Biochemical Pharmacology, Vol. 29, pp. 1799–1805.

0006-2952/80/0615–1799 $02.00/0

EFFECTS OF ADENOSINE ANALOGS AND ADENINE NUCLEOTIDES ON ADENOSINE 5′-DIPHOSPHATE-INDUCED RAT PLATELET AGGREGATION*

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(*Received* 1 *November* 1979; *accepted* 16 *January* 1980)

Abstract—Various adenosine analogs and adenine nucleotides have been tested as inhibitors of ADP-induced aggregation of rat platelets. The potent inhibitors of human platelet aggregation, adenosine, 2-fluoroadenosine, 2-chloroadenosine, carbocyclic adenosine and N^6-phenyl adenosine, had little effect on rat platelet aggregation (0–30 per cent inhibition). The effects of adenosine or its analogs on ADP-induced aggregation of cross-species platelet-rich plasmas (PRPs) (human platelets suspended in rat plasma or rat platelets in human plasma) were similar to those with the native PRPs, indicating that these species differences were due to intrinsic factors in the platelets and not in the plasma. When these analogs were tested in the presence of the cyclic AMP phosphodiesterase inhibitor papaverine, strong inhibiton of rat platelet ADP-induced aggregation was seen. 2′-Deoxyadenosine and 3′-deoxyadenosine were not inhibitory to ADP-induced aggregation of rat PRP even in the presence of papaverine. Adenosine 5′-tetraphosphate strongly inhibited both human and rat platelet aggregation. AMP, like adenosine, did not inhibit rat platelet aggregation but became strongly inhibitory in the presence of papaverine. This inhibitory effect was abolished by preincubating rat PRP with an adenylate cyclase inhibitor, 2′,5′-dideoxyadenosine or adenosine deaminase. In the later case, however, if the adenosine deaminase inhibitor 2′-deoxycoformycin was included in the incubation mixture, the inhibition by AMP plus papaverine was similar to adenosine plus papaverine. About 50 per cent of [^{14}C]AMP was converted to [^{14}C]adenosine in rat platelet-free plasma or PRP after a 10-min incubation. α,β-Methylene-ADP and β,γ-methylene-ATP (200 μM) inhibited rat platelet aggregation by 50 and 64 per cent, respectively. Cyclic AMP phosphodiesterase of rat and human platelets gave comparable K_m and V_{max} values (K_m 0.53 and 0.21μM and V_{max} 6.0 and 6.7 pmoles/min/10^7 platelets, respectively).

Striking differences are seen in the behavior of blood platelets from humans and rats in response to substances that induce or block aggregation [1–13]. Although aggregation is induced in both species by ADP, in the rat it is induced by ATP and is not blocked by adenosine or its analogs. By contrast, in human platelets, aggregation is blocked by both ATP and adenosine-like compounds [14–18]. Recent reports [13, 19] from this laboratory demonstrated that the induction of aggregation with rat platelets by ATP is due to a plasma enzyme, probably creatine kinase, that converts ATP to ADP. Rat platelets suspended in human plasma no longer respond to ATP, whereas human platelets suspended in rat plasma become susceptible to ATP-induced aggregation. On the other hand, the capacity of adenosine to block ADP-induced aggregation is retained in human platelets suspended in rat plasma and is not acquired when rat platelets are suspended in human plasma. Thus, the difference in the response to adenosine-type compounds seems to be an intrinsic property of the platelets of each species and not due to an extrinsic factor. The present report explores further the reasons for the inactivity of adenosine-type compounds as blockers of ADP-induced aggregation of rat platelets. A preliminary report of these findings has been presented [13].

* Supported by USPHS Grant CA 07340.

† Abbreviations used: Ado, adenosine; Cl-Ado, 2-chloroadenosine; F-Ado, 2-fluoroadenosine; C-Ado, carbocyclic adenosine, 9-[β-DL-2α,3α-dihydroxy-4β-(hydroxymethyl)-cyclopentyl]-adenine; 2′-dAdo, 2′-deoxyadenosine; 3′-dAdo, 3′-deoxyadenosine; 2′,5′-DDA, 2′,5′-dideoxyadenosine; dCF, 2′-deoxycoformycin; ADA, adenosine deaminase (EC 3.5.4.4); cyclic AMP, adenosine 3′,5′-cyclic phosphate; AP_4, adenosine 5′-tetraphosphate; α,β-Me-ADP, α,β-methylene adenosine 5′-diphosphate; β,γ-Me-ATP, β,γ-methylene adenosine 5′-triphosphate; and PRP, platelet-rich plasma.

