Biochemical Pharmacology, Vol. 37, No. 4, pp. 621–626, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ADENOSINE METABOLISM IN HUMAN WHOLE BLOOD

EFFECTS OF NUCLEOSIDE TRANSPORT INHIBITORS AND PHOSPHATE CONCENTRATION*

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(*Received* 18 *September* 1986; *accepted* 6 *July* 1987)

Abstract—Adenosine (Ado, 10 μM) was metabolized in whole blood within 1 min, primarily to hypoxanthine and ATP. The concentration of Ado, the activities of adenosine deaminase (ADA) and Ado kinase, the K_m values for Ado with ADA and Ado kinase, and the substrate inhibition of Ado kinase are factors that govern the Ado metabolism between deamination and phosphorylation. If ADA activity was blocked by 2′-deoxycoformycin (dCF, 5 μM), a tight-binding inhibitor of ADA, most of the Ado (96%) was incorporated into adenine nucleotides, whereas if Ado kinase activity was blocked with 5-iodotubercidin (10 μM), Ado was mainly (95%) metabolized into hypoxanthine. A high phosphate concentration (25 mM) caused marked increases in the formation of IMP. The nucleoside transport inhibitors dilazep (1 μM), dipyridamole (10 μM) and nitrobenzylthioinosine (NBMPR, 1 μM) strongly blocked cellular Ado metabolism. In the presence of nucleoside transport inhibitors, Ado which slowly enters the cell was metabolized principally by Ado kinase rather than ADA. Dilazep, NBMPR and dipyridamole were more effective in blocking Ado uptake and metabolism by erythrocytes suspended in a protein-free medium than by cells suspended in plasma.

Adenosine (Ado)§ is an important regulator of many physiologic functions including vascular blood flow, platelet aggregation, lipolysis and neurotransmission [1–3]. Many Ado effects are mediated by its binding to specific receptors coupled to membrane-bound adenylate cyclase. Depending on the cell or tissue type, Ado may stimulate or inhibit adenylate cyclase [2, 3].

Many tissues such as vascular endothelium, brain, kidney and skeletal muscle produce Ado [3]. However, in humans, plasma Ado levels are low (< 0.4 μM) presumably due to rapid cellular transport and metabolism [4]. Several studies have shown that erythrocytes quickly take up and metabolize Ado via adenosine deaminase (ADA) and adenosine kinase (Ado kinase) [5–8]. However, most prior studies examined erythrocytic Ado uptake and metabolism under non-physiologic conditions such as high concentrations of Ado and/or in artificial media. The present studies investigate the metabolism of Ado in whole blood and thus reflect more closely *in vivo* events. Also examined are the effects of phosphate concentration and the nucleoside transport inhibitors dilazep, dipyridamole and nitrobenzylthioinosine (NBMPR) on Ado metabolism in whole blood. *In vitro*, these nucleoside transport inhibitors display high affinity for the nucleoside transport system, i.e. apparent K_i (inhibition constant of nucleoside transport) values of 50 nM or less [9–12]. Dilazep and dipyridamole are used in the clinic as vasodilators and antiplatelet drugs [13–16]. Recent evidence from this laboratory indicates that these agents do not directly exert antiplatelet actions but rather inhibit the membrane transport of Ado and thus cellular metabolism of Ado [17–19]. Presumably this elevates the steady-state plasma levels of Ado to the vasodilatory and antiplatelet range.

This report emphasizes the measurement of Ado and its metabolites during the first minute of interaction with whole blood and determines the synthesis of intraerythrocytic nucleotides under various experimental conditions.

MATERIALS AND METHODS

Adenosine (Ado) and dipyridamole (Persantine) were obtained from the Sigma Chemical Co., St. Louis, MO. Dilazep (Cormelian) was supplied by Hoffmann–LaRoche, Inc., Nutley, NJ. RA 233 was a gift from Dr. Karl Thomae GmbH, Biberach an der Riss, Germany. Nitrobenzylthioinosine (NBMPR) was supplied by Dr. A. R. P. Paterson, University of Alberta, Alberta, Canada. 5-Iodotubercidin was a gift from Dr. L. B. Townsend, University of Michigan, Ann Arbor, MI. 2′-Deoxycoformycin (dCF, Pentostatin) was obtained from the Drug Development Branch, Division of Cancer Treatment of the National Cancer Institute, Bethesda, MD. [8-

* This work was supported by PHS Grants CA 07340 and 09204 and by funds from G. D. Searle & Co.

