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Effects of Adenosine on Levels of Adenosine Cyclic 3',5'-Monophosphate in Human Blood Platelets in Relation to Adenosine Incorporation and Platelet Aggregation

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SUMMARY

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Cyclic 3',5'-[^{14}C]AMP was measured in platelets that had first been incubated with [^{14}C]adenine. Maximum increases of 2-4-fold were observed 0.5 min after addition of 10-40 μM adenosine. Smaller increases were obtained with higher concentrations of adenosine. In 0.5-min incubations 2-chloroadenosine was less effective than adenosine at concentrations below 20 μM and more effective at concentrations above 100 μM. Incorporation of 1-10 μM adenosine into platelets was inhibited at least 96% by *p*-nitrobenzylthioguanosine without any effect on the increase in cyclic [^{14}C]AMP caused by these concentrations of adenosine, suggesting that adenosine acts at an extracellular site. With higher adenosine concentrations, *p*-nitrobenzylthioguanosine was less effective in inhibiting incorporation of adenosine but blocked the decline in cyclic [^{14}C]AMP levels observed on increasing the adenosine concentration above 40 μM. This inhibitory effect of high adenosine concentrations on the accumulation of cyclic [^{14}C]AMP was more easily detected when adenosine was added with prostaglandin E_1 and represents a second, possibly intracellular action of adenosine unrelated to its effect in increasing cyclic AMP levels. Papaverine markedly potentiated the increase in platelet cyclic [^{14}C]AMP observed with all concentrations of adenosine, indicating that adenosine activates platelet adenylate cyclase. The kinetics of this activation were studied in intact platelets incubated for short intervals in the presence of papaverine. Adenosine (K_A = 1 μM) activated platelet adenylate cyclase up to a maximum of 8-10-fold. This action of adenosine was competitively inhibited by caffeine (K_i = 72 μM) or theophylline (K_i = 25 μM). No inhibitory effect of high adenosine concentrations on cyclic [^{14}C]AMP formation was observed in intact platelets in the presence of papaverine. The platelet-aggregating agents ADP and epinephrine, but not vasopressin, markedly inhibited the increase in platelet cyclic [^{14}C]AMP with adenosine. ADP was found to be a noncompetitive inhibitor (K_i = 0.9 μM) of the effect of adenosine on adenylate cyclase in intact platelets. Some close correlations were observed between the effects of adenosine on platelet cyclic [^{14}C]AMP levels and on platelet aggregation. Caffeine partially blocked the inhibition of aggregation by adenosine. As a whole the results show that platelets possess a specific extracellular membrane receptor for adenosine, which is distinct from that for ADP and which mediates the inhibition of platelet function by adenosine by activating platelet adenylate cyclase.

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INTRODUCTION

Adenosine and some closely related compounds, particularly 2-substituted adenosines, are potent inhibitors of the aggregation of blood platelets induced by agonists such as ADP, epinephrine, serotonin, and vasopressin (1–5). The mechanism responsible for this inhibition has been the subject of considerable controversy. Proposed mechanisms have included antagonism by adenosine of the action of ADP on its receptor (6, 7), mechanisms dependent on the uptake and metabolism of adenosine (8–10), and, more recently, an increase in platelet cyclic 3′,5′-AMP as a result of inhibition of cyclic AMP phosphodiesterase by adenosine (11, 12) or activation of adenylate cyclase by adenosine (13). In an investigation of this problem it has already been shown that adenosine activates adenylate cyclase in a particulate fraction isolated from platelets (14). We now present a detailed study of the mechanisms by which adenosine affects cyclic AMP levels in intact platelets and of the relationships of these effects to the uptake and metabolism of adenosine by platelets in platelet-rich plasma. In these experiments we have used *p*-nitrobenzylthioguanosine, a compound known to inhibit the transport of nucleosides into red cells (15), to inhibit uptake of adenosine by platelets. These studies are relevant to an understanding of the mechanism by which adenosine increases cyclic AMP levels in brain slices (16, 17) and in a variety of cells in tissue culture (18–23). As suspensions of blood platelets provide a homogeneous system in which biochemical changes can readily be related to pharmacological effects, we also investigated the relationship between platelet cyclic AMP levels and the inhibition of platelet aggregation by adenosine. Some of our findings have been briefly described (24).

