British Journal of Pharmacology (1996) 118, 369-377

Pharmacokinetic-haemodynamic relationships of 2-chloroadenosine at adenosine A_1 and A_{2a} receptors *in vivo*

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1 The purpose of the present study was to develop an experimental strategy for the quantification of the cardiovascular effects of non-selective adenosine receptor ligands at the adenosine A_1 and A_{2a} receptor *in vivo*. 2-Chloroadenosine (CADO) was used as a model compound.

2 Three groups of normotensive conscious rats received an short intravenous infusion of 1.4 mg kg^{-1} CADO during constant infusions of the A_1-selective antagonist, 8-cyclopentyltheophylline (CPT; 20 $\mu g\ min^{-1}\ kg^{-1}$), the A_{2a}-selective antagonist, 8-(3-chlorostyryl)caffeine (CSC; 32 $\mu g\ min^{-1}\ kg^{-1}$) or the vehicle. The heart rate (HR) and mean arterial blood pressure (MAP) were recorded continuously during the experiment and serial arterial blood samples were taken for analysis of drug concentrations. The ratio MAP/HR was also calculated, which may reflect changes in total peripheral resistance on the assumption that no changes in stroke volume occur.

3 During the infusion of CPT, CADO produced a reduction in both blood pressure and MAP/HR by activation of the A_{2a} receptor. The concentration-effect relationships were described according to the sigmoidal E_{max} model, yielding potencies based on free drug concentrations ($EC_{50,u}$) of 61 and 68 ng ml^{-1} (202 and 225 nM) for the reduction of blood pressure and MAP/HR, respectively. During the infusion of CSC, an $EC_{50,u}$ value of 41 ng ml^{-1} (136 nM) was observed for the A_1 receptor-mediated reduction in heart rate. The *in vivo* potencies correlated with reported receptor affinities ($K_i(A_1) = 300$ nM and $K_i(A_{2a}) = 80$ nM). The maximal reductions in MAP/HR and heart rate were comparable to those of full agonists, with the E_{max} values of $-12 \pm 1 \times 10^{-2}$ mmHg b.p.m.$^{-1}$ and -205 b.p.m. respectively.

4 It is concluded that this integrated pharmacokinetic-pharmacodynamic approach can be used to obtain quantitative information on the potency and intrinsic activity of new non-selective adenosine receptor agonists at different receptor subtypes *in vivo*.

Keywords: Adenosine receptor; 2-chloroadenosine (CADO); cardiovascular effects; pharmacokinetic-pharmacodynamic modelling; intrinsic activity; *in vivo* potency

Introduction

Adenosine produces its physiological effects by interaction with membrane-bound receptors, called P_1 receptors, of which currently four subtypes have been defined, A_1, A_{2a}, A_{2b} and A_3 (Abbrachio *et al.*, 1993). The cardiovascular responses mediated by A_1 and A_{2a} receptor subtypes have been studied in conscious normotensive and hypertensive rats (Webb *et al.*, 1990; 1991; Abiru *et al.*, 1991; Milavec-Krizman *et al.*, 1991). In these studies, the hypotensive effect elicited was independent of the A_1- and A_{2a}-selectivity of the compounds, whereas the opposite was observed for the effect on heart rate. Selective A_1 receptor agonists caused significant bradycardia, whereas A_{2a} agonists promoted a strong reflex tachycardia. It has therefore been suggested that adenosine receptor agonists with a balanced A_1- and A_2-selectivity may be useful as antihypertensive drugs, since hypotension may be produced without an unfavourable effect on heart rate (Milavec-Krizman *et al.*, 1991). In principle, these drugs may be partial agonists and/or full agonists for the two receptor subtypes. In order to assess the most favourable ratio of potency and intrinsic activity, it is necessary to develop and experimental strategy which allows the cardiovascular effects, mediated by the different receptor subtypes, to be quantified separately.

Recently, an integrated pharmacokinetic-pharmacodynamic model has been reported for the characterization of the haemodynamic effects of the A_1-selective agonist N^6-cyclopentyladenosine (CPA) in conscious normotensive rats (Mathôt *et al.*, 1994). Estimates of the *in vivo* potency and intrinsic activity of CPA were obtained in individual animals by relating blood concentrations to the reduction in heart rate. The observed *in vivo* potency of CPA was in good agreement with the *in vitro* receptor affinity, indicating that bradycardia is a relevant index of *in vivo* activation of A_1 receptors. In a comparable study with the prototypic A_{2a}-selective agonist CGS 21680, the reduction in blood pressure was found to be an appropriate pharmacodynamic endpoint for the *in vivo* activation of A_{2a} receptors (Mathôt *et al.*, 1995a). Application of these pharmacokinetic-pharmacodynamic models has been proven useful in the *in vivo* quantification of the cardiovascular effects of newly synthesized selective agonists (IJzerman *et al.*, 1994a; Mathôt *et al.*, 1995d).

The purpose of the present study was to develop an experimental strategy for the *in vivo* quantification of the cardiovascular effects of non-selective adenosine receptor agonists. 2-Chloroadenosine (CADO) was selected as a model compound. In radioligand binding studies, this compound has comparable affinities for rat A_1 and A_{2a} receptors ($K_i(A_1) = 300$ nM; $K_i(A_{2a}) = 80$ nM), whereas the affinity for the A_3 receptor is low (K_i 1900 nM) (IJzerman *et al.*, 1994b; Van Galen *et al.*, 1994). The quantification of the cardiovascular effects of CADO is complex due to activation of both A_1 and A_{2a} receptors, which both cause typical effects on heart rate and blood pressure. In the present study, selective antagonists were used to distinguish between A_1 and A_{2a} receptor-mediated effects. Conscious normotensive rats received an intravenous bolus infusion of CADO during continuous infusion of either 8-cyclopentyltheophylline (CPT) or 8-(3-chlorostyryl)caffeine

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(CSC). CPT is a selective antagonist for the A_1 receptor ($K_i(A_1) = 11$ nM; $K_i(A_{2a}) = 1400$ nM), whereas CSC is selective for A_{2a} receptors ($K_i(A_{2a}) = 54$ nM; $K_i(A_1) = 28200$ nM) (Bruns *et al.*, 1986; Jacobson *et al.*, 1993). In addition CSC has been found to be 150 fold selective for A_{2a} versus A_{2b} receptors. CPT and CSC have been demonstrated to antagonize competitively the cardiovascular effects of selective adenosine receptor agonists with *in vivo* potencies bases on free concentrations ($EC_{50,u}$) of 2.0 and 48 nM, respectively (Appel *et al.*, 1995; Mathôt *et al.*, 1995b). In the present experiments, the time courses of heart rate and blood pressure were monitored continuously in conjunction with serial arterial blood sampling. The time profile of the ratio of mean arterial pressure over heart rate (MAP/HR) was derived as an additional cardiovascular parameter. This ratio may reflect changes in total peripheral resistance on the assumption that no changes in stroke volume occur (Mathôt *et al.*, 1995a). The *in vivo* potency and intrinsic activity of CADO for the A_1 and A_{2a} receptor were determined by relating the haemodynamic responses to CADO blood concentration during blockade of either the A_{2a} or the A_1 receptor.

