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Processing of Adenosine Receptor Agonists in Rat and Human Whole Blood

Barbara Pavan and A. P. IJzerman†*

Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300RA Leiden, The Netherlands

ABSTRACT. A stability study of adenosine receptor agonists in rat and human whole blood was performed. The compounds were incubated at 37° in fresh blood, and aliquots of the incubation mixture were hemolyzed at regular time intervals and analyzed with HPLC. N^6-cyclopentyladenosine (CPA) and N^6-cyclobutyladenosine (CBA) were degraded, whereas N^6-cyclohexyladenosine, N^6cycloheptyladenosine and N^6-sulfophenyladenosine were not. 2-Chloroadenosine had a half-life very similar to that of CPA. However, the 2′-, 3′-, and 5′-deoxyribose derivatives of CPA remained intact. The nucleoside transport inhibitor nitrobenzylthioinosine attenuated CBA and CPA metabolism in rat blood as did the inhibitor of adenosine deaminase erythro-9-(2-hydroxy-3-nonyl)adenine, albeit at relatively high concentrations. Complete blockade of CBA and CPA degradation was achieved by a preincubation of rat and human blood with the adenosine kinase (AK) inhibitor 5′-amino-5′-deoxyadenosine. We conclude that the two adenosine analogues are metabolized by AK both in rat and in human whole blood. BIOCHEM PHARMACOL **56**;12:1625–1632, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. adenosine receptor agonist; N^6-cyclopentyladenosine; adenosine kinase; adenosine deaminase; nucleoside transport protein; 5′-amino-5′-deoxyadenosine

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 [1]. The levels of adenosine available for stimulation of adenosine receptors are controled by various enzymes, whereas its release from and uptake into cells is governed by membrane-bound transport proteins. In many species, the nucleoside transport system represents the first step in the rapid removal of adenosine from its site of action. In the circulation, for instance, adenosine is rapidly cleared from plasma with a half-life of 1 to 2 sec due to its uptake into vascular endothelium and red blood cells. In the cytosol, the nucleoside can either be phosphorylated by AK‡ (EC 2.7.1.20) to AMP, deaminated by ADA (EC 3.5.4.4) to inosine, or converted to *S*-adenosyl-L-homocysteine by *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1) [2]. Because of adenosine's extremely short half-life, adenosine analogues that are resistant to adenosine-metabolizing enzymes have been synthesized [3, 4]. Thus far, all known adenosine receptor agonists are closely related to adenosine itself, with modifications predominantly at the N^6-, 2-, and 5′-positions. The N^6-substituted analogues of adenosine have generally proven to be potent and selective agonists for the A_1 receptor, with CPA and CHA being 780- and 280-fold A_1/A_{2A}-selective, respectively.

So far, few reports on the pharmacokinetics of synthetic adenosine receptor agonists have appeared in the literature. Recently, a sensitive HPLC method was developed which allows the determination of the pharmacokinetics of CPA in the rat. It was shown that the prototypic A_1-selective agonist CPA is degraded in rat whole blood with a half-life of 24 min [5]. The half-life of CPA in conscious rats was determined to be 7 min [6]. Apparently, the reference agonist for the adenosine A_1 receptor has a poor pharmacokinetic profile.

Following these observations, we decided to analyze the pharmacokinetic profile of adenosine receptor agonists with substitutions in either the N^6-position, the ribose ring, or in the purine moiety by testing their stability in rat and human whole blood. Subsequently, the enzymes involved in the metabolism of some of the adenosine receptor agonists were investigated. We studied the effects of an adenosine uptake inhibitor, NBTI, and of the ADA inhibitor, EHNA. We also determined the effects of AMDA, a strong inhibitor of the enzyme AK. The results obtained strongly suggest that AK is the enzyme responsible for the metabolism of the adenosine derivatives in rat and human whole blood.

* Present address: Università degli Studi di Ferrara, Dip. Di Biologia, Via L. Borsari, 46, I-44100 Ferrara, Italy.

† Corresponding author: Dr. A. P. IJzerman, LACDR, Div. of Medicinal Chemistry, PO Box 9502, 2300 RA Leiden, The Netherlands. Tel. 31-71-5274651; FAX 31-71-5274565.