MATERIALS AND METHODS

Materials. [8-^{14}C]Adenosine (47 mCi/mmole), [*U*-^{14}C]adenine (287 mCi/mmole), and [^{3}H]inulin (300 mCi/mmole) were obtained from Amersham/Searle Corporation, and cyclic [8-^{3}H]AMP (28 Ci/mmole), from Schwarz/Mann. These were used at the specific activities at which they were obtained unless stated otherwise. [*U*-^{14}C]Adenine was purified before use by thin-layer chromatography in two dimensions on cellulose (50 μCi/plate), using solvent systems described previously (25). Heparin, adenosine, nucleotides, *l*-epinephrine bitartrate, [8-arginine]vasopressin, caffeine, theophylline, and 3′,5′-cyclic nucleotide phosphodiesterase were obtained from Sigma Chemical Company. Papaverine was obtained from BDH Chemicals. Cation-exchange resin (AG 50W-X8, 200–400 mesh, H^+ form) was obtained from Bio-Rad Laboratories, and cellulose powder for thin-layer chromatography (MN 300 HR), from Brinkmann Instruments (Canada), Ltd. PGE_1[1] was very kindly provided by Dr. J. Pike of the Upjohn Company; NBTGR, by Dr. R. K. Robins of the ICN Nucleic Acid Research Institute; and 2-chloroadenosine, by Dr. S. C. Smith of Imperial Chemical Industries, Ltd., Pharmaceuticals Division. As NBTGR was only sparingly soluble in aqueous solutions it was dissolved in DMSO, which was present at a final concentration of 0.2% in all experiments with NBTGR and had no effect at this concentration on any of the parameters studied.

Heparinized human platelet-rich plasma containing more than 350,000 platelets/mm^3 was used throughout this study. It was prepared as described previously (5), stored at 37° in a closed, siliconized flask, and used within 4 hr of preparation.

Incorporation of [8-^{14}C]adenosine into platelets. Samples of platelet-rich plasma (0.85 ml) were added to mixtures of 0.002 ml of DMSO with or without dissolved NBTGR and 0.148 ml of 0.154 M NaCl containing 0.25 μCi of [^{3}H]inulin and 0.047 μCi of [8-^{14}C]adenosine. Unlabeled adenosine was included with final adenosine concentrations above 1 μM. Incubations were carried out at 37° and terminated by centrifugation at 12,000 × *g* for 0.5 min in an

[1] The abbreviations used are: PGE_1, prostaglandin E_1; NBTGR, *p*-nitrobenzylthioguanosine; DMSO, dimethyl sulfoxide.

Eppendorf 3200 centrifuge. The supernatant plasma was rapidly removed, and 0.05 ml was counted for ^{14}C and ^{3}H. The platelet pellet was disrupted by sonication in 0.5 ml of H_2O and transferred quantitatively to liquid scintillation vials for counting. The ^{14}C in the platelet pellets was corrected for extracellular ^{14}C on the basis of the [^{3}H]inulin present and the ^{14}C:^{3}H ratio in the plasma. The incorporation of adenosine was calculated in terms of nanomoles per 10^9 platelets, allowing for variations in its specific activity.

Metabolism of [8-^{14}C]adenosine in platelet-rich plasma. Incubation mixtures were similar to those used for measurement of adenosine incorporation into platelets but contained 0.5 μCi of [^{3}H]inulin and 0.47 μCi of [8-^{14}C]adenosine. After centrifugation the supernatants and pellets were separately extracted with ice-cold perchloric acid (final concentration, 0.5 N) containing unlabeled adenosine metabolites in amounts giving final concentrations of 0.8 mM in the extract. The acid extracts were neutralized with 2 N KOH, and, after removal of $KClO_4$ by centrifugation at 0°, 0.02-ml samples were subjected to thin-layer chromatography in two dimensions on cellulose as described elsewhere (25). The positions of individual metabolites were identified under ultraviolet light, and appropriate areas of cellulose were scraped into liquid scintillation vials for counting of ^{14}C. Aliquots of neutralized extract were also counted directly and, from the ^{14}C:^{3}H ratios obtained and the distribution of different metabolites in the supernatant plasma, the ^{14}C in each metabolite measured in the platelet pellets was corrected for contamination by extracellular material. About 10% of the ^{14}C chromatographed was unaccounted for, either as a result of handling losses or because of the presence of unidentified metabolites. The amounts of adenosine converted to each compound isolated were determined from the ^{14}C in each, expressed as a percentage of the plasma or platelet ^{14}C chromatographed, and the total ^{14}C in the plasma or platelets (measured separately as described above).

