

The guinea pig atrial A₁ adenosine receptor reserve for the direct negative inotropic effect of adenosine

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Abstract. Although the A₁ adenosine receptor (A₁ receptor), the main adenosine receptor type in cardiac muscle, is involved in powerful cardioprotective processes such as ischemic preconditioning, the atrial A₁ receptor reserve has not yet been quantified for the direct negative inotropic effect of adenosine. In the present study, adenosine concentration-effect (*E/c*) curves were constructed before and after pretreatment with FSCPX (8-cyclopentyl-N³-[3-(4-(fluorosulfonyl)benzoyloxy)propyl]-N¹-propylxanthine), an irreversible A₁ receptor antagonist, in isolated guinea pig atria. To prevent the intracellular elimination of the administered adenosine, NBTI (S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine), a nucleoside transport inhibitor, was used. As expected, NBTI alone and FSCPX-pretreatment alone shifted the adenosine *E/c* curve to the left and right, respectively. However, in the presence of NBTI, FSCPX-pretreatment appeared to increase the maximal response to adenosine. By means of the receptorial responsiveness method (RRM), our recently developed procedure, adenosine *E/c* curves generated in the presence of NBTI were corrected for the bias caused by the endogenous adenosine accumulated by NBTI. The corrected curves indicate a substantial A₁ receptor reserve for the direct negative inotropy evoked by adenosine. In addition, our results suggest that accumulation of an endogenous agonist may bias the *E/c* curve constructed with the same or similar agonist that can lead to seemingly paradoxical results.

Key words: A₁ adenosine receptor — Atrium — Heart — Inotropy — Receptorial responsiveness method

Introduction

The A₁ adenosine receptor (A₁ receptor) is expressed in almost every mammalian tissue, in which it mediates regulatory, retaliatory (energy consumption limiting), adaptive and regenerative functions (Fredholm et al. 2001, 2011; Burnstock et al. 2010; Headrick et al. 2011). By virtue of their capacity to evoke these actions, several A₁ receptor agonists are in late stage clinical development or approved for clinical

use at the time of this writing (Elzein and Zablocki 2008; Manjunath and Sakhare 2009; Schenone et al. 2010; Müller and Jacobson 2011; Szentmiklosi et al. 2011; Albrecht-Küpper et al. 2012). Since an A₁ receptor agonist, administered for any reason, can bind to any A₁ receptor, differences in tissue responsiveness have great practical significance. In the traditional receptor theory, an important determinant of tissue responsiveness is the so-called receptor reserve (Dhalla et al. 2003; Kenakin 2009).

The term receptor reserve refers to a phenomenon whereby stimulation of only a fraction of the whole receptor population apparently elicits the maximal effect achievable in a particular tissue. The existence (and magnitude) of receptor reserve depends on the agonist (efficacy), tissue (signal am-

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plification ability) and measured effect (pathways activated to cause signal amplification) (Brown and Goldstein 1986; Srinivas et al. 1997; Kenakin 2009). High-efficacy agonists usually act on most tissues expressing the given receptor as a full agonist. In turn, low-efficacy agonists exert significant effect only in tissues with large receptor reserve (Ruffolo 1982; Dhalla et al. 2003; Kenakin 2009). Thus, use of low-efficacy agonists can ensure tissue selectivity in a sense that they will not evoke biologically significant effect in tissues with small (or no) receptor reserve.

In cardiomyocytes, most effects of adenosine are mediated by the A₁ receptor (Ford and Broadley 1997; Headrick et al. 2011). Previously, some authors of the present study used slowly metabolizing synthetic A₁ receptor full agonists (NECA, CPA, CHA) and adenosine, the physiological full agonist, to determine the A₁ receptor reserve related to the direct negative inotropic effect in the guinea pig atrium (Gesztelyi et al. 2013). In the case of the synthetic agonists, the A₁ receptor reserve belonging to the direct negative inotropic effect was found to be considerably greater than historical A₁ receptor reserve values appertaining to any other effects in the heart (determined with the use of identical or similar agonists). However, no reliable receptor reserve value could be calculated for adenosine (Gesztelyi et al. 2013). This outcome was attributed to the short half-life (<10 s) of adenosine in living tissues (Wilbur and Marchlinski 1997) that is primarily due to the rapid cellular uptake and subsequent intracellular elimination of adenosine (Deussen et al. 1999). Another consequence of the short half-life of adenosine is that it is difficult to calculate or directly measure adenosine levels in the microenvironment of the A₁ receptors (Deussen 2000a, 2000b; Karsai et al. 2006; Ramakers et al. 2008). Thus, it is difficult, if not impossible, to interpret an adenosine concentration-response (*E/c*) curve as correctly as an *E/c* curve constructed with a slowly-eliminating synthetic adenosine receptor agonist.

Therefore, the aim of the present study was to develop a method enabling characterization of A₁ receptor reserve for the direct negative inotropic effect of adenosine, by overcoming difficulties caused by the degradability of adenosine. Our strategy started from the observation that physiological adenosine transport is directed into the cell interior, the main site for adenosine elimination (Deussen et al. 1999). In the myocardium, the major pathway for adenosine across the cytoplasmic membrane is the equilibrative and NBTI-sensitive nucleoside transporter (ENT1) (Clanachan et al. 1987; Thorn and Jarvis 1996). Accordingly, NBTI, a selective and strong inhibitor of ENT1, was found to significantly elevate extracellular adenosine levels in the guinea pig heart under well-oxygenated conditions (Deussen et al. 1999). Therefore, in the present study, adenosine administered during the construction of *E/c* curves was protected against the degradation by inhibiting ENT1 with the use of NBTI.