‡ *Abbreviations:* ADA, adenosine deaminase; AK, adenosine kinase; AMDA, 5′-amino-5′-deoxyadenosine; CADO, 2-chloroadenosine; CBA, N^6-cyclobutyladenosine; CHA, N^6-cyclohexyladenosine; CHeptA, N^6-cycloheptyladenosine; CPA, N^6-cyclopentyladenosine; EHNA, erythro-9-(hydroxy-3-nonyl)adenine; NBTI, nitrobenzylthioinosine; NECA, N-ethyl-5′-carboxamidoadenosine; R-PIA,(R)-N^6-(1-methyl-2-phenylethyl) adenosine; and SPA, N^6-(*p*-sulfophenyl)adenosine.

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MATERIALS AND METHODS

Chemicals

CPA, R-PIA, and ADA were obtained from Boehringer Mannheim. CHA, CADO, NECA, NBTI, and AMDA were obtained from Sigma. CBA and CHeptA were synthesized by Prof. G. Cristalli, Camerino, Italy. SPA was a gift from Research Biochemicals Inc. 2′-Deoxy-CPA and 3′-deoxy-CPA were synthesized by Dr. E.M. Van der Wenden at the Department of Medicinal Chemistry of the Leiden/Amsterdam Center for Drug Research. 5′-Deoxy-CPA was kindly provided by Parke-Davis. EHNA was obtained from Burroughs Wellcome Co. Methanol and acetonitrile (HPLC grade) were purchased from Rathburn. Ethyl acetate was purchased from Baker Chemicals. Tetrabutylammonium hydroxide was obtained from Acros Organics. Water was drawn from a Milli-Q UF plus water purification system (Millipore). All other chemicals used were of analytical grade.

Kinetic Experiments in Whole Blood

The stability of drugs was investigated by a slightly modified method previously described by Mathôt *et al.* [5]. Compounds were incubated at 37° in fresh whole blood, obtained from different rats (Wistar WU, Sylvius breeding facilities) weighing 220–250 g, and from healthy human volunteers. Blood was directly transferred to heparinized glass tubes. Two mL of blood was spiked with drug solutions resulting in final concentrations of 2 μM. This concentration was chosen for two reasons. First, at this concentration all compounds showed maximal activity in earlier pharmacodynamic studies [6–9]. Second, this concentration was sufficient to ensure first-order kinetics in the degradation experiments. During the experiment, blood was continuously and gently shaken in an oscillating water-bath. At regular time intervals, 100-μL blood samples were taken, immediately hemolyzed in Eppendorf tubes prefilled with 500 μL of millipore water (0°), and stored at −20° until analysis.

When tested, the enzyme inhibitors were preincubated with whole blood for 45 min. Pharmacokinetic profiles were then recorded in the absence and presence of each inhibitor. When studied, the nucleoside transporter inhibitor NBTI was added 15 min after the addition of the enzyme inhibitors to allow their admission into the blood cells.

CPA, CHA, CBA, CHeptA, 2′-dCPA, 3′-dCPA, 5′-dCPA, and 2-CADO Analysis

Blood degradation of CPA, CBA, CHA, and CHeptA was assessed by reversed phase HPLC. To hemolyzed blood samples 50 μL of 3M sodium hydroxide and 50 μL of internal standard (1 μg/mL of CHA for CPA and CHeptA, 1 μg/mL of CPA for CBA and CHA, 1 μg/mL of R-PIA for 2′-dCPA, 3′-dCPA, and 5′-dCPA or 1 μg/mL of NECA for 2-CADO) were added. The samples were extracted twice with 1 mL of water-satured ethyl acetate. After 15 min of centrifugation at 2000 *g*, the organic layer was filtered through cotton, dried with magnesium sulphate, and evaporated to dryness on a vortex vacuum evaporator (Speed® Vac Plus, SC 110 A, Savant). The residue was dissolved in 250 μL of mobile phase and 100 μL was injected into the HPLC system.

The HPLC system was from Gilson and consisted of two 305 and 306 piston pumps, a 234 autosampling injector, and a 115 variable wavelength UV detector set at 269 nm, all operated by 712 HPLC System Controller Software (Version 1.0). Chromatography was performed at room temperature on a reversed-phase column (Econosphere C-18 3U cartridge column, 100 mm × 4.6 mm I.D.; Alltech Applied Science BV) equipped with a guard column packed with C-18 material (Alltech). The mobile phase consisted of a ternary mixture of acetonitrile, methanol, and 10 mM acetate buffer (pH 4.0) with a ratio of 4/40/56 (v/v/v) for the analysis of CPA, CHA, CBA, and CheptA in the absence or presence of enzyme and nucleoside transport inhibitors. The flow rate was 0.6 mL/min. The retention times of CPA, CHA, CBA, and CHeptA were 5.9, 8.8, 4.2, and 10.9 min, respectively.