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§ Abbreviations: Ado, adenosine; Hx, hypoxanthine; Ino, inosine; Nuc, total nucleotides; ADA, adenosine deaminase; Ado kinase, adenosine kinase; dCF, 2′-deoxycoformycin; and NBMPR, nitrobenzylthioinosine.

[^{14}C]Ado (sp. act. = 59 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA, and was greater than 95% pure as shown by thin-layer chromatography (TLC) on polyethyleneimine (PEI)-cellulose sheets (J. T. Baker Chemical Co., Phillipsburg, NJ) with potassium chloride (100 mM) as the eluent. All other materials were obtained from commercially available sources.

Blood collection. Blood was drawn from healthy donors who had not taken any medication for at least 7 days prior to blood collection. The blood was dispensed into plastic tubes containing 0.1 volume of sodium citrate (3.8%) to prevent coagulation. Ado uptake and metabolism were studied in citrated whole blood (hematocrits = 34–38%) or in suspensions of erythrocytes in cell-free plasma or in a low-phosphate (1 mM), protein-free medium. For these studies, citrated whole blood was centrifuged (300 *g*, 10 min) and platelet-rich plasma was removed. Platelet-rich plasma was centrifuged (1200 *g*, 2 min) to obtain cell-free plasma. The buffy coat was removed, and the erythrocytes were suspended in cell-free plasma or thrice washed in a medium containing potassium phosphate (1 mM, pH 7.4), sodium chloride (128 mM), magnesium chloride (2 mM) and glucose (10 mM). Washed erythrocytes were resuspended in this low-phosphate, protein-free medium to the same hematocrit as that in plasma. Concentrations of Ado and nucleoside transport inhibitors are based on total volumes not extracellular volumes. To convert these concentrations to plasma concentrations at zero time, multiplication by a factor of about 1.5 seems appropriate. It must be noted, however, that in the absence of transport inhibitors, the equilibration of adenosine between plasma and erythrocytes is very rapid, whereas in the presence of nucleoside transport inhibitors the plasma concentration decreases much more slowly, e.g. $T_{\frac{1}{2}} \cong 5$ min (Fig. 4). Thus, it would be necessary to determine Ado concentrations directly in timed samples. Similarly, the plasma levels of free nucleoside transport inhibitors are affected by nonspecific binding and cellular uptake. Once again, direct measurement of free inhibitor levels in timed plasma samples would be necessary to determine actual plasma concentrations. Experiments were completed within 4 hr after blood collection.

Ado uptake and metabolism. [^{14}C]Ado metabolism was determined at 37° in whole blood or in mixtures of erythrocytes suspended in cell-free plasma or a low-phosphate, protein-free medium. After adding [^{14}C]Ado (10 μM, sp. act. = 10 μCi/μmol), aliquots (100 μl) were removed at various times and immediately added with rapid mixing to ice-cold perchloric acid (PCA) (25 μl, 20%). After removing the precipitated proteins by centrifugation (12,000 *g*, 4 min, 4°), aliquots (75 μl) of supernatant fraction were removed and added to tubes containing potassium phosphate (50 mM, pH 7.4) and phenol red (0.0005%). Samples were then neutralized with potassium hydroxide (5 N), and the precipitated potassium perchlorate was removed by centrifugation.

