Biochemical Pharmacology, Vol. 34, No. 22, pp. 3965–3972, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ROLE OF ADENOSINE UPTAKE AND METABOLISM BY BLOOD CELLS IN THE ANTIPLATELET ACTIONS OF DIPYRIDAMOLE, DILAZEP AND NITROBENZYLTHIOINOSINE*

DOLORETTA D. DAWICKI, KAILASH C. AGARWAL† and ROBERT E. PARKS, JR.
Section of Biochemical Pharmacology, Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

(*Received* 3 *October* 1984; *accepted* 12 *April* 1985)

Abstract—Adenosine (Ado, 10 μM) did not inhibit ADP-induced human platelet aggregation in whole blood. However, if the blood was preincubated with dipyridamole (10 μM), a potent inhibitor of the erythrocytic nucleoside transport system (NTS), Ado acted as a strong inhibitor of platelet aggregation. Similarly, Ado inhibited platelet aggregation in whole blood in the presence of other potent NTS inhibitors, dilazep (1 μM) and *p*-nitrobenzylthioinosine (NBMPR, 1 μM). RA 233 (10 μM), an analog of dipyridamole which is a potent inhibitor of platelet cAMP phosphodiesterase (PDE), did not evoke the Ado effect in whole blood. However, in platelet-rich plasma (PRP), RA 233 potentiated strongly Ado-mediated inhibition, whereas dipyridamole, dilazep and NBMPR were without activity. 5′-Methylthioadenosine (MTA), an Ado receptor antagonist, reversed the inhibition produced by a nucleoside transport system inhibitor plus Ado in whole blood. Dipyridamole (10 μM), dilazep (1 μM) or NBMPR (1 μM) blocked [^{14}C]Ado (10 μM) uptake by blood cells in whole blood, whereas RA 233 (10 μM) was not effective. The combination of 2′-deoxycoformycin (dCF, 5 μM), a tight-binding inhibitor of adenosine deaminase (ADA), plus 5-iodotubercidin (ITu, 10 μM), a potent inhibitor of adenosine kinase (Ado kinase), gave comparable Ado-mediated inhibition of platelet aggregation in whole blood as was obtained when the blood was pretreated with dilazep. These studies suggest that the *in vivo* antiplatelet actions of drugs such as dipyridamole and dilazep result from their abilities to block erythrocytic Ado uptake and subsequent metabolism, thus elevating the extracellular steady-state concentration of the physiologically occurring, antiplatelet agent, Ado.

Blood platelets play a key role in normal hemostasis. Since platelet functions are greatly altered in a number of pathological conditions [1, 2], it is often useful to assess platelet functions *in vitro*. For many years the study of platelet activity relied heavily on the measurement of platelet aggregation by the optical method of Born [3], which employs platelet-rich plasma (PRP‡) devoid of erythrocytes and leukocytes. Recently, Cardinal and Flower [4] developed a whole blood aggregometer which measures the increase in electrical impedance caused by adherence and aggregation of platelets on electrodes. This new method enables the study of various drugs on platelet behavior in whole blood and permits examination of the interactions of platelets with the other formed elements of blood, which is not possible with optical aggregometry.

Adenosine (Ado) is a naturally occurring purine metabolite which modulates a variety of physiological processes such as vascular blood flow, platelet aggregation, neural functions and lymphocyte differentiation [5, 6]. Many of these functions are mediated by the binding of Ado to specific receptors that are coupled to membrane-bound adenylate cyclase [5, 7]. Ado is continuously produced by many tissues such as vascular endothelium, heart, brain, and kidney [8, 9]. However, physiologically, only trace amouts [10] of Ado are present in extracellular fluids. Ado is taken up rapidly via transport systems in many cells including erythrocytes and is metabolized by cellular adenosine deaminase (ADA) and adenosine kinase (Ado kinase) [11, 12].

Although dipyridamole, a vasodilatory and antiplatelet drug [13], has been used in the clinic for many years, its antithrombotic mechanism of action remains unclear. Agents that increase intraplatelet cAMP levels inhibit aggregation. Dipyridamole is an inhibitor of platelet cAMP phosphodiesterase (PDE) with a K_i value for the low K_m cAMP PDE in the range of 12–20 μM [14, 15]. Dipyridamole binds extensively to plasma protein [16] with considerable individual variation in blood levels of the drug [16, 17]. Peak therapeutic blood levels of dipyridamole are usually in the range of 1–4 μM [16–18]. Therefore, it appears that clinically relevant blood

* This work was supported by PHS Grants CA 07340 and HD 11343 and funds from the G.D. Searle Co. D. D. D. was supported by Training Grant CA 09204 awarded by the National Cancer Institute, DHHS.

† Author to whom all correspondence should be addressed.

