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INHIBITION OF UPTAKE OF ADENOSINE INTO HUMAN BLOOD PLATELETS

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Abstract—Adenosine transport into human blood platelets is mediated by two independent systems with different affinities. Both systems transport only purine nucleosides and no pyrimidine nucleosides. In experiments with differently substituted purine nucleosides, purines and analogues, differences in carrier specificity could be shown. For both uptake systems an intact purine ring system is required. Substituents at position 6 are of importance for the low affinity carrier. The more bulky this substituent, the less is the affinity. Double substitution, position 2 being an amino group and position 6 either being an amino, hydroxyl or mercapto group gave opposite effects for both carriers: the high affinity system being less inhibited, the low affinity system being more inhibited in changing from amino to mercapto. The 2′ and 3′ position of the ribose ring appeared to be of importance for both high and low affinity carriers. The requirement of a 2′ hydroxyl group oriented opposite the purine ring with respect to the ribose ring is suggested for the low affinity system. This, together with an appropriate substituent at position 6 of the ring system, is typical for a substance being a substrate for the low affinity carrier. On the other hand, the presence of a hydroxyl group at the 2′ position is of less importance for the high affinity carrier: here the 3′ position appears to be the more important one. The effects of dipyridamole (RA 8) and some related drugs (RA 233, RA 433, VK 744 and VK 774) upon adenosine uptake were investigated. Only the pyrimido pyrimidine compounds RA 8, RA 233 and RA 433 had an effect upon adenosine transported through the high affinity system. RA 8 appeared to be a competitive inhibitor of adenosine uptake by the high affinity system.

Adenosine plays an important role as building block for purine nucleotides in all living species. It also has vasodilator properties in mammals [1]. Myocardial cells release adenosine into the blood [2, 3] and it may than be degraded by adenosine deaminase [4] or taken up into various cells in or in contact with blood. The transport processes involved in uptake of adenosine have been described in detail [5–9]. Adenosine is transported into almost all of these cells by a carrier mediated high affinity uptake system (K_m near 10 μm) and one or several uptake systems of low affinity [6–9]. Rabbit polymorphonuclear leukocytes form a notable exception in that only the high affinity system was observed [5]. In these cells and in dog myocardium [10] the steric requirements for the adenosine high affinity carrier molecular have been studied.

In our previous study on the uptake and metabolism in human blood platelets, we observed specific inhibition by papaverine of the high affinity system but also of the single low affinity transport system for adenosine present in these cells [8]. We thought it of interest to compare the steric requirements for inhibition of both uptake systems.

Inhibition of adenosine uptake has been a guiding principle [11] in the development of a series of platelet function inhibitors [12, 13], the pyrimido pyrimidines. In the present study we include some data on inhibition caused by these agents.

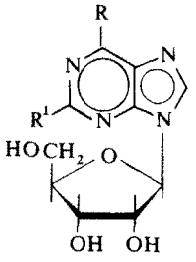
MATERIALS AND METHODS

[2.8-^{3}H]Adenosine, sp. act. 30 Ci/mmole, was purchased from New England Nuclear Corp, Boston, U.S.A. High voltage electrophoresis of the adenosine showed that at least 92 per cent was adenosine. It was diluted with non radioactive adenosine before use and made up to 300 mOsmol by adding solid sodium chloride. All purines and purine ribosides* used were from Sigma Chemical Co., St. Louis, MO U.S.A., with the exception of 4-amino-5-imidazole carboxamide, 4-amino-5-imidazole carboxamide riboside, formycin, adenine arabinoside which were from Calbiochem. San Diego, CA, U.S.A. Psicofuranine and adenine xyloside were a gift from Dr. H. Holmsen, Specialized Center for Thrombosis Research, Temple University, Philadelphia, PA.