Aliquots (10 μl, 1200–1500 cpm) containing [^{14}C]Ado and its metabolites (approx. 60 pmol) were applied on TLC silica gel plates, and the metabolites (hypoxanthine, Hx; inosine, Ino; and total nucleotides, Nuc) were separated as described [19]. Nucleotides (AMP, ADP, ATP and IMP) were separated by TLC on PEI-cellulose plates using a modification of the method of Leech and Newsholme [20]. PEI-cellulose plates were first developed with methanol (50%) and then with a mixture of formic acid (1.0 M) and lithium chloride (0.25 M). Individual spots corresponding to Ado and metabolites were visualized in ultraviolet light and cut out, and the ^{14}C-activity was determined in aqueous counting scintillant (ACS, Amersham, Arlington Heights, IL) using a Packard Tri-Carb liquid scintillation spectrometer (model 460 C). Radioactivity was eluted from the PEI-cellulose plates with hydrochloric acid (1 ml, 1 N) before adding ACS.

To determine the effects of various agents on Ado metabolism, citrated whole blood (1 ml) or suspensions of erythrocytes in plasma or medium was preincubated for 3 min at 37° with dipyridamole (1 and 10 μM), dilazep (1 and 10 μM), NBMPR (1 and 3 μM) or RA 233 (10 μM) or for 5 min with 5-iodotubercidin (10 μM), dCF (5 μM) or potassium phosphate (25 mM, pH 7.4) before adding [^{14}C]Ado. The reactants were added in a total volume of 25 μl, and normal saline or ethanol (0.2%, v/v) replaced drugs in control reactions. Total reaction volumes were 1.025 ml. The percentages of total cpm were obtained by dividing the cpm associated with Ado and each metabolite by the total cpm spotted on the TLC plates and multiplying by 100.

Ado kinase assay. Hemolysate was prepared from washed erythrocytes isolated from freshly drawn human blood. An aliquot of washed erythrocyte suspension (40%) was diluted 5-fold with potassium phosphate buffer (50 mM, pH 7.4) and hemolyzed by freezing and thawing twice. The stroma were removed by centrifugation at 12,000 *g* for 5 min at 4°. Ado kinase activity was measured in the hemolysate after 10-fold dilution with Tris–HCl (100 mM, pH 7.4).

The reaction mixture (200 μl) contained Tris–HCl, pH 7.4, 100 mM; $MgCl_2$, 1 mM; GTP, 1 mM; dCF, 5 μM; and Ado, 1.5 μM. The reaction was started by adding hemolysate (20 μl). The assay method was similar to one published by Plagemann *et al.* [8] except for the dCF concentration (5 μM) and the reaction temperature (37°). Adenine nucleotides were separated from Ado using PEI-cellulose plates and 50% methanol as eluent.

RESULTS AND DISCUSSION

Figure 1 demonstrates the rapid removal of high levels of Ado (10 μM) from whole blood. In 15 sec, more than 50% of the Ado had disappeared. This $T_{\frac{1}{2}}$ for 10 μM Ado (< 15 sec) is consistent with earlier reports [21]. In 1 min, metabolism was complete, the main (60%) product being hypoxanthine. This is explained by the high levels of purine nucleoside phosphorylase (13.5 EU/ml) activity in human erythrocytes [22]. About 40% of the Ado was phosphorylated to adenine nucleotides, mainly ATP. Ado (10 μM) was preferentially deaminated by ADA rather than phosphorylated by Ado kinase. This may be partly explained by substrate inhibition of Ado

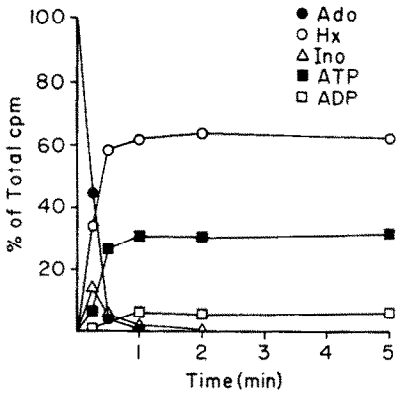

Fig. 1. Ado metabolism in whole blood. Human whole blood was incubated with [^{14}C]Ado (10 μM), for 0–5 min at 37°. Ado and its metabolites were measured in PCA extracts by a TLC method as described in Materials and Methods. The values represent the means of three experiments performed in duplicate.