‡ Abbreviations: PRP, platelet-rich plasma; NTS, nucleoside transport system; Ado, adenosine; NBMPR, *p*-nitrobenzylthioinosine, 6-(*p*-nitrobenzyl)mercaptopurine ribonucleoside; dCF, 2′-deoxycoformycin; ITu, 5-iodotubercidin; MTA, 5′-methylthioadenosine; ADA, adenosine deaminase, adenosine aminohydrolase (EC 3.5.4.4); Ado kinase, adenosine kinase, ATP:adenosine 5′-phosphotransferase (EC 2.7.1.20); PDE, phosphodiesterase; and EU, enzyme units.

levels are too low to inhibit platelet cAMP PDE. Dilazep is another vasodilator [19, 20] that has been reported to have antiplatelet activity at high concentrations (>100 μM) [21–23]. Dipyridamole and dilazep are potent inhibitors of the nucleoside transport system (NTS) of a variety of cells, with apparent K_i values lower than 50 nM [24–26]. It has been suggested that the vasodilatory properties of dipyridamole and dilazep result from their activities as inhibitors of nucleoside transport [26, 27]. Thus, it is possible that dipyridamole and dilazep block platelet aggregation indirectly by decreasing the rate of Ado metabolism by erythrocytes (and perhaps other tissues), thus prolonging elevated concentrations of Ado in the bloodstream. In examining this possibility, we have used various compounds that have specific biochemical actions: dilazep and *p*-nitrobenzylthioinosine (NBMPR), which are potent inhibitors of the NTS [24–26, 28]; RA 233, a dipyridamole analog, with high anti-PDE activity (K_i = 0.6 μM) [14]; dipyridamole, which is a potent inhibitor of the NTS with only moderate inhibition of PDE; 2′-deoxycoformycin (dCF), a tight-binding inhibitor of ADA ($K_i = 2.5 \times 10^{-12}$ M) [29]; and 5-iodotubercidin (ITu), an inhibitor of Ado kinase (K_i for the rabbit liver enzyme ≤20 nM) [30]. The structures of these compounds are shown in Fig. 1. Preliminary reports of these findings have been presented [31–33]. Subsequent to our first preliminary report [31], a paper has appeared that independently confirms certain of these findings [34].

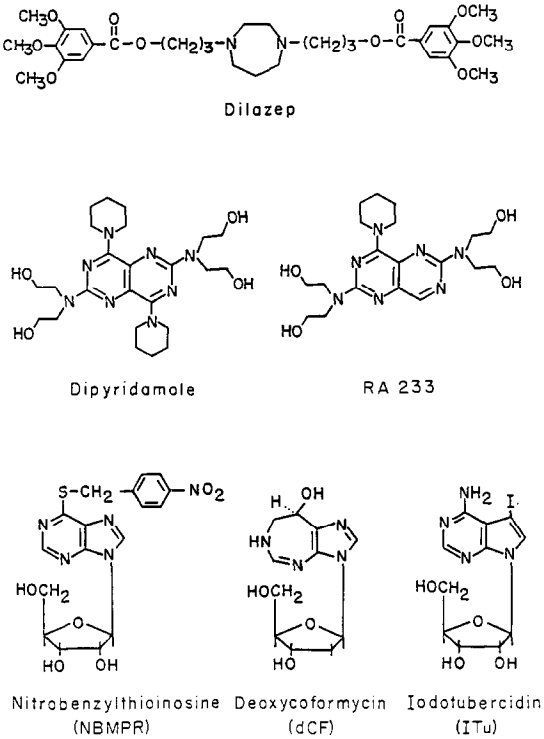

Fig. 1. Structures of inhibitors of the NTS (dilazep, dipyridamole and NBMPR), of platelet cAMP PDE (dipyridamole and RA 233) and of the Ado-metabolizing enzymes ADA (dCF) and Ado kinase (ITu).

MATERIALS AND METHODS

ADP, Ado, MTA and dipyridamole (Persantine) were obtained from the Sigma Chemical Co., St. Louis, MO. Dilazep (Cormelian) was a gift from Hoffmann-LaRoche, Inc., Nutley, NJ. RA 233 was a gift from Dr. Karl Thomae, Boehringer Ingelheim, Ridgefield, CT. dCF (Pentostatin) was obtained from the Drug Development Branch, Division of Cancer Treatment of the National Cancer Institute, Bethesda, MD. NBMPR was a gift from Dr. A. R. P. Paterson, University of Alberta, Alberta, Canada. ITu was a gift from Dr. L. B. Townsend, University of Michigan, Ann Arbor, MI. [8-^{14}C]Ado (sp. act. = 59 mCi/mmole) was obtained from Moravek Biochemicals, Brea, CA, and was >95% pure as shown by thin-layer chromatography on polyethyleneimine-cellulose sheets with potassium chloride (100 mM) as the eluent.

Platelet aggregation. Blood was drawn from healthy donors who had not taken antiplatelet drugs for at least 10 days. The blood was dispensed into plastic tubes containing 0.1 vol. of sodium citrate (3.8%) to prevent coagulation. PRP and platelet-poor plasma were obtained by differential centrifugation [35]. Platelet aggregation was measured in PRP by the turbidimetric method of Born [3] and in whole blood by the impedance technique of Cardinal and Flower [4] using a Chrono-Log (Havertown, PA) Whole Blood Aggregometer (model 500).

As indicated in the table and figure legends, many experiments used blood or PRP containing dCF (5 μM). For whole blood, dCF was present in the sodium citrate solution at the time of blood collection. For PRP, dCF was added when PRP was obtained.

Uptake of adenosine by blood cells. To simplify the analyses, the Ado uptake studies described below were performed on whole blood pretreated with dCF (5 μM) to inactivate the ADA. In the presence of uninhibited ADA, substantial amounts of the Ado that are transported into the cell are deaminated to form inosine which may leave the cell by facilitated diffusion or be phosphorolyzed rapidly by the high levels of purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) found in human erythrocytes (*ca.* 13 EU/ml of cells) [36]. The hypoxanthine formed can react with hypoxanthine-guanine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) producing IMP or be transported from the cell into the plasma. When the erythrocytic ADA is inhibited by dCF, the rate-limiting metabolic enzyme is Ado kinase, the product of which, AMP, is trapped within the cell. It should be noted that the activity of ADA in human erythrocytes is 8- to 10-fold greater than that of Ado kinase, i.e. 0.2 to 0.3 EU vs 0.03 EU per ml of cells [24, 36]. Thus, inactivation of ADA probably increases the reported $T_{\frac{1}{2}}$ for 10 μM Ado of <10 sec [18].