Blood from donors who had used no drugs during the preceding 5 days, was collected into 0.027 M EDTA in 0.15 M NaCl, pH 7.4 (1 vol. to 9 vol. blood), in polyethylene tubes. After centrifugation (275 g_{max} for 10 min at room temperature), the platelets in the supernatant platelet rich plasma were washed twice with a buffer containing 0.103 M NaCl, 0.04 M NaH_2PO_4, 0.0047 M KH_2PO_4 0.005 M glucose and 0.005 M EDTA, and adjusted to pH 7.6, according to Gaintner *et al.* [14]. Bovine albumin (5 mg/ml) was added to this buffer [15]. Centrifugation during the washing procedure was carried out at 1000 g_{max} for 10 min at 4°. Platelet numbers were determined with the aid of a Coulter

* For names and structural formules of the used substances see Tables 1, 2 and 3. The names adenine xyloside and psicofuranine refer to adenine-9-β-D-xylofuranoside and adenine-9-β-D-ribo-hex-2-ulofuranoside. All other compounds shown in Table 1 belong to the 9(H) purinyl-β-D pentofuranosides.

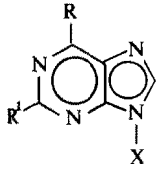
Table 1. Purine nucleoside compounds used for investigating the influence of changes in structure on adenosine uptake in human blood platelets

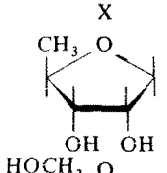


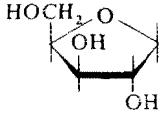
R′	R^1	Inhibitor (concentration 1mM)	Adenosine concentration 5 μM % of control ± S.E.M.	5 mM % of control ± S.E.M.
NH_2	H	Adenosine	100	100
$N(CH_3)_2$	H	6.6-*N*,*N*-Dimethylaminopurine riboside	33.0 ± 4.1**	71.2 ± 3.3
H	H	Purine riboside	37.8 ± 3.7**	72.3 ± 2.6
NH_2	H	Adenosine-N^1-oxide	36.6 ± 1.6**	94.6 ± 4.1
NH_2	Cl	2-Chloroadenosine	36.2 ± 3.6**	66.4 ± 4.1
SH	H	6-Mercaptopurine riboside	37.6 ± 1.6**	104.0 ± 6.9
$NHCH_3$	H	6-*N*-Methylaminopurine riboside	42.1 ± 2.8**	83.2 ± 4.6
$O_2NC_6H_4S$	NH_2	6-Nitrobenzylthioguanine riboside (100 μM)	45.8 ± 3.3**	93.3 ± 7.1
$O_2NC_6H_4S$	H	6-Nitrobenzylthioinosine (100 μM)	41.3 ± 6.1**	88.1 ± 2.3
OH	NH_2	Guanosine	44.6 ± 7.1**	44.2 ± 5.3**
OH	H	Inosine	43.2 ± 2.6**	107.7 ± 4.6
SH	NH_2	5-Mercaptoguanosine	58.6 ± 3.4**	91.5 ± 5.2
NH_2	H	8-Bromoadenosine	64.3 ± 7.0*	74.6 ± 6.1*

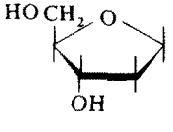
(a) Changes in groups attached to the purine ring (adenosine is shown in the syn conformation)

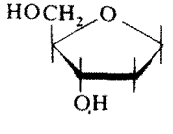
(b) Changes in the ribose moiety of the nucleosides



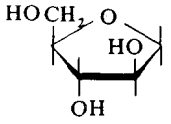
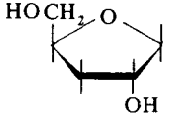
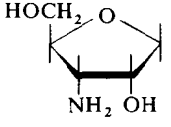
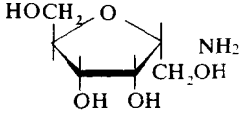




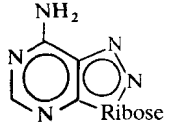
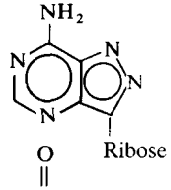
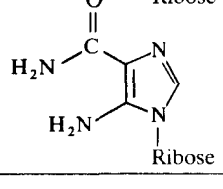