kinase by Ado concentrations greater than 5 μM [7, 8, 23] and the higher activity of ADA (0.2 to 0.3 EU/ml) than Ado kinase (0.03 EU/ml) in erythrocytes [5, 23, 24]. Previously, we showed that, at low concentration (200 nM), Ado is almost completely (> 95%) phosphorylated in blood cells to adenine nucleotides in 1 min [19]. Therefore, Ado, at low (200 nM) or high (10 μM) concentration, is rapidly metabolized in whole blood. However, the pathway(s) of metabolism for a high concentration of Ado is different from that for a low concentration of Ado. The K_m values for Ado with Ado kinase and ADA are < 1 μM and 25–30 μM respectively [24–26]. Thus, the relative proportions of Ado metabolism by ADA and Ado kinase, at specific Ado concentrations, may be explained by the K_m values for Ado with ADA and Ado kinase, the relative enzyme activities, and substrate inhibition of Ado kinase. As Plagemann *et al.* [8] have reported, the first-order rate constants for Ado transport and phosphorylation are about the same, whereas that for deamination is about 10-fold lower. During tissue breakdown, when marked increases in Ado concentrations may occur, deamination by ADA should be favored, whereas under normal physiologic conditions phosphorylation by Ado kinase should predominate. Interestingly, under the present experimental conditions, very small amounts of hypoxanthine were converted to IMP by hypoxanthine-guanine phosphoribosyl transferase which is in disagreement with prior findings with washed erythrocytes [5, 24].

When the ADA activity was blocked by dCF (5 μM), a tight-binding inhibitor of ADA [27], Ado (10 μM) was incorporated into the adenine nucleotides with an ATP:ADP ratio of 8:1 (Fig. 2A). However, when the blood was preincubated with 5-iodotubercidin (10 μM) to block Ado kinase [28], the major metabolite (95%) was hypoxanthine rather than IMP, whereas synthesis of adenine nucleotides was not detected (Fig. 2B). The earlier studies, in which substantial IMP formation was seen, examined erythrocytic Ado metabolism in a plasma-free medium containing high phosphate concentrations (18–50 mM) [5, 24]. In the present studies with whole blood, the addition of phosphate (25 mM) resulted in high levels of IMP synthesis (Fig. 3). Phosphate-dependent IMP synthesis has also been observed by Hawkins *et al.* [7] and Plagemann [29] in plasma-free media. These findings are in agreement with the concept that high phosphate concentrations are needed to overcome the feedback inhibition of 5-phosphoribosyl-1-pyrophosphate synthetase by erythrocytic ADP, GDP and 2,3-diphosphoglycerate [30].

In the presence of the nucleoside transport inhibitors dilazep (1 μM), dipyridamole (10 μM) or NBMPR (1 μM), the rate of Ado disappearance was reduced (Fig. 4). Interestingly, although the transport inhibitors have apparent K_i (inhibition constant of nucleoside transport) values of 50 nM or less *in vitro* [9–12], there were definite differences in their