[^{14}C]Ado uptake by blood cells was determined at 37° in whole blood containing dCF (5 μM). After adding [^{14}C]Ado (10 μM, sp. act. = 10 μCi/μmole), aliquots (50 μl) were removed at various times and immediately added, with rapid mixing, to 2 ml of ice-

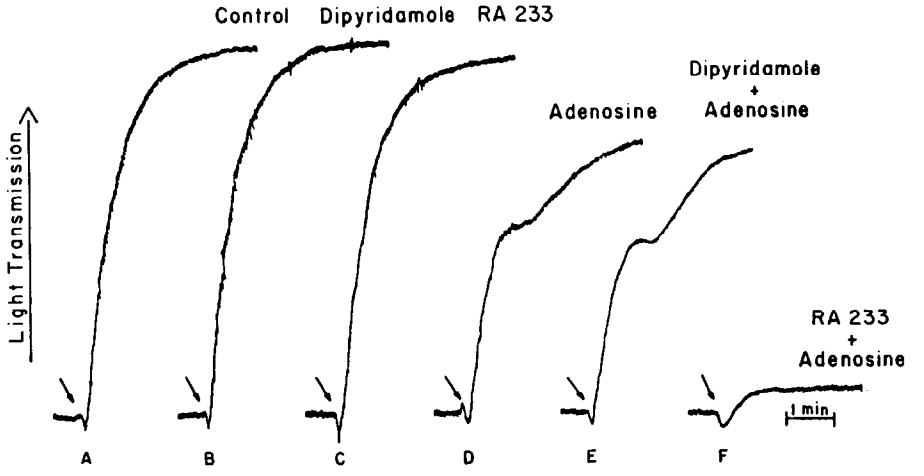

Fig. 2. Effects of dipyridamole, RA 233 and/or Ado on ADP-induced human platelet aggregation in PRP. PRP (0.35 ml) containing dCF (5 μM) was incubated at 37° with (A) normal saline or ethanol (0.24%, v/v) for 8 min; (B) dipyridamole, 10 μM, for 8 min; (C) RA 233, 10 μM, for 8 min; (D) Ado, 10 μM, for 5 min; (E) dipyridamole, 10 μM, for 3 min and then Ado, 10 μM, for 5 min; and (F) RA 233, 10 μM, for 3 min and then Ado, 10 μM, for 5 min. After incubations, ADP (10 μM) was added (↓) to induce aggregation. Tracings represent results from one typical experiment of Table 1.

cold stopping solution (potassium phosphate buffer, 50 mM, pH 7.4; sodium chloride, 75 mM; magnesium chloride, 2 mM; glucose, 10 mM; and NBMPR, 5 μM). Cells were centrifuged (1500 *g*, 5 min) and washed twice with 2 ml of ice-cold stopping solution. Cells were lysed with water (0.5 ml), and perchloric acid extracts were made by adding ice-cold perchloric acid (0.5 ml, 9%). After removing the precipitated proteins by centrifugation (1200 *g*, 10 min, 4°), an aliquot (0.5 ml) of supernatant fluid was removed and [^{14}C]activity was determined.

RESULTS

Figure 2 compares the effects of dipyridamole (10 μM) and RA 233 (10 μM), an analog of dipyridamole, on Ado inhibition of ADP-induced human platelet aggregation in PRP. RA 233, which was inactive alone, greatly potentiated Ado inhibition, whereas dipyridamole was without effect at this concentration (10 μM). This is consistent with earlier findings that greater potentiation is produced by RA 233 [37] and that RA 233 is a more potent inhibitor of platelet cAMP PDE than dipyridamole [14, 37].

In contrast to the findings with PRP, if whole blood was incubated with Ado (10 μM) for 5 min, platelet aggregation induced by ADP was not inhibited (Fig. 3). However, if the NTS inhibitor, dipyridamole (10 μM), was added to the blood 3 min before the Ado, potent inhibition of ADP-induced aggregation occurred. On the other hand, RA 233 (10 μM) which is active in PRP, did not evoke Ado activity in whole blood which is consistent with the concept that RA 233, at the concentration studied, does not block the NTS and thus the degradation of Ado by erythrocytes.

These studies were extended using other potent NTS inhibitors: NBMPR and dilazep. As shown