Methods

Animals and surgical preparation

Adult male normotensive SPF rats of Wistar descent (200–250 g) were obtained from the Sylvius Laboratory Breeding Facility (Leiden, The Netherlands). They were housed individually in plastic cages under a normal 12-h light (07 h 00 min–19 h 00 min)-dark cycle and had free access to standard lab food (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TH, Hope Farms, Woerden, The Netherlands) and tap water.

Two days before experimentation, in-dwelling cannulae were implanted under light ether anaesthesia as has been described previously (Mathôt *et al.*, 1994; 1995a). The abdominal aorta was cannulated by an approach through the left and the right femoral arteries for the measurement of the arterial blood pressure and the collection of serial blood samples, respectively. The right jugular vein was cannulated with a cannula consisting of two separate tubings (PE45, Media BV,'s Hertogenbosch, The Netherlands) for the short infusion of CADO and the continuous administration of solutions of either CPT, CSC or the vehicle. The cannulae were pulled subcutaneously to emerge on the nape of the neck. The cannulae for blood collection and administration of CSC were protected from light during the experiments in order to prevent photoisomerization of CSC (Mathôt *et al.*, 1995b).

Cardiovascular monitoring

Arterial blood pressure was measured from the left femoral catheter in the abdominal aorta with a miniature strain gauge P10EZ transducer, equipped with a TA1017 CritiFlo diaphragm dome (Viggo-Spectramed BV, Bilthoven, The Netherlands). During the experiment the diaphragm dome was flushed regularly with heparinized saline (20 iu ml^{-1}) to avoid clotting in the cannula. The pressure transducer was placed at the level of the animals heart and connected to a polygraph amplifier console (RMP6018, Nihon Kohden Corp., Tokyo, Japan). Heart rate was determined from the pressure signal, which was coupled to a tachograph. Signals were recorded on a polygraph and concurrently converted in a CED1401 interface (Cambridge Electronic Design Ltd., Cambridge, England) and transferred into a 80486 computer (INTEB, Sassenheim, The Netherlands). The data were stored on hard disk for off-line analysis. Data acquisition and reduction were performed with Spike2 computer software (Cambridge Electronic Design Ltd.). The ratio of mean arterial blood pressure over heart rate (MAP/HR) was calculated off-line. All experiments were started between 10 h 00 min and 11 h 00 min in order to exclude diurnal variation in base-line cardiovascular responses. Cardiovascular recording started 60 min prior to the beginning of the constant infusion and continued for 5.5 h.

Pharmacokinetic-pharmacodynamic experiments

The effect of constant infusions of CPT, CSC or the vehicle on the concentration-effect relationship of CADO was investigated in 18 individual rats. The animals were randomly allocated to three groups that received a continuous intravenous infusion of either 20 $\mu g\ min^{-1}\ kg^{-1}$ (81 nmol $kg^{-1}\ min^{-1}$) of CPT, 32 $\mu g\ min^{-1}\ kg^{-1}$ (97 nmol $kg^{-1}\ min^{-1}$) of CSC or the vehicle (Harvard infusion pump 22, Plato, Diemen, The Netherlands). The CSC infusion solutions were prepared in a room illuminated only by sodium light and protected from light during the experiment. CSC and CPT were dissolved in DMSO (dimethyl sulphoxide) and the infusion rate was 1.0 $\mu l\ min^{-1}$ for all treatments. After 150 min of constant infusion, the animals received an intravenous infusion of 1.4 mg kg^{-1} (4.6 $\mu mol\ kg^{-1}$) of CADO over 15 min (Braun infusion pump, Melsungen, Germany). CADO was dissolved in 765 μl of saline (0.9% (w/v). Blood sampling schedules were similar for the three groups. Arterial samples for the measurement of CADO blood concentrations were withdrawn at 2, 5, 10, 15, 16, 18 (20 μl), 20, 25 (50 μl), 30, 35 (100 μl), 40 and 45 min (200 μl) after the start of the infusion of CADO. Blood samples with a volume of 50 μl were taken at −5, 8, 30, 60, and 120 min for the determination of CPT or CSC blood concentrations. Blood samples were immediately haemolyzed in glass centrifuge tubes containing 400 μl of water at 0°C. CSC samples were protected from light. The samples were stored at −35°C until h.p.l.c. analysis. The influence of the bolus infusion and the volume of blood withdrawn on baseline cardiovascular responses was evaluated in a separate group of 6 rats. The group received a continuous infusion of DMSO and a bolus infusion of 765 μl saline. The blood sampling schedule was identical to that of the other treatment groups.

The plasma-to-blood (P/B) ratio and the free fraction in plasma (f_u) of CADO, CPT and CSC were determined for each individual rat. Blood samples with a volume of 300 μl were withdrawn at 8, 15 and 120 min after the start of the infusion of CADO. P/B and f_u values of the antagonists were determined in the blood samples which were taken at $t = 8$ and 120 min; the values of CADO were determined at $t = 15$ min. The blood samples were directly transferred to heparinized tubes on ice. CSC samples were protected from light and were handled under sodium light. The plasma was separated at 4°C and a sample of 20 μl was retained for analysis. A volume of 120 μl of the supernatant was subjected to ultrafiltration for 10 min at 1090 *g* at 37°C to separate unbound drug from plasma protein bound drug (Amicon Micropartition System, Amicon Division, Danvers, MA, U.S.A.). Unbound concentrations of CADO, CPT and CSC were determined in 50 μl of the ultrafiltrate.

Drug analysis

The concentrations of CADO in blood, plasma and ultrafiltrate were determined by a reversed-phase high-performance liquid chromatographic procedure, based on a method described earlier by Mathôt *et al.* (1993). Calibration standards were prepared by adding 50 μl of solutions of CADO to 100 μl blood in 400 μl water, producing a blood concentration range of 0.020 to 2.0 $\mu g\ ml^{-1}$. Standard solutions were prepared in water and were stable over a period of 2 months when stored at 4°C. NECA (0.2 μg in 50 μl water) was added as an internal standard. Following the addition of 50 μl 3.0 M NaOH, the samples were extracted for 60 s with 5 ml of ethyl acetate by using a vortex mixer. After centrifugation for 15 min at 2000 *g* the organic layer was transferred to another tube and evaporated under reduced pressure at 40°C. The residue was dissolved in 150 μl of the mobile phase and 50 μl were injected into the chromatographic system.