MATERIALS AND METHODS

Adenosine, 2-chloroadenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, inosine, papaverine, bovine serum albumin, adenosine deaminase (calf intestional mucosa, sp. act. 270 units/mg), and the sodium salts of AMP, ADP, ATP and adenosine 5′-tetraphosphate were purchased from the Sigma Chemical Co., St. Louis, MO. Cyclic AMP was obtained from Plenum Scientific Research, Hackensack, NJ. [^{3}H]Cyclic AMP (sp. act. 38.7 Ci/mmole) and [U-^{14}C]AMP (sp. act. 377 mCi/mmole) were purchased from New England Nuclear, Boston, MA. α,β-Methylene-ADP and β,γ-methylene-ATP were purchased from P-L Biochemicals, Milwaukee, WI. N^6-Phenyl-Ado† was obtained from Dr. M. H. Fleysher

of Roswell Park Memorial Institute, Buffalo, NY. Samples of 2-fluoroadenosine (F-Ado) and 2',5'-dideoxyadenosine (2',5'-DDA) were supplied by Dr. Harry B. Wood, Jr., from the Drug Development Branch, Division of Cancer Treatment of the National Cancer Institute. The F-Ado was synthesized by the method of Montgomery and Hewson [20]. Carbocyclic adenosine (C-Ado) was the gift of Dr. L. Lee Bennett, Jr., of Kettering Meyer Laboratory, Southern Research Institute, Birmingham, AL, and was synthesized by the method of Shealy and Clayton [21]. 2'-Deoxycoformycin (Pentostatin, dCF) was obtained from Dr. H. W. Dion of Parke, Davis & Co., Detroit, MI. Polyethyleneimine (PEI) cellulose plates were purchased from the Fisher Scientific Co., Fairlawn, NJ.

Isolation of platelets. Human blood was drawn into 0.1 vol. of sodium citrate (3.8%) from healthy volunteers who had not taken aspirin or other drugs presently known to affect platelets for at least 1 week. Human platelet-rich plasma (PRP) was obtained by centrifugation of citrated whole blood at 377 *g* for 8 min. Platelet-poor plasma (PPP) was obtained by recentrifuging the remaining packed cells at 1349 *g* for 10 min. Platelet-free plasma (PFP) was obtained by centrifuging PPP at 12,000 *g* for 10 min at 4°. Albino rats (CD strain, Charles River, Wilmington, MA), weighing 200–300 g, were anesthetized with ether. Whole blood was obtained by cardiac puncture in 0.1 vol. sodium citrate for anticoagulation. Rat PRP and PPP were separated by centrifugation of the whole blood, similarly, as in the case of human blood. To obtain similar platelet counts in rat and human PRP, rat PRP was diluted with autologous PPP. Platelet counts were determined by use of a coulter counter (model B, Coulter Electronics, Hialeah, FL).

To obtain cross-species PRPs (human platelets in rat plasma or rat platelets in human plasma), PRP from one species was mixed with 0.15 vol. ACD (Acid-Citrate-Dextrose, U.S.P. Formula A) and centrifuged at 1200 *g* for 10 min at 4°. Plasma was removed carefully by a Pasteur pipette and the test tube walls were wiped with a cotton swab. The platelets were then suspended gently with a Pasteur pipette in cell-free plasma of the opposite species with restoration of the initial PRP volume. As a control, PRPs (human and rat) were centrifuged and the platelets were resuspended in a similar manner in autologous cell-free plasma. All PRP samples were placed at room temperature for at least 10 min prior to aggregation studies.