R	R^1			
NH_2	H	5′-Deoxyadenosine	6.0 ± 1.6**	7.0 ± 2.2*
NH_2	H	Adenine xyloside	11.2 ± 1.3**	26.7 ± 2.4**
NH_2	NH_2	2,6-Diaminopurine-2′-deoxyriboside	36.0 ± 2.6**	76.2 ± 1.4
NH_2	H	2′-Deoxyadenosine	33.2 ± 2.4**	104.9 ± 2.9

	NH_2	H	Adenine arabinoside	53.0 ± 3.3*	90.2 ± 4.9
	NH_2	H	3'-Deoxyadenosine	47.0 ± 5.6**	42.3 ± 1.4**
	$N(CH_3)_2$	H	Puromycin aminonucleoside	89.8 ± 4.2	87.8 ± 5.5
	NH_2	H	Psicofuranine	95.3 ± 6.5	94.8 ± 3.6

(c) Changes in the purine ring itself

	Tubercidin	32.4 ± 2.8**	72.4 ± 3.8*
	Formycin	66.0 ± 5.5	84.4 ± 6.2
	(4-Amino-5-imidazole)carboxamide riboside	79.6 ± 4.3*	68.7 ± 4.8*

Values shown are mean percentages of control ± S.E.M. (N = 4). *$0.005 < P < 0.05$, **$P < 0.005$, with respect to control as determined with *t*-test.

Counter (Coulter Electronics, Harpenden, England). Adenosine uptake was measured with the pellet technique as described previously [8] with adaptation of the volumes for addition of the inhibitor: 0.9 ml platelet suspension and 0.1 ml inhibitor in buffer (final concentration of inhibitor: 1 mM) together with 0.25 ml radioactive adenosine solution. The inhibitor was preincubated for 5 min with the platelets at 37°. The uptake of adenosine was expressed as pmoles/min/10^9 platelets and calculated as initial rate from the linear part of the time course curve.

In Tables 1, 2 and 3 given values designate mean percentage of control, of four experiments with S.E.M., adenosine uptake without inhibitor being 100 per cent.

The effect of most inhibitors on the high affinity system was studied at an adenosine concentration of 5 μM. For calculation of the amounts of adenosine transported through the high affinity system at 5 μM and 5 mM, use was made of previously determined K_m and V_{max} values for the low affinity system [8] while for the K_m and V_{max} values of the high affinity system 9.8 μM and 398 pmoles/min/10^9 platelets were used. These last two values were calculated for the pellet technique [8] utilized in the present study.

On the basis of these calculations, 70 per cent of total transport at 5 μM adenosine is through the high affinity uptake system while at 5 mM adenosine, 99 per cent of transport is through the low affinity uptake system. Significance of differences was calculated with Student's t test for paired observations.

RESULTS

The effects of various nucleosides on adenosine transport are shown in Table 1 (a–c). Most nucleosides at the concentration of 1 mM were powerful inhibitors ($P < 0.001$) in the 5 μM adenosine experiments. It should be remembered that 30 per cent of the transport at this concentration is through the low affinity system. Complete inhibition of the high affinity system without effect on the low affinity system would lead to a transport of 30 per cent of control.

Guanosine (Table 1a), 5′-deoxyadenosine and adenine xyloside and 3′-deoxyadenosine (Table 1b) were not only powerful inhibitors of the low affinity system ($P < 0.0005$) but also of the high affinity system ($P < 0.001$).

Three of the most interesting substances, 2-chloroadenosine, 6-nitrobenzylthioguanine riboside and 6-nitrobenzylthioinosine were studied in more detail. All three were competitive inhibitors of the high affinity system. The data for 2-chloroadenosine are shown in Fig. 1. The K_i value was 75.3 ± 23.2 μM(mean ± S.E.M., n = 7). For the other two compounds similar curves were found (not shown). The K_i value for 6-nitrobenzylthioguanine riboside was 21.7 ± 9.1 μM (mean ± S.E.M., n = 4) and that for 6-nitrobenzylthioinosine 9.2 ± 6.2 μM (mean ± S.E.M., n = 4). The pyrimidines (uracil, cytosine, thymine) had no and the pyrimidine nucleosides (uridine, cytidine, thymidine) had little inhibitory effect on adenosine transport through both uptake systems (not shown).