For the analysis of 2′-dCPA and 3′-dCPA, the mobile phase consisted of a ternary mixture of acetonitrile, methanol, and acetate buffer (pH 4.0) with a ratio of 4/44/52 (v/v/v) and a ratio of 4/47/49 (v/v/v) for the analysis of 5′-dCPA [7]. The flow rate was 0.5 mL/min, and the retention times for 2′-dCPA, 3′-dCPA, and 5′-dCPA were 7.9, 7.0, and 8.5 min, respectively. The retention times of the internal standard R-PIA were 11 and 14 min with the mobile phase ratios of 4/47/49 and 4/44/52, respectively.

The analysis of 2-CADO was based on the method of Mathôt *et al.* [8]. Briefly, 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 0.5 mL/min and the retention times for 2-CADO and internal standard NECA were 5.8 and 6.5 min, respectively.

To determine SPA degradation in blood, ion pair reversed-phase HPLC with UV detection at 302 nm was performed, based on the method described by Van Schaick *et al.* [9]. The mobile phase consisted of a mixture of 20 mM acetate buffer (pH 4.0) and acetonitrile in a ratio of 82/18 (v/v) to which 20 mM tetrabutylammoniumhydroxide was added as ion-pairing reagent. At a flow rate of 0.5 mL/min, the retention times of SPA and the internal standard CPA were 7 and 9 min, respectively.

The SPA blood samples were prepared for HPLC analysis according to the following procedure. To hemolyzed blood internal standard (50 μL of 18 μg/mL CPA solution) was added. Samples were deproteinated by adding 2 mL of acetonitrile, mixing on a vortex, and centrifugation at 2000 *g*. The supernatant was transferred to a clean tube, and 200 μL of acetate buffer (20 mM, pH 4.0) and 50 μL of 1 M

hydrochloric acid were added. This mixture was extracted with 5 mL of ethyl acetate on a vortex for 1 min. After centrifugation (10 min, 2000 *g*) 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 (Speed® Vac Plus, SC 110 A, Savant) at 40°. The residue was dissolved in 250 μL of acetate buffer containing 20 mM of TBAH, and 100 μL was injected into the chromatographic system.

In all cases, the within-day coefficients of variation were less than 4%. The between-day variations, measured over a period of 9 months, were also less than 4%. The detection limits were 3–5 ng/mL (signal to noise ratio of 3).

Data Analysis

The peak areas in the chromatograms were calculated by means of the Gilson 712 HPLC System Controller software (version 1.0). The half-life of each adenosine analogue was calculated from a semilogarithmic plot of the peak area ratio between the compound and internal standard, expressed as percentage, versus incubation time, using the computer program PRISM (version 2.0, Graph Pad Inc.).

RESULTS

Pharmacokinetic Profile of Adenosine Analogues in Rat Fresh Whole Blood

The structures of the adenosine analogues used in the present study are shown in Fig. 1. These include N^6-substituted derivatives (Fig. 1A), a 2-substituted derivative (Fig. 1B), and deoxyribose analogues (Fig. 1C).

In Fig. 2, HPLC chromatograms are shown for CPA (Fig. 2A) and CHA (Fig. 2B) at the beginning (t = 0 min) and conclusion (t = 120 min) of incubations in rat fresh whole blood at 37°. The peak corresponding to CPA had disappeared after 120 min of incubation, whereas at the same time point, the CHA peak was not modified. This is more evident in Fig. 3, where degradation–time relationships for 2 μM CBA, 2 μM CPA, and 2 μM CHA are shown. The degradation rate decreased with increasing cycloalkyl substituent, i.e. highest for cyclobutyl, intermediate for cyclopentyl, and negligible for a cyclohexyl group. The half-life values (means ± SE) of CBA and CPA were 0.97 ± 0.17 min and 25 ± 1 min, respectively. Two other adenosine analogues with larger substituents (CHeptA and SPA) were resistant to metabolism.