Experiments of this study were conducted using isolated and paced guinea pig left atria, the contractile force of which was recorded. Results obtained this way are reliable and easy to interpret, because, in this simple model, negative tropic effects mediated by the A₁ receptor manifest only in a decrease of the contractile force. No “priming” (previous adenylyl cyclase stimulation) was made, thus the direct component of negative inotropy, characteristic of the atrial A₁ receptor (Belardinelli et al. 1995), was measured. According to Furchgott’s method for receptor reserve estimation (Furchgott 1966), two adenosine *E/c* curves were generated, one before and another one after the permanent inactivation of a fraction of the A₁ receptors. For this purpose, we used FSCPX, a selective, strong and irreversible A₁ receptor antagonist (Srinivas et al. 1996; Morey et al. 1998).

Based on the initial results, the original strategy had to be refocused, so further *E/c* curves were generated with CPA in the presence and absence of NBTI, and with and without a pretreatment with FSCPX. The CPA *E/c* curves were evaluated according to a procedure called receptorial responsiveness method (RRM) (Gesztelyi et al. 2004).

Materials and Methods

Materials

The following chemicals were used: adenosine, N⁶-cyclopentyladenosine (CPA), 8-cyclopentyl-N³-[3-(4-(fluoro sulfonyl)benzoyloxy)propyl]-N¹-propylxanthine (FSCPX) and S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine (NBTI), purchased from Sigma (St. Louis, MO, USA).

Adenosine was dissolved in 36°C Krebs solution. CPA was dissolved in ethanol:water (1:4) solution (v/v). Dimethyl-sulfoxide (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 used to achieve 3 mM concentration in the bathing medium. For this purpose, 20 mM adenosine solution was prepared freshly before use. When appropriate, stock solutions were diluted with modified Krebs-Henseleit buffer (Krebs solution). The composition of Krebs solution in mM was: NaCl 118, KCl 4.7, CaCl₂ 2.5, NaH₂PO₄ 1, MgCl₂ 1.2, NaHCO₃ 24.9, glucose 11.5, ascorbic acid 0.1; dissolved in redistilled water.

Animals, preparations and groups

The protocols were approved by the Committee of Animal Research, University of Debrecen, Hungary (DE MÁB 35/2007). Male Hartley guinea pigs weighting 500–700 g were guillotined, then left atria were quickly removed and mounted at 10 mN resting tension in 10 ml vertical organ

chambers (Experimetria TSZ-04) containing Krebs solution, oxygenated with 95% O₂ and 5% CO₂ (36°C; pH = 7.4). Atria were paced by platinum electrodes (3 Hz, 1 ms, twice the threshold voltage) with the use of a built-in 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 measured by a transducer (Experimetria SD-01) and strain gauge (Experimetria SG-01D), and recorded by a polygraph (Medicor R-61 6CH Recorder).

The atria were divided into six groups: P1 (in which Protocol 1 was carried out), P2 (a group for Protocol 2), P3-Control and P3-NBTI (two groups for Protocol 3), P4-Control and P4-FSCPX (two groups for Protocol 4).

Protocol 1 (demonstration of the effect of FSCPX on the adenosine E/c curve)

Control adenosine E/c curve: Atria ($n = 8$) were allowed to equilibrate in Krebs solution for 25 min, and then they were exposed to 100 μM of adenosine for 1 min followed by a 15 min long washout with Krebs solution. Afterward, a cumulative *E/c* curve with adenosine (from 1 nM to 3 mM) was constructed (P1-Control curve).

FSCPX-pretreatment: After a 15 min washout, atria were subjected to 10 μM FSCPX for 45 min followed by a 75 min long washout with Krebs solution.

Second adenosine E/c curve: After the FSCPX-pretreatment, a cumulative *E/c* curve with adenosine (from 1 nM to 3 mM) was generated (P1-FSCPX curve).

Protocol 2 (attempt to determine the A₁ receptor reserve for adenosine)

Control adenosine E/c curve: Atria ($n = 7$) were allowed to equilibrate in Krebs solution for 25 min, and then they were exposed to 100 μM adenosine for 1 min followed by a 15 min long washout with Krebs solution. Subsequently, a cumulative *E/c* curve with adenosine (from 1 nM to 3 mM) was constructed (P2-Control curve).

NBTI treatment: After washout with Krebs solution (15 min), atria were incubated in 10 μM NBTI for 15 min.

Second adenosine E/c curve: A cumulative *E/c* curve with adenosine (from 1 nM to 3 mM) was generated in the presence of 10 μM NBTI (P2-NBTI curve).

FSCPX+NBTI treatment: After washout with Krebs solution (20 min), atria were subjected to 10 μM FSCPX for 45 min followed by a 60 min long washout with Krebs solution. Then, preparations received 10 μM NBTI and were incubated for 15 min.