Aggregation studies. Platelet aggregation was performed at 37° in PRP stirred at 900 r.p.m. and measured by the turbidometric method of Born [22] as described by Mustard and Glynn [23] using a Payton aggregometer (Payton Associates, Inc., Buffalo, NY) attached to a recorder. The extent of aggregation was measured by the net maximal percentage increase in light transmission during the 4-min period after addition of ADP. PPP was used as a blank to represent 100 per cent light transmission. The per cent inhibition of aggregation by various agents was calculated by the difference in the maximal extent of aggregation after 4 min.

Platelet lysates and phosphodiesterase assay. Human PRP and rat PRP were mixed with 0.15 vol. ACD and centrifuged at 1200 *g* for 15 min at 4°. The platelet pellets were washed once with saline (0.9% NaCl). Washed platelets were resuspended in saline containing Tris–HCl buffer (50 mM, pH 8.0) to obtain a count of about 1×10^9 platelets/ml. Platelet lysates were obtained by sonication followed by freezing and thawing of the washed platelet suspension. Sonication was carried out for 15–20 sec at 110 W of acoustic energy in an ice bath using a Sonifier Cell Disruptor (model W 185D). The lysate was then centrifuged at 27,000 *g* for 40 min at 4°, and the supernatant fluid was employed for enzymatic assays. The cyclic AMP phosphodiesterase activity was measured by a slight modification of the method of Rangel-Aldao *et al.* [24]. Cyclic AMP and its metabolites (AMP, Ado and inosine) were separated on 20 × 20 cm PEI cellulose plates developed for 60 min using KCl (100 mM) as the eluent.

RESULTS

The inhibiton of rat and human ADP-induced platelet aggregation by Ado, adenine nucleotides and a number of their analogs (Fig. 1) is shown in Table 1. In confirmation of previous observation, Ado and AMP were considerably less effective as inhibitors (< 10 per cent) of the aggregation of rat platelets than of human platelets. In addition, Cl-Ado, F-Ado, C-Ado (100 μM) and N^6-ϕ-Ado (50 μM) were only weak inhibitors (10–30 per cent) of rat platelet aggregation, but were potent inhibitors of human platelet aggregation (45–95 per cent). The deoxynucleosides, 2'-dAdo and 3'-dAdo (100 μM), were only weak inhibitors (< 20 per cent) with both species. Only AP_4, of the compounds studied, strongly inhibited both human and rat platelet ADP-induced aggregation. For AP_4 studies, fresh AP_4 solutions were employed and were found to be > 95 per cent pure when analyzed by high pressure liquid chromatography. As reported previously, ATP (100 μM) strongly inhibited (97 per cent) human and rabbit platelet aggregation [25, 26] but, in contrast, induced aggregation of rat platelets [9]. The ADP

Table 1. Effects of adenosine and adenine nucleotide analogs on ADP-induced aggregation of human and rat PRP*

Analogs	Conc (μM)	Inhibition (%) Human	Rat
Ado	100	85	5
F-Ado	100	95	16
Cl-Ado	100	95	30
Carbocyclic-Ado	100	78	10
N^6-Phenyl-Ado	50	45	14
2'-Deoxy-Ado	100	5	5
3'-Deoxy-Ado	100	18	5
AMP	100	59	10
ATP	100	97	†
AP_4	100	91	95
α,β-Methylene-ADP	200	10	50
β,γ-Methylene-ATP	200	75	64

* Human or rat PRP was incubated with the analog for 10 min at 37° followed by addition of ADP (human, 8 μM; rat, 4 μM).

† Aggregates platelets of rat PRP.