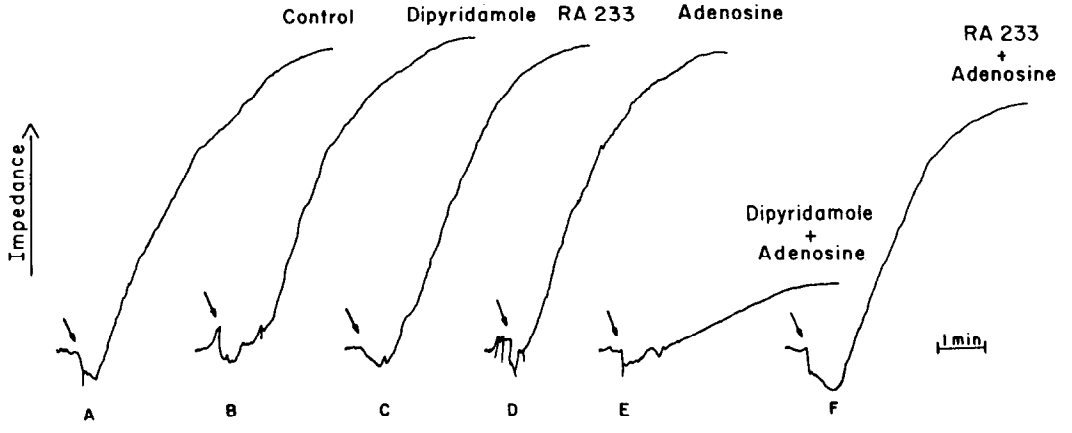

Fig. 3. Effects of dipyridamole, RA 233 and/or Ado on ADP-induced human platelet aggregation in whole blood. Incubations are the same as described in the legend of Fig. 2 except that 1 ml of whole blood was used.

Table 1. Effects of nucleoside transport inhibitors, cAMP phosphodiesterase inhibitors and/or adenosine (Ado) on human platelet aggregation in whole blood or PRP*

Inhibitors	Concn (μM)	% Inhibition in whole blood		% Inhibition in PRP	
		−Ado	+Ado†	−Ado	+Ado†
			11 ± 3 (20)		38 ± 4 (23)
Dilazep	1	7 ± 3 (11)	84 ± 6 (22)	3 ± 1 (6)	40 ± 9 (6)
NBMPR	1	15 ± 7 (10)	80 ± 6 (22)	2 ± 1 (6)	44 ± 9 (6)
Dipyridamole	10	19 ± 6 (12)	92 ± 2 (14)	1 ± 1 (6)	44 ± 10 (6)
RA 233	10	28 ± 8 (13)	32 ± 7 (13)	3 ± 1 (6)	89 ± 2 (6)

* Whole blood (1 ml) or PRP (0.35 ml) containing dCF (5 μM) was incubated with an inhibitor for 8 min; with Ado for 5 min; or with the inhibitor for 3 min and then Ado for 5 min. For controls, incubations were performed using normal saline or ethanol (0.24%, v/v). After the preincubations, platelet aggregation was induced by ADP (10 μM). Percent inhibition of platelet aggregation in whole blood or PRP represents the difference between the percent aggregation of the control (100%) and the drug-treated samples. Values are $\bar{X}$ ± S.E.; the N value is given in parenthesis.

† Ado, 10 μM.

in Table 1, dilazep (1 μM), NBMPR (1 μM) and dipyridamole (10 μM), which display only slight inhibitory activity both in whole blood and PRP, evoked Ado-mediated inhibition of platelet aggregation in whole blood but caused no potentiation in PRP. Thus, at the concentrations studied, it appears that these agents act primarily, if not solely, as NTS inhibitors, with little, if any, effect on platelet cAMP PDE. Also, RA 233 caused striking potentiation of the Ado inhibitory action in PRP but was without effect in whole blood, suggesting that this compound acts primarily as a cAMP PDE inhibitor with little or no inhibition of the NTS.

The inhibition of platelet aggregation in whole blood caused by Ado (10 μM) in the presence of the NTS inhibitor, dilazep (1 μM), was a time-dependent phenomenon (Fig. 4). Maximal inhibition (*ca.* 90%) occurred in 3 min. This agrees with the earlier finding with PRP where Ado [38] or forskolin [39] requires several minutes of preincubation to elicit full inhibition. These agents stimulate platelet adenylate cyclase, and several minutes are needed to achieve the elevation in cAMP levels required for full inhibition of aggregation.

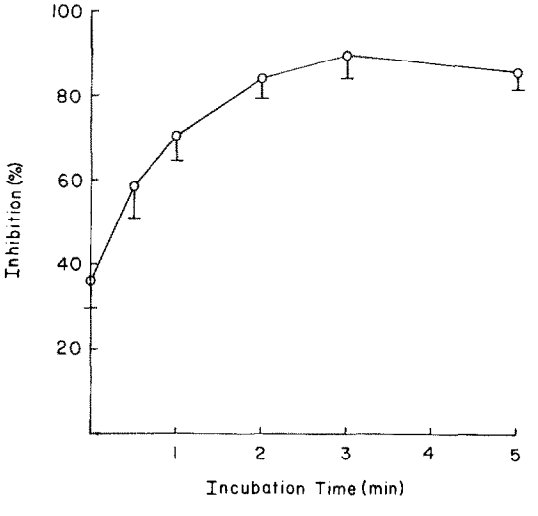

Fig. 4. Time-dependent inhibition of ADP-induced human platelet aggregation in whole blood by dilazep plus Ado. Whole blood (1 ml) containing dCF (5 μM) was incubated at 37° with dilazep, 1 μM, for 3 min and then Ado, 10 μM. After various times of incubation with Ado, ADP (10 μM) was added to induce aggregation. Results are the $\bar{X}$ ± S.E. (N = 12). Percent inhibition of platelet aggregation was calculated as described in the legend of Table 1.

Previously it has been shown that 5'-methylthioadenosine (MTA) antagonizes Ado inhibition of platelet aggregation in PRP [35], presumably by blocking the Ado receptor on the regulatory subunit of adenylate cyclase [7]. Consistent with findings with PRP [35], MTA (100 μM) which is noninhibitory alone, reversed Ado inhibition in whole blood (Fig. 5). This suggests that the inhibition of aggregation caused by dipyridamole plus Ado in whole blood was due primarily to the Ado. MTA also reversed the inhibitions produced by the combinations of NBMPR or dilazep plus Ado (data not shown).