Third adenosine E/c curve: A cumulative *E/c* curve with adenosine (from 1 nM to 3 mM) was generated in the presence of 10 μM NBTI (P2-FSCPX+NBTI curve).

Protocol 3 (data collection to determine c_x , the CPA concentration that is equieffective with the surplus endogenous adenosine accumulated interstitially in the presence of NBTI)

Adenosine E/c curve: Atria were allowed to equilibrate in Krebs solution for 40 min. Then, a cumulative *E/c* curve was constructed with adenosine (from 10 nM to 1 mM) to assess the responsiveness of atrial A₁ receptors. (Adenosine is especially suitable for this purpose owing to its rapid elimination without yielding confounding byproducts (Wilbur and Marchlinski 1997).)

Control or NBTI treatment: After washout with Krebs solution (15 min), atria were randomized into two groups. Atria in the P3-Control group ($n = 8$) received 10 μl DMSO, the vehicle of NBTI, then they underwent a 15 min long incubation period. Atria in the P3-NBTI group ($n = 8$) were incubated in the presence of 10 μM NBTI for 15 min.

CPA E/c curve: A cumulative *E/c* curve was generated with CPA (from 0.1 nM to 100 μM) in the presence of 10 μl DMSO (P3-Control group) or 10 μM NBTI (P3-NBTI group). (Due to the slow elimination of CPA from tissues (Pavan and IJzerman 1998; Gesztelyi et al. 2004), only one CPA *E/c* curve was constructed in each preparation in order to avoid the prolonged washout period that would have been necessary between two CPA *E/c* curves.)

Protocol 4 (data collection to compute the negative inotropic effect of c_x on the FSCPX-pretreated atria)

Adenosine E/c curve: Atria were allowed to equilibrate in Krebs solution for 40 min. Afterward, a cumulative *E/c* curve was constructed with adenosine (from 10 nM to 1 mM) to determine the responsiveness of atrial A₁ receptors.

Control or FSCPX-pretreatment: After washout with Krebs solution (15 min), atria were randomly divided into two groups. Atria in the P4-Control group ($n = 11$) received 10 μl DMSO, the solvent of FSCPX, for 45 min followed by a 75 min long washout with Krebs solution. Atria in the P4-FSCPX group ($n = 12$) were subjected to 10 μM FSCPX for 45 min, succeeded by a 75 min long washout with Krebs solution. (Similarly to the Protocol 3, separate control and treated groups were generated because of the use of CPA.)

CPA E/c curve: A cumulative *E/c* curve was generated with CPA (from 0.1 nM to 100 μM).

Empirical characterization of E/c curves

The effect was defined as the percentage decrease in the initial contractile force of atria. All *E/c* curves were fitted to the Hill equation (Hill 1910):

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

where: E , the effect of the agonist; c , the concentration of the agonist (administered for the E/c curve); E_{max} , the maximal effect achievable by the agonist; EC_{50} , the agonist concentration producing half-maximal effect; n , the Hill coefficient.

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 applied for statistical analysis. Empirical parameters of the averaged CPA E/c curves of the P3-Control group and P4-FSCPX group were used for the mathematical correction (see below).

Strategy of the mathematical correction

Effect values of E/c curves generated in the presence of NBTI were considered to be biased by an increase in interstitial concentration of endogenous adenosine that was produced by NBTI. This surplus interstitial adenosine concentration (above the resting level) biased the E/c curves because it was unknown and was not taken into account (it was “neglected”). The biased adenosine E/c curves were not suitable for the assessment of A_1 adenosine receptor reserve, the goal of the present study. Therefore, effects of adenosine E/c curves biased by NBTI were corrected by means of RRM, our method validated for quantifying changes in agonist concentrations in the microenvironment of the receptors (Gesztelyi et al. 2004; Greczner et al. 2010a, 2010b).

Because NBTI strongly affects the fate of exogenous adenosine used to generate an E/c curve (Szentmiklosi et al. 1982; Gesztelyi et al. 2003a), adenosine could not be used as an agonist for the E/c curves essential for RRM (Karsai et al. 2006). However, RRM enables replacement of a degradable agonist with a stable one (Gesztelyi et al. 2004; Greczner et al. 2010a, 2010b). Thus, the surplus interstitial adenosine accumulated by NBTI was quantified using E/c curves generated with CPA, an A_1 receptor agonist with a significantly longer half-life in living tissues than adenosine (see Eq. 2 below) (Pavan and IJzerman 1998; Gesztelyi et al. 2004). The replacement of adenosine with CPA was based on the assumption that all E/c curves constructed in the presence of NBTI were biased by the same surplus adenosine concentration, irrespectively of whether adenosine or CPA was used to generate the E/c curves. Due to the relative stability of CPA, the change of the CPA E/c curve in response to NBTI was predominantly mediated by the surplus interstitial concentration of endogenous adenosine, which was quantified by the equieffective CPA concentration (c_x). From c_x , the effect elicited by the surplus adenosine concentration was computed (E_x). As c_x itself did not depend on the number of intact A_1 receptors, the same value was valid for all NBTI-treated atria. In contrast, c_x could exert different

effects on atria having naïve A_1 receptors and on atria with depleted A_1 receptor population. Thus, different E_x values were calculated for the correction of adenosine E/c curves before and after a pretreatment with FSCPX, an irreversible A_1 receptor antagonist (see Eq. 3 below). Finally, the correct effects were computed from E_x and the biased effects with the use of the relationship between biased and correct effects (see Eq. 4 below), which is the fundamental equation of RRM (Gesztelyi et al. 2004; Greczner et al. 2010a).