The chromatographic system consisted of a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsay, NJ, U.S.A.), a WISP-712B autosampler (Millipore-Waters, Milford, MA, U.S.A.) and a Spectroflow 757 variable-wavelength u.v. detector (λ 269 nm) (Applied Biosystems, Ramsey, NJ, U.S.A.). Chromatography was performed at 27°C using a stainless-steel Microsphere C18 3 μm cartridge column (100 mm × 4.6 mm i.d.) (Chrompack Nederland BV, Bergen Op Zoom, The Netherlands) equipped with a guard column (20 mm × 2 mm i.d.) (Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with C18 (particle size 20–40 μm) (Chrompack Nederland BV). Data processing was performed with a Chromatopack C-R3A reporting integrator (Shimadzu, Kyoto, Japan). The mobile phase consisted of a mixture of 0.10% (w/v) triethylamine in water, with the pH adjusted to 4.0 by addition of glacial acetic acid, and acetonitrile with a ratio of 89.5/10.5 (v/v). The flow rate was 0.50 ml min^{-1} and the retention times of CADO and NECA were 5.4 and 6.3 min, respectively. The calibration curves were constructed by weighted linear regression (weight factor: 1/(peak-height-ratio of CADO and NECA)) and were linear ($r > 0.999$). The within-day and between-day coefficients of variation were less than 10 and 5% ($n = 6$), respectively, for CADO concentrations of 0.021, 0.047 and 1.9 μg ml^{-1}. The extraction yield of CADO was greater than 60% in the same concentration-range ($n = 3$).

Concentrations of the adenosine receptor antagonists CPT and CSC were determined by h.p.l.c. according to procedures described earlier (Mathôt *et al.*, 1993; 1995b).

Data analysis

The individual blood concentration-time profiles after intravenous infusion of CADO were fitted to a poly-exponential equation by using the non-linear least squares programme Siphar (Simed, Creteil, France):

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \cdot T} \cdot (1 - e^{-\lambda_i \cdot t}) \qquad t \leq T \qquad (1A)$$

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \cdot T} \cdot (e^{-\lambda_i \cdot (t-T)} - e^{-\lambda_i \cdot t}) \qquad t > T \qquad (1B)$$

$C(t)$ is the blood concentration of CADO at time t, T is the infusion duration, and C_i and λ_i are coefficients and exponents of the equation, respectively. The most suitable exponential model was chosen according to standard criteria (Akaike, 1974; Yamaoka *et al.*, 1978). The computer-generated estimates of the coefficients and exponents were used to calculate pharmacokinetic parameters for CADO, such as the area under the curve (AUC), total blood clearance (Cl), mean residence time (MRT), volume of distribution at steady-state (V_{ss}) and the terminal half-life ($t_{1/2,n}$) according to standard equations (Gibaldi & Perrier, 1982; Mathôt *et al.*, 1994). The blood clearances of CPT and CSC were calculated by dividing the infusion rate by the steady-state concentration. Individual steady-state blood concentrations of the antagonists were obtained by averaging concentrations at $t = 8$, 30 and 60 min for each rat.

The poly-exponential pharmacokinetic fit was used to calculate the CADO blood concentration at the time of the averaged cardiovascular effect measurement. Time-effect points were obtained by averaging 60 s of consecutive heart rate, blood pressure or MAP/HR data. For each individual rat, concentrations were correlated to cardiovascular responses according to the sigmoidal E_{max} pharmacodynamic model with a fixed no-drug response (Holford & Sheiner, 1981)

$$E = E_0 + \frac{E_{max} \cdot C^{n_H}}{EC_{50}{}^{n_H} + C^{n_H}} \qquad (2)$$

where E is the cardiovascular response at CADO concentration C, E_0 is the no-drug response, EC_{50} is the blood concentration that corresponds to 50% of the maximum effect (E_{max}) and n_H expresses the sigmoidicity of the curve (Hill factor). Values of the no-drug response were obtained by averaging the cardiovascular effect from 60 to 90 min. EC_{50} values based on free CADO concentrations ($EC_{50,u}$) were obtained after correction for binding of the compound to blood cells and plasma proteins. The values of the E_{max} and EC_{50} of CADO at the A_1 and A_{2a} receptor subtypes were compared to the values of selective A_1 and A_{2a} agonists obtained in previous investigations (Mathôt *et al.*, 1995a; 1995d).

Student's t tests or one-way analysis of variance (ANOVA) were performed for comparison of the cardiovascular responses, and the pharmacokinetic and pharmacodynamic parameter estimates obtained after the different treatments; the corresponding non-parametric tests were used in case of non-homogeneity of the data. A significance level of 5% was selected. All data are reported as mean ± s.e., unless indicated otherwise.

Chemicals

2-Chloroadenosine (CADO) was purchased from Sigma (St. Louis, MO, U.S.A.). 8-Cyclopentyltheophylline (CPT), N^6-cyclohexyladenosine (CHA) and 5'-N-ethylcarboxamido-adenosine (NECA) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). (E)-8-(3-Chlorostyryl)caffeine (CSC) and (E)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KF 17837) were kindly donated by Dr K.A. Jacobson (NIDDK, National Institutes of Health, Bethesda, MD, U.S.A.) and Dr F. Suzuki (Kyowa Hakko Kogyo, Japan), respectively. Ethyl acetate was purchased from Baker Chemicals Deventer, The Netherlands) and distilled prior to use. Acetonitrile (h.p.l.c. grade) was obtained from Westburg (Leusden, The Netherlands). All the other chemicals used were of analytical grade (Baker, Deventer, The Netherlands).

Results

The average concentration-time profiles after the intravenous administration of 1.4 mg kg^{-1} CADO during 15 min are shown in the Figure 1a, b and c. For all treatment groups, a bi-exponential function satisfactorily described the time course of CADO concentration. CPT and CSC did not influence the pharmacokinetic parameters of CADO (Table 1). Averaged over the three treatments, the total blood clearance, the apparent volume of distribution at steady-state and the terminal half-life were 150 ± 10 ml min^{-1} kg^{-1}, 630 ± 30 ml kg^{-1} and 6.7 ± 0.5 min, respectively ($n = 18$). The average blood concentration-time profiles of CPT and CSC are shown in Figure 1b and c, respectively. Over the period from 8 to 60 min, the average concentrations of CPT and CSC were 260 ± 30 and 860 ± 40 ng ml^{-1}, respectively, resulting in the corresponding total blood clearance values of 90 ± 11 and 41 ± 2 ml min^{-1} kg^{-1} ($n = 6$). After the administration of CADO, blood concentrations of CPT were not changed (Figure 1b), whereas CSC concentrations were significantly increased (Figure 1c). The post-administration clearance of CSC ($t = 120$ min) was significantly lower than the pre-administration clearance ($t = -5$ min) with values of 36 ± 2 and 52 ± 5 ml min^{-1} kg^{-1}, respectively ($n = 6$; $P < 0.01$).