Various purines were also tested. They showed little inhibition of the high affinity system (Table 2 a,b) as demonstrated by the small decrease caused by these substances, in a concentration of 1 mM at an adenosine concentration of 5 μM. Relatively more potent inhibition was seen at high adenosine concentration. The most powerful inhibitor was

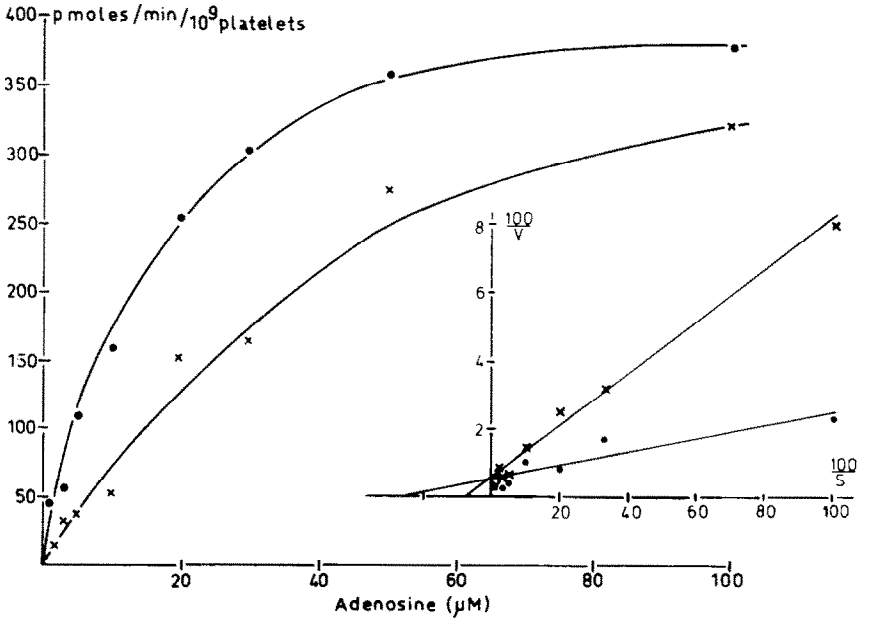
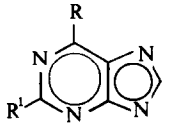


Fig. 1. Influence of 2-chloroadenosine on the uptake of adenosine in the micromolar range. ●——● Adenosine control uptake; ×——× adenosine uptake in the presence of 75 μM 2-chloroadenosine. In this figure, total adenosine uptake has been corrected for the low affinity uptake, by subtraction of the rectilinear part (for details see ref. [8]). Inset: Lineweaver–Burk plot of both curves show a competitive relationship. A representative experiment is shown.

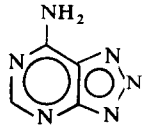
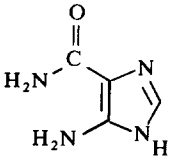
Table 2. Purines used for the study of inhibition of adenosine uptake

(a) Changes in groups attached to the purine ring



R	R^1	Inhibitor (concentration 1mM)	Adenosine concentration 5 μM % of control ± S.E.M.	5 mM
NH_2	H		52.3 ± 7.8*	6.8 ± 1.1**
$NHCH_3$	H	Adenine 6-*N*-Methylaminopurine	54.9 ± 2.4*	27.6 ± 0.4**
$N(CH_3)_2$	H	6,6-*N*,*N*- Dimethylaminopurine	51.1 ± 2.8*	56.4 ± 2.1*
NH_2	H	Adenine-N^1-oxide	64.8 ± 8.8	28.5 ± 2.0**
NH_2	H	8-Bromoadenine	49.0 ± 9.1*	31.2 ± 3.0**
SH	H	6-Mercaptopurine	82.6 ± 3.0	51.2 ± 7.2*
OH	H	Hypoxanthine	87.3 ± 0.7	59.4 ± 8.8*
H	H	Purine	82.4 ± 4.4	61.8 ± 1.0*
OH	NH_2	Guanine	84.3 ± 7.1	40.1 ± 5.8**
SH	NH_2	6-Mercaptoguanine	77.5 ± 2.2	41.0 ± 2.4**
NH_2	NH_2	2,6-Diaminopurine	71.4 ± 4.4	75.6 ± 2.7*

