British Journal of Pharmacology (1997) 122, 525–533

Pharmacokinetic-pharmacodynamic modelling of the anti-lipolytic and anti-ketotic effects of the adenosine A_1-receptor agonist N^6-(p-sulphophenyl)adenosine in rats

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1 The purpose of this study was to develop and validate an integrated pharmacokinetic-pharmacodynamic model for the anti-lipolytic effects of the adenosine A_1-receptor agonist N^6-(p-sulphophenyl)adenosine (SPA). Tissue selectivity of SPA was investigated by quantification of haemodynamic and anti-lipolytic effects in individual animals.

2 After intravenous infusion of SPA to conscious normotensive Wistar rats, arterial blood samples were drawn for determination of blood SPA concentrations, plasma non-esterified fatty acid (NEFA) and β-hydroxybutyrate levels. Blood pressure and heart rate were monitored continuously.

3 The relationship between the SPA concentrations and the NEFA lowering effect was described by the indirect suppression model. Administration of SPA at different rates and doses (60 μg kg^{-1} in 5 min and 15 min, and 120 μg kg^{-1} in 60 min) led to uniform pharmacodynamic parameter estimates. The averaged parameters (mean$\pm$s.e., $n=19$) were E_{max}: $-80\pm2\%$ (% change from baseline), EC_{50}: 22 ± 2 ng ml^{-1}, and Hill factor: 2.2 ± 0.2.

4 In another group, given 400 μg kg^{-1} SPA in 15 min, pharmacodynamic parameters for both heart rate and anti-lipolytic effect were derived within the same animal. The reduction in heart rate was directly related to blood concentration on the basis of the sigmoidal E_{max} model. SPA inhibited lipolysis at concentrations lower than those required for an effect on heart rate. The EC_{50} values (mean$\pm$s.e., $n=6$) were 131 ± 31 ng ml^{-1} and 20 ± 3 ng ml^{-1} for heart rate and NEFA lowering effect, respectively.

5 In conclusion, the relationship between blood SPA concentrations and anti-lipolytic effect was adequately described by the indirect suppression model. For SPA a 6 fold difference in potency was observed between the effects on heart rate and NEFAs, indicating some degree of tissue selectivity *in vivo*.

Keywords: N^6-(p-sulphophenyl)adenosine; adenosine A_1 receptor; pharmacokinetic; pharmacodynamic; tissue selectivity; heart rate; lipolysis; fatty acids; ketone body

Introduction

Adenosine has long been known to inhibit lipolysis through activation of adenosine A_1-receptors on adipocytes (Londos *et al*., 1980). This anti-lipolytic effect may be of therapeutic value for several metabolic disorders, in which increased levels of plasma non-esterified fatty acids (NEFA) can be found. In non-insulin dependent diabetes mellitus (NIDDM) increased fatty acid concentrations have been shown to lead to a diminished insulin-stimulated glucose uptake (Randle *et al*., 1969; Ferrannini *et al*., 1983) and an increased glucose production in the liver (Reaven & Cheng, 1988). Lowering of NEFA concentrations has been demonstrated to decrease blood glucose levels in diabetic rats (Reaven *et al*., 1988) and man (Fulcher *et al*., 1992). Furthermore, Saloranta and co-workers demonstrated that a decreased NEFA availability enhanced the suppression of hepatic glucose production by insulin in Type 2 (non-insulin dependent) diabetic patients (Saloranta *et al*., 1991).

Although selective adenosine A_1-receptor agonists may be used as new anti-lipolytics in diabetes (Foley, 1994), their lack of selectivity is a major drawback. Synthetic adenosine A_1-receptor agonists have been shown to inhibit lipolysis and consequently lower plasma NEFA concentrations *in vitro* (Londos *et al*., 1980; Kennedy *et al*., 1992) and *in vivo* (Hoffman *et al*., 1986; Strong *et al*., 1993). Some pre-clinical studies have demonstrated that the agonists N^6-**R**-phenylisopropyladenosine (**R**-PIA) and N^6-[1**S**,*trans*)-2-hydroxycyclopentyl]-adenosine (GR79236) were able to lower blood glucose levels in both normal (Strong *et al*., 1993; Merkel *et al*., 1995) and diabetic rats (Reaven *et al*., 1988). However, due to the ubiquity of adenosine A_1-receptors, it is difficult to separate beneficial effects from side effects. Besides anti-lipolytic effects, most adenosine A_1-receptor agonists also have strong cardiovascular actions (Gardner *et al*., 1994; Merkel *et al*., 1995). Therefore, for successful use of adenosine analogues, compounds need to be developed which have a selective action *in vivo*.

In vivo, selectivity of action is determined by a combination of pharmacokinetic and pharmacodynamic factors. Pharmacokinetic properties of the adenosine ligands are of major importance, since protein binding, distribution, and metabolism may determine the concentration of the drug at the site of the receptor. For example, the ability of an analogue to pass the blood-brain barrier is necessary for action at central adenosine A_1-receptors. Regarding pharmacodynamic effects, receptor subtype selectivity, efficiency of receptor-effector coupling, or the influence of homeostatic control are important for a selective action. Based on tissue-differences in receptor-effector coupling or receptor reserve, activation of adenosine A_1-receptors can ultimately lead to a tissue- or organ-dependent response intensity (Kenakin, 1993).

In this study the anti-lipolytic effects of the adenosine A_1-receptor agonist N^6-(p-sulphophenyl)adenosine (SPA) have been quantified on the basis of an integrated pharmacokinetic-

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pharmacodynamic model. The effects of intravenous infusions of SPA on plasma NEFA concentrations and blood β-hydroxybutyrate concentrations were determined in fasted, conscious rats. The relationship between the SPA blood concentrations and the anti-lipolytic effect was described by a physiological indirect effect model. The consistency of the parameters estimated by this model was investigated by administration of SPA at different doses and rates. In this manner, possible time- or dose-dependencies or incorrect use of the model could be investigated. Tissue selectivity of SPA was studied by simultaneous measurement of haemodynamic effects in the same rats. By quantification of both effects on the basis of an integrated PK/PD model, differences in *in vivo* potency or intrinsic activity of SPA for its haemodynamic and anti-lipolytic actions were investigated.

Methods

Animals and surgical procedures

Normotensive male Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 220–280 g, were used. The animals were housed individually in plastic cages with a normal 12-h light-dark cycle and fed on laboratory chow (Standard Laboratory Rat, Mouse and Hamster Diets, SMR-A, Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*.