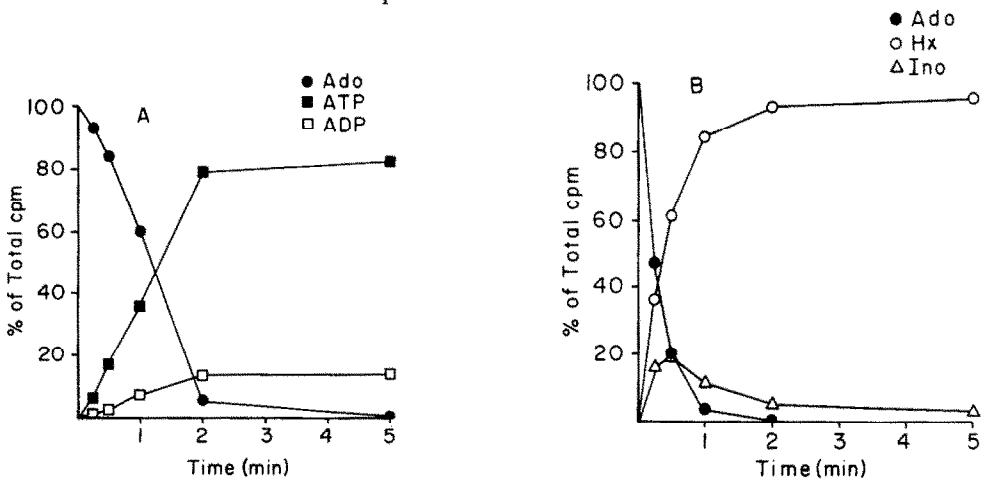

Fig. 2. Effects of dCF (A) and 5-iodotubercidin (B) on Ado metabolism in whole blood. Human whole blood was preincubated with dCF (5 μM) or 5-iodotubercidin (10 μM) for 5 min at 37° before the incubation with Ado (10 μM). Ado and its metabolites were measured in PCA extracts by a TLC method as described in Materials and Methods. The values represent the means of three experiments performed in duplicate.

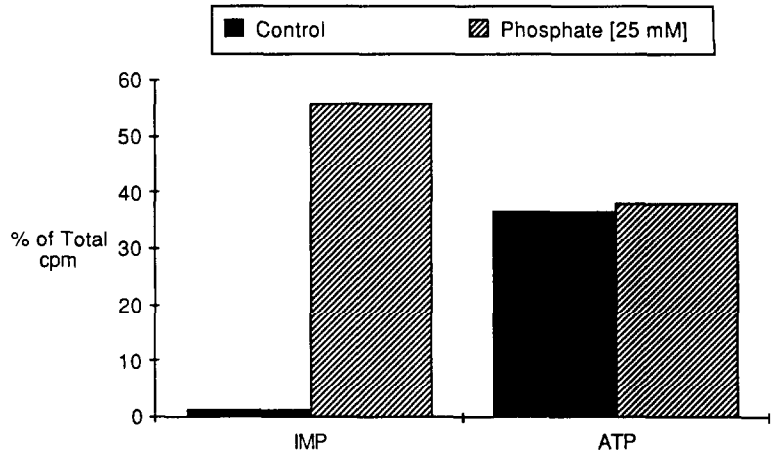

Fig. 3. Effect of phosphate on Ado metabolism into IMP and ATP in whole blood. Human whole blood was preincubated with potassium phosphate (25 mM, pH 7.4) for 5 min at 37° before the incubation with Ado (10 μM). The nucleotides were measured in PCA extracts by a TLC method as described in Materials and Methods. The values represent the means of one experiment performed in duplicate.

capacities to block Ado metabolism in whole blood at concentrations greater than ten times their K_i values. These results may reflect differences in non-specific binding to plasma proteins and to cells. As reported by Mahony *et al.* [31], the average binding of dipyridamole to plasma proteins is 99.13 ± 0.24% with the free drug ranging from 0.55 to 1.19%. Furthermore, concentrations of nucleoside transport inhibitors that are much greater than reported K_i values [9–12] are required to block nucleoside uptake in whole blood; this may be explained in part by the large number of NBMPR binding sites (nucleoside transport sites) in the erythrocytes of whole blood, i.e. about 10^{-7} molar equivalents per liter [18]. RA 233 (10 μM), a close structural analog of dipyridamole, did not block Ado disappearance, which indicates that the piperidine ring on C-8 of the pyrimidopyrimidine compound is important for inhibition of nucleoside transport.

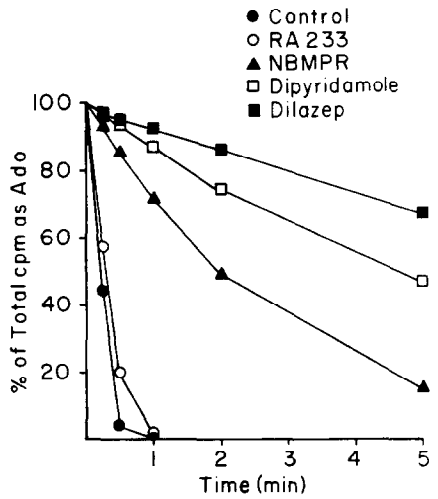

Fig. 4. Effects of RA 233, NBMPR, dipyridamole and dilazep on Ado disappearance in whole blood. Human whole blood was preincubated with RA 233 (10 μM), NBMPR (1 μM), dipyridamole (10 μM) or dilazep (1 μM) for 3 min before the incubation with Ado (10 μM). Ado was measured in PCA extracts by a TLC method as described in Materials and Methods. The values represent the means of three experiments performed in duplicate.