CADO, the only 2-substituted derivative included in the present study, showed a degradation rate comparable to CPA (22 ± 1 min and 25 ± 1 min, respectively; Fig. 3). This behavior was also observed *in vivo* (6.7 ± 0.5 min and 6.9 ± 0.5 min for CADO and CPA, respectively) [5, 8]. Degradation of the 2′-, 3′-, and 5′-deoxyribose analogues of CPA in rat blood was negligible.

Influence of Enzyme and Transport Inhibitors on CBA and CPA Degradation in Rat Fresh Whole Blood

In Table 1, the half-life values of CBA and CPA are shown as a function of the presence of enzyme and transport inhibitors. The nucleoside transport inhibitor NBTI slowed down the degradation rate of CBA and CPA depending on the concentration applied. The effect of NBTI at high concentration (20 μM) for both compounds is also depicted in Fig. 4.

Table 1 also summarizes the data obtained in the presence of the ADA inhibitor EHNA at two concentrations. At low concentration (2 μM), EHNA did not modify the kinetics of CBA and CPA disappearance, but the half-life values significantly increased for both adenosine analogues at high EHNA concentration (20 μM; see also Fig. 4). The combination of 20 μM NBTI and 20 μM EHNA produced a further metabolic stabilization.

Finally, Fig. 4 and data from Table 1 show that the AK inhibitor AMDA (20 μM) completely prevented CBA and CPA degradation in rat whole blood whether alone or in combination with the nucleoside transport blocker NBTI.

Pharmacokinetic Profile of Adenosine Analogues in Human Whole Blood

The half-life values of CBA, CPA, and CHA after incubation in human fresh whole blood were also determined. CBA and CPA were degraded at a rate similar to that observed in rat blood, i.e. 0.81 ± 0.05 min and 15 ± 3 min, respectively. Moreover, in human blood a modest degradation of CHA was obtained (half-life = 250 ± 20 min).

After a 45-min preincubation with 20 μM AMDA, no degradation for any of these compounds was noticed in human blood.

DISCUSSION

We analyzed the stability of CPA in rat whole blood, confirming the half-life value of 24 min previously reported [5]. Consecutively, we tested the stability of a similarly potent and selective adenosine A_1 receptor agonist, CHA. Unexpectedly, CHA was not degraded in rat whole blood, despite the minor structural difference of one carbon atom between the two compounds. Therefore, we decided to explore the structure–stability relationships of adenosine receptor agonists in more detail. We observed a very rapid degradation of CBA (N^6-substituent = cyclobutyl) in rat and human whole blood, with no degradation at all of CHeptA (N^6-substituent = cycloheptyl) and SPA (N^6-substituent = sulfophenyl). We conclude that the size of the N^6-substituent strongly affects the degradation rate of adenosine derivatives. In particular, their degradation is fully avoided if the N^6-substituent is at least a cyclohexyl.

A chlorine atom in the 2-position of the purine moiety increased the terminal half-life of adenosine in rat blood. The half-life values of CADO and CPA *in vitro* appeared