Four days before experimentation indwelling cannulae were implanted into the right jugular vein for drug administration (polythene, 13.5 cm, 0.58 mm i.d.), and the right and left femoral artery (polythene, 18 cm, 0.58 mm i.d. + 4.5 cm, 0.28 mm i.d.) under light ether anaesthesia (Mathôt *et al*., 1994). The arterial cannulae were guided through the femoral artery into the abdominal aorta and were used for blood sampling and recording of arterial blood pressure, respectively. All cannulae were tunnelled subcutaneously to the back of the neck and exteriorized. After the operation the cannulae were filled with a 25% (w/v) solution of polyvinylpyrrolidone (Brocacef, Maarssen, The Netherlands) in 0.9% (w/v) sodium chloride containing 50 iu ml^{-1} heparin.

One day before the experiment the animals were deprived of food. During the experiment the animals were conscious, freely moving, and allowed to drink tap water *ad libitum*.

Experimental design

Animals were randomly assigned to five groups of 6–7 animals, each receiving 60 μg kg^{-1} SPA in 5 min, 60 μg kg^{-1} SPA in 15 min, 120 μg kg^{-1} in 60 min, 400 μg kg^{-1} in 15 min or vehicle (0.9% saline) in 15 min. A 0.2 mg ml^{-1} solution of SPA in water was prepared and stored at 4°C until use. On the day of the experiment this solution was diluted to the final dose. A motor driven infusion pump (Braun Melsungen, Germany) was used to infuse a constant volume of 638, 765 and 1146 μl during 5, 15 and 60 min, respectively.

All experiments were started between 9 h 00 min and 10 h 00 min in the morning to minimize the contribution of diurnal rhythm in haemodynamics. Arterial blood pressure was measured from the cannula in the left femoral artery by a miniature strain gauge P10EZ transducer, connected to a plastic diaphragm dome (TA1017, Disposable Critiflo Dome) (both Viggo-Spectramed B.V., Bilthoven, The Netherlands). The pressure transducer was connected to a polygraph amplifier console (RMP6018, Nihon Kohden Corporation, Tokyo, Japan). Heart rate was captured from the blood pressure signal. The signals were passed through a CED 1401 interface (Cambridge Electronic Design Ltd, Cambridge, U.K.) into a 80486 computer by use of the data acquisition programme Spike 2 (Spike 2 Software, Version 3.1, Cambridge, U.K.) and stored on diskette. During the experiments the catheter connected to the pressure transducer was flushed continuously with a 0.9% sodium chloride solution at a flow rate of 500 μl h^{-1} (Syringe infusion pump 22, Harvard apparatus, Plato B.V., Diemen, The Netherlands) to ensure a continuous recording of the blood pressure.

Small arterial blood samples were drawn frequently for determination of SPA blood concentrations and measurement of metabolic parameters. A total number of 14 arterial blood samples was drawn for the determination of the pharmacokinetic profile of SPA. Sampling times and sample volume (50, 100 or 200 μl) depended on the dose administered. After sampling the blood samples were haemolyzed directly in 400 μl water and kept on ice until storage at −20°C. For determination of plasma non-esterified fatty acid (NEFA) and blood β-hydroxybutyrate concentrations, 24 small blood samples were drawn according to a predefined time-schedule. For determination of NEFA concentrations, 50 μl of blood was sampled into plastic tubes prefilled with 50 μl saline containing 5% (w/v) EDTA. After centrifugation 70 μl of plasma was pipetted into a clean tube and stored at −20°C until analysis. Hydroxybutyrate was determined in a 20 μl blood sample. Blood was added to 75 μl perchloric acid (2.9% w/v). After centrifugation 70 μl supernatant was transferred to a clean tube, neutralized by the addition of 10 μl of a saturated potassium hydrogen carbonate solution and stored.

After sampling the arterial line was flushed with a few microliters of saline containing 20 iu ml^{-1} heparin to prevent clotting.

Plasma protein binding

The plasma-to-blood ratio (P/B) and the free fraction in plasma (f_u) of SPA were determined in a separate group of 4 rats which had received 0.4 mg kg^{-1} SPA in 15 min. The concentration-dependence of protein binding was examined by determination at two different SPA concentrations. Blood samples with a volume of 1 ml were drawn at 17 and 90 min after the start of the infusion and transferred directly to heparin-treated tubes on ice. An aliquot of 50 or 100 μl of blood was haemolyzed in 400 μl millipore water. The remaining blood was centrifuged at 4°C to separate the plasma. A sample of 50 or 100 μl was retained for analysis and the remaining plasma was subjected to ultrafiltration. Free compound was separated from plasma protein bound compound by filtration of the supernatant at 1090 *g* at 37°C, by use of the Amicon Micropartition System in combination with an YMT ultrafiltration membrane (Amicon Divisions, Danvers, MA, USA). Unbound SPA concentrations were determined in 100 μl of the ultrafiltrate. Corresponding SPA concentrations in blood, plasma and ultrafiltrate were determined in each sample.

H.p.l.c. analysis of SPA

The blood concentrations of SPA were determined by ion-pair reversed phase high performance liquid chromatography (h.p.l.c.) with u.v. detection at 302 nm. The h.p.l.c. system consisted of a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, U.S.A.), a WISP 710B automatic sample injector (Millipore-Waters, Milford, MA, U.S.A.) and a Spectroflow 757 u.v. detector (Applied Biosystems, Ramsey, NJ, U.S.A.). A stainless-steel Microsphere C-18 cartridge column (100 mm, 4.6 mm i.d., 3 μm particle size) (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 C-18 material (Chrompack Pellicular, particle size 20–40 μm, Chrompack Nederland BV) were used. Data processing was performed with a Chromatopack C-R3A reporting integrator (Shimadzu, Kyoto, Japan). The mobile phase consisted of a mixture of 20 mM acetate buffer (pH 4) and acetonitrile in a ratio of 82/18 (v/v) to which 20 mM tetrabutyl-ammoniumhydroxide was added as ion-pairing reagent. At a flow rate of 0.5 ml min^{-1} the retention times of SPA and the internal standard (CPA) were 7 and 9 min, respectively. SPA blood concentrations were calculated by use of the SPA/CPA peak-height ratio in the calibration curve. The detection limit was 3 ng ml^{-1} (signal-to-noise ratio

of 3). In the concentration range of 7.5 ng ml^{-1} to 500 ng ml^{-1} the within-day coefficient of variation was less than 4%. The between-day variation, measured over a period of one year, was 26% and 4% for 10 and 350 ng ml^{-1}, respectively.