The influence of plasma on the behavior of the nucleoside transport inhibitors was investigated. Table 1 presents the effects of dilazep, NBMPR and dipyridamole on Ado uptake and metabolism by washed erythrocytes resuspended in cell-free plasma or in protein-free medium containing low phosphate (1 mM). Within 1 min, the Ado (10 μM) was metabolized completely by erythrocytes in both plasma and artificial medium. This suggests that platelets and leukocytes contribute minimally to the disappearance of Ado from whole blood. Plasma proteins had a slight effect on the ability of dilazep (10 and 1 μM) to block the uptake and metabolism of Ado. Some protein binding may occur, however, as evidenced by the slight increase in the level of Ado and the decreased amount of nucleotides formed by erythrocytes in artificial medium as compared with the cells suspended in plasma. Typical plasma samples deaminated Ado (10 μM) at a rate of about 0.5 $nmol \cdot ml^{-1} \cdot min^{-1}$. In the presence of dilazep, about 15% of the Ado was removed in 5 min. This decrease was presumably due to non-mediated diffusion which transport inhibitors do not block [10, 12]. In contrast to the findings with dilazep, the activities of both NBMPR and dipyridamole were considerably lower both in the presence and absence of plasma. Previously, we have noted the apparent discrepancy between the relatively low K_i values of the nucleoside transport inhibitors ($< 10^{-8}$ M) and the substantially higher levels required to show inhibition in whole blood ($> 1 \times 10^{-6}$ M) [17, 18]. Although the relatively high concentration of erythrocyte nucleoside transporters (about 10^{-7} molar equivalents/liter of whole blood) offers a partial explanation, the present results indicate important roles for both non-specific binding to erythrocytes and to plasma proteins.

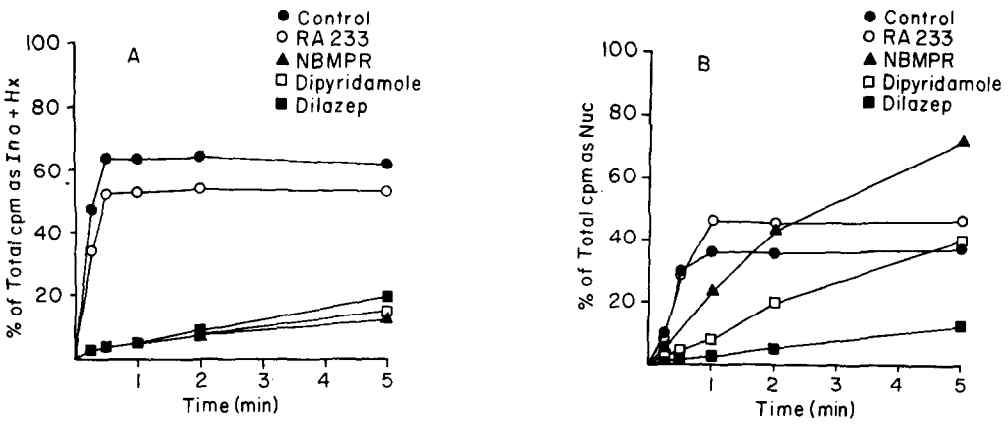

Fig. 5. Effects of RA 233, NBMPR, dipyridamole and dilazep on Ado metabolism into (A) inosine (Ino) and hypoxanthine (Hx) and (B) total nucleotides (Nuc) in whole blood. Human whole blood was preincubated with RA 233 (10 μM), NBMPR (1 μM), dipyridamole (10 μM) or dilazep (1 μM) for 3 min at 37° before the incubation with Ado (10 μM). The metabolites were measured in PCA extracts by a TLC method as described in Materials and Methods. The values represent the means of three experiments performed in duplicate.

