai D., Gesztelyi R., Zsuga J., Jakab A., Szendrei L., Juhász B., Bak I., Szabo G., Lekli I., Vecsernyes M., Varga E., Szentmiklósi A. J., Tosaki A. (2007): Influence of hyperthyroidism on the effect of adenosine transport blockade assessed by a novel method in guinea pig atria. *Cell. Biochem. Biophys.* **47**, 45–52
- Katz B. (1978): Archibald Vivian Hill, 26 September 1886 – 3 Jun 1977. *Biogr. Mem. Fellows R. Soc.* **24**, 71–149
<http://dx.doi.org/10.1098/rsbm.1978.0005>

- Kenakin T. P. (2009): *A Pharmacology Primer: Theory, Applications, and Methods*. pp. 104–110, Elsevier Academic Press
<http://dx.doi.org/10.1016/B978-0-12-374585-9.00013-X>
- Kreuzberg U., Theissen P., Schicha H., Schroder F., Mehlhorn U., de Vivie E. R., Boknik P., Neumann J., Grohe C., Herzig S. (2000): Single-channel activity and expression of atrial L-type Ca²⁺ channels in patients with latent hyperthyroidism. *Am. J. Physiol. Heart Circ. Physiol.* **278**, H723–730
- Kurachi Y., Nakajima T., Sugimoto T. (1986): On the mechanism of activation of muscarinic potassium channels by adenosine in isolated atrial cells: Involvement of GTP-binding proteins. *Pflügers Arch.* **407**, 264–274
<http://dx.doi.org/10.1007/BF00585301>
- Lazareno S., Birdsall N. J. (1993): Estimation of competitive antagonist affinity from functional inhibition curves using the Gaddum, Schild and Cheng-Prusoff equations. *Br. J. Pharmacol.* **109**, 1110–1119
<http://dx.doi.org/10.1111/j.1476-5381.1993.tb13737.x>
- Linden J. (2005): Adenosine in tissue protection and tissue regulation. *Mol. Pharmacol.* **67**, 1385–1387
<http://dx.doi.org/10.1124/mol.105.011783>
- Motulsky H. J., Christopoulos A. (2004): Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting. GraphPad Software Inc., San Diego
- Nabbout L. A., Robbins R. J. (2010): The cardiovascular effects of hyperthyroidism. *Methodist Debakey Cardiovasc. J.* **6**, 3–8
- Ojamaa K., Kenessey A., Klein I. (2000a): Thyroid hormone regulation of phospholamban phosphorylation in the rat heart. *Endocrinology* **141**, 2139–2144
<http://dx.doi.org/10.1210/en.141.6.2139>
- Ojamaa K., Klein I., Sabet A., Steinberg S. F. (2000b): Changes in adenylyl cyclase isoforms as a mechanism for thyroid hormone modulation of cardiac beta-adrenergic receptor responsiveness. *Metabolism* **49**, 275–279
[http://dx.doi.org/10.1016/S0026-0495\(00\)92262-5](http://dx.doi.org/10.1016/S0026-0495(00)92262-5)
- Pantos C., Mourouzis I., Cokkinos D. V. (2011): New insights into the role of thyroid hormone in cardiac remodeling: time to reconsider? *Heart Fail. Rev.* **16**, 79–96
<http://dx.doi.org/10.1007/s10741-010-9185-3>
- Pavan B., IJzerman A. P. (1998): Processing of adenosine receptor agonists in rat and human whole blood. *Biochem. Pharmacol.* **56**, 1625–1632
[http://dx.doi.org/10.1016/S0006-2952\(98\)00270-6](http://dx.doi.org/10.1016/S0006-2952(98)00270-6)
- Pearl J., Willems L., Headrick J. P. (2003): Receptor and non-receptor-dependent mechanisms of cardioprotection with adenosine. *Am. J. Physiol.* **284**, H519–527
<http://dx.doi.org/10.1113/jphysiol.2003.041541>
- Ranasinghe A. M., Bonser R. S. (2010): Thyroid hormone in cardiac surgery. *Vascul. Pharmacol.* **52**, 131–137
<http://dx.doi.org/10.1016/j.vph.2009.11.004>
- Robeva A. S., Woodard R. L., Jin X. W., Gao Z. H., Bhattacharya S., Taylor H. E., Rosin D. L., Linden J. (1996): Molecular characterization of recombinant human adenosine receptors. *Drug Dev. Res.* **39**, 243–252
[http://dx.doi.org/10.1002/\(SICI\)1098-2299\(199611/12\)39:3/4<243::AID-DDR3>3.0.CO;2-R](http://dx.doi.org/10.1002/(SICI)1098-2299(199611/12)39:3/4<243::AID-DDR3>3.0.CO;2-R)
- Rubinstein I., Binah O. (1989): Thyroid hormone modulates membrane currents in guinea-pig ventricular myocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**, 705–711