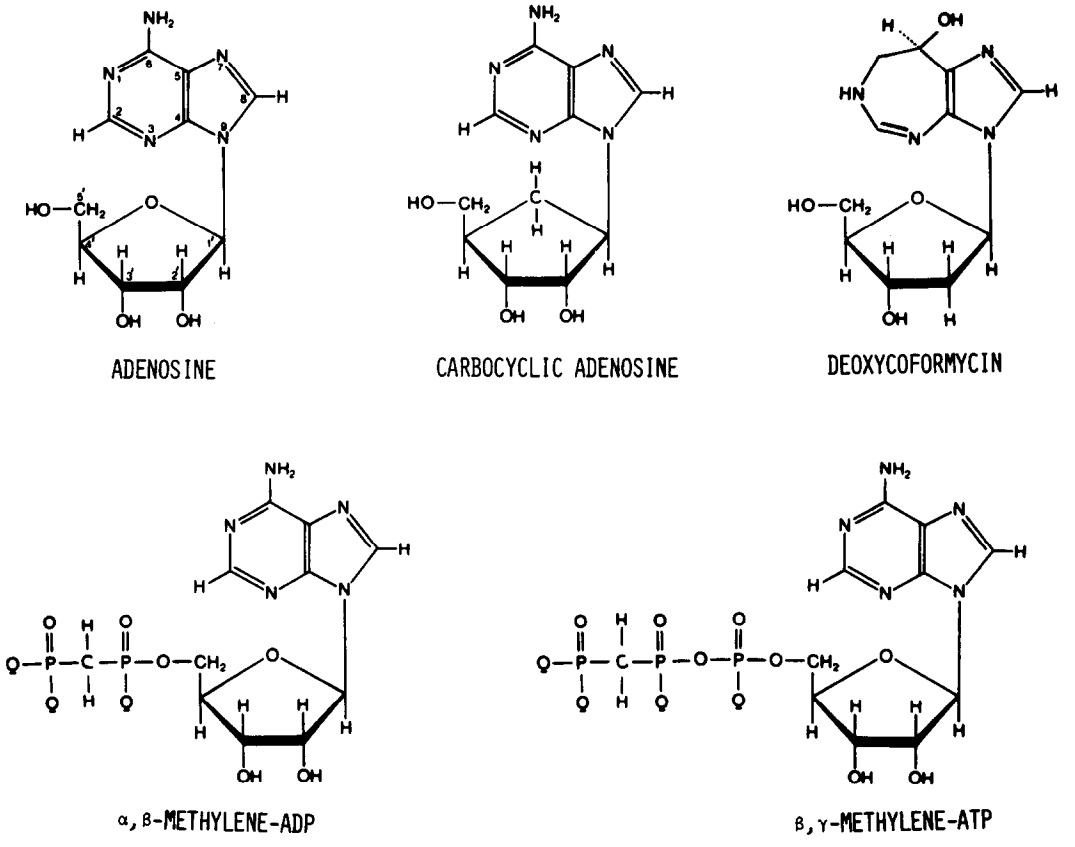

Fig. 1. Structures of adenosine and adenine nucleotide analogs.

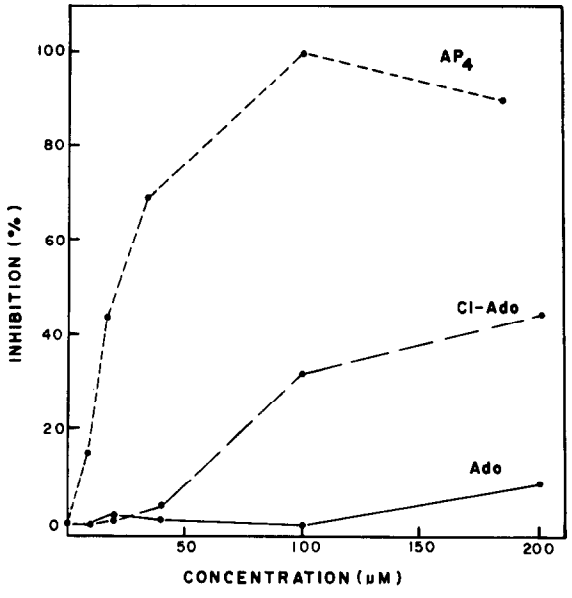

Fig. 2. Effects of adenosine (Ado), 2-chloroadenosine (Cl-Ado) and adenosine 5′-tetraphosphate (AP_4) on aggregation of rat platelets. Fresh PRP was incubated at 37° with Ado, Cl-Ado or AP_4 (0–200 μM) and, after 10 min, aggregation was induced by addition of ADP (4 μM). The results are expressed as per cent inhibition of aggregation compared to that of control samples where the addition of the analog solution to PRP was replaced by an equal volume of normal saline.

analog α,β-Me-ADP (200 μM) did not cause aggregation of either human or rat platelets, but moderately inhibited (50 per cent) rat platelet aggregation. The ATP analog β,γ-Me-ATP (200 μM) inhibited both human and rat platelet aggregation to similar extents (75 and 64 per cent, respectively).