Measurement of platelet cyclic [^{14}C]AMP. To label platelet adenine nucleotides, platelet-rich plasma was incubated at 37° with purified 2 μM [*U*-^{14}C]adenine for 80–90 min before each experiment, by which time the uptake of ^{14}C by platelets had reached a maximum of about 90%. Samples (0.85–0.90 ml) were then mixed with 0.15–0.10 ml of additions and incubated at 37° for appropriate times. Incubations were terminated by addition of 0.2 ml of 3 N $HClO_4$ with 0.01 μCi of cyclic [8-^{3}H]AMP, followed by cooling to 0°. All incubations were performed during the 20–30-min period following labeling of the platelets, to minimize any changes in the specific activities of platelet adenine nucleotides. The total ^{14}C incorporated into the platelets was measured immediately before and after each experiment by counting the ^{14}C in samples of platelet-rich plasma and platelet-free plasma prepared by centrifuging platelet-rich plasma for 1 min at 12,000 × *g* (Eppendorf centrifuge). The mean value for the ^{14}C incorporated was used in calculating the percentage of platelet ^{14}C in cyclic [^{14}C]AMP. After extraction for 20–30 min at 0°, the acidified incubation mixtures were centrifuged for 4 min at 12,000 × *g* (Eppendorf centrifuge). Two methods were used for isolation of cyclic AMP from the supernatants. In some experiments cyclic AMP was isolated from columns of Bio-Rad AG 50W-X8 resin (200–400 mesh) by elution with water and further purified by thin-layer chromatography on cellulose (method 1), as fully described elsewhere (26). In other experiments (method 2) a minor modification of the method of Krishna *et al.* (27) was used, which gave the same results as method 1 provided that the [*U*-^{14}C]adenine used to label the platelets was purified as described above. In this procedure 1 ml of acid extract was placed on a column containing a packed volume of 1.5 ml of Bio-Rad AG 50W-X8 resin (200–400 mesh, H^+ form). Cyclic AMP was eluted in the 6–15-ml fraction with 1 mM KH_2PO_4 adjusted to pH 7.3 with KOH. The eluate was adsorbed twice with nascent $BaSO_4$-$Zn(OH)_2$ [0.3 ml of 0.25 M $Ba(OH)_2$ plus 0.3 ml of 0.25 M $ZnSO_4$]. The supernatant was lyophilized and counted for ^{14}C and ^{3}H. The cyclic [^{14}C]AMP found by either method was corrected for the recovery of cyclic

[^{3}H]AMP (50–70%) and expressed as a percentage of the platelet-bound ^{14}C. Except when indicated otherwise, the statistical significance of small differences in platelet cyclic [^{14}C]AMP levels was evaluated by unpaired Student's *t*-tests.

In some experiments the radiochemical purity of material isolated as cyclic [^{14}C]AMP by method 2 was determined. Platelet extract was neutralized with KOH, incubated with cyclic nucleotide phosphodiesterase (0.1 unit/ml) in the presence of 1.5 mM $MgSO_4$ and 56 mM Tris-HCl (pH 7.4), reacidified with perchloric acid containing unlabeled cyclic AMP for determination of percentage recovery (from E_{260}), and finally subjected to the usual isolation procedure for cyclic [^{14}C]AMP (method 2). Under conditions in which exogenous cyclic [^{3}H]AMP was completely degraded by the phosphodiesterase, the presumptive cyclic [^{14}C]AMP found in control samples of platelet-rich plasma was degraded by 80–90% indicating the presence of a residual minor impurity. Other experiments suggested that this had been added with the [*U*-^{14}C]adenine and remained in the plasma compartment, which contained no true cyclic [^{14}C]AMP. The increases in presumptive cyclic [^{14}C]AMP observed in the presence of adenosine or PGE_1 were completely degraded by cyclic nucleotide phosphodiesterase.