P/B and f_u of CADO were not influenced by the continuous infusion of CPT or CSC resulting in the corresponding average values of 0.92 ± 0.05 and 78 ± 2% ($n = 18$) in the blood concentration range of 500 to 1100 ng ml^{-1}. The presence of CADO did not affect binding of the antagonists to blood cells and plasma proteins. P/B and f_u of both compounds were similar at $t = 8$ and $t = 120$ min. P/B and f_u of CPT were 0.54 ± 0.05 and 29 ± 3% ($n = 12$), respectively, in the blood concentration-range of 95 to 460 ng ml^{-1}; the corresponding values of CSC were 0.75 ± 0.04 and 34 ± 2% ($n = 12$) in the concentration range of 520–1300 ng ml^{-1}.

The absence of intrinsic activity of the antagonists was

evaluated during the constant infusion of the compounds prior to the administration of CADO. The continuous infusion of the vehicle (DMSO; 1 μl min^{-1}) did not affect baseline heart rate or blood pressure. Heart rate and blood pressure values of the antagonist-treated groups were not significantly different from those of the vehicle-treated group.

The influence of CPT and CSC on the chronotropic effect of CADO is depicted in Figure 2a. During the continuous infusion of the vehicle, the administration of CADO produced a rapid reduction of heart rate. After the infusion was ended, heart rate gradually returned to pre-administration values. The bradycardic effect was reversible; heart rate was not significantly different from the placebo group after 30 min. The volume of the infusion and the volume of blood withdrawn had no significant effect on baseline heart rate. During the constant infusion of CSC, CADO also exerted a negative chronotropic effect. However, the reduction in heart rate upon the bolus infusion of CADO was significantly greater than for the vehicle-treated group. Constant infusion of CPT inhibited the negative chronotropic effect. Heart rate increased during

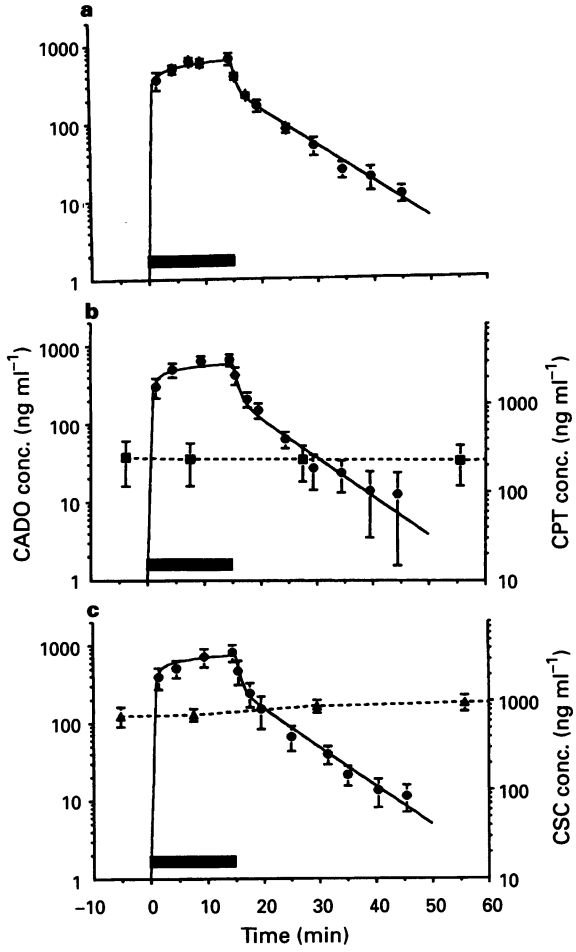

Figure 1 Average time profiles of blood concentrations of CADO after intravenous infusion of 1.4 mg kg^{-1} over 15 min (black bar) during continuous intravenous administration of (a) the vehicle (DMSO), (b) 20 μg min^{-1} kg^{-1} CPT and (c) 32 μg min^{-1} kg^{-1} CSC. Blood concentrations of CADO (●), CPT (■) and CSC (▲) are presented as mean ± s.d. (n = 6) and the solid fitted lines are the means of 6 distinct curves.

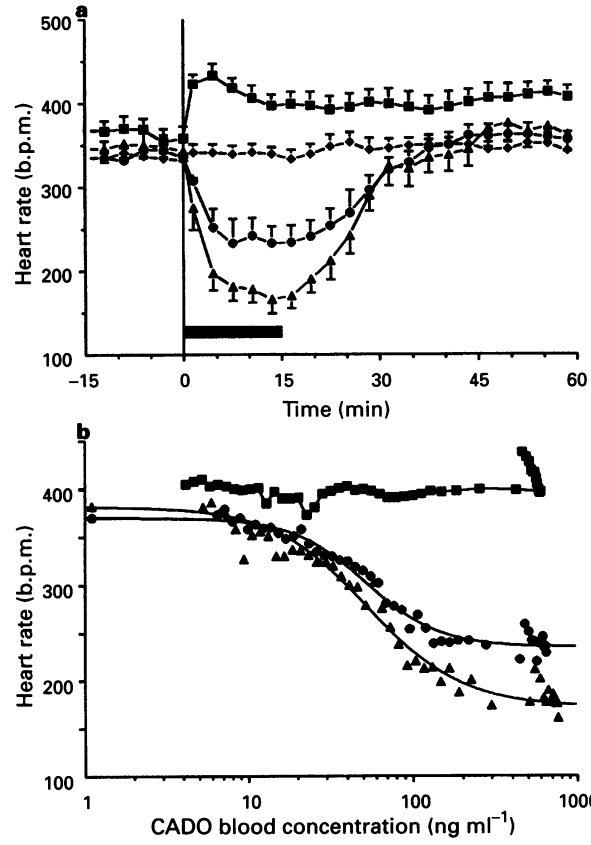

Figure 2 (a) Average time profiles of heart rate and (b) average concentration-heart rate relationships of all individual rats, which had received 1.4 mg kg^{-1} CADO over 15 min during continuous infusion of the vehicle (DMSO) (●), 20 μg min^{-1} kg^{-1} CPT (■) and 32 μg min^{-1} kg^{-1} CSC (▲). Control rats received a continuous infusion of the vehicle (DMSO) and a short infusion of saline with a blood sampling schedule identical to that of the other groups (◆). The sigmoidal E_{max} model was fitted to the concentration-effect data of vehicle- and CSC-treated rats with the no-drug responses fixed to values of 370 and 381 b.p.m., respectively.