Typical dose–response inhibition curves of ADP-induced rat platelet aggregation for Ado, Cl-Ado and AP_4 are shown in Fig. 2. At a high concentration (200 μM), inhibition by Ado remained low (< 10 per cent) and Cl-Ado was only 45 per cent inhibitory. A plot of the inhibition by AP_4 was linear from 0 to 40 μM, with complete inhibition occurring at 100 μM.

To determine whether the different effects of Ado and its analogs on human and rat PRP resulted from inherent platelet or plasma factors, the platelets from one species were suspended in plasma of the other species. As a control, the centrifuged platelets from each species were resuspended in autologous plasma. The effect of Cl-Ado on ADP-induced aggregation of platelets in control and cross-species PRPs is shown in Fig. 3 (A and B). The extent of aggregation inhibition by Cl-Ado of rat platelets suspended in human plasma was similar to that found with native rat PRP, whereas the inhibition by Cl-Ado of human platelets suspended in rat plasma closely resembled that seen with human PRP. As expected, the control experiments with human and rat platelets resuspended in autologous plasma exhibited inhibition patterns similar to those with original PRPs. Similar results were found with Ado and F-Ado.

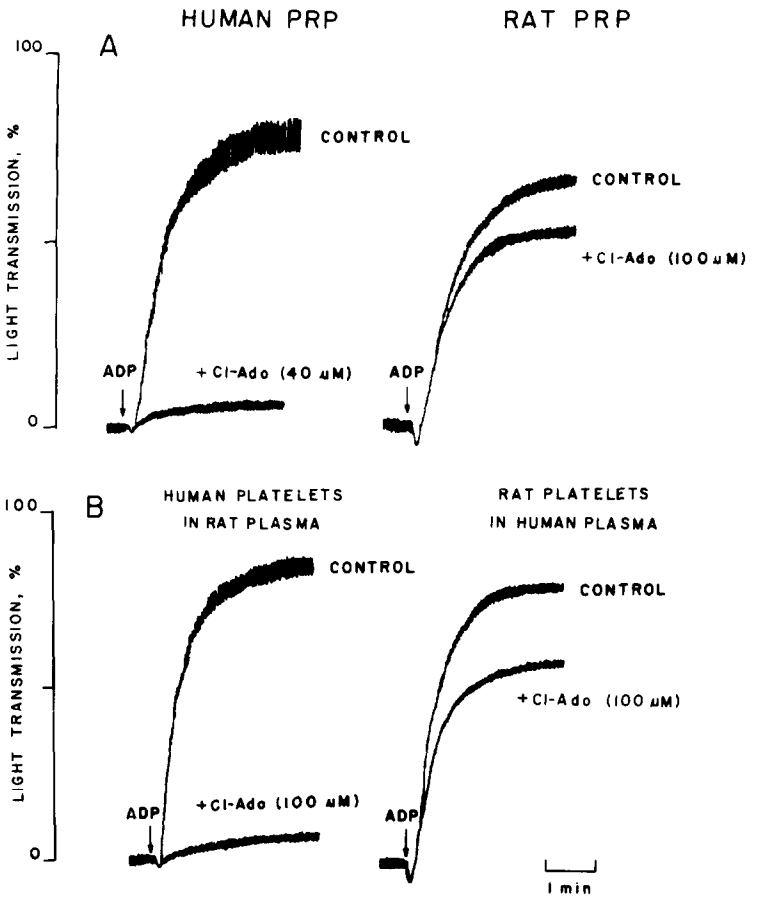

Fig. 3. Effect of 2-chloroadenosine (Cl-Ado) on human and rat platelet aggregation. (A) Human PRP and rat PRP were incubated at 37° with Cl-Ado (40 μM with human and 100 μM with rat PRP) and, after 10 min, aggregation was induced by addition of ADP (8 μM). (B) Cross-species PRPs (human platelets in rat plasma or rat platelets in human plasma) were incubated with Cl-Ado (100 μM) and, after 5 min, aggregation was induced by addition of ADP (8 μM).