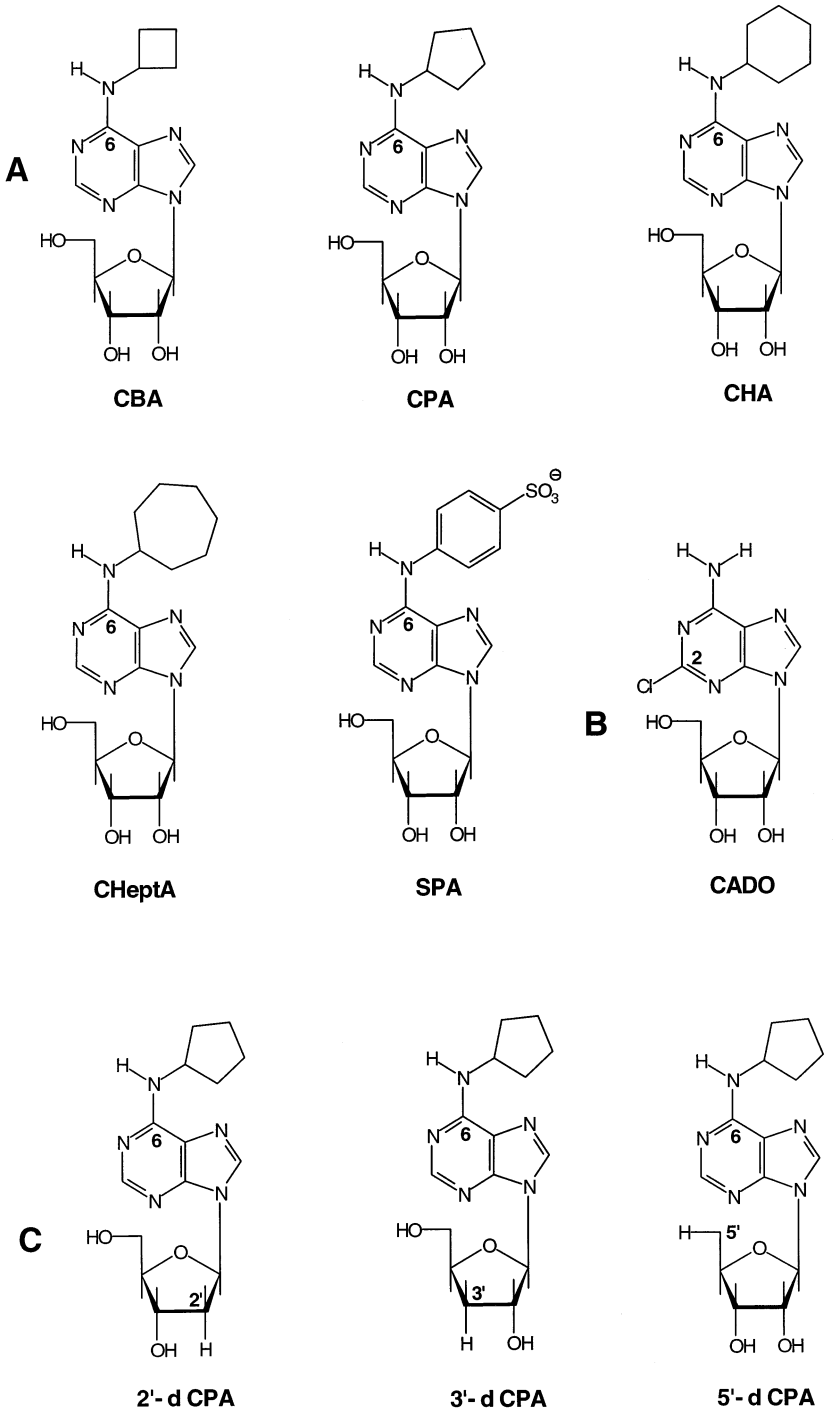

FIG. 1. Chemical structures of the adenosine analogues used in the present study: (A) N^6-substituted derivatives, (B) CADO, a 2-substituted derivative, and (C) deoxyribose derivatives.

very similar (25 ± 1 min and 22 ± 1 min, respectively) as had been observed *in vivo* (6.9 ± 0.5 min and 6.7 ± 0.5 min, respectively). For both compounds, the *in vivo* studies also underlined the relative importance of extrahepatic clearance with respect to a small extraction ratio of CPA and CADO in the liver and kidney [6, 8].

Recently, a series of deoxyribose analogues of CPA was synthesized and tested in radioligand binding studies on rat brain membranes [10]. It was shown that the removal of the 2′-, 3′-, or 5′-hydroxyl group reduced both the affinity and intrinsic activity of these compounds for the adenosine A_1 receptor. In particular, 2′- and 3′-deoxyribose derivatives proved partial agonists for the adenosine A_1 receptor. Rat *in vivo* studies [7] revealed an increase in the elimination half-lives of the deoxyribose analogues compared to CPA. In our *in vitro* study in rat blood, the 2′-, 3′-, and