The biological samples were prepared for h.p.l.c. analysis according to the procedure described below. Fifty microliters of internal standard (18 μg ml^{-1} CPA) were added to haemolyzed blood (samples either from the experiment or blood spiked with SPA standard solution). Samples were deproteinized by addition of 2 ml acetonitrile, mixing on a vortex and centrifugation at 5000 r.p.m. After the supernatant had been transferred to a clean tube, 200 μl acetate buffer (20 mM, pH 4) and 50 μl 1 M hydrochloric acid were added. This mixture was extracted with 5 ml ethyl acetate on a vortex for 1 min. After centrifugation (10 min, 5000 r.p.m.) the organic phase was discarded. Following the addition of 50 μl of 1 M sodium hydroxide the aqueous layer was evaporated to dryness in a vacuum vortex at 40°C. The residue was dissolved in 100 μl acetate buffer containing 20 mM TBAH and 50 μl was injected into the chromatographic system.

Biochemical assays

Plasma non-esterified fatty acid (NEFA) concentrations were determined by use of the Wako NEFA C-kit (Wako Chemicals GmbH, Neuss, Germany). The method was slightly adapted to allow measurement of small samples. The sensitivity of the assay was substantially improved by using small volumes (50 μl of sample and 50 and 100 μl of reagent) on a 96-well microtitreplate. The assay was linear in the concentration range of 0.025 to 0.3 mM. Within this concentration range the within-day and between-day coefficients of variation were less than 5% and 6%, respectively.

Blood β-hydroxybutyrate concentrations were determined according to the method described by Williamson & Hellanby (1974). A calibration curve was constructed in the concentration range of 0.075 and 0.06 mM and was linear. Within this concentration range the within-day and between-day coefficients of variation were less than 7% and 14%, respectively.

Chemicals

N^6-(p-sulphophenyl)adenosine was a gift from RBI through the NIMH programme and N^6-cyclopentyladenosine was purchased from RBI (Research Biochemicals Inc., Natick, MA). Ethyl acetate (Baker Chemicals, Deventer, The Netherlands) was distilled before use. Acetonitrile (h.p.l.c. grade) was obtained from Westburg (Leusden, The Netherlands) and tetrabutylammonium-hydroxide from Aldrich (Axel, The Netherlands). Water was used from a Milli-Q system (Millipore SA, Molsheim, France). All other chemicals were of analytical grade (Baker, Deventer, The Netherlands).

Data analysis

The pharmacokinetics and pharmacodynamics of SPA were quantified in individual rats. The blood concentration-time profiles of SPA were described by a poly-exponential equation for intravenous infusion (Gibaldi & Perrier, 1982):

$$C_t = \sum_{i=1}^{n} \frac{C_i}{\alpha_i \cdot T} \cdot (1 - e^{-\alpha_i \cdot t}) \qquad t \le T \qquad (1A)$$

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

where C_t is the concentration at time t, T is the infusion duration, C_i and α_i are, respectively, the coefficients and exponents of the equation. Different exponential models were investigated and the most suitable model was chosen on the basis of the Akaike information criterion (Akaike, 1974). Total blood clearance (Cl), the elimination half-life ($t_{½,n}$), and the volume of distribution at steady state (V_{dss}) were calculated following standard procedures, by use of the coefficients and exponents of the fitted function (Gibaldi & Perrier, 1982). The functions were fitted to the data with weight $1/y^2$ by use of the non-linear least squares regression programme Siphar (Simed SA, Creteil, France). In each individual rat the fitted function of the concentration-time profile was used to calculate the concentrations at the measured effect-time points.

The relationship between SPA blood concentration and the anti-lipolytic effect has been quantified by a physiological pharmacokinetic-pharmacodynamic model, which describes the change in NEFA concentrations as being an indirect response to the inhibition of the factors controlling it. The model is schematically depicted in Figure 1 and is similar to the indirect suppression model proposed by Dayneka *et al*. (1993). The rate of change of the NEFA concentrations can be described as:

$$\frac{dN}{dt} = k_s \cdot f(C) - k_e \cdot N \qquad (2)$$

where k_s represents the zero-order rate constant for the synthesis of NEFAs, k_e the first-order rate constant for the elimination of NEFAs and N the plasma NEFA concentration. The function f(C) represents the fractional inhibitory effect according to the sigmoidal E_{max} model:

$$f(C) = 1 - \frac{E_{max} \cdot C^n}{EC_{50}^n + C^n} \qquad (3)$$

where C is the SPA concentration. E_{max} is the maximal inhibition of lipolysis, EC_{50} is the SPA concentration at half-maximal inhibition and n is the Hill factor expressing the steepness of the curve. Solving equation 2 resulted in the function to which the NEFA data were fitted:

$$N(t) = N_0 \cdot (1 - f(C)) \cdot e^{-k_e \cdot t} + N_0 \cdot f(C) \qquad (4)$$

where N_0 is the baseline NEFA concentration.

Heart rate has been shown to be a sensitive and appropriate pharmacodynamic endpoint for the cardiovascular effects of A_1-receptor agonists (Mathôt *et al*., 1994; 1995b). The relationship between the SPA blood concentrations and heart rate was described on the basis of the sigmoidal E_{max} model (Holford & Sheiner, 1982):

$$E(C) = E_0 + \frac{E_{max} \cdot C^n}{EC_{50}^n + C^n} \qquad (5)$$

where E(C) is the observed effect at blood concentration C,E_0 is the baseline heart rate, E_{max} is the maximal effect, EC_{50} is the blood concentration at half-maximal effect and n is a constant expressing the sigmoidicity of the concentration-effect relationship (Hill factor). The equations were fitted to the data by use of the non-linear least squares regression programme Siphar (Simed SA, Creteil, France).