(b) Changes in the purine ring itself

	8-Azaadenine	93.5 ± 2.0	77.4 ± 5.3
	(4-Amino-5-imidazole) carboxamide	76.0 ± 4.0*	74.7 ± 2.2

Values shown are mean percentages of control ± S.E.M. (n = 4).
* $0.005 < P < 0.05$, ** $P < 0.005$, with respect to control as determined with *t*-test.

adenine, it was shown to be a competitive inhibitor with the low K_i of 5.8 ± 2.9 μM (mean ± S.E.M., n = 3) [8].

The results obtained with the pyrimido-pyrimidines are summarized in Table 3. Of these substances only the pyrimido-pyrimidines were inhibitory at 5 μM adenosine, while the thienopyrimidines had no effect on adenosine transport. Dipyridamole, the best known substance of these series was studied in detail. It appeared to be a competitive inhibitor of the high affinity uptake system (K_i 47.2 ± 28.7 μM, mean ± S.E.M., n = 3).

DISCUSSION

In our previous paper on adenosine uptake and metabolism in human blood platelets [8] we described that adenosine is taken up by two independent, carrier mediated transport systems, one of which had a high affinity for adenosine (K_m 9.8 μM) while the other had a much lower affinity (K_m 9.4 mM). We previously reported that the low affinity adenosine uptake system is different from the uptake system for adenine [16].

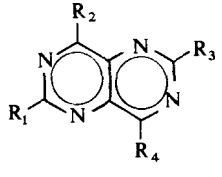
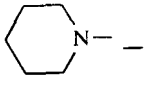
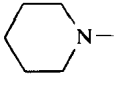
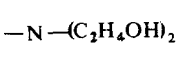
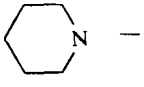
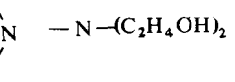
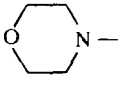
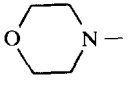
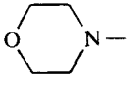
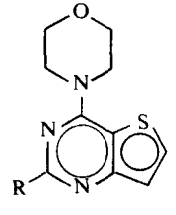
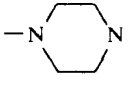
In this paper we present further evidence for differences in both adenosine uptake systems. This evidence is based on differences in steric requirements for both carrier molecules.

Specificity of the high affinity system

The nucleosides shown in Table 1 are grouped according to structural differences in groups attached to the purine ring (1a), differences in the ribose moiety (1b) or differences in the purine ring itself (1c). The purines in Table 2 are also grouped in the order of variations in the attached groups (2a) or in the purine ring (2b).

Gross comparison between Tables 1 and 2 indicates that the high affinity system is inhibited by nucleosides but not by purines. Changes in position 6 due to the presence of hydrogen, a negatively charged mercapto group, a non charged methylamino or dimethyl-amino or even a bulky nitrobenzylthio group did not influence the inhibitory potential. Introduction of an amino-group at position 2 increased the sensitivity for the electronegativity of the charge at position 6, as shown by the decrease in inhibition obtained by 2,6 diaminopurine 2′-deoxyriboside compared with 6-mercaptoguanosine ($P < 0.005$; Table 1a and b).