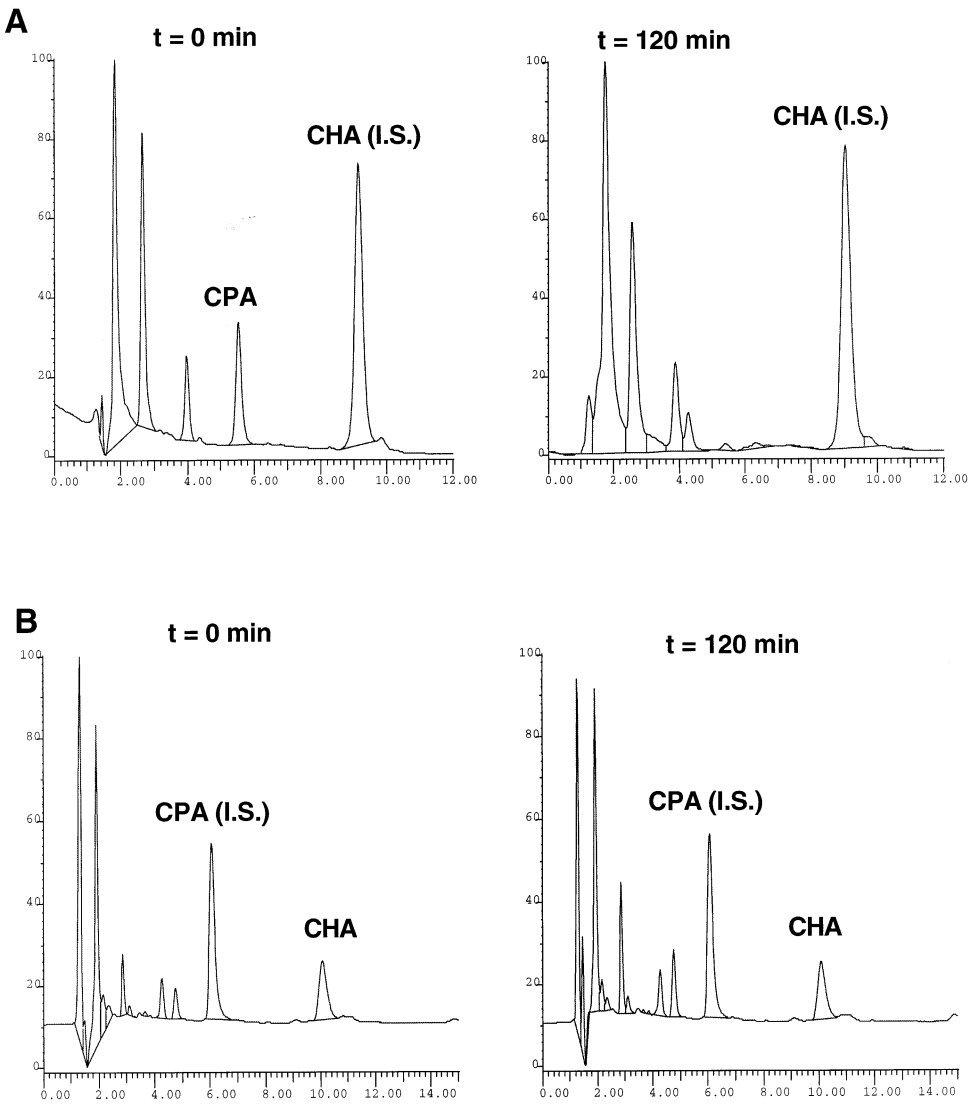

FIG. 2. HPLC chromatograms for samples collected at t = 0 (1) and t = 120 min (2) after addition of CPA (2 μM; A) and CHA (2 μM; B) to rat whole blood. I.S. = internal standard. Y-axis in % peak intensity, x-axis in min.

5′-deoxyribose CPA derivatives remained intact. Apparently, extrahepatic metabolism of CPA needs all the hydroxyl groups of the ribose moiety so that enzymatic degradation can occur. The half-life values obtained *in vitro* have significant impact on those determined *in vivo*. As a first conclusion, the results suggest that at an early stage of development of adenosine receptor agonists, the metabolic stability determined from *in vitro* studies may be a useful parameter to avoid a poor pharmacokinetic profile.

What, then, could be the molecular mechanism involved in this extrahepatic metabolism? Mathôt *et al.* [5] have demonstrated that blood cells are necessary to degrade CPA, because no degradation of CPA was observed in plasma in the temperature range of 0–37°. The entry of nucleosides and nucleoside analogues into mammalian cells is mediated by specific membrane-bound transport proteins. Two major classes are discerned based on their mechanisms of action: (1) Active, sodium ion-dependent transport,