Statistical analysis

The pharmacokinetic and pharmacodynamic parameter estimates of the different groups were statistically compared by

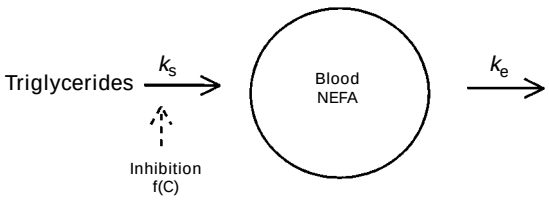

Figure 1 Schematic representation of the pharmacodynamic model for the indirect effect of blood SPA concentrations on NEFA concentrations; blood NEFA concentrations are controlled by both synthesis (k_s) and elimination (k_e). Synthesis (lipolysis) of NEFAs is inhibited by SPA on the basis of the inhibitory function f(C).

use of the parametric one-way analysis of variance (ANOVA) or a non-parametric Kruskall-Wallis test, if more appropriate. Parameters for heart rate and NEFA obtained in an individual rat were compared by a paired *t* test. A significance level of 5% was selected. All data are presented as mean ± s.e., unless indicated otherwise.

Results

Pharmacokinetics

The averaged blood SPA concentration-time profiles after intravenous administration of SPA for 5, 15 and 60 min are shown in Figure 2. Administration of 400 μg kg^{-1} SPA for 15 min resulted in the highest blood concentrations, whereas the other doses resulted in approximately equal SPA peak concentrations. In all rats the concentration-time profiles were described by a bi-exponential function. The pharmacokinetic parameters are summarized in Table 1. No relevant differences between the parameter estimates were observed for the various infusion rates. Averaged over all four different infusions, clearance, volume of distribution at steady state and terminal half-life were 8.5 ± 0.5 ml min^{-1} kg^{-1}, 381 ± 24 ml kg^{-1} and 35 ± 3 min, respectively (mean ± s.e., $n = 25$).

The plasma-to-blood ratio (P/B) and the free fraction of the drug in plasma (f_u) were determined at two different SPA blood concentrations: 923 ± 41 and 341 ± 37 ng ml^{-1} (mean ± s.e.). Since no concentration-dependence was observed, the values for P/B and f_u were averaged and appeared to be 1.68 ± 0.12 and 40 ± 3% (mean ± s.e., $n = 8$), respectively.

Anti-lipolytic effect

Figure 3a depicts the effect of SPA on plasma NEFA levels after intravenous administrations of 60 μg kg^{-1} SPA for 5 min (12 μg kg^{-1} min^{-1}), 60 μg kg^{-1} for 15 min (4 μg kg^{-1} min^{-1}), 120 μg kg^{-1} for 60 min (2 μg kg^{-1} min^{-1}) or the vehicle to fasted rats. The decrease in fatty acid concentrations is depicted as the relative change to baseline NEFA concentrations. The baseline values were determined by averaging the four samples taken in the 45 min period before drug administration. Following administration of the vehicle a slight increase in NEFA concentration was observed. However, this increase was not significantly different from initial baseline values. Each infusion caused a significant decrease in NEFA concentrations as compared to the vehicle-treated group. The onset of the decrease in NEFA concentrations was slow and occurred at a single rate irrespective of the infusion rate of the adenosine agonist. The return of the effect to baseline values was dose-dependent. Higher doses, resulting in higher blood SPA concentrations, led to longer durations of the anti-lipolytic effect. In the two highest doses of 120 and 400 μg kg^{-1} SPA, a maximal decrease in NEFA levels was observed. These profiles clearly showed that lipolysis could not be inhibited completely, but only by about 80%.

In each individual rat the time profile of the NEFA concentrations could be adequately described by the indirect suppression model. The pre-administration NEFA concentrations were averaged and used as the value for N_0 in the indirect suppression model. Figure 4a shows the plasma NEFA concentrations and the predicted time course of the effect on the basis of this model in an individual rat. The pharmacodynamic

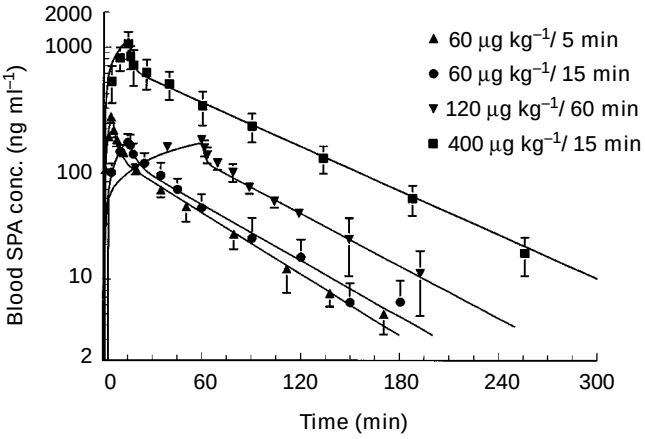

Figure 2 Blood concentration time profiles after intravenous administration of SPA in four different doses and infusion rates to rats; the dosage regimen is presented as total amount of drug per infusion time. Data are presented as mean and the fitted lines are the averages of 6–7 curves; vertical lines show s.d.

Table 1 Pharmacokinetic parameter estimates obtained after intravenous infusion of SPA at different doses and rates

Dose/ infusion time (μg kg^{-1}/min)	n	$t_{½,n}$ (min)	*Cl* (ml min^{-1} kg^{-1})	V_{dss} (ml kg^{-1})
60/5	7	35 ± 3	8.8 ± 0.4	381 ± 24
60/15	6	39 ± 4	8.7 ± 0.8	399 ± 16
120/60	6	44 ± 6	9.2 ± 0.4	414 ± 28
400/15	6	48 ± 2	7.0 ± 0.6*	432 ± 43

$t_{½,n}$, terminal half-life; *Cl*, clearance; V_{dss}, volume of distribution at steady state. *Significantly different from 120/60 (ANOVA, $P < 0.05$).