Measurement of total cyclic AMP. Cyclic AMP in perchloric acid extracts was partially purified by elution from Bio-Rad AG 50W-X8 resin with water as in method 1. The eluates were lyophilized. As described elsewhere (28), the cyclic AMP was taken up in a small volume of buffer, its recovery was determined, and the remainder was assayed by a modification of a specific protein binding method (29).

Liquid scintillation counting. ^{3}H and ^{14}C were counted in a Beckman LS 230 scintillation counter in a mixture comprising 1.5 ml of aqueous solution (including the radioactive sample) and 10.5 ml of a 1,4-dioxane-based phosphor (30). Thixotropic gel powder (Packard, 50 g/liter) was included when insoluble material (e.g., cellulose) was present. Counting efficiencies were approximately 33% and 50% for ^{3}H and ^{14}C, respectively, when single isotopes were counted, and 16% and 50%, respectively, under dual-isotope counting conditions. Results were corrected for background radioactivity, channel crossover (when present), and variations in quenching.

Platelet aggregation studies. An aggregation module (Payton Associates, Ltd., Scarborough, Ontario) was used in which a 1-ml mixture of platelet-rich plasma with additions could be maintained at 37° and stirred magnetically at 1100 rpm with a small, nickel-plated stir bar. Platelet aggregation was recorded by this apparatus as the decrease in light extinction by platelet-rich plasma after addition of an aggregating agent. Superimposable aggregation recordings were obtained with the same additions when the same platelet-rich plasma was used. With platelet-rich plasma samples from different donors the same qualitative effects were always obtained, but quantitative variations in the changes in extinction occurred which were related to the initial platelet count. Each experiment was repeated with platelet-rich plasma from at least three different donors. When quantitative results were required, the decrease in extinction after exactly 0.5 min was measured from the recordings obtained.

RESULTS

Uptake and metabolism of adenosine by platelets in platelet-rich plasma in the presence and absence of NBTGR. Preliminary experiments showed that 1 μM NBTGR inhibited incorporation of 1–10 μM [8-^{14}C]adenosine into platelets by over 95% and was more effective than either 0.1 μM NBTGR or 100 μM papaverine. NBTGR was no more effective at 10 μM, but this concentration was used in later experiments to reduce any competitive effect of high adenosine concentrations. The incorporation of [8-^{14}C]adenosine into platelets during 10-min incubations was measured over a concentration range of 1–400 μM adenosine in the presence and absence of 10 μM NBTGR (Fig. 1). In the absence of NBTGR a complex relationship between adenosine concentration and incorporation, indicative of three separate processes affecting incorporation, was observed. In-

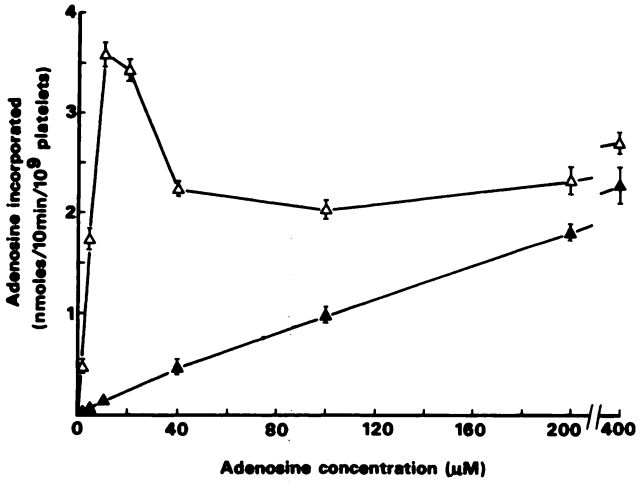

FIG. 1. *Incorporation of different concentrations of adenosine into platelets in the presence and absence of NBTGR*

Samples of platelet-rich plasma (0.85 ml; 557,000 platelets/mm³) were incubated for 10 min with 0.002 ml of DMSO with or without NBTGR (final concentration, 10 μM) and 0.148 ml of 0.154 M NaCl containing [³H]inulin and various concentrations of [8-¹⁴C]adenosine. Values for adenosine incorporated are means ± standard errors from three identical incubation mixtures. △, NBTGR absent; ▲, NBTGR present.