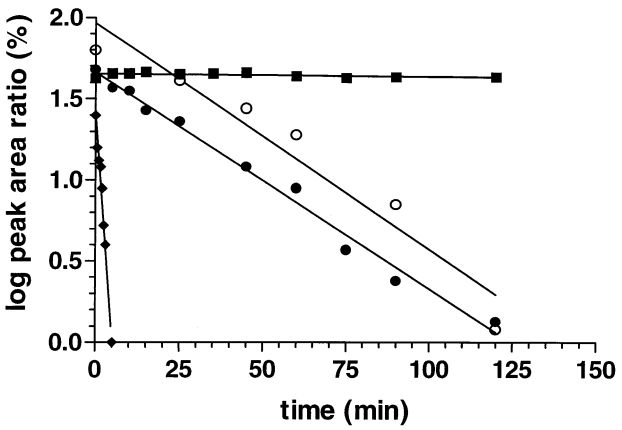

FIG. 3. Time-courses of degradation of CBA (◆), CPA (●), CHA (■), and CADO (○) in rat whole blood. The semilogarithmic plot shows the peak area ratio between any single compound and its internal standard, expressed as percentage, versus time. Results are the means of three independent experiments, performed in duplicate (SEM less than 10%).

TABLE 1. Half-life values *in vitro* obtained by incubating CBA and CPA at 37° in rat fresh whole blood in the presence of the nucleoside transporter inhibitor NBTI, the ADA inhibitor EHNA, and the AK inhibitor AMDA

Inhibitor added	Half-life CBA in rat blood (min.)	Half-life CPA in rat blood (min.)
—	0.97 ± 0.17	25 ± 1
2 μM NBTI	1.7 ± 0.1	40 ± 2
20 μM NBTI	4.6 ± 0.2	88 ± 5
2 μM EHNA	1.6 ± 0.1	28 ± 1
20 μM EHNA	4.8 ± 0.2	122 ± 2
20 μM NBTI + 20 μM EHNA	7.9 ± 0.1	302 ± 2
20 μM AMDA	∞	∞
20 μM NBTI + 20 μM AMDA	∞	∞

Values are reported as means ± SE. ∞: no degradation observed.

predominantly in kidney and small intestine epithelium, is mediated by at least four different proteins [11]. No inhibitors have been found yet for these active transport systems. (2) Passive transport by means of facilitated diffusion is mediated by proteins both sensitive and insensitive towards the reference inhibitor NBTI [2]. The sensitivity of the nucleoside transport system to NBTI has been shown to be species- and cell-dependent [12]. In rat erythrocytes, both NBTI-sensitive and NBTI-insensitive components of nucleoside transport exist in the cell population.

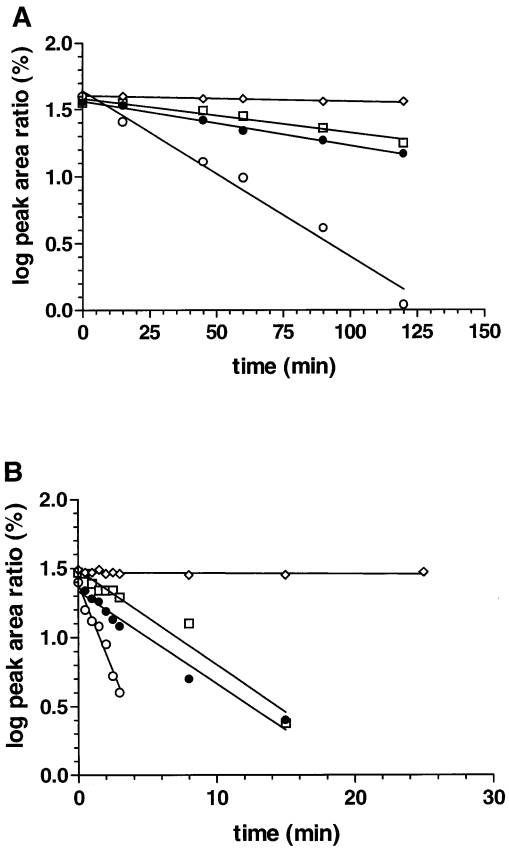

FIG. 4. Time-courses of degradation of CPA (A) and CBA (B) in rat whole blood in the absence (○) and presence of the nucleoside transport inhibitor NBTI (20 μM, ●), the ADA inhibitor EHNA (20 μM, □), and the AK inhibitor AMDA (20 μM, ◇). Results are the means of three independent experiments, performed in duplicate (SEM less than 10%).