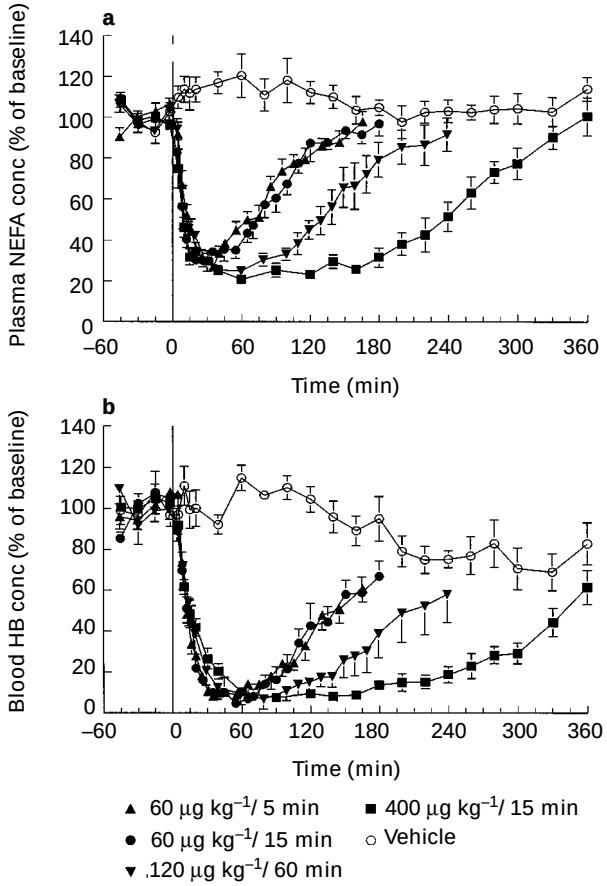

Figure 3 Time profiles of the plasma NEFA concentrations (a) and blood β-hydroxybutyrate (HB) concentrations (b) after intravenous administration of the four different dosage regimens of SPA to conscious, fasted rats. The vehicle treated group received 765 μl of saline for 15 min. Data are presented as mean ($n = 6–7$); vertical lines show s.e.

parameter estimates for the reduction in NEFA concentration are summarized in Table 2. For the two lowest doses (60 μg kg^{-1} SPA in 5 min and 15 min) the model failed to estimate the E_{max} value, since an insufficient number of samples were available during this period of maximal suppression. In the modelling procedure, the E_{max} value was fixed to the average value found in the other two groups.

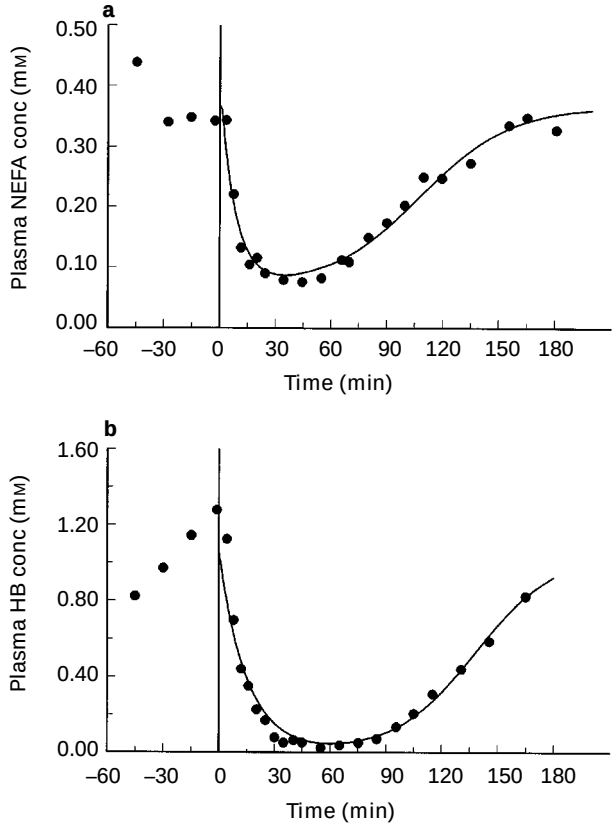

Figure 4 (a) Time course of the plasma NEFA concentrations for a rat which had received an i.v. infusion of 60 μg kg^{-1} SPA for 15 min and (b) blood β-hydroxybutyrate (HB) concentrations for a rat which had received an i.v. infusion of 120 μg kg^{-1} SPA for 60 min. The solid lines represent the effect as described by the indirect suppression model.

Administration of SPA at different doses and rates resulted in consistent pharmacodynamic parameter estimates with no statistically significant differences between the groups (Table 2). The mean values after combining the results of the four groups were: $EC_{50} = 22 \pm 1.8$ ng ml^{-1}, $k_e = 0.11 \pm 0.01$ min^{-1}, $N_0 = 0.26 \pm 0.01$ mM and Hill factor 2.2 ± 0.2 (mean $\pm$ s.e., $n = 25$). The mean value for E_{max} was 0.80 ± 0.02 (mean $\pm$ s.e., $n = 12$) as determined from the two groups which received 120 μg kg^{-1} SPA in 60 min and 400 μg kg^{-1} SPA in 15 min.

The effects of intravenous administration of SPA on β-hydroxybutyrate (β-HB) levels are shown in Figure 3b). The decrease in the β-HB levels is depicted as the relative change to baseline. In the vehicle-treated group the β-HB levels were stable during the first two hours of the experiment but decreased slightly towards the end of the experiment. Administration of SPA resulted in a dose-dependent decrease in blood β-HB levels. Again, the onset of this effect was independent of the infusion rate of SPA. Although the effect on β-HB concentrations appeared to be similar to the effect on NEFA concentrations, there were some important differences. Firstly, in all four groups the β-HB levels decreased to levels below detection, indicating a complete inhibition of the release of β-HB. Secondly, there seemed to be an additional lag time in the effect. At 5 min after the start of the infusion the β-HB levels were still at baseline levels. Finally, the return in the β-HB levels succeeded the return in the NEFA concentrations.

In each rat the indirect suppression model could adequately describe the time course of the anti-ketotic effect. Figure 4b shows the β-HB concentrations and the predicted time course of the effect on the basis of the integrated PK/PD model in one individual animal. Since complete suppression of the β-HB levels occurred, the E_{max} value in the inhibitory function (equation 4) was replaced by the value 1. The estimated pharmacodynamic parameters are summarized in Table 3. Between the treatments no significant differences were observed in the various parameters. The mean values obtained from all four groups were: $EC_{50} = 8.3 \pm 1.3$ ng ml^{-1}, $k_e = 0.063 \pm 0.005$ min^{-1}, $N_0 = 1.1 \pm 0.06$ mM and Hill factor $= 1.6 \pm 0.2$ (mean $\pm$ s.e., $n = 25$).