The effects of dilazep, NBMPR, dipyridamole and RA 233 on the uptake of [^{14}C]Ado (10 μM) by blood cells in whole blood pretreated with dCF (5 μM) are shown in Fig. 6. In the absence of NTS inhibition, most [^{14}C]activity (90–100%) was found in the blood cells after 1.5 min which explains why Ado did not inhibit platelet aggregation after 5 min of incubation with whole blood. RA 233 (10 μM) did not inhibit [^{14}C]Ado uptake by the blood cells which explains its inability to restore Ado inhibition in whole blood. NBMPR (1 μM), dilazep (1 μM) and dipyridamole (10 μM), all of which restore Ado inhibition of platelet aggregation in whole blood, blocked [^{14}C]Ado uptake by blood cells. The inhibition of [^{14}C]Ado uptake was approximately 80% after 1.5 min and about 50% after 5 min. This indicates that sufficient concentrations of Ado (approximately 8 μM Ado based on an average hematocrit of 35% and an average uptake of [^{14}C]Ado of 50%) are present in the plasma after 5 min to inhibit strongly ADP-induced platelet aggregation in whole blood, as measured by the impedance technique.

Figure 7 compares the effects of Ado in the presence of inhibitors of Ado-metabolizing enzymes (ADA and Ado kinase) or of the NTS on ADP-

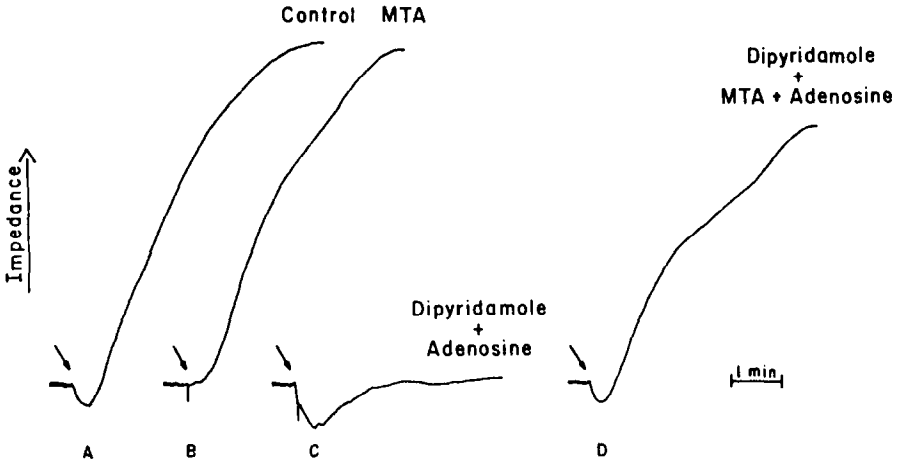

Fig. 5. Reversal by MTA of the inhibition produced by dipyridamole plus Ado on ADP-induced human platelet aggregation in whole blood. Whole blood (1 ml) containing dCF (5 μM) was incubated at 37° with (A) normal saline or ethanol (0.24%, v/v) for 8 min; (B) MTA, 100 μM, for 7 min; (C) dipyridamole, 10 μM, for 3 min and then Ado, 10 μM, for 5 min; and (D) dipyridamole, 10 μM for 1 min, then MTA, 100 μM, for 2 min and then Ado, 10 μM, for 5 min. After incubations, ADP (10 μM) was added ($\downarrow$) to induce aggregation. Tracings are representative of typical results.

induced platelet aggregation in whole blood. Ado at a high concentration (50 μM) did not inhibit aggregation. However, if the blood was pretreated with dCF (5 μM), a tight-binding inhibitor of ADA, Ado strongly inhibited platelet aggregation (IC_{50} = 22 μM). Preincubation of blood with ITu (10 μM), an inhibitor of Ado kinase, did not evoke Ado-mediated inhibition. However, when the actions of both ADA and Ado kinase were blocked by the addition of dCF and ITu, the IC_{50} of Ado was reduced to about 1.5 μM. Comparable inhibition (IC_{50} = 2.2 μM) was produced when the blood was preincubated with the NTS inhibitor dilazep (1 μM). The presence of dCF did not alter the Ado-mediated inhibition seen after preincubation of blood with dilazep. This suggests that, in the presence of a nucleoside transport inhibitor, inactivation of ADA does not further delay the transport and metabolism of Ado. Essentially similar effects were also seen with dipyridamole (IC_{50} = 1.7 μM) or NBMPR