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

To characterize the surplus interstitial adenosine concentration by RRM, the averaged CPA E/c curve of the P3-NBTI group was fitted to Eq. 2 (see below). Since RRM compares the biased curve to a control one (generated under identical conditions excepting the cause of bias), Eq. 2 contained the empirical parameters of the averaged CPA E/c curve of the P3-Control group (to read more details for this procedure, see: Karsai et al. 2006):

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

where: E' , the effect value of the averaged CPA E/c curve of the P3-NBTI group (the biased effect); E_{max} , EC_{50} , n , the empirical parameters of the averaged CPA E/c curve of the P3-Control group; c , the concentration of CPA (administered for the E/c curve); c_x , the CPA concentration that is equieffective with the surplus interstitial concentration of endogenous adenosine produced by NBTI (a “surrogate parameter” for the present investigation). The c_x is the only variable parameter of Eq. 2.

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

The effect belonging to c_x could be determined 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 interstitial adenosine accumulated by NBTI; c_x , the theoretical 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 correcting the averaged P2-NBTI curve, empirical parameters of the averaged CPA E/c curve of the P3-Control group were substituted into Eq. 3. In turn, when E_x was calculated for correcting the averaged P2-FSCPX+NBTI curve, empirical parameters of the averaged CPA E/c curve of the P4-FSCPX group were written into Eq. 3.

From the biased effects and their corresponding E_x values, correct effects were computed by means of the following equation (which is the rearranged form of the originally published relationship, see: Gesztelyi et al. 2004; Greczner et al. 2010a):

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

where: E , the correct(ed) effect (belonging to the averaged P2-NBTI or P2-FSCPX+NBTI curve); E' , the biased effect (related to the averaged P2-NBTI or P2-FSCPX+NBTI curve); E_x , the effect of the surplus interstitial adenosine produced by NBTI (in atria with naive or depleted A₁ receptor population, respectively).

The corrected effect values yielded by Eq. 4 reflected the action of NBTI on the adenosine E/c curve without the bias caused by the extracellularly accumulated endogenous adenosine.

Data analysis

Each atrium had to meet three criteria to be included 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 to 10 μ M adenosine obtained from the first E/c curve had to be within the mean \pm 2 SD range (i.e. outliers were excluded). The mean and SD were computed using samples meeting the first two criteria. All data obeying the three criteria were processed.

According to the recommendation of Motulsky and Christopoulos (2004), concentrations (agonist concentration, EC_{50} and c_x) in the equations used for curve fitting were expressed as common logarithms (as described previously: Greczner et al. 2010a, 2010b).

Two data sets (passed the normality test) were compared with paired t-test. More than two data sets (passed the normality test) 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 2010.

Results

Initial adenosine E/c curves

Adenosine concentration-dependently decreased the atrial contractile force. Empirical parameters of the adenosine E/c curves, which were generated at the beginning of each experimental protocol, did not show significant differences among the experimental groups. This observation indicates the homogeneity of atria in the different groups (Fig. 1).

Adenosine E/c curves of Protocols 1 and 2

As expected, FSCPX, a selective and irreversible A₁ receptor antagonist, caused a pronounced rightward shift in the adenosine E/c curve in comparison to the control curve (Fig. 2). Also as expected, NBTI, a selective inhibitor of adenosine transport across the cell membrane, produced an extensive leftward displacement in the adenosine E/c curve

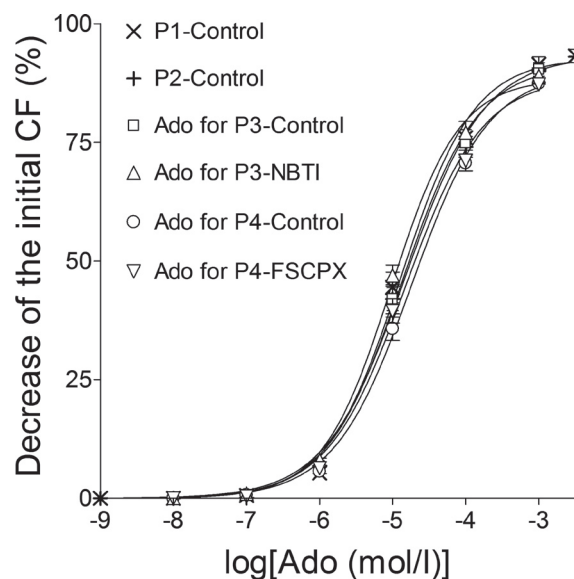


Figure 1. The direct negative inotropic effect of adenosine (Ado) in isolated guinea pig left atria divided into six groups (P1; P2; P3-Control; P3-NBTI; P4-Control; P4-FSCPX). In groups P1 and P2, the first adenosine E/c curve is shown (the P1-Control curve and P2-Control curve), while in groups P3-Control, P3-NBTI, P4-Control and P4-FSCPX, 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 axis x shows the common logarithm of adenosine concentration (in mol/l), and the axis y indicates the effect as a percentage decrease of the initial contractile force (CF). The symbols show the responses to adenosine averaged within the groups (\pm SEM), and the curves illustrate the fitted Hill equation (Eq. 1).