Haemodynamic effects

Intravenous administration of SPA resulted in pronounced cardiovascular effects. The lower doses of SPA produced sub-

Table 2 Pharmacodynamic parameter estimates for the reduction in plasma NEFA concentrations after intravenous infusion of SPA to fasted rats

Dose/infusion time (μg kg^{-1}/min)	n	EC_{50} (ng ml^{-1})	E_{max} (fraction of baseline)	*Hill factor*	k_e (min^{-1})	N_0 (mM)
60/5	7	23 ± 4	0.80[a]	2.5 ± 0.5	0.09 ± 0.01	0.26 ± 0.03
60/15	6	25 ± 3	0.80[a]	2.4 ± 0.7	0.11 ± 0.01	0.29 ± 0.02
120/60	6	22 ± 4	0.81 ± 0.03	2.0 ± 0.3	0.10 ± 0.01	0.25 ± 0.01
400/15	6	20 ± 3	0.78 ± 0.02	2.1 ± 0.3	0.13 ± 0.03	0.23 ± 0.01

Data are presented as mean ± s.e. [a]E_{max} was fixed in the modelling procedure.

Table 3 Pharmacodynamic parameter estimates for the reduction in blood β-hydroxybutyrate concentrations after intravenous infusion of SPA to fasted rats

Dose/infusion time (μg kg^{-1}/min)	n	EC_{50} (ng ml^{-1})	E_{max}[a] (fraction of baseline)	*Hill factor*	k_e (min^{-1})	N_0 (mM)
60/5	7	8.0 ± 2.4	1	1.7 ± 0.2	0.06 ± 0.01	0.98 ± 0.11
60/15	6	10 ± 2.5	1	1.9 ± 0.4	0.07 ± 0.01	1.15 ± 0.09
120/60	6	8.8 ± 3.0	1	1.4 ± 0.3	0.06 ± 0.01	0.98 ± 0.21
400/15	6	4.6 ± 1.3	1	0.9 ± 0.2	0.05 ± 0.01	1.13 ± 0.09

Data are presented as mean ± s.e. [a]E_{max} was fixed in the modelling procedure.

maximal decreases in both heart rate and blood pressure (data not shown). At maximal SPA concentrations (at the end of the infusion) both heart rate and mean arterial pressure were reduced by −57, −53 and −87 beats min^{-1}, and −9, −11 and −3 mmHg (for the infusions of 60 μg kg^{-1} SPA in 5 min, 60 μg kg^{-1} in 15 min and 120 μg kg^{-1} in 60 min, respectively). Administration of 400 μg kg^{-1} SPA for 15 min resulted in a rapid decrease of heart rate to a level which was approximately 40% of pre-administration level (Figure 5). After termination of the infusion, the maximal effect was maintained for several minutes and gradually returned to baseline with decreasing blood SPA concentrations. Figure 5 also depicts the average effect on plasma NEFA levels in this treatment group. It is clearly shown that the effect on the plasma NEFA levels is far more sensitive. At 180 min after the start of the infusion, the heart rate was almost back to baseline levels, whereas the NEFA concentrations were still maximally suppressed.

Both the anti-lipolytic and negative chronotropic effect could be related to blood SPA concentrations on the basis of an integrated pharmacokinetic-pharmacodynamic model. The negative chronotropic effect was directly related to blood SPA concentrations, since no delay in the effect was observed. The sigmoidal E_{max} model adequately described the effect on heart rate. The pharmacodynamic parameter estimates for the effect on heart rate are summarized in Table 4. The values for EC_{50}, E_{max}, Hill factor, and E_0 were 131 ± 31 ng ml^{-1}, -197 ± 18 beats min^{-1}, 2.2 ± 0.3, and 363 ± 27 beats min^{-1}, respectively (mean ± s.e., $n = 6$). The difference in sensitivity between the effects is reflected in the EC_{50} values. The EC_{50} for the effect on plasma NEFA levels was approximately 6 fold lower than the EC_{50} for heart rate. Individual EC_{50} values for heart rate and NEFA concentrations are shown in Figure 6.

Since it is the free drug that acts on the adenosine A_1-receptor, the EC_{50} values were corrected for the extent of plasma protein and blood cell binding. The mean $EC_{50,u}$ values, EC_{50} values based on free drug concentrations, were 13 ± 2 ng ml^{-1} (26 ± 4 nM) and 88 ± 21 ng ml^{-1} (168 ± 40 nM) for the effect on plasma NEFA concentrations and heart rate, respectively.

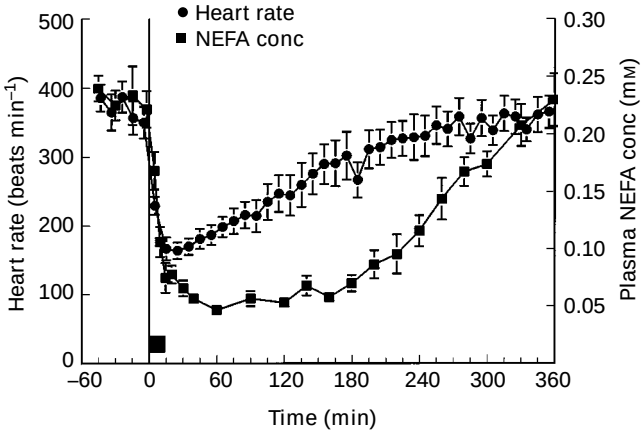

Figure 5 The effect of an i.v. infusion of 400 μg kg^{-1} SPA for 15 min on plasma NEFA concentration and heart rate in conscious, fasted rats. Data are presented as mean ($n = 6$); vertical lines show s.e.

Table 4 Comparison of the pharmacodynamic parameter estimates for the effect on plasma NEFA concentrations and heart rate after intravenous infusion of 400 μg kg^{-1} SPA for 15 min to fasted, conscious rats

Parameter	*NEFA*	*Heart rate*
EC_{50} (ng ml^{-1})	20 ± 3	131 ± 31
$EC_{50,u}$ (ng ml^{-1})	13 ± 2	88 ± 21
E_{max}	0.78 ± 0.02	-197 ± 18 beats min^{-1}
Hill factor	2.1 ± 0.3	2.2 ± 0.3
E_0 (beats min^{-1})		363 ± 27
N_0 (mM)	0.23 ± 0.01	
k_e (min^{-1})	0.13 ± 0.03	

Data are presented as mean ± s.e., $n = 6$.

Discussion

In the present study an integrated pharmacokinetic-pharmacodynamic model has been developed to describe the anti-lipolytic and anti-ketotic effects of the adenosine A_1 receptor agonist N^6-(*p*-sulphophenyl)adenosine in fasted and conscious rats. Recently, the approach of pharmacokinetic-pharmacodynamic modelling has been successfully used to describe the haemodynamic effects of selective adenosine A_1- and A_{2A}-receptor agonists (Mathôt *et al*., 1994; 1995a). By determination of *in vivo* concentration-effect relationships, estimates of potency and intrinsic activity of the adenosine analogues were obtained, which correlated well with *in vitro* data obtained in radioligand binding. In these studies a direct effect model was used for the cardiovascular effects. However, for the anti-lipolytic effect a physiological indirect effect model appeared to be more appropriate. The adequacy of this model was tested by administration of the adenosine agonist in different doses and rates.