Table 1 Effect of continuous infusions of CPT (20 μg min^{-1} kg^{-1}) and CSC (32 μg min^{-1} kg^{-1}) on the pharmacokinetic parameters of CADO following the i.v. administration of 1.4 mg kg^{-1} to normotensive conscious rats

Compound	n	C_1 (μg ml^{-1})	λ_1 (min^{-1})	C_2 (μg ml^{-1})	λ_2 (min^{-1})	*AUC* (μg min ml^{-1})	*Cl* (ml min^{-1} kg^{-1})	*MRT* (min)	V_{ss} (ml kg^{-1})	$t_{½,n}$ (min)
Vehicle	6	7.8 ± 1.4	1.4 ± 0.3	0.55 ± 0.08	0.11 ± 0.01	10 ± 1	140 ± 10	4.8 ± 0.3	670 ± 40	6.9 ± 1.0
CPT	6	6.4 ± 1.2	1.1 ± 0.3	0.44 ± 0.09	0.11 ± 0.02	10 ± 1	150 ± 10	4.2 ± 0.4	630 ± 40	6.8 ± 1.0
CSC	6	9.6 ± 3.3	1.3 ± 0.4	0.58 ± 0.16	0.11 ± 0.01	12 ± 1	150 ± 10	4.0 ± 0.3	600 ± 60	6.4 ± 0.8

The individual concentration-time courses of CADO were decribed with a bi-exponential pharmacokinetic function with coefficients C_1 and C_2 and exponents λ_1 and λ_2 (equation 1). These coefficients and exponents were used to calculate the area under the blood concentration-time curve (AUC), the systemic blood clearance (Cl), the mean residence time (MRT), the volume of distribution at steady-state (V_{ss}) and the terminal half-life ($t_{½,n}$). Values are reported as mean ± s.e. Data were analyzed statistically by a one-way ANOVA or a Kruskal-Wallis test, if more appropriate. No significant differences were detected between the different treatments.

the first 5 min of CADO administration and then decreased during the rest of the infusion. After the administration was ended, heart rate returned slowly to a constant value, which was significantly higher than that of the placebo group.

The bi-exponential pharmacokinetic function was used to calculate the CADO blood concentrations at the time points of effect measurement. The average concentration-heart rate relationships of the different treatment groups are depicted in Figure 2b. No time-delay or hysteresis was observed between concentration and effect. The sigmoidal E_{max} model (equation 2) adequately described the CADO concentration-heart rate relationships for all individual rats which had received a continuous infusion of either CSC or the vehicle. During the infusion of CPT, no sigmoidal relationship was observed between blood concentrations of CADO and heart rate. The influence of CSC on the pharmacodynamic parameters of CADO is summarized in Table 2. In comparison with the vehicle-treated group, CSC significantly increased the maximal bradycardic effect from -130 ± 29 to -205 ± 13 b.p.m. ($P<0.05$), whereas the no-drug response (E_0), *in vivo* potency ($EC_{50,u}$) and Hill factor were not affected.

Figure 3a shows the influence of the antagonists on the hypotensive effect of CADO. Infusion of CADO induced a decrease in blood pressure in all three treatment groups. Upon the start of the infusion, the effect rapidly reached a maximum and maintained that level during the infusion. The maximal hypotensive effect during the continuous infusion of CPT was significantly less than during the infusions of CSC and the vehicle. After the infusion was ended, blood pressure gradually increased. The blood pressure of the CPT-, CSC- and vehicle-treated rats was not significantly different from placebo-treated rats after 30, 60 and 45 min, respectively. As no hysteresis was observed in the different treatment groups, blood concentrations of CADO were directly correlated to the reduction in blood pressure. The sigmoidal E_{max} model described the concentration-hypotensive effect relationship for all individual rats. The influence of CPT and CSC on the concentration-blood pressure relationship of CADO is shown in Figure 3b. The pharmacodynamic parameters are given in Table 3. In comparison with the infusion of the vehicle, infusion of the antagonists did not affect E_0 and n_H. Infusion of CSC did not alter the maximal reduction in blood pressure, whereas CPT significantly reduced E_{max} from -50 ± 3 to -42 ± 1 mmHg. Infusion of CPT and CSC significantly increased the $EC_{50,u}$ value from 35 ± 2 ng ml^{-1} to 61 ± 5 and 86 ± 24 ng ml^{-1}, respectively.

The average time-profiles of MAP/HR of the different treatment groups are depicted in Figure 4a. For the vehicle- and CPT-treated rats, maximal reductions in MAP/HR were observed within 5 min after the start of the CADO infusion. After the infusion was stopped, MAP/HR values gradually elevated to levels that were significantly lower than those of the vehicle-treated rats. Continuous infusion of CSC resulted in a biphasic time-course of MAP/HR. The concentration-MAP/HR relationships could be described with the sigmoidal E_{max} model for each individual rat that had received a constant infusion of CPT or the vehicle (Figure 4b). During the infusion of CSC, no sigmoidal relationship was observed between blood concentrations of CADO and MAP/HR. The pharmacodynamic parameters are listed in Table 4. In comparison with the vehicle-treated rats, continuous infusion of CPT did not in-

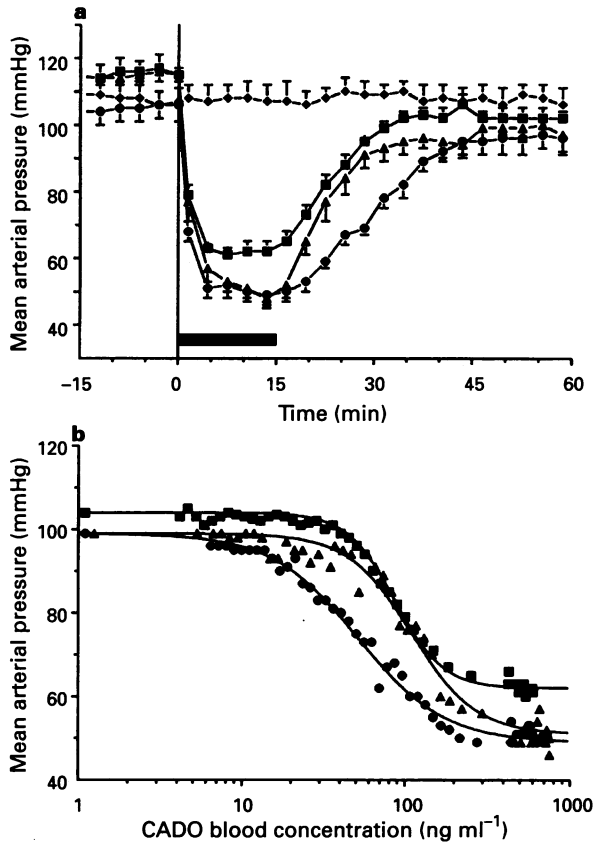

Figure 3 (a) Average time profiles of mean arterial pressure and (b) average concentration-mean arterial pressure relationships of all individual rats, which had received 1.4 mg kg^{-1} CADO over 15 min during continuous infusion of the vehicle (DMSO) (●), 20 μg min^{-1} kg^{-1} CPT (■) and 32 μg min^{-1} kg^{-1} CSC (▲). Control rats had received a continuous infusion of the vehicle (DMSO) and a short infusion of saline with blood sampling identical to that of the other groups (◆). The sigmoidal E_{max} model was fitted to the concentration-effect data of all treatment groups with the no-drug responses fixed to values of 99 (●), 104 (■) and 99 mmHg (▲).