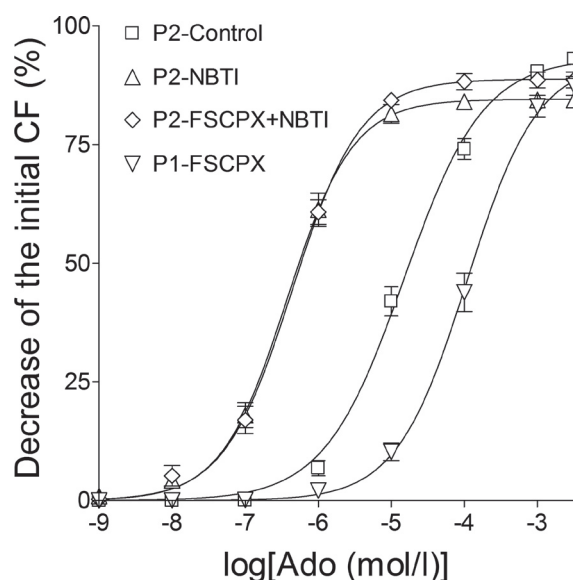


Figure 2. The direct negative inotropic effect of adenosine (Ado) before and after an FSCPX-pretreatment, and in the absence and presence of NBTI (alone and in combination). The curve names refer to the protocol used (Protocols 1 or 2) and to the applied *in vitro* treatment (control, NBTI, FSCPX or FSCPX+NBTI). For the sake of simplicity, the P1-Control curve (not differing significantly from the P2-Control curve) is omitted. The axis x indicates the common logarithm of adenosine concentration (in mol/l), and the axis y shows the effect as a percentage decrease of the initial contractile force (CF). The symbols represent the responses to adenosine averaged within the groups (\pm SEM), and the curves illustrate the fitted Hill equation (Eq. 1).

with a moderate decrease in E_{\max} (Fig. 2). Astonishingly, however, FSCPX-pretreatment slightly but in a statistically significant manner counteracted the effect of NBTI on E_{\max} by enhancing the response to higher adenosine concentrations, without affecting other parameters of the adenosine E/c curve (Fig. 2, Table 1).

CPA E/c curves

Similarly to adenosine, CPA concentration-dependently reduced the atrial contractile force, which effect was blunted by both NBTI and FSCPX. NBTI decreased E_{\max} as well as Hill coefficient and increased $\log EC_{50}$. In contrast, FSCPX augmented only $\log EC_{50}$, mimicking the behavior of a competitive A_1 receptor antagonist (Fig. 3, Table 1).

By means of RRM, the surplus interstitial adenosine accumulated by NBTI was found to be equieffective with 45.08 nM CPA. This result came from the best-fit value provided by Eq. 2 that was $\log(c_x) = -7.346$ (with a 95% confidence interval from -7.454 to -7.237).

Corrected effects of adenosine E/c curves generated in the presence of NBTI

After correcting for the bias caused by the surplus interstitial adenosine accumulated by NBTI, effect values of the P2-NBTI and P2-FSCPX+NBTI curves changed in their relationship to each other. As the total (endogenous + exogenous) interstitial adenosine concentration in the microenvironment of A_1 receptors was unknown, the cor-

Table 1. The influence of FSCPX-pretreatment and NBTI (alone or together) on the direct negative inotropic effect of adenosine or CPA, characterized by the Hill equation in isolated guinea pig left atria

Agonist	Curve/group	E_{\max} (%)	$\log EC_{50}$	EC_{50}	n
adenosine	P1-Control	93.54 \pm 1.01	-4.86 \pm 0.07	13.8 μ M	0.93 \pm 0.09
	P1-FSCPX	92.6 \pm 1.8 (ns)	-3.96 \pm 0.07 (***)	109.65 μ M	0.98 \pm 0.07 (ns)
	P2-Control	93.28 \pm 0.82 (ns)	-4.83 \pm 0.08 (ns)	14.79 μ M	0.82 \pm 0.03 (ns)
	P2-NBTI	84.6 \pm 1.44 (***)	-6.43 \pm 0.07 (***)	0.37 μ M	1 \pm 0.06 (ns)
	P2-FSCPX+NBTI	88.92 \pm 1.77 (*; \neq)	-6.36 \pm 0.05 (***; ns)	0.44 μ M	0.98 \pm 0.1 (ns; ns)
CPA	P3-Control	92.54 \pm 1.54	-7.56 \pm 0.07	27.54 nM	1 \pm 0.03
	P3-NBTI	82.06 \pm 2.34 (***)	-7.16 \pm 0.11 (*)	69.34 nM	0.78 \pm 0.07 (*)
	P4-Control	90.22 \pm 1.21 (ns)	-7.59 \pm 0.06 (ns)	25.76 nM	0.89 \pm 0.03 (ns)
	P4-FSCPX	91.78 \pm 0.8 (ns; \neq)	-6.7 \pm 0.13 (***; \neq)	201.37 nM	0.83 \pm 0.04 (ns; ns)