corporation reached a maximum of about 3.6 nmoles/10⁹ platelets/10 min with 10 μM [8-¹⁴C]adenosine and then decreased about 45% to a minimum at 100 μM, above which a secondary gradual increase occurred. This complex pattern was not observed in the presence of NBTGR, when incorporation increased approximately linearly with increasing adenosine concentration to reach roughly the same level as in the absence of NBTGR with 400 μM adenosine. Thus the inhibitory effect of NBTGR on incorporation was very marked with adenosine concentrations up to 10 μM (96% inhibition) but decreased rapidly with higher concentrations. With short incubations, such as were used in many of the experiments on cyclic AMP formation, uptake of adenosine could only be measured with tolerable accuracy at low adenosine concentrations, because of the high proportion of extracellular ¹⁴C in the platelet pellets. Table 1 shows that, within the error of the experiment, NBTGR completely prevented incorporation of 1–10 μM [8-¹⁴C]adenosine into platelets over 0.5 min. Assuming that the total adenine nucleotides in the metabolic pool of platelets amounted to about 33 nmoles/10⁹ platelets (10), these results also indicate that incubation with 10 μM unlabeled adenosine would not dilute intracellular labeled adenine nucleotides by more than about 1%/min.

TABLE 1

Effect of 10 μM NBTGR on incorporation of adenosine into platelets during 0.5-min incubations

Samples of platelet-rich plasma (0.85 ml; 470,000 platelets /mm³) were incubated for 0.5 min with 0.002 ml of DMSO with or without NBTGR and 0.148 ml of 0.154 M NaCl containing [³H]inulin and various concentrations of [8-¹⁴C]adenosine. Values for adenosine incorporated are means ± standard errors for the numbers of identical incubation mixtures indicated in parentheses.

Adenosine concentration	Adenosine incorporated	
	−NBTGR	+NBTGR
μM	*nmole/10⁹ platelets*	
1	0.044 ± 0.002 (3)	0.002 ± 0.005 (3)
3	0.119 ± 0.006 (3)	−0.008 ± 0.011 (2)
10	0.143 ± 0.013 (3)	−0.001 ± 0.005 (2)

The metabolism of [8-¹⁴C]adenosine in platelet-rich plasma was also studied in the presence and absence of NBTGR. In a representative experiment (Table 2) approximately 75% and 60% of 10 μM and 40 μM adenosine, respectively, were metabo-

TABLE 2

Metabolism of adenosine in platelet-rich plasma in the presence and absence of NBTGR

Incubation mixtures contained 0.85 ml of platelet-rich plasma (PRP) (485,000 platelets/mm^3), 0.002 ml of DMSO with or without NBTGR (final concentration, 10 μM), and 0.148 ml of 0.154 M NaCl containing [^{3}H]inulin and [8-^{14}C]adenosine. Each was incubated for 10 min. The total incorporation of ^{14}C into the platelets was measured, and the metabolism of [8-^{14}C]adenosine was studied in separate incubation mixtures in which, after centrifugation, supernatants (S) and platelets (P) were each extracted with perchloric acid and the extracts chromatographed (see MATERIALS AND METHODS). For comparative purposes the metabolism of [8-^{14}C]adenosine was also studied in platelet-free plasma (PPP), prepared by centrifuging platelet-rich plasma at 2000 × *g* for 15 min at room temperature.

Additions	Fraction analyzed	Final distribution of adenosine + metabolites	Amounts of individual compounds							Total accounted for
			ATP	ADP	AMP	IMP	Adenosine	Inosine	Hypoxanthine	
		nmoles				*nmoles*				%
Adenosine (10 μM)	PRP (P)	1.371	0.875	0.206	0.009	0.049	0	0	0.028	85.1
	PRP (S)	8.63	0	0	0	0	2.48	3.90	1.39	90.0
	PPP	10.0	0	0	0	0	3.50	5.23	0.30	90.3
Adenosine (10 μM) + NBTGR	PRP (P)	0.050	0.032	0.006	0	0.005	0	0	0.002	90.0
	PRP (S)	9.95	0	0	0	0	3.24	4.83	0.22	83.3
Adenosine (40 μM)	PRP (P)	0.872	0.537	0.110	0	0.021	0	0	0.108	89.0
	PRP (S)	39.1	0	0	0	0	16.2	13.6	4.8	88.5
	PPP	40.0	0	0	0	0	19.5	15.8	1.1	91.0
Adenosine (40 μM) + NBTGR	PRP (P)	0.169	0.135	0.014	0	0.010	0	0	0.003	95.8
	PRP (S)	39.8	0	0	0	0	20.2	15.9	0.6	92.2