Table 2 Pharmacodynamic parameters for the reduction in heart rate after intravenous infusion of CADO to conscious normotensive rats during continuous infusion of the vehicle, CPT or CSC

Compound	*Concentration* (ng ml^{-1})	n	E_0 (b.p.m.)	E_{max} (b.p.m.)	EC_{50} (ng ml^{-1})	$ED_{50,u}$ (ng ml^{-1})	n_H
Vehicle	–	6	370±9	−130±29	49±10	35±8	3.0±1.2
CPT[a]	230±40	6	–	–	–	–	–
CSC	860±40	6	382±13	−205±13*	52±12	41±13	2.1±0.4

The individual CADO blood concentration-heart rate relationships were fitted to equation 2, yielding estimates for the no-drug response (E_0), intrinsic activity (E_{max}), potency (EC_{50}) and Hill factor (n_H). EC_{50} values were corrected for blood cell and plasma protein binding, yielding potencies based on free CADO concentrations ($EC_{50,u}$). Values are reported as mean±s.e. Data were analyzed statistically by a Student's *t*-test or a Wilcoxon rank sum test, if more appropriate. [a]The sigmoidal E_{max} model did not describe the concentration-effect data. *Significantly different from the vehicle-treated group ($P<0.05$).

Table 3 Pharmacodynamic parameters for the reduction in mean arterial blood pressure after intravenous infusion of CADO to conscious normotensive rats during continuous infusions of the vehicle, CPT or CSC

Compound	Concentration ($ng\,ml^{-1}$)	n	E_0 (mmHg)	E_{max} (mmHg)	EC_{50} ($ng\,ml^{-1}$)	$ED_{50,u}$ ($ng\,ml^{-1}$)	n_H
Vehicle	–	6	99±4	−50±3	49±2	35±2	1.8±0.3
CPT	230±40	6	104±4	−42±1*	89±7**	61±5**	2.3±0.4
CSC	860±40	6	99±3	−51±3	108±22**	86±24**	2.3±0.6

The individual CADO blood concentration-blood pressure relationships were fitted to equation 2, yielding estimates for the no-drug response (E_0), intrinsic activity (E_{max}), potency (EC_{50}) and Hill factor (n_H). EC_{50} values were corrected for blood cell and plasma protein binding, yielding potencies based on free CADO concentrations ($EC_{50,u}$). Values are reported as mean±s.e. Data were analyzed statistically by a one-way ANOVA or a Kruskal-Wallis test, if more appropriate. *Significantly different from the vehicle- and CSC-treated group ($P<0.05$); **significantly different from the vehicle-treated group ($P<0.01$).

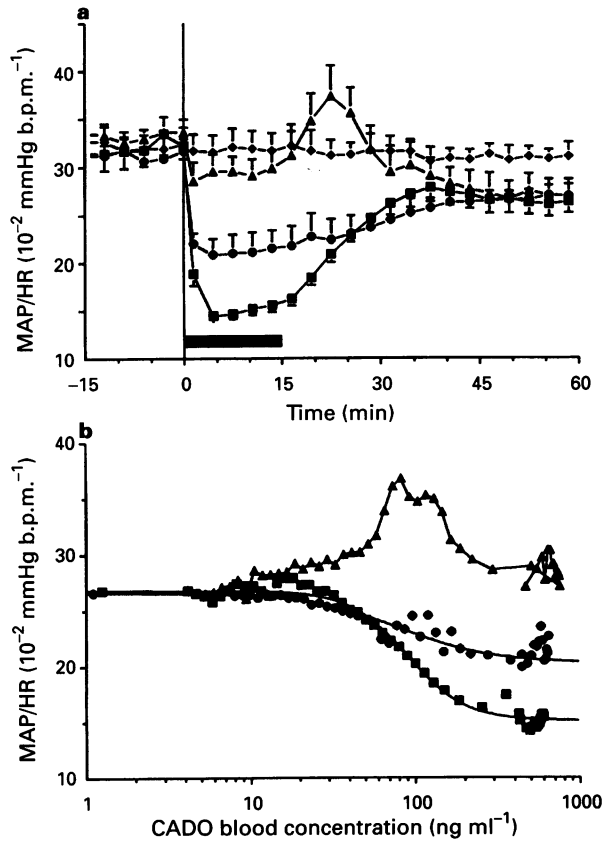

Figure 4 (a) Average time profiles of the ratio of mean arterial pressure over heart rate, MAP/HR and (b) averaged concentration-MAP/HR relationship of all individual rats, which had received $1.4\,mg\,kg^{-1}$ CADO over 15 min during continuous infusion of the vehicle (DMSO) (●), $20\,\mu g\,min^{-1}\,kg^{-1}$ CPT (■) and $32\,\mu g\,min^{-1}\,kg^{-1}\,kg^{-1}$ CSC (▲). Control rats received a continuous infusion of the vehicle (DMSO) and a short infusion of saline with blood sampling identical to that of the other groups (◆). The sigmoidal E_{max} model was fitted to the concentration-effect data of vehicle- and CPT-treated rats with the corresponding no-drug responses fixed to values of 26.6 and $26.8\times10^{-2}\,mmHg\,bpm^{-1}$.

fluence the E_0 and the Hill factor. CPT slightly decreased the observed potency and significantly increased the intrinsic activity from -6.9 ± 1.3 to $-12\pm1\times10^{-2}$ mmHg bpm^{-1}.

Discussion

The purpose of the present investigation was to characterize quantitatively the cardiovascular effects of a non-selective adenosine receptor agonist in rats by using an integrated pharmacokinetic-pharmacodynamic approach. CADO was selected as a model compound because of the comparable affinity for the A_1 and the A_{2a} receptor combined with the low affinity for the A_3 receptor. Simultaneous activation of A_1 and A_{2a} receptors produces complex cardiovascular effects. The separate contributions of A_1 and A_{2a} receptor activation to the overall cardiovascular effects of CADO were therefore distinguished by continuous infusion of CSC or CPT, which are selective antagonists for the A_{2a} and A_1 receptor, respectively. This approach allowed the assessment of the potency and intrinsic activity of CADO for the separate receptor subtypes *in vivo*.