E_{\max} , $\log EC_{50}$ and n (mean \pm SEM) are best-fit values of the Hill equation (Eq. 1) that were derived from individually fitted E/c curves. EC_{50} is the antilog of $\log EC_{50}$ (mean). The level of statistical significance is indicated: ns, not significant; one or three marks (* or \neq), $p < 0.05$ or $p < 0.001$, respectively. For the adenosine E/c curves: * the curve generated after FSCPX-pretreatment (P1-FSCPX) or in the presence of NBTI (P2-NBTI) or both (P2-FSCPX+NBTI) vs. the corresponding control adenosine E/c curve (P1-Control or P2-Control); \neq , the P2-FSCPX+NBTI curve vs. the P2-NBTI curve. For the CPA E/c curves: * the P3-NBTI group or the P4-FSCPX group vs. the corresponding control group (P3-Control or P4-Control); \neq the P4-FSCPX group vs. the P3-NBTI group. (Differences between the P1-Control curve and P2-Control curve were not significant, similarly to the differences between the P3-Control group and P4-Control group.)

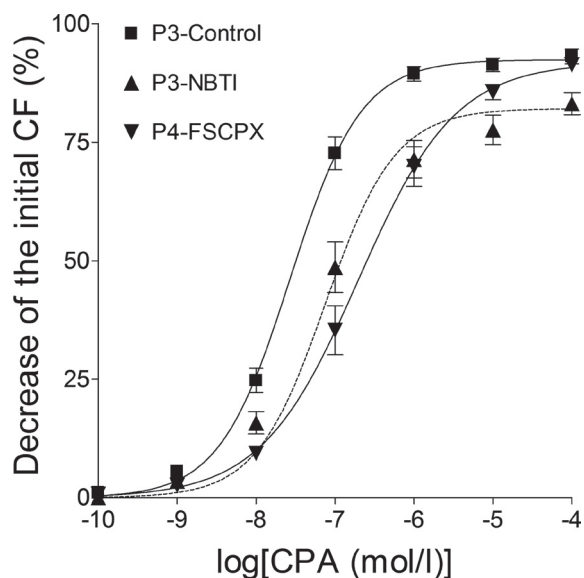


Figure 3. The direct negative inotropic effect of CPA with or without an FSCPX-pretreatment, furthermore in the presence and absence of NBTI. The group names refer to the protocol used (Protocols 3 or 4) and to the applied *in vitro* treatment (control, NBTI or FSCPX). For simplicity, the P4-Control group (not differing significantly from the P3-Control group) is omitted. The axis x denotes the common logarithm of CPA concentration (in mol/l), and the axis y shows the effect as a percentage decrease of the initial contractile force (CF). The symbols indicate the responses to CPA averaged within the groups (\pm SEM). The continuous curves represent the fitted Hill equation (Eq. 1), while the dotted curve illustrates the fitted model of RRM (Eq. 2).

rected effect values of the P2-NBTI and P2-FSCPX+NBTI curves could only be plotted against the concentration of exogenous adenosine in the bathing medium (Fig. 4). Consequently, the corrected *E/c* curves were not suitable for assessing exact receptor reserve values. Nevertheless, the inherently correct and corrected effects in the P2 group that belonged to the same x value could be compared to one another. The maximal effects related to 3 mM adenosine were as follows: 93.06%, 93.36% and 91.33% for the P2-Control curve, P2-NBTI curve and P2-FSCPX+NBTI curve, respectively. Thus, the corrected P2-NBTI and P2-FSCPX+NBTI curves changed places with each other as compared to the original curves, according to the expectations concerning the action of FSCPX, an irreversible A₁ receptor antagonist (Fig. 4). On the other hand, the final (saturated) parts of the corrected P2-NBTI and P2-FSCPX+NBTI curves got very close to each other, indicating great A₁ receptor reserve for the direct negative inotropic effect of adenosine in the guinea pig atrium.

For the sake of comparison, the P2-Control curve (considered to be correct without any transformation) was plotted