Table 2. Effect of adenosine in the presence and absence of various agents on ADP-induced rat platelet aggregation*

Adenosine (8 μM)	Papaverine (56 μM)	2′,5′-Dideoxy-adenosine (200 μM)	Adenosine deaminase (0.054 units/ml)	Inhibition (%)
+	−	−	−	< 5 (a)
−	+	−	−	< 10 (b)
+	+	−	−	60 (c)
+	+	+	−	10 (d)
+	+	−	+	< 5 (e)

* Agents were added to rat PRP in the order described in each case. After incubation, aggregation was induced by addition of ADP (4 μM): (a) adenosine, 5 min; (b) papaverine, 5 min; (c) papaverine, 5 min; and adenosine, 5 min; (d) 2′,5′-dideoxyadenosine, 2 min; papaverine, 5 min; and adenosine, 5 min; and (e) adenosine deaminase, 1 min; papaverine, 5 min; and adenosine, 5 min.

Table 3. Effects of adenosine analogs and AMP in the presence and absence of papaverine on ADP-induced rat platelet aggregation*

Analogs	Conc (μM)	Inhibition (%) −Papaverine	Inhibition (%) +Papaverine (56 μM)
Ado	8	0†	60
Cl-Ado	8	< 5	60
F-Ado	10	< 5	75
Carbocyclic-Ado	50	0†	56
2'-Deoxy-Ado	100	0†	10
3'-Deoxy-Ado	100	< 5	7
AMP	10	0	64

* PRP was preincubated at 37° with papaverine solution or 0.9% NaCl for 5 min followed by incubation with the analog for 5 min. Platelet aggregation was induced by ADP (4 μM).

† No inhibiton or sometimes 0–15 per cent potentiation of aggregation was noticed.

In order to examine more closely the inability of Ado and its analogs to inhibit rat platelet aggregation, papaverine, a cyclic AMP phosphodiesterase inhibitor [27, 28], was employed (Table 2). Papaverine (56 μM) or Ado (8 μM) individually did not significantly inhibit (< 10 per cent) ADP-induced aggregation in rat PRP. However, preincubation of rat PRP with papaverine caused Ado to become inhibitory (60 per cent). This synergistic effect was abolished by preincubation of the PRP for 2 min with 2',5'-DDA (200 μM), an adenylate cyclase inhibitor, or with adenosine deaminase (ADA), which converts Ado into inosine.

Since papaverine has been reported to inhibit nucleoside transport in platelets [29, 30], the question was raised whether its action resulted from effects on transport rather than on cyclic AMP degradation. That this is not true is indicated by studies with the nucleoside transport inhibitors [31] *p*-nitrobenzylthioinosine (10 μM) and dipyridamole (50 μM). Neither transport inhibitor produced any effect on the response of rat platelets to Ado.

The effect of Ado analogs and AMP on ADP-induced rat platelet aggregation in the presence and absence of papaverine is shown in Table 3. At low concentrations (8–10 μM), Cl-Ado, F-Ado and AMP did not inhibit rat platelet aggregation but became strongly inhibitory (60–75 per cent) in the presence of papaverine. Under similar conditions, C-Ado (50 μM) produced 56 per cent inhibition. However, 2'-dAdo and 3'-dAdo remained non-inhibitory at an even higher concentration (100 μM).

The effect of AMP in the presence of papaverine, 2',5'-DDA and ADA on rat platelet ADP-induced aggregation is shown in Fig. 4. Papaverine (56 μM) or AMP (8 μM) was non-inhibitory (< 10 per cent) when added individually to rat PRP. However, when rat PRP was preincubated with papaverine, AMP became strongly inhibitory (about 65 per cent). As with Ado plus papaverine, the inhibition by AMP was abolished by preincubation with the adenylate cyclase inhibitor 2',5'-DDA. Also, addition of ADA to PRP overcame the inhibition of AMP in the presence of papaverine. However, when the tight-binding ADA inhibitor dCF was added with ADA, the inhibition by AMP plus papaverine on ADP-induced aggregation was restored.