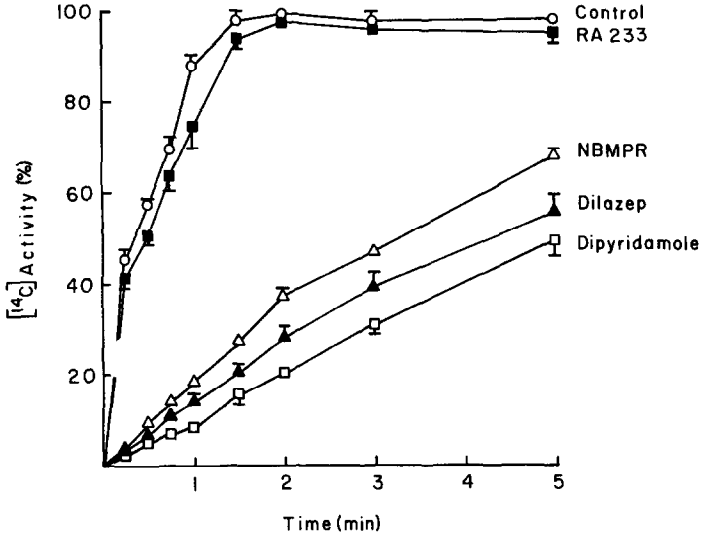

Fig. 6. Effects of RA 233, NBMPR, dilazep and dipyridamole on [^{14}C]Ado uptake by blood cells in human whole blood. Whole blood (1 ml) containing dCF (5 μM) was incubated for 3 min at 37° with (○) normal saline or ethanol (0.24%, v/v); (■) RA 233, 10 μM; (△) NBMPR, 1 μM; (▲) dilazep, 1 μM; or (□) dipyridamole, 10 μM. Then [^{14}C]Ado (10 μM, sp. act. = 10 μCi/μmole) was added, and aliquots (50 μl) were removed at various time periods. The [^{14}C]activity in cell pellets was determined as described in Materials and Methods. [^{14}C]Activity (%, fraction of total activity added) is the $\bar{X} \pm$ S.E. of three experiments performed in duplicate.

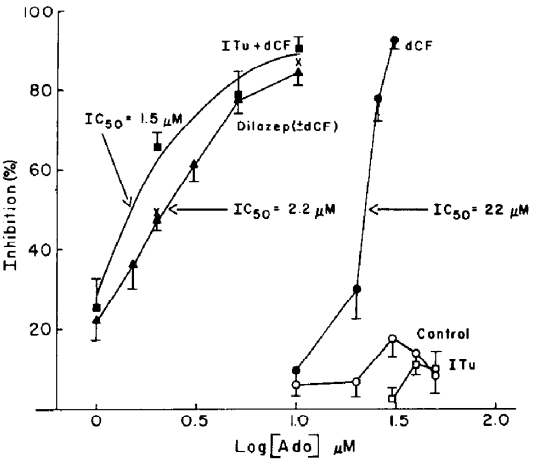

Fig. 7. Effects of Ado in the presence of inhibitors of Ado-metabolizing enzymes (ADA and Ado kinase) or of the NTS on ADP-induced human platelet aggregation in whole blood. Whole blood (1 ml) with (●, ▲, ■) or without (□, ○, X) dCF, 5 μM, was incubated at 37° with (□) ITu, 10 μM, for 5 min; (○) normal saline for 5 min; (●) dCF, 5 μM; (▲) dilazep, 1 μM, for 3 min; (X) dilazep, 1 μM, for 3 min; or (■) ITu, 10 μM, for 5 min; then Ado (1–50 μM) was added. After 5 min, ADP (10 μM) was added to induce aggregation. dCF was present in the sodium citrate solution at the time of blood collection. Results are the $\bar{X} \pm$ S.E. (N = 6–12). Percent inhibition of platelet aggregation was calculated as described in the legend of Table 1.

(IC_{50} = 3.2 μM), i.e. marked potentiation of the anti-aggregatory action of Ado in whole blood. These studies demonstrate that the loss of Ado-mediated inhibition of platelet aggregation in whole blood resulted from the uptake and metabolism of Ado by erythrocytes and was restored by blocking these processes.

DISCUSSION

These studies demonstrate the importance of inhibition of erythrocytic nucleoside transport in the antiplatelet actions of drugs such as dipyridamole and dilazep. Although the antiaggregatory effect and the decreased rate of degradation of exogeneous adenosine in whole blood *in vitro* caused by dipyridamole was observed about 20 years ago [40, 41], the definitive mechanism of the antiplatelet action of this agent is still not clear. Many hold the view that it acts by inhibiting platelet cAMP PDE, with a resulting potentiation of the elevation in cAMP levels caused by stimulators of adenylate cyclase such as Ado or prostacyclin [42–44]. It must be noted, however, that dipyridamole is a relatively weak PDE inhibitor (reported K_i values are 12 and 20 μM) [14, 15], and pharmacologically active plasma levels are usually well below these values. Of significance are the results of studies with RA 233, NBMPR and dilazep. RA 233, an analog of dipyridamole, is a more potent PDE inhibitor (K_i = 0.6 μM) [14] but was not an inhibitor of the NTS at the concentration studied (see Fig. 6). On the other hand, dilazep and NBMPR are potent NTS inhibitors but are probably devoid of PDE inhibitory activity at the concentration studied, since no potentiation of Ado inhibition occurred in PRP (Table 1). Thus, although RA 233 potentiated the antiaggregatory action of Ado in PRP, it was without effect in whole blood since it did not delay Ado metabolism. In contrast, dipyridamole, dilazep and NBMPR impeded Ado metabolism by blocking its influx into erythrocytes, thus evoking the Ado antiaggregatory effect in whole blood. Thus we propose that a major component of the antiplatelet effects of drugs such as dipyridamole and dilazep resides in their ability to inhibit potently the NTS, thus elevating the steady-state extracellular concentrations of the vasodilatory and antiplatelet agent, Ado. This concept is supported by the findings that MTA, which blocks Ado-induced inhibition in PRP [35], also exhibited this effect in whole blood pretreated with a nucleoside transport system inhibitor. In addition, when the two key enzymes of erythrocytic Ado metabolism, ADA and Ado kinase, were both blocked by the specific inhibitors, dCF and ITu, inhibition of aggregation by Ado was potentiated markedly (see Fig. 7).