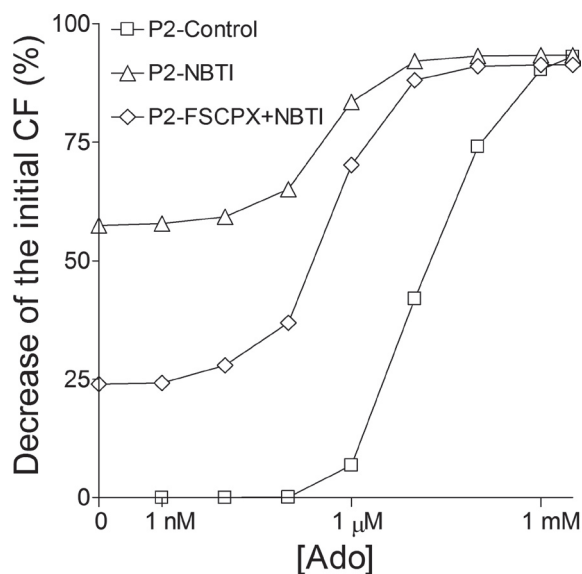


Figure 4. The corrected effect values of the P2-NBTI curve and P2-FSCPX+NBTI curve, furthermore the original effect values of the P2-Control curve (regarded to be inherently correct), all plotted against the concentrations of administered adenosine (Ado) developed in the bathing medium of atria. The curve names refer to the protocol used (Protocols 2) and to the applied *in vitro* treatment (control, NBTI or FSCPX+NBTI). The effects of the P2-NBTI curve and P2-FSCPX+NBTI curve were corrected using regression parameters yielded by the averaged CPA *E/c* curves of Protocols 3 and 4 (except for the P4-Control group that served only to survey the influence of the long incubation period on the response to CPA). The axis x shows the concentration of (exogenous) adenosine on a logarithmic scale, and the axis y indicates the effect as a percentage decrease of the initial contractile force (CF). The symbols represent responses to adenosine averaged within the groups.

together with the corrected *E/c* curves. In addition to the fact that both corrected *E/c* curves showed considerable sinistral displacement in comparison with the control curve, the corrected P2-NBTI curve ran above the control curve all along, while the corrected P2-FSCPX+NBTI curve got below the control curve at the highest (exogenous) adenosine concentration (3 mM) (Fig. 4). Although differences among the three curves at 3 mM adenosine are quite small, these observations are in agreement with the previous findings that NBTI enhances the response to adenosine by preventing its intracellular metabolism, in contrast to FSCPX that suppresses the effect of adenosine by inactivating the A₁ receptors.

The initial effect values of the corrected P2-NBTI and P2-FSCPX+NBTI curves (at zero exogenous adenosine concentration) were 57.44% and 23.97%, respectively (Fig. 4). These values were computed as the negative inotropic

effect of c_x (i.e. E_x) in atria with naive and depleted A_1 receptor populations, respectively. The remarkably smaller E_x developed in the FSCPX-pretreated atria (Fig. 4) gives an explanation why the bias caused by NBTI was smaller in the original P2-FSCPX+NBTI curve than in the original P2-NBTI curve. The smaller E_x and the resultant smaller bias in the conventionally plotted adenosine E/c curve led to the paradoxical phenomenon that E_{\max} of the original P2-FSCPX+NBTI curve was bigger than that of the original P2-NBTI curve (Fig. 2).

Discussion

The major finding of the present study is that A_1 receptor reserve in the guinea pig supraventricular myocardium appertaining to the direct negative inotropic effect of the physiological ligand adenosine is considerably great. This finding corroborates our earlier results obtained with the use of NECA, CPA and CHA, synthetic A_1 receptor agonists. In addition, the present study indicates that nucleoside transport blockade exerts a dual effect on the adenosine E/c curve that can lead to seemingly paradoxical results.

Adenosine, a ubiquitous purine nucleoside, primarily exerts its protective effects against ischaemia, stress and tissue injury *via* activation of cell-surface adenosine receptors (Fredholm et al. 2011; Headrick et al. 2011). Although studies of adenosinergic mechanisms require accurate data, rapid metabolic clearance of adenosine (Wilbur and Marchlinski 1997; Pavan and IJzerman 1998) makes reliable quantification of interstitial adenosine levels challenging (Ramakers et al. 2008), especially in mechanically highly active organs, such as the heart (Karsai et al. 2006). An accepted strategy to overcome this uncertainty is to blunt the rapid adenosine turnover by inhibiting adenosine-handling enzymes and/or transporters (Szentmiklosi et al. 1982, 2011; Deussen et al. 1999; Ramakers et al. 2008).

In earlier studies, inhibition of nucleoside transport was found to augment the response to adenosine that is primarily reflected by a leftward shift (decreased EC_{50}) of the adenosine E/c curve (Szentmiklosi et al. 1982; Gesztelyi et al. 2003a). This phenomenon can be explained by the fact that, under well-oxygenated conditions, the net transmembrane adenosine transport is directed into the cardiomyocytes due to the rapid intracellular elimination of adenosine (Deussen et al. 1999; Deussen 2000a, 2000b). Thus, nucleoside transport blockade, by preventing the intracellular metabolism of adenosine administered for the E/c curve, elevates the interstitial adenosine level leading to an enhanced stimulation of the cell-surface adenosine receptors.

In contrast, when slowly metabolizing adenosine receptor agonists (such as CPA) were used to produce E/c curves, nucleoside transport inhibitors were found to decrease E_{\max}

and increase EC_{50} (Gesztelyi et al. 2003b; Karsai et al. 2006, 2007). This outcome is due to an elevation in interstitial concentration of endogenous adenosine, which is a known consequence of inhibiting adenosine transport (Deussen et al. 1999; Deussen 2000a, 2000b). Surplus adenosine, made available by nucleoside transport blockade, interact with an unaccounted fraction of adenosine receptors, and thereby in part consumes the response capacity of the system before the construction of the E/c curve (Karsai et al. 2006, 2007). This phenomenon appears if the exogenous agonist is only minimally eliminated by the cells, so there is no significant inward drive for the exogenous agonist. In this case, blockade of the transmembrane transport barely increases the quantity of the exogenous agonist available for the cell-surface adenosine receptors.