A sensitive h.p.l.c. method was developed to determine accurately the pharmacokinetics of SPA in the rat. Following intravenous administration, the time course of the SPA concentrations in blood was adequately described by a bi-exponential function (Figure 2). SPA exhibited a low blood clearance of 8.5 ml min^{-1} kg^{-1}, which was smaller than the clearance value observed for other adenosine A_1-agonists (Mathôt *et al*., 1995b; Van Schaick *et al*., unpublished observations). SPA had a small volume of distribution of 380 ml kg^{-1}. This value was not different from the value for the more lipophilic analogue CPA (Mathôt *et al*., 1994). The low clearance value resulted in a relatively long terminal half-life for SPA of approximately 40 min. The estimated pharmacokinetic parameters were independent of the rate of administration, indicating that these parameters are related to the structure of the compound and not to the rate of administration. The differences in the estimated parameters between the treatment groups were only small and not significant. By relating the observed pharmacological effect to the individual

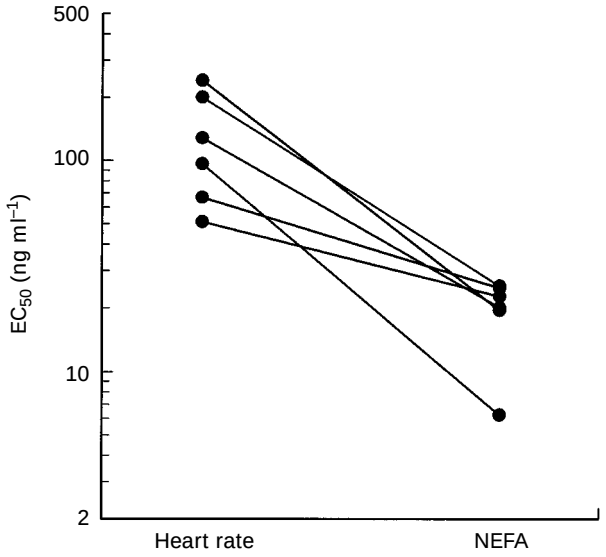

Figure 6 Individual EC_{50} values for the effect on NEFA and heart rate for the group of rats which had received 400 μg kg^{-1} SPA for 15 min. Values obtained in the same rat are connected. Mean EC_{50} values were 20 ± 2.9 and 131 ± 31 ng ml^{-1} for NEFA and heart rate, respectively.

pharmacokinetic profiles, the variability will ultimately be accounted for in determining the concentration-effect relationship.

Intravenous administration of SPA resulted in inhibition of lipolysis and consequently in a decrease of plasma NEFA levels. The anti-lipolytic effect of the adenosine A_1-agonist was reversible and dose-dependent. Higher doses, resulting in higher blood concentrations of SPA, caused a more prolonged inhibition of lipolysis. The NEFA-time profiles of the two lowest infusion rates were almost superimposable. This resemblance was the result of almost identical concentration-time profiles for SPA after these infusion rates (Figure 2). The observed effect of SPA on fatty acid concentrations is in agreement with the other studies in which **R**-PIA (Hoffman *et al*., 1986), GR79236 (Strong *et al*., 1993; Merkel *et al*., 1995) and WAG994 (Bell *et al*., 1994) were used.

After the onset of drug infusion, NEFA levels decreased slowly and a maximal decrease was reached after approximately 35 min. The slow onset of the effect occurred independently of the infusion rate of SPA, which demonstrated that the infusion rate of SPA was not the driving force for the development of the effect. The delay between agonist blood concentrations and free fatty acid concentrations is largely determined by a time-dependent turnover of fatty acids rather than by slow distribution of SPA to the site of action. Therefore, the NEFA lowering effect should preferably be quantified on the basis of a physiological substance model rather than an effect-compartment model (Holford, 1992; Dayneka *et al*., 1993; Levy, 1994). Recently, physiological substance models have proved useful for the characterization of indirect drug effects, such as inhibition or stimulation of the production or dissipation of factors controlling the measured effect (e.g. plasma free fatty acid concentrations) (Dayneka *et al*., 1993; Jusko & Ko, 1994). Examples of such indirect drug effects include anticoagulation by warfarin (Holford, 1986), corticosteroid effect on basophil trafficking (Wald *et al*., 1992) and aldose reductase inhibition (Van Griensven *et al*., 1995).

In each individual rat the time course of the plasma NEFA concentrations was described satisfactorily by a physiological indirect effect model. The relationship between agonist blood concentrations and the inhibition of lipolysis was found to be adequately described by the sigmoidal E_{max} equation, and so this equation was used as an inhibitory function in the physiological PK/PD model. Investigation of the various time profiles revealed the necessity of a variable for E_{max} in this inhibitory function.

In most inhibition models to date no variable for E_{max} (the parameter expressing maximal suppression) has been included, assuming that synthesis can be blocked completely (Jusko & Ko, 1994). However, administration of the highest doses of SPA (120 μg kg^{-1} for 60 min and 400 μg kg^{-1} for 15 min) showed that a maximal reduction in plasma NEFA levels of only 80% can be obtained *in vivo*. This observation is in agreement with the results from other studies, in which a maximal suppression of NEFA and glycerol levels of approximately 70–80% was observed (Strong *et al*., 1993; Gardner *et al*., 1994). The observation of an E_{max} value deviating from one, also demonstrates the importance of validation and application of the PK/PD model to different doses. With only the lower doses it would not have been feasible to detect this maximal effect. The individual effect-time profiles were not corrected for the increase in NEFA concentrations observed after administration of the vehicle, since this increase was not significant and merely the result of variation in the vehicle group.

The pharmacodynamic parameter estimates were independent of the infusion rate of SPA. The averaged EC_{50} for SPA was 22 ± 1.8 ng ml^{-1}. The parameter reflecting the elimination of fatty acids from the central compartment (k_e) was also dose-independent with a value of 0.097 ± 0.007 min^{-1}. This elimination rate constant corresponds to a half-life of approximately 7 min. The consistency of the parameters between the different dosing regimens supports the usefulness of the model to quantify the anti-lipolytic effects of adenosine A_1-agonists *in vivo*.