A puzzling question relates to the observation that, although several NTS inhibitors display high affinity *in vitro*, i.e. apparent K_d or K_i values of 50 nM or less, it is necessary to employ concentrations of 1 μM or greater to achieve inhibitions in whole blood or *in vivo*. In preliminary studies of the inhibition of platelet aggregation in whole blood, 1 μM dipyridamole was ineffective and a 10 μM level was required to obtain inhibition equivalent to that seen with 1 μM dilazep or NBMPR. The answer appears to lie in two areas. First, it has been shown that non-specific binding can alter significantly the effective free drug levels of compounds such as NBMPR [25, 28] and dipyridamole [25]. For example, the blood levels of free dipyridamole are affected markedly by binding to plasma proteins and can vary substantially from one individual to another [16, 17]. As reported by Mahony *et al.* [16], the average binding of dipyridamole to plasma protein is 99.13 ± 0.24% with the free drug ranging from 0.55 to 1.19%. A second important factor is the absolute number of NTS sites in the erythrocytes of whole blood. It has been shown that each erythrocyte has 10^4 NTS sites [24, 28]. Since a liter of erythrocytes contains about 10^{13} cells, there are about 10^{17} NTS sites per liter of erythrocytes. From Avogadro's number, it may be determined that each liter of erythrocytes contains about 1.67×10^{-7} moles of NTS sites and that a liter of whole blood (hematocrit ≃ 45%) would contain about 0.75×10^{-7} moles of NTS sites. Therefore, concentrations of free drug greater than 1×10^{-7} M would be required to titrate all of the erythrocytic NTS sites. The combined factors of non-specific drug binding and the absolute number of erythrocytic NTS sites may account for the unexpectedly high concentrations of NTS inhibitors required for therapeutic activity. For further consideration of the above topic see Ref. 33.

A recent publication offers findings consistent with the above conclusions [18]. It was shown that the metabolism of Ado (10 μM) by human blood diluted 12-fold with isotonic saline is strongly inhibited by 1×10^{-6} M dipyridamole. The threshold concen-

tration at which inhibition appears is about 1×10^{-7} M. In the present study with undiluted human blood, Ado-mediated inhibition first appeared at about 1×10^{-6} M dipyridamole with full effects seen at 1×10^{-5} M.

Since the impedance method for studying platelet aggregation in whole blood is relatively new, it is not yet fully understood. From the above studies, it appears that the Ado inhibitory effect was greater in whole blood as measured by impedance changes than in PRP as measured by changes in light transmission (see Table 1). It is likely that the impedance results are a composite of platelet adhesion as well as aggregation [4], whereas the densitometric method measures primarily changes in platelet morphology and aggregation [3]. Consequently, antiplatelet agents can display different potencies in whole blood and PRP depending on the technique employed.

Although these studies point to the importance of erythrocytic Ado uptake and metabolism in modulating the antiaggregatory effect of Ado, an additional component of potential importance is the role played by vascular endothelium. Recently, Nees and Gerlach [9] reported that cultured guinea pig coronary endothelial cells can secrete Ado into the medium and the rate of delivery is considerably increased by physiologically important factors, e.g. elevated pCO_2, decreased pO_2, and histamine and adrenergic agonists. Thus it appears that, through its ability to take up purine nucleosides and synthesize Ado, vascular endothelium may play a key role *in vivo*, not only in the phenomenon of vasodilatation, but also in the control of blood platelet aggregation. For a recent discussion of many aspects of the regulatory role of Ado, see Ref. 45.

Since there is widespread concern about controlling the activity of blood platelets in a wide range of disease conditions (e.g. ischemic heart disease, thromboembolism, and diabetes), there is obvious interest in designing new therapeutic agents that are superior in activity to dipyridamole. An important consideration in the clinical effectiveness of dipyridamole is the fact that plasma levels of free drug vary greatly among patients receiving the same dose [16, 17]. It seems possible that the techniques used above could be adapted to the monitoring of the effectiveness of dipyridamole dosages in the clinic. The above studies also permit the speculation that an ideal antiplatelet agent should have the ability to inhibit both the NTS and platelet PDE at pharmacologically achievable tissue levels. It is proposed to test this concept by using combinations of drugs that combine these properties, e.g. dilazep plus RA 233.