In order to examine the conversion of AMP to Ado, rat PFP and PRP were incubated with dCF

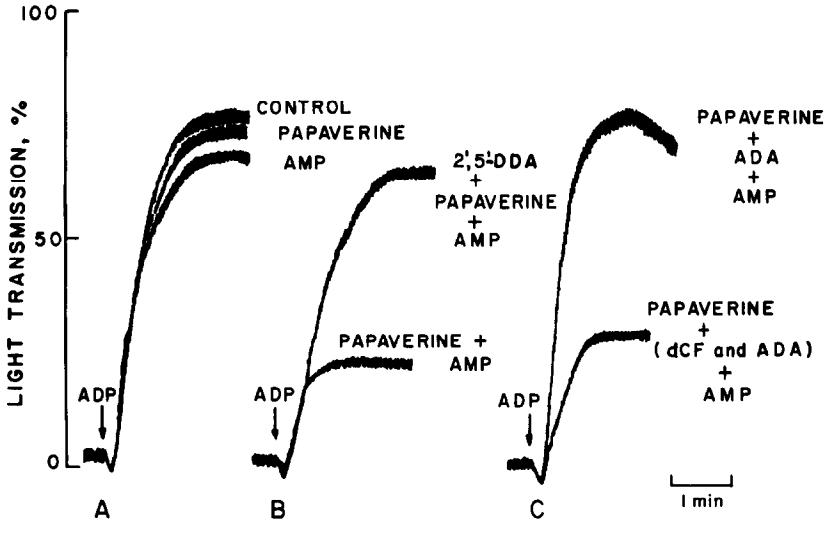

Fig. 4. Effect of AMP in the presence of papaverine, 2',5'-dideoxyadenosine (2',5'-DDA), adenosine deaminase (ADA) and 2'-deoxycoformycin (dCF) on rat platelet aggregation. These agents were added to rat PRP in the order described below, and, after incubation, aggregation was induced by addition of ADP (8 μM). (A) AMP (8 μM, 5 min); papaverine (56 μM, 5 min); control (5 min). (B) Papaverine + AMP: papaverine (56 μM, 5 min) and AMP (8 μM, 5 min); 2',5'-DDA + papaverine + AMP: 2',5'-dideoxyadenosine (100 μM, 2 min), papaverine (56 μM, 5 min) and AMP (8 μM, 5 min). (C) Papaverine + (dCF and ADA) + AMP: papaverine (56 μM, 5 min), dCF and ADA (2'-deoxycoformycin, 2.2 μM, and adenosine deaminase, 0.027 units; this mixture was preincubated for 15 min) and AMP (8 μM, 5 min); Papaverine + ADA + AMP: papaverine (56 μM, 5 min), ADA (adenosine deaminase, 0.027 units, 1 min) and AMP (8 μM, 5 min).

Table 4. Per cent conversion of AMP to adenosine in rat platelet-free plasma (PFP) and platelet-rich plasma (PRP)*

Incubation (min)	% AMP conversion to Ado PFP	PRP
2	13.6	15.0
5	27.0	29.3
10	47.0	49.7
15	63.0	66.0

* Aliquots (1.35 ml) of PFP and PRP were preincubated with 2′-deoxycoformycin (5 μM) for 15 min. Samples (0.15 ml) of [U-^{14}C]AMP (1 mM) (sp. act. 6.7 μCi/μmole) were added, and the reaction mixtures were incubated at 37° in a shaking water-bath. Aliquots (0.3 ml) were removed after 2, 5, 10 and 15 min and added immediately to equal volumes of ice-cold perchloric acid (9%) and stirred vigorously on a Vortex mixer. Precipitated proteins were removed after centrifugation and the supernatant fluids were neutralized with KOH (5 N). Thin-layer chromatography on PEI plates was performed to separate AMP, Ado and inosine as described in Materials and Methods. At each time point, the amount of AMP that disappeared could be accounted for by formation of Ado and inosine. Levels of inosine remained less than 1 per cent throughout the 15-min incubation.

and [U-^{14}C]AMP, as shown in Table 4. After 10 min, 47–50 per cent of the [U-^{14}C]AMP was converted to [U-^{14}C]Ado. Levels of [U-^{14}C]inosine remained less than 1 per cent throughout the 15-min incubation. These results demonstrate that AMP inhibits ADP-induced platelet aggregation in the presence of papaverine as a result of its dephosphorylation to Ado.