<http://dx.doi.org/10.1007/BF00717748>
- Sakaguchi Y., Cui G., Sen L. (1996): Acute effects of thyroid hormone on inward rectifier potassium channel currents in guinea pig ventricular myocytes. *Endocrinology* **137**, 4744–4751
<http://dx.doi.org/10.1210/en.137.11.4744>
- Schild H. O. (1947): pA, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* **2**, 189–206
<http://dx.doi.org/10.1111/j.1476-5381.1947.tb00336.x>
- Shenoy R., Klein I., Ojamaa K. (2001): Differential regulation of SR calcium transporters by thyroid hormone in rat atria and ventricles. *Am. J. Physiol. – Heart Circ. Physiol.* **281**, H1690–1696
- Shimoni Y., Banno H. (1993): Thyroxine effects on temperature dependence of ionic currents in single rabbit cardiac myocytes. *Am. J. Physiol.* **265**, H1875–1883
- Smolenski R. T., Yacoub M. H., Seymour A. M. (1995): Hyperthyroidism increases adenosine transport and metabolism in the rat heart. *Mol. Cell. Biochem.* **143**, 143–149
<http://dx.doi.org/10.1007/BF01816948>
- Sunagawa M., Yamakawa M., Shimabukuro M., Higa N., Takasu N., Kosugi T. (2005): Electrophysiologic characteristics of atrial myocytes in levo-thyroxine-treated rats. *Thyroid*. **15**, 3–11
<http://dx.doi.org/10.1089/thy.2005.15.3>
- Szentmiklosi A. J., Cseppento A., Szabo J. Z., Nosztray K., Szegi J. (1992): Myocardial and vascular actions of purinoceptor activators are reduced after thyroxine treatment. *Pharmacol. Res.* **25** (Suppl. 2), 171–172
[http://dx.doi.org/10.1016/1043-6618\(92\)90345-C](http://dx.doi.org/10.1016/1043-6618(92)90345-C)
- Tawfik-Schlieper H., Klotz K. N., Kreye V. A., Schwabe U. (1989): Characterization of the K(+) -channel-coupled adenosine receptor in guinea pig atria. *Naunyn Schmiedeberg's Arch. Pharmacol.* **340**, 684–688
<http://dx.doi.org/10.1007/BF00717745>
- Tesmer J. J., Dessauer C. W., Sunahara R. K., Murray L. D., Johnson R. A., Gilman A. G., Sprang S. R. (2000): Molecular basis for P-site inhibition of adenylyl cyclase. *Biochemistry* **39**, 14464–14471
<http://dx.doi.org/10.1021/bi0015562>
- Watanabe H., Washizuka T., Komura S., Yoshida T., Hosaka Y., Hatada K., Aizawa Y., Chinushi M., Yamamoto T., Ma M., Watanabe K. (2005): Genomic and non-genomic regulation of L-type calcium channels in rat ventricle by thyroid hormone. *Endocr. Res.* **31**, 59–70
<http://dx.doi.org/10.1080/07435800500229227>
- Waud D. R. (1975): Analysis of dose-response curves. In: *Methods in Pharmacology*. (Eds. Daniel E. E. and Paton D. M.), Vol. 3, pp. 471–506, Plenum Press, New York
- Waud D. R. (1976): Analysis of dose-response relationships. In: *Advances in General and Cellular Pharmacology*. (Eds. Narahashi T. and Bianchi C. P.), Vol. 1, pp. 145–178, Plenum Press, New York
http://dx.doi.org/10.1007/978-1-4615-8198-7_4
- Waud D. R., Son S. L., Waud B. E. (1978): Kinetic and empirical analysis of dose-response curves illustrated with a cardiac example. *Life Sci.* **22**, 1275–1285
[http://dx.doi.org/10.1016/0024-3205\(78\)90096-6](http://dx.doi.org/10.1016/0024-3205(78)90096-6)

- Wilbur S. L., Marchlinski F. E. (1997): Adenosine as an antiarrhythmic agent. *Am. J. Cardiol.* **79**, 30–37
[http://dx.doi.org/10.1016/S0002-9149\(97\)00261-0](http://dx.doi.org/10.1016/S0002-9149(97)00261-0)
- Wilken A., Tawfik-Schlieper H., Klotz K. N., Schwabe U. (1990): Pharmacological characterization of the adenylate cyclase-coupled adenosine receptor in isolated guinea pig atrial myocytes. *Mol. Pharmacol.* **37**, 916–920
- Wyllie D. J., Chen P. E. (2007): Taking the time to study competitive antagonism. *Br. J. Pharmacol.* **150**, 541–551
<http://dx.doi.org/10.1038/sj.bjp.0706997>

Received: October 16, 2011

Final version accepted: June 11, 2012