In a previous investigation conducted without the use of transport (or enzyme) inhibitors, adenosine proved to be inadequate for quantifying A_1 receptor reserve by means of functional assays (specifically: the operational model of agonism and Furchgott's method) (Gesztelyi et al. 2013). In this previous study, failure to quantify the A_1 receptor reserve was attributed to the rapid tissue elimination of the exogenous adenosine. Thus, a major design feature of the present study was to repeat these previous experiments in the presence of NBTI, a selective and efficacious nucleoside transport inhibitor (Thorn and Jarvis 1996), in order to diminish the elimination of adenosine and thereby to obtain reliable estimates of the magnitude of A_1 receptor reserve for the direct negative inotropic effect of adenosine.

In the present study, however, pretreatment with FSCPX, a strong, selective and irreversible A_1 receptor antagonist (Srinivas et al. 1996; Morey et al. 1998), was found to increase E_{\max} of the conventionally plotted adenosine E/c curves that were constructed in the presence of NBTI (Table 1, Fig. 2). This observation contradicting common pharmacological logic raised the possibility that interstitial accumulation of endogenous adenosine, an effect of NBTI, cannot be ignored in this case.

In previous studies, an effect was designated to be "biased", if a fraction of the receptor population had been already stimulated by a neglected agonist concentration, when the effect in question was elicited by another agonist concentration that was taken into account (Gesztelyi et al. 2004; Greczner et al. 2010a). Based on this definition, adenosine E/c curves generated in the presence of NBTI as part of the present study were considered to be biased.

Theoretically, the best way to correct the above mentioned bias would be to determine the neglected agonist concentration and to include it (together with its effect) in the evaluation. However, to date there is no method, with the use of which the interstitial adenosine concentration could be measured with accuracy sufficient for our purpose. Indeed, the current methods provide estimates for cardiac

interstitial adenosine levels *in vivo* or *ex vivo* that range from nano- to micromolar (!) concentrations (Karsai et al. 2006). Due to the lack of feasible methods, mathematical modeling may be used to correct for the bias. One such model is RRM that provides a surrogate parameter that can be precisely determined in our experimental setting, simply *via* constructing conventional *E/c* curves (Gesztelyi et al. 2004; Greczner et al. 2010a, 2010b). So, using RRM, the surplus interstitial adenosine accumulated by NBTI was quantified by the equieffective CPA concentration (c_x), by means of which the effects biased by NBTI could be then corrected.

The corrected effects clearly show that the partial inactivation of the A₁ receptor population by FSCPX in fact reduced the maximal response to adenosine even in the presence of NBTI (Fig. 4). However, this reduction was quite small suggesting that the guinea pig atrial A₁ receptor reserve appertaining to the direct negative inotropy is as great for adenosine as for the synthetic A₁ receptor full agonists investigated previously (NECA, CPA, CHA) (Gesztelyi et al. 2013). This means that the signal amplification machinery linking the A₁ receptor to the contractile system of the atrial cardiomyocyte works with similarly high efficiency for adenosine as for synthetic full agonists.

The major limitation of the present study is that the exact concentrations of interstitial adenosine (the resting level and its changes) remained unknown; therefore the corrected effects could only be plotted *versus* the concentrations of administered (exogenous) adenosine evolved in the organ bath (Fig. 4). This fact, unfortunately, impeded the exact quantification of A₁ receptor reserve by methods used for functional data (e.g. the operational model of agonism or Furchgott's method). However, the quasi-*E/c* curves created from the mathematically corrected effects are unique in that they are free of the bias caused by the interstitial accumulation of endogenous adenosine, despite the repressed adenosine elimination. These quasi-*E/c* curves are fully saturated, so maximal responses to adenosine can be well demonstrated (Fig. 4). In addition, our findings show that ignorance of accumulation of an endogenous agonist caused by inhibition of its handling enzymes and/or carriers can lead to deceptive results. This is especially true if these results are obtained from *E/c* curves (Fig. 2), unless they are corrected for the bias (Fig. 4).

A further limitation of this study is its pure functional nature. Nevertheless, this study gives evidence about the great sensitivity of atrial contractility to A₁ receptor stimulation evoked by adenosine, the physiological ligand. This is particularly important in light of the fact that, in addition to synthetic adenosine receptor agonists, numerous drugs enhancing the actions of endogenous adenosine are in the pipeline (Elzein and Zablocki 2008; Schenone et al. 2010; Fredholm et al. 2011; Szentmiklosi et al. 2011).

In summary, to the best of our knowledge, the present study is the first to characterize the atrial A₁ receptor reserve for the direct negative inotropic effect of adenosine. A considerably great A₁ receptor reserve has been found in the guinea pig supraventricular myocardium. This finding draws attention to a possible side effect of A₁ receptor positive modulators (enhancers), namely they might decrease the atrial contractile force. Additionally, our results demonstrate that the nucleoside transport blockade simultaneously exerts an enhancing and blunting effect on the *E/c* curve generated with exogenous adenosine. This observation can be explained by the fact that NBTI prevents intracellular elimination of adenosine (Deussen et al. 1999) that affects both exogenous and endogenous adenosine. In general, a change in the level of an endogenous agonist in response to an agent may significantly bias an *E/c* curve if it is constructed in the given biological system with the same agonist or with another one for the same receptor (or for another receptor having largely overlapping signaling).

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