The phosphodiesterase activities in both rat and human washed platelet lysates were measured (Table 5). The K_m and V_{max} values for cyclic AMP were determined by Lineweaver–Burk plots using cyclic AMP concentrations ranging from 0.03 to 2 μM. The K_m values for rat and human platelet enzymes were 0.53 and 0.21 μM, respectively. The V_{max} values obtained were about 6 pmoles cyclic AMP hydrolyzed/min/10^7 platelets for both rat and human platelets.

DISCUSSION

These studies demonstrate conclusively that the failure of Ado and its analogs to inhibit ADP-induced aggregation in rat platelets is an inherent property of the platelet and is not due to external factors in the plasma. Important use was made of the cyclic AMP phosphodiesterase inhibitor papaverine and the adenylate cyclase inhibitor 2′,5′-DDA. In the presence of papaverine, the behavior of rat platelets after the addition of Ado or its analogs was similar to that of human platelets in the absence of papaverine. Thus, it appears that papaverine may convert insensitive rat platelets into an acceptable animal model for study of the aggregation of human platelets. Furthermore, the effects of papaverine on rat platelets were completely reversed by the adenylate cyclase inhibitor 2′,5′-DDA. This finding is consistent with the hypothesis that papaverine acts by blocking the degradation of cyclic AMP to AMP, thus permitting the cyclic nucleotide concentrations to rise to inhibitory levels. These results are consistent with the earlier studies of Michel *et al.* [10], who showed that the elevation of platelet cyclic AMP levels in response to the addition of Ado is much less pronounced in rat than in human platelets.

Table 5. Kinetic parameters (K_m and V_{max}) for platelet cyclic AMP phosphodiesterase

Platelet phosphodiesterase	K_m (μM)	V_{max} (pmoles/min/10^7 platelets)
Human	0.21	6.7
Rat	0.53	6.0

The fact that papaverine caused rat platelets to become sensitive to the effects of compounds such as Ado, F-Ado, Cl-Ado and C-Ado but not to 2′-dAdo or 3′-dAdo (see Table 3) and to the inhibitory effects of 2′,5′-DDA is in agreement with the concept that the adenylate cyclase of rat platelets has both stimulatory and inhibitory sites for Ado and its analogs as has been postulated for human platelets [32, 33].

Kinetic studies performed with the cyclic AMP phosphodiesterases of high speed centrifugation supernatant fractions of sonicates of rat and human platelets gave comparable K_m and V_{max} values. In both cases linear Lineweaver–Burk plots were obtained. Although to date we have not undertaken separation and study of possible forms of kinetically different cyclic AMP phosphodiesterases in rat platelets as had been reported for human platelets [28], superficially these enzymatic activities appear similar. Ado analogs have been reported to inhibit cyclic AMP phosphodiesterase with high K_i values (70–80 μM for Cl-Ado and 400–3800 μM for Ado) [28]. Since Ado and its analogs in the presence of papaverine inhibit rat platelet aggregation at low concentrations (8–10 μM), and this inhibitory effect is abolished by an adenylate cyclase inhibitor 2′,5′-DDA, it is more likely that Ado analogs inhibit platelet aggregation by affecting the platelet adenylate cyclase system than by affecting cyclic AMP phosphodiesterase activity. Caution must be exercised, however, before concluding that papaverine exerts its effects directly through inhibition of cyclic AMP phosphodiesterase. In studies [34] with C-6 astrocytoma cells, papaverine was shown to cause marked rapid decreases in intracellular concentrations of ATP, creatine phosphate and glycogen. At low papaverine concentrations (5 μM) respiration was depressed 50 per cent. Therefore, studies of the effect of papaverine on the energy metabolism of platelets should be performed before drawing conclusions about its site of action.

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