REFERENCES

1. M. A. Packham and J. F. Mustard, in *Blood Platelet Function and Medicinal Chemistry* (Ed. A. Lasslo), p. 86. Elsevier Science, New York (1984).
2. M. A. Packham, *Thromb. Haemostas.* **40**, 175 (1978).
3. G. V. R. Born, *J. Physiol., Lond.* **162**, 67P (1962).
4. D. C. Cardinal and R. J. Flower, *J. pharmac. Meth.* **3**, 135 (1980).
5. A. C. Newby, *Trends biochem. Sci.* **9**, 42 (1984).
6. J. R. S. Arch and E. A. Newsholme, *Essays Biochem.* **14**, 82 (1978).
7. J. W. Daly, *J. med. Chem.* **25**, 197 (1981).
8. R. M. Berne, R. M. Knabb, S. W. Ely and R. Rubio, *Fedn Proc.* **42**, 3146 (1983).
9. S. Nees and E. Gerlach, in *Regulatory Functions of Adenosine* (Eds. R. M. Berne, T. W. Rall and R. Rubio), p. 347. Martinus Nijhoff, The Hague (1983).
10. A. Sollevi, J. Ostergren, P. Hjemdahl, B. Fredholm and B. Fagrell, *J. clin. chem. clin. Biochem.* **20**, 420 (1982).
11. R. E. Parks, Jr. and P. R. Brown, *Biochemistry* **12**, 3294 (1973).
12. J. Schrader, in *Regulatory Functions of Adenosine* (Eds. R. M. Berne, T. W. Rall and R. Rubio), p. 142. Martinus Nijhoff, The Hague (1983).
13. L. A. Harker, *Thromb. Res.* Suppl. IV, 39 (1983).
14. F. A. McElroy and R. B. Philp, *Life Sci.* **17**, 1479 (1975).
15. T. Asano, Y. Ochiai and H. Hiroyoski, *Molec. Pharmac.* **13**, 400 (1977).
16. C. Mahony, K. M. Wolfram, D. M. Cocchetto and T. D. Bjornsson, *Clin. Pharmac. Ther.* **31**, 330 (1982).
17. S. M. Rajah, M. J. Crow, A. F. Penny, R. Ahmad and D. A. Watson, *Br. J. clin. Pharmac.* **4**, 129 (1977).
18. R. E. Klabunde, *Eur. J. Pharmac.* **93**, 21 (1983).
19. J. P. Buyniski, M. Losada, M. E. Bierwagen and R. W. Gardier, *J. Pharmac. exp. Ther.* **181**, 522 (1972).
20. M. Marzilli, I. Simonetti, D. Levantes, M. G. Trivella, M. De Nes, A. Perissinotto, R. Puntoni, G. Buzzigoli, C. Boni, C. Michelassi and A. L'Abbate, *Am. Heart J.* **108**, 276 (1984).
21. F. Kuzuya, *Arzneimittel-Forsch.* **29**, 539 (1979).
22. O. Ponari and C. Manotti, *Arzneimittel-Forsch.* **31**, 97 (1981).
23. S. Colli, P. Maderna, G. Morazzoni, C. Speroni and E. Tremoli, *Pharmac. Res. Commun.* **15**, 593 (1983).
24. S. F. Chen, J. D. Stoeckler and R. E. Parks, Jr., *Biochem. Pharmac.* **33**, 4069 (1984).
25. S. M. Jarvis, D. McBride and J. D. Young, *J. Physiol. Lond.* **324**, 31 (1982).
26. A. R. P. Paterson, E. S. Jackobs, E. R. Harley, N. Fu, M. J. Robins and C. E. Cass, in *Regulatory Functions of Adenosine* (Eds. R. M. Berne, T. W. Rall and R. Rubio), p. 203. Martinus Nijhoff, The Hague (1983).
27. S. J. Mustafa, *Biochem. Pharmac.* **28**, 2617 (1979).
28. C. E. Cass, L. A. Gaudette and A. R. P. Paterson, *Biochim. biophys. Acta* **345**, 1 (1974).
29. R. P. Agarwal, T. Spector and R. E. Parks, Jr., *Biochem. Pharmac.* **26**, 359 (1977).
30. T. P. Zimmerman, R. D. Deeprose, G. Wolberg, C. R. Stopford, G. S. Duncan, W. H. Miller, R. L. Miller, M. Lim, W. Ren and R. S. Klein, *Biochem. Pharmac.* **32**, 1211 (1983).
31. K. C. Agarwal, D. D. Dawicki and R. E. Parks, Jr., *Fedn Proc.* **42**, 1990 (1983).
32. K. C. Agarwal, D. D. Dawicki and R. E. Parks, Jr., *IUPHAR Ninth International Congress of Pharmacology Abstracts*, 1743P. Macmillan Press, London.
33. R. E. Parks, Jr., D. D. Dawicki, K. C. Agarwal, S. F. Chen and J. D. Stoeckler, *Ann. N.Y. Acad. Sci.*, in press.
34. J. Arnout, J. Vermylen and M. Verstraete, *Thromb. Haemostas.* **50**, 852 (1983).
35. K. C. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **29**, 2529 (1980).
36. R. E. Parks, Jr., G. W. Crabtree, C. M. Kong, R. P. Agarwal, K. C. Agarwal and E. M. Scholar, *Ann. N.Y. Acad. Sci.* **255**, 412 (1975).
37. M. C. Rozenburg and C. M. Walker, *Br. J. Haemat.* **24**, 409 (1973).
38. K. C. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 2239 (1975).
39. K. C. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **31**, 3713 (1982).

40. P. R. Emmons, M. J. G. Harrison, A. J. Honour and J. R. A. Mitchell, *Lancet* **1**, 603 (1965).
41. R. D. Bunag, C. R. Douglas, S. Imai and R. M. Berne, *Circulation Res.* **15**, 83 (1964).
42. S. Moncada and R. Korbut, *Lancet* **1**, 1286 (1978).
43. G. Di Minno, S. Villa, V. Bertele and G. de Gaetano, in *Diet and Drugs in Atherosclerosis* (Eds. G. Noseda, B. Lewis and R. Paoletti), p. 121. Raven Press, New York (1980).
44. J. P. Kistler, A. H. Ropper and R. C. Heros, *New Engl. J. Med.* **311**, 100 (1984).
45. R. M. Berne, T. W. Rall and R. Rubio (Eds.), *Regulatory Functions of Adenosine.* Martinus Nijhoff, The Hague (1983).