The pharmacokinetic parameters of CADO during the infusion of the vehicle were similar to those of the antagonist-treated groups, indicating that CPT and CSC did not affect the distribution and elimination of the compound. The total blood clearance of CADO (150 ml min^{-1} kg^{-1}) exceeded the typical hepatic blood flow found in rats (60–100 ml min^{-1} kg^{-1}), which suggests that extrahepatic clearance may occur. In this respect, CADO has been reported to be transported by the nucleoside transporter, which is present at the surface of endothelial cells (Plagemann *et al.*, 1988). Therefore, uptake by the endothelium may be an alternative elimination mechanism of CADO. The observed blood clearance of CPT (90 ml min^{-1} kg^{-1}) was not influenced by the administration of CADO, and was in good accordance with earlier reported values (Appel *et al.*, 1995). Administration of CADO appeared to affect the blood clearance of CSC, which significantly decreased from 52 ($t=-5$ min) to 36 ml min^{-1} kg^{-1} ($t=120$ min). Nevertheless, the pre-administration value was still considerably lower than the previously reported value of 74 ml min^{-1} kg^{-1}, which has also been obtained during continuous infusion of the compound (Mathôt *et al.*, 1995b). Since CSC concentrations in the present study were higher than those in the referred study (860 versus 400 ng ml^{-1}, respectively), saturation of elimination may be a possible explanation for the reduced clearance. Consequently, non-linear elimination (i.e. a reduction in clearance with increasing concentrations) may predominantly account for the constant increase of CSC concentrations during the experiment. The elimination of CPT has been demonstrated to be saturable as well (Mathôt *et al.*, 1993). In the present experiments, however, concentrations of CPT were well below the reported Michaelis-Menten constant of 1.6 μg ml^{-1}.

Intravenous administration of CADO during the constant infusion of the vehicle produced reductions in both heart rate and blood pressure (Figures 2a and 3a), which corresponded with earlier observations by Webb *et al.* (1990) and Abiru *et al.* (1991). The sigmoidal E_{max} model adequately described the relationship between CADO blood concentrations and both effects (Figures 2b and 3b). The bradycardic response clearly demonstrates the activation of A_1 receptors in the vehicle-treated group. The observed hypotension may be produced by activation of A_1, A_{2a} and/or A_{2b} receptors respectively. A_1

Table 4 Pharmacodynamic parameters for the reduction in the ratio of mean arterial pressure over heart rate (MAP/HR) after intravenous infusion of CADO to conscious normotensive rats during continuous infusions of the vehicle, CPT or CSC

Compound	Concentration ($ng\,ml^{-1}$)	n	E_0 (10^{-2} mmHg bpm^{-1})	E_{max} (10^{-2} mmHg bpm^{-1})	EC_{50} ($ng\,ml^{-1}$)	$ED_{50,u}$ ($ng\,ml^{-1}$)	n_H
Vehicle	–	6	27±1	−6.9±1.3	73±21	58±8	2.8±0.7
CPT	230±40	6	27±1	−12±1*	101±10	68±5	2.5±0.9
CSC[a]	860±40	6	–	–	–	–	–

The individual CADO blood concentration-MAP/HR relationships were fitted to equation 2, yielding estimates for the no-drug response (E_0), intrinsic activity (E_{max}), potency (EC_{50}) and Hill factor (n_H). EC_{50} values were corrected for blood cell and plasma protein binding, yielding potencies based on free CADO concentrations ($EC_{50,u}$). Values are reported as mean±s.e. Data were analyzed statistically by a Student's *t* test or a Wilcoxon rank sum test, if more appropriate. [a]Sigmoidal E_{max} model did not describe the concentration-effect data. *Significantly different from the vehicle-treated group ($P<0.05$).

agonists have been proposed to produce hypotension by a decrease in cardiac output, whereas activation of the A_{2a} and A_{2b} receptor substypes produces hypotension by vasodilatation (Webb *et al.*, 1990; 1991). In the present study it was demonstrated that CSC competitively inhibits the hypotensive effect of CADO at a mean steady-state plasma concentration of 860 ng ml^{-1}. In *in vitro* studies a 150 fold selectivity of CSC for the A_{2a} versus the A_{2b} receptor has been demonstrated (Daly & Jacobson, 1994). The EC_{50} of CSC at the A_{2a} receptor *in vivo* is 60 ng ml^{-1} (Mathôt *et al.*, 1995b). Thus the concentrations of CSC required to block also the A_{2b} receptor are much higher than those observed in the present investigation. These findings indicate that the vasodilator effect of CADO is mediated predominantly through the A_{2a} receptor subtype. Stimulation of A_{2a} receptor has been reported to evoke reflex tachycardia (Webb *et al.*, 1991). Activation of A_{2a} receptors in the present study may be demonstrated by comparison of the concentration-effect curves, as obtained during the infusion of the vehicle, with those of A_1-selective agonists as obtained in a previous pharmacokinetic-pharmacodynamic study (Mathôt *et al.*, 1995d). In the latter study, administration of A_1-selective agonists was reported to cause similar relative maximal reductions in heart rate and blood pressure, whereas EC_{50} values for bradycardia were 2 to 20 fold lower than the corresponding values for hypotension. In the present study, however, the E_{max} for the hypotensive effect was greater than the E_{max} for the negative chronotropic effect (E_{max}: 50 and 35%, respectively), which indicates that the A_1 receptor-mediated bradycardia is attenuated by reflex tachycardia. Furthermore, CADO exerted equipotent effects on heart rate and blood pressure (EC_{50} values: 49 and 49 ng ml^{-1}; Tables 2 and 3), indicating that activation of the A_{2a} receptor contributes to the hypotensive effect.