The effect of SPA on blood β-hydroxybutyrate levels resembled the effect on plasma NEFA levels. From the onset of the intravenous infusions the β-hydroxybutyrate concentrations decreased gradually and returned to baseline, depending on the concentration profile of SPA. Although the NEFA lowering and anti-ketotic effect seemed similar, there were some characteristic differences. Firstly, β-hydroxybutyrate concentration could be suppressed completely leading to a complete disappearance of ketone bodies at maximal inhibition. Apparently, a partial suppression of fatty acids of 80% results in a complete inhibition of the formation of ketone bodies in the liver. Secondly, both the decline and the return to baseline of the β-hydroxybutyrate concentrations always occurred later than with the NEFA concentrations.

The effect of the adenosine agonist SPA on the blood β-hydroxybutyrate concentrations could also be described by the indirect suppression model. The estimated pharmacodynamic parameters were independent of the dose and rate of administration, again indicating the relationship between SPA concentrations and the anti-ketotic effect. Although the decrease in ketone bodies is a secondary effect to the inhibition of lipolysis, the time-course of this effect is still adequately described on the basis of the model. The apparently longer lasting effect on hydroxybutyrate levels was caused by a significantly lower EC_{50} for this effect in comparison to the EC_{50} for the NEFA lowering effect (8.3 ng ml^{-1} versus 22 ng ml^{-1}, respectively).

Continuous monitoring of heart rate and blood pressure during the experiments allowed the opportunity to compare haemodynamic effects of the adenosine A_1-agonist with metabolic effects. Administration of SPA resulted in a decrease in both blood pressure and heart rate that was only maximal in the group receiving 400 μg kg^{-1} SPA for 15 min. Administration of the three lower doses of SPA resulted in short and less pronounced reductions in heart rate, whereas still a maximal inhibition of lipolysis was observed. The effects on blood pressure of these low doses were marginal and in most rats not significantly different from the vehicle-treated group. These results are in line with recent observations that orally administered GR79236 can exert profound anti-lipolytic effects with minimal effects on heart rate (Gardner *et al*., 1994).

In contrast to the metabolic effects, the effect on heart rate was related directly to the blood SPA concentrations on the basis of the sigmoidal E_{max} model. No hysteresis loop was observed in the concentration-heart rate relationship of SPA, indicating a rapid equilibration between SPA concentrations in blood and those at the receptors. Although the model for the bradycardiac effect of SPA is structurally different, the estimates of potency and intrinsic activity are similar to the ones obtained from the model for the anti-lipolytic effects. The intrinsic activity of SPA (E_{max}) was -197 ± 18 beats min^{-1}, which was similar to the activity of other full agonists (Mathôt *et al*., 1995b). The EC_{50} value of SPA for the negative chronotropic effect was 131 ± 31 ng ml^{-1}, which appeared to be an approximately 6 fold higher value than the EC_{50} for the NEFA lowering effect. This difference is also apparent when the values within the same animal are compared. In each rat the EC_{50} for the inhibition of lipolysis was lower than the EC_{50} for heart rate (Figure 6). These *in vivo* results are in good agreement with the 5 fold difference in EC_{50} of 5′-*N*-ethylcarboxamidoadenosine (NECA) between rat adipocytes and right atria observed *in vitro* (Gurden *et al*., 1993).

After correcting for protein binding both values for *in vivo* potency could be compared with receptor affinity as determined in radioligand binding experiments. Both EC_{50} values (29 nM and 168 nM for anti-lipolytic and bradycardiac effect, respectively) were lower than the apparent K_i value (K_i from displacement of antagonist binding in the absence of GTP) of 1410 nM as determined in rat brain (Van Schaick *et al*., un-

published observations). Jacobson and coworkers (1992) obtained a K_i for SPA of 74 nM, as determined by agonist displacement in rat brain membrane. This value correlates well to our *in vivo* estimate of potency.

The 6 fold difference in potency between the two effects is the result of a tissue-difference in receptor density and receptor-effector coupling. Brain and adipose tissue have been shown to have the highest densities of adenosine A_1-receptors, whereas significantly lower receptor densities (approximately 20 fold) are found in heart and kidney (Lohse *et al*., 1986; Klotz *et al*., 1989). Although the receptor density can be a controlling factor for the response, this effect can be superseded by the amplification caused by receptor-effector coupling (receptor reserve). In adipose tissue the adenosine A_1-receptor has been shown to be efficiently coupled to the effector system, resulting in a large number of spare receptors (Lohse *et al*., 1986). In contrast, studies by Dennis *et al*. (1992) revealed a relatively low number of spare A_1-adenosine receptors in the guinea-pig AV node. In tissues with a low number of spare receptors, higher concentrations of a drug are needed to produce a maximal effect, leading to a higher EC_{50} value and an apparently lower sensitivity of the agonist (Kenakin, 1993).

The outcome of a certain degree of tissue selectivity of SPA may be very important for therapeutic application of adenosine A_1-receptor agonists. Due to the abundance of the adenosine A_1-receptor in many tissues, receptor-subtype selectivity cannot be a basis for selectivity of action *in vivo*. However, separation of haemodynamic from metabolic effects on the basis of differences in receptor-reserve may be feasible. In this respect, application of compounds with reduced intrinsic efficacy may result in an even higher selectivity. Centrally-mediated adenosine A_1-receptor effects may be prevented by additional modification of the adenosine analogues with a polar substituent (like the sulphophenyl-group in SPA), which may preclude the compounds from entering into the brain.

In conclusion, the relationship between blood SPA concentrations and metabolic effect could be adequately described by the indirect suppression model, yielding estimates of potency and intrinsic activity of the adenosine A_1 agonist *in vivo*. Administration of SPA in various rates and doses did not influence the estimated pharmacodynamic parameters, which supports the usefulness of the physiological indirect effect model. The 6 fold difference in potency of SPA between the effects on heart rate and NEFAs, indicates that separation of metabolic from haemodynamic effects may be feasible.

This work was financially supported by a grant from Glaxo Wellcome, U.K. N^6-(p-sulphophenyl)adenosine was provided by Research Biochemicals International as part of the Chemical Synthesis Programme of the National Institute of Mental Health, Contract NO1MH30003.

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(Received February 13, 1997
Revised May 15, 1997
Accepted July 4, 1997)