As reported in Table 1, NBTI slowed the disappearance of CPA and CBA in rat blood, but did not completely prevent it. This observation may indicate that adenosine analogues cross the cell membrane despite the inhibition of the NBTI-sensitive nucleoside transport system.

The ADA inhibitor EHNA slowed CPA and CBA degradation in rat blood at 20 μM, but not at 2 μM. This latter concentration, however, is usually sufficient to inhibit ADA [13]. To directly verify whether ADA is capable of degrading CBA and CPA, we incubated CBA and CPA at 37° in Tris–HCl buffer (pH 7.4) in the presence of 2 IU/mL of ADA (data not shown). The concentration of intact CPA remained unchanged for 120 min, whereas CBA was slightly degraded with a half-life of 80–87 min (n = 2). Moreover, this slight degradation of CBA was prevented if ADA was blocked with EHNA at 2 μM. According to these data, the decrease in the degradation rate of CPA and CBA in rat whole blood by 20 μM of EHNA could depend on nonspecific actions of the ADA inhibitor. This conclusion is further supported by the following considerations. ADA activity has been thought to be mainly localized in the cytoplasm, although recent evidence suggests that ecto-ADA activity is present in a number of tissues including erythrocytes, lymphocytes, and endothelial cells [14]. Furthermore, inhibitors of ADA influence many other aspects of nucleoside metabolism [15–17]. Therefore, at a higher concentration, EHNA could interact with other enzymes such as AK or interfere with NBTI-modulated or other membrane transport systems [18]. Indeed, the addition of 20 μM EHNA to rat blood preincubated with 20 μM NBTI caused a further attenuation of the rate of CBA and, in particular, CPA metabolism. These findings suggest that CPA and CBA deamination in rat whole blood is negligible. Finally, preincubation of rat whole blood with the inhibitor of AK, AMDA, fully prevented CBA and CPA degradation. AK catalyzes the phosphorylation of the 5′-hydroxyl group of adenosine by transfer of the γ-phosphoryl group of either ATP or GTP. Adenosine has been found to be mainly

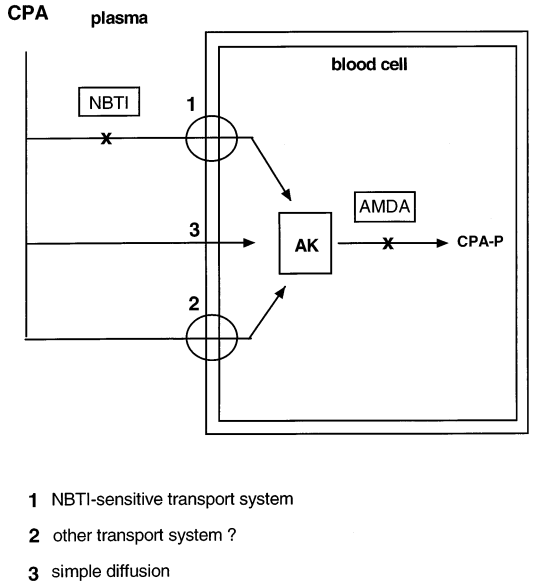

FIG. 5. Hypothetical model describing the mechanism of degradation of adenosine analogues in whole blood. (CPA-P: CPA-5′-monophosphate).

rephosphorylated by AK under basal conditions [18] and deaminated by ADA under ATP-depleted conditions, where adenosine levels were increased [19]. AMDA has high, although variable, affinity for AK, with an IC_{50} value of 21 nM for rabbit liver AK [20]. According to our data, a hypothetical model is suggested (Fig. 5), in which the adenosine A_1 receptor agonists CBA and CPA are phosphorylated by AK in rat whole blood. The enzymatic phosphorylation probably occurs inside the blood cells, since AK is known as a cytosolic enzyme.

No marked differences in the degradation rate of adenosine analogues were observed between rat and human blood. The half-lives in human blood tended to be a little shorter, with some degradation of CHA. Also, in human blood AK seems to be the enzyme responsible for the metabolism of adenosine analogues.

In summary, the results obtained in the present study suggest the relevance of the type and position of substituents to affect the degradation rate of adenosine receptor agonists. In particular, compounds having very similar pharmacodynamic parameters, such as receptor affinity (CPA and CHA), show important pharmacokinetic differences, such as half-life. Furthermore, the main metabolism in both rat and in human whole blood appears to occur via the enzyme adenosine kinase.

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