Table 3. Pyrimido pyrimidines and thieno pyrimidines

	R_1	R_2	R_3	R_4		Adenosine concentration 5 μM % of control ± S.E.M.	Adenosine concentration 5 mM % of control ± S.E.M.
	$-N-(C_2H_4OH)_2$		$-N-(C_2H_4OH)_2$		RA 8 (Dipyridamole)	45.6 ± 2.4*	73.0 ± 5.0*
				H	RA 233	39.9 ± 7.4*	84.2 ± 5.4
				H	RA433	32.1 ± 4.9*	71.0 ± 3.5
	R				VK 744	104.8 ± 15.2	89.8 ± 5.6
					VK 774	99.0 ± 12.5	97.1 ± 4.8

Inhibitory influence on adenosine uptake.
* Significant at the $0.005 < P < 0.05$ level with respect to control.

The lack of inhibition by 8-bromoadenosine is also of interest. The bulky bromide at position 8 probably hinders the free rotation around the N-9-C-1′ bond and forces adenosine into the syn-conformation [10] instead of the usual anti-conformation.

The furanose ring seems required for inhibition of the high affinity system. The 5′-carbinol group is of no importance for substrate recognition as demonstrated by the powerful inhibition by 5′-deoxyadenosine. More important are the 2′ and 3′ hydroxyl groups, although the 2′ hydroxyl group itself is not crucial, as demonstrated by the inhibition caused by several 2′deoxyribosides. The moderate inhibition by adenine arabinoside in comparison with adenine xyloside indicates that the orientation of the 2′ hydroxyl group with respect to the 2′-3′ carbon bond may be of importance.

The replacement of the 3′ hydroxyl group by either a hydrogen (3′ deoxyadenosine) or an amino group (puromycine amino nucleoside) causes a loss of inhibitory potential, indicating the importance of this hydroxyl group.

Our experiments allow no definite conclusions as to the importance of the erythro versus the threo configurations, although it should be mentioned that adenine xyloside is a powerful inhibitor.

A bulky carbinol group attached to the 1′ carbon atom (psicofuranine) causes a complete loss of inhibition. This may indicate that this 1′ carbon atom is of importance but steric hindrance for position 2′ cannot be excluded (Table 1b).

Changes in the purine ring system show that the only tolerated change is at position 7 (tubercidin). When position 8 and 9 are involved, as in formycin, there is much less inhibition. That changes at the 9 position are more important might be concluded from the inhibition by 8-bromoadenine (Table 2a). This compound, although it is not a very powerful inhibitor at low adenosine concentration, is at least as strong as adenine itself, in inhibiting the high affinity system. The pyrimidine ring of the purine ring must be intact for substrate recognition of both uptake systems, as shown by the absence of effect of (4-amino-5-imidazole) carboxamide and its riboside (Table 1c and 2b).

Specificity of the low affinity system

Comparison of Tables 1 and 2 shows that the low affinity adenosine uptake mainly is influenced by purines. This raises the question whether the low affinity transport system represents a purine transport system. In our earlier report on adenosine uptake and metabolism in human blood platelets [8] we presented evidence suggesting that this is not the case, as far as adenine is concerned. The possibility that guanine or hypoxanthine are handled by this transport system needs to be elucidated.

The substituents attached to position 6 of the purine ring play an appreciable role in substrate recognition of the low affinity system. The importance of an amino group (adenine) is stressed by the difference in inhibition found for a mercapto or hydroxyl group or a hydrogen atom: there is a decrease in inhibition. The bulkiness of the group at position 6 is also important. 6-Aminopurine (adenine) inhibits more than 6-*N*-methylaminopurine and this in turn more than 6,6-*N*,*N*-dimethylaminopurine ($P < 0{,}001$ for both with respect to adenine). Purines with substitution at the 2 and 6 position show more inhibition, when the group at position 6 is more electronegative, e.g. guanine and 6-mercaptoguanine are more powerful inhibitors of the low affinity system than 2,6-diaminopurine (Table 2a). The importance of the purine ring itself is shown by the lack of inhibition by compounds with changes in the purine ring (azaadenine, (4-amino-5-imidazole)-carboxamide, formycin and tubercidin; Tables 1c and 2b).