The relative amounts of Ado deaminated and phosphorolyzed were about the same when the blood was preincubated with dilazep, dipyridamole or NBMPR (Fig. 5A). In the presence of a nucleoside transport inhibitor, most of the Ado that entered the cell was metabolized by Ado kinase to adenine nucleotides (Fig. 5B) in agreement with the earlier reports of Agarwal and Parks [32] and Plagemann [29]. Presumably, in the presence of nucleoside transport inhibitors, Ado enters cells slowly so that the intracellular Ado concentration falls in the range of the K_m for Ado kinase, rather than for ADA, without inhibiting Ado kinase. Consequently, the amount of nucleotides formed in the presence of nucleoside transport inhibitors varies inversely with the degree of inhibition of Ado uptake. We have reported earlier the effects of the nucleoside transport inhibitors on the metabolism of a lower concentration of Ado (200 nM) [19]. Similar rates of Ado uptake and metabolism were seen at an initial Ado concentration of 200 nM as at 10 μM. The nucleoside transport inhibitors dilazep (10 μM), dipyridamole (10 μM) and NBMPR (6.6 μM) had no inhibitory effects on human erythrocytic Ado kinase (data not shown).

Previously, on the basis of marked differences in the incorporation of Ado into the nucleotide pools of washed human erythrocytes in high-phosphate medium in the presence and absence of the nucleoside transport inhibitor *p*-nitrobenzylthioguanosine [32], it was postulated that ADA may be physically

Table 1. Effects of nucleoside transport inhibitors on erythrocytic adenosine metabolism in plasma and protein-free medium*

Inhibitor	Concn (μM)	Time (min)	RBCs in plasma† (% of total cpm) Ado	Hx	Ino	Nuc	RBCs in medium† (% of total cpm) Ado	Hx	Ino	Nuc
Control		1	<1	57	6	36	<1	57	6	36
		5	<1	61	3	36	<1	60	3	37
Dilazep	10	1	86	6	6	2	91	5	1	3
		5	63	7	22	8	84	6	4	6
	1	1	85	4	6	4	88	6	2	4
		5	54	6	20	19	79	6	4	10
NBMPR	3	1	79	6	6	8	88	5	2	5
		5	45	7	19	29	72	6	6	16
	1	1	70	4	6	20	86	5	2	8
		5	22	8	12	58	55	5	4	36
Dipyridamole	10	1	79	6	5	10	89	4	2	6
		5	40	7	18	34	78	4	4	13
	1	1	32	13	6	48	65	4	1	28
		5	2	18	2	78	12	4	2	82

* Suspensions of erythrocytes (1 ml, 40% hematocrits) in cell-free plasma and a low-phosphate (1 mM), protein-free medium were incubated with dilazep, NBMPR or dipyridamole for 3 min at 37°. [^{14}C]Ado (10 μM, sp. act. = 10 μCi/μmol) was added, and aliquots (100 μl) were removed at 1 min and 5 min and immediately added with mixing to ice-cold perchloric acid (25 μl, 20%). ^{14}C-Activity associated with Ado and metabolites was determined as described in Materials and Methods.

† Values are the average from two experiments. Abbreviations: RBCs, red blood cells, Ado, adenosine; Hx, hypoxanthine; Ino, inosine; Nuc, total nucleotides; and NBMPR, nitrobenzylthioinosine.

associated with the cellular membrane at sites close to the nucleoside transporters. Other investigators, in a variety of non-erythrocytic tissues, have offered evidence for the association of ADA with cellular membranes [33–35]. The above evidence and other recent findings in this laboratory do not support this concept with human erythrocytes. The differences in nucleotide pool distributions can be explained adequately by the differences in K_m values and activities of ADA and Ado kinase rather than by a postulated localization of ADA at the cellular membrane. In studies of the kinetics of inactivation of ADA by dCF in intact erythrocytes, it was found that the inhibitor enters the cell by the nucleoside transport system, that the rate of ADA inactivation is linear until more than 80% of the enzyme is titrated, and that there is a 1:1 stoichiometry between dCF molecules that enter the cell and ADA molecules inactivated [12]. If a portion of the ADA molecule in the erythrocyte had been bound to the membrane, one might have expected bi- or multiphasic, rather than linear, rates of enzyme inactivation.

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