Selective antagonists were used to determine the separate effects of CADO at each of the receptor subtypes. In order to characterize the effects mediated by the A_{2a} receptor, CADO was administered in the presence of a high concentration of the selective A_1 receptor antagonist, CPT. Increasing steady-state concentrations of CPT have been shown to produce parallel shifts of the concentration-heart rate relationship of the prototypic A_1 agonist CPA, indicating competitive antagonism at the A_1 receptor *in vivo* (Appel *et al.*, 1995). In the latter study, an $EC_{50,u}$ value of 0.5 ng ml^{-1} was derived for CPT. In the present study, the average unbound CPT concentration was 41 ng ml^{-1} (i.e. $[260 \times P/B \times f_u/100]$). This resulted in an approximate 82 fold increase of the EC_{50} of CADO for the A_1 receptor, which indicates that the receptor was effectively blocked. Continuous infusion of CPT produced profiles of heart rate and blood pressure which are typical for the *in vivo* activation of the A_{2a} receptor (Figures 2a and 3a). As mentioned above, A_{2a} agonists produce hypotension by a reduction in peripheral resistance. The hypotensive effect is attenuated by a concomitant reflex tachycardia (Webb *et al.*, 1991). In comparison with vehicle-treated rats, continuous infusion of CPT decreased the E_{max} for the hypotensive effect of CADO (Table 3), which may demonstrate the counteracting effect of the reflex increase in cardiac output. No direct relationship existed between the increase in heart rate and blood concentrations of CADO (Figure 2b). The A_{2a}-mediated tachycardia has been postulated to be partially the result of baroreceptor activation of cardiac sympathetic nerves (Webb *et al.*, 1991). The activation of the baroreceptor reflex has been indicated to be dependent on both the level of the blood pressure and its rate of change (Guyton *et al.*, 1972; Francheteau *et al.*, 1993). The immediate rise and subsequent fall in heart rate during the administration of CADO may therefore be explained by the initial rapid fall in blood pressure, which produces a transient and additional activation of the baroreceptor reflex. During the infusion of CPT, CADO produced a reversible hypotensive effect, whereas heart rate remained elevated. Similar observations have been reported for the selective A_{2a} agonist, CGS 21680 (Webb *et al.*, 1991; Mathôt *et al.*, 1995a). The mechanism of this sustained tachycardia is still unclear, but appears not to be mediated by direct activation of the A_{2a} receptor (Jackson *et al.*, 1993).

In a previous study, estimates of the *in vivo* potency and intrinsic activity of the A_{2a}-selective full agonist, CGS 21680, have been obtained by relating the hypotensive effect to the concentration in blood (Mathôt *et al.*, 1995a). In comparison with CGS 21680, CADO produced a greater maximal reduction in blood pressure (E_{max}: −32 and −42 mmHg, respectively), whereas the heart rate level at the end of the bolus infusion was smaller (510 versus 405 bpm, respectively). The reduced tachycardic response in the present study may be explained by activation of A_1 receptors, which, despite the presence of CPT, might occur at hight CADO concentrations. The larger intrinsic activity of the hypotensive effect of CADO may therefore be caused by a reduced reflex increase of cardiac output. This confounding influence can be circumvented by the use of the ratio of mean arterial pressure over heart rate (MAP/HR) as an index of A_{2a} activation. This parameter reflects the total peripheral resistance on the condition that stroke volume does not change. Since A_{2a} agonists primarily cause vasodilatation, total peripheral resistance has been suggested to be a more relevant pharmacodynamic endpoint for the *in vitro* activation of A_{2a} receptor than blood pressure (Mathôt *et al.*, 1995a). In the latter study, a consistent concentration-MAP/HR relationship was obtained for CGS 21680, which was independent of the administered dose. The observed E_{max} in the present study (-12×10^{-2} mmHg bpm^{-1}) was similar to the value reported for CGS 21680 (-13×10^{-2} mmHg bpm^{-1}), indicating that CADO and CGS 21680 are both full agonists for the A_{2a} receptor. The observed *in vivo* potencies of CADO for the hypotensive effect and the reduction in MAP/HR during blockade of A_1 receptors were comparable. The corresponding $EC_{50,u}$ values were 61 and 68 ng ml^{-1} (202 and 225 nM) and correlated well with the A_{2a} affinity (K_i: 80 nM), as has been found in radioligand binding studies (IJzerman *et al.*, 1994b).

The *in vivo* potency and intrinsic activity of CADO for the

A_1 receptor were determined in the group that had received a continuous infusion of the A_{2a} antagonist, CSC. Potent A_{2a} antagonists have only recently become available and CSC is the most selective representative of this class of compounds (Jacobson *et al.*, 1993). CSC has been demonstrated to antagonize competitively the cardiovascular effects of the A_{2a}-selective agonist, CGS 21680 with an *in vivo* potency ($EC_{50,u}$) of 16 ng ml^{-1} (Mathôt *et al.*, 1995b). In the present study, constant infusion of CSC resulted in an average unbound concentration of 220 ng ml^{-1} over the period from 8 to 60 min (i.e. $[860 \times P/B \times f_u/100]$), thereby producing an approximate 14 fold increase of the EC_{50} of CADO at the A_{2a} receptor. Despite its moderate *in vivo* potency, CSC inhibited the A_{2a}-mediated attenuation of the bradycardiac response as produced by the stimulation of the A_1 receptor (Figure 2a). When given alone, CADO appeared to be a partial agonist for the A_1 receptor, since the maximal reduction in heart rate was less than the values reported for A_1-selective full agonists (Mathôt *et al.*, 1995d). However, during the infusion of CSC, E_{max} values were similar, indicating that CADO is a full agonist for the A_1 receptor as well. Inhibition of A_{2a} receptor-mediated vasodilatation produced an increase of the EC_{50} value for hypotensive effect (Table 3). As a result, CADO exerted a two fold greater *in vivo* potency for the negative chronotropic effect than for the hypotensive effect, whereas similar proportional maximal reductions were observed. These findings were consistent with results reported for A_1-selective agonists (see above) and confirm that the negative chronotropic effect is a more relevant and sensitive pharmacodynamic endpoint for the *in vivo* activation of A_1 receptors than the hypotensive effect (Mathôt *et al.*, 1994; 1995c,d; Appel *et al.*, 1995). The bradycardic potency based on free CADO concentrations ($EC_{50,u}$: 41 ng ml^{-1} or 136 nM) was similar to the reported K_i value for the adenosine A_1 receptor (300 nM; IJzerman *et al.*, 1994b).

As mentioned earlier, MAP/HR may reflect the total peripheral resistance upon the assumption that no change in stroke volume occurs, Since A_1 agonists have been reported to produce cardiac depression, changes in stroke volume can be expected during activation of A_1 receptors (Olsson & Pearson, 1990). Consequently, no conclusions can be drawn from the time profiles as observed during the infusion of CSC or the vehicle. As shown above, MAP/HR may only have physiological relevance when A_1 receptors are blocked, as in the case of continuous infusion of CPT.

In conclusion, this study has shown that the cardiovascular effects of the non-selective adenosine receptor agonist, CADO, could be quantified by use of an integrated pharmacokinetic-pharmacodynamic model. Estimates of the *in vivo* potency and intrinsic activity for both the A_1 and A_{2a} receptor were obtained during selective blockade of either of the receptor subtypes. The developed experimental approach offers the possibility of characterizing the haemodynamic effects of new non-selective adenosine receptor agonists *in vivo*.

The authors are grateful for the technical assistance of Mariska W.E. Langemeijer.

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(Received September 11, 1995
Revised January 15, 1996
Accepted January 30, 1996)