Purines are stronger inhibitors than nucleosides, but changes in the riboside ring can also play a role. Presence of the 2′-hydroxyl group seems essential. Only nucleosides with a hydroxyl group in this position (5′-deoxyadenosine, adenosine xyloside, 3′deoxy adenosine and guanosine) were inhibitory.

Substitution at the 1′ position by a carbinol group as in psicofuranine abolished inhibition, either because the 1′ hydrogen atom is essential or due to a steric effect at the 2′ position.

The substituent at the 3′ position is less important: 3′deoxyadenosine and adenine xyloside are relatively good inhibitors ($P < 0.0005$) (Table 1b).

The observations, described in this paper, regarding the carrier specificity of both adenosine uptake systems correspond to a large extent to the adenosine uptake system described for rabbit polymorphonuclear leukocytes, [5] and a membrane carrier for adenosine in canine heart [10]. Although in the leukocytes pyrimidines could also be transported, substitution on the purine ring caused a decrease in affinity and the 3′ hydroxyl appeared to be the most important place of the furanose ring. In canine heart, the same holds true for the purine ring, but the 2′ and 3′ hydroxyls of the furanose ring were equally important. The finding that purines may be important for substrate recognition has recently been demonstrated [9].

In rabbit erythrocytes an intracellular adenine analogue binding protein has recently been demonstrated [16]. Although the existence of such a protein in human platelets is doubtful, the possibility cannot be excluded that adenine displaces adenosine from this protein, thus diluting the specific radioactivity of the added adenosine and consequently mimicking inhibition of adenosine transport. However, it should be mentioned that no efflux of once taken up radioactive adenosine was ever observed [8]. Another argument against adenine displacing adenosine from intracellular binding sites may be derived from adenine transport investigations [17, 18]. No evidence of free adenine inside the platelets was found in these studies.

Some of the compounds tested are inhibitors or substrates for adenosine metabolizing enzymes [19]. One of these enzymes, adenosine kinase, might be directly involved in transport of adenosine [8]. However, the K_m found of this enzyme (2 μM, ref. [20] differs from the K_m found for transport (9.8 μM) indicating that transport of adenosine is rate limiting. Other adenosine metabolizing enzymes are probably located intracellularly [20] and consequently play no role in inhibition of transport.

One might speculate about the physiological

importance of two different adenosine uptake systems, one of which is probably of minor relevance, since at physiological adenosine concentrations (0.3 μM, ref [21]) only a small part (< 10 per cent) of total adenosine taken up is transported by the low affinity uptake system. This uptake system is unlikely to be involved in adenine uptake taking into account the mutual differences in K_m values for uptake and K_i values for inhibition [8, 18, 22]. The actual role of the low affinity adenosine uptake system in human blood platelets needs further investigation.

Dipyridamole is a drug originally developed as a coronary vasodilator [23]. In the course of time several mechanisms were offered to explain its action. Among these are influence on deamination of adenosine in blood [24], inhibition of phosphodiesterase from human platelets [25, 26], and inhibition of the elimination of adenosine from blood by uptake in different cells.

It was shown that adenosine uptake was inhibited by dipyridamole in human red cells [6, 11], in human blood platelets [27, 28] and in cultured pig endothelial cells [9]. The site of interaction of dipyridamole with platelets was suggested to be located at the platelet membrane [28–30]. The inhibition of adenosine uptake by dipyridamole and some related substances, as described in this paper, is a confirmation of an earlier report [28]. However, only one adenosine concentration was used in this study. We have found that the pyrimido-pyrimidines inhibit only the high affinity component of adenosine uptake. The thienopyrimidine compounds VK 744 and VK 774 (Table 3) had no effect on either high and low affinity adenosine uptake. Of special interest is the finding that dipyridamole is a competitive inhibitor of the high affinity adenosine uptake system. This is a further [28–30] confirmation that the site of interaction of dipyridamole with human platelets probably is located at the platelet membrane.

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