lized in 10 min. Only slightly less was removed in incubations of platelet-free plasma or of platelet-rich plasma containing NBTGR, indicating that most of the adenosine metabolism in platelet-rich plasma occurs extracellularly. In the plasma compartment of platelet-rich plasma the only major metabolites were inosine and hypoxanthine, as observed by others (31). However, in platelet-free plasma and in platelet-rich plasma containing NBTGR, more inosine and much less hypoxanthine were present, indicating that the platelets were largely responsible for converting the former into the latter and that NBTGR prevented this conversion, probably by blocking membrane transport of inosine (15) as well as of adenosine. Within the platelets the principal adenosine metabolites were ATP, ADP, IMP, and hypoxanthine. As expected, much less ATP, ADP, and IMP were formed from 40 μM than from 10 μM adenosine, but with the higher concentration the amount of intracellular hypoxanthine was increased about 4-fold, as were the extracellular hypoxanthine and inosine. In the presence of NBTGR the intracellular accumulation of all these metabolites was inhibited. Neither adenosine nor inosine was observed intracellularly, although very small quantities metabolized during isolation of the platelet pellets could have been present. With extracellular adenosine concentrations above 40 μM, it was technically impossible to determine whether or not some intracellular adenosine was present. The results are consistent with previous evidence (31) that incorporation of adenosine into platelet nucleotides depends on rapid phosphorylation by adenosine kinase of adenosine transported across the platelet membrane. Only the intracellular hypoxanthine would be likely to be derived from extracellular inosine. Most importantly, the results indi-

cated that most of the added adenosine would still be present as extracellular adenosine throughout the 0.5-min incubations used in most of the succeeding studies on platelet cyclic AMP levels. The major metabolites that could potentially affect cyclic AMP levels, particularly in longer incubations, were inosine and hypoxanthine.

Effects of adenosine and 2-chloroadenosine on platelet cyclic [^{14}C]AMP in the absence of inhibitors of cyclic AMP phosphodiesterase. Cyclic [^{14}C]AMP levels reached a maximum about 0.5 min after addition of 10 μM adenosine or 10 μM 2-chloroadenosine, and then declined slowly (Fig. 2). The decline was more pronounced with adenosine than with 2-chloroadenosine, so that although after 0.5 min adenosine had increased cyclic [^{14}C]AMP more than 2-chloroadenosine, in 10-min incubations there was significantly less cyclic [^{14}C]AMP in the presence of adenosine ($2p < 0.05$). NBTGR had no effect on the increases in cyclic [^{14}C]AMP with 10 μM adenosine in incubations of 2 min or less. In a separate experiment, when labeled platelet-rich plasma was incubated for up to 2 min with 40 μM adenosine or 2-chloroadenosine, less than 10% of the increase in cyclic [^{14}C]AMP was found in platelet-free plasma prepared by rapid centrifugation (20 sec at 12,000 × g).

The effects of different adenosine concentrations on cyclic [^{14}C]AMP levels were studied in 0.5-min incubations. A maximum increase in cyclic [^{14}C]AMP to 2–4 times the control level, depending on the platelet-rich plasma preparation used, was observed with 10–40 μM adenosine (e.g., Fig. 3). Higher concentrations of adenosine caused slightly smaller increases in cyclic [^{14}C]AMP. With 2-chloroadenosine (Fig. 3) there was significantly less accumulation of cyclic [^{14}C]AMP at 10 μM and below ($2p < 0.05$), and significantly more at 200 μM ($2p < 0.01$; paired t-test). NBTGR had no effect on either the basal level of cyclic [^{14}C]AMP or the levels found with adenosine concentrations up to 10 μM. Above this concentration the curves diverged (Fig. 3), so that significantly more cyclic [^{14}C]AMP was found with 100 or 200 μM adenosine in the presence than in the absence of NBTGR ($2p < 0.01$; paired t-test). No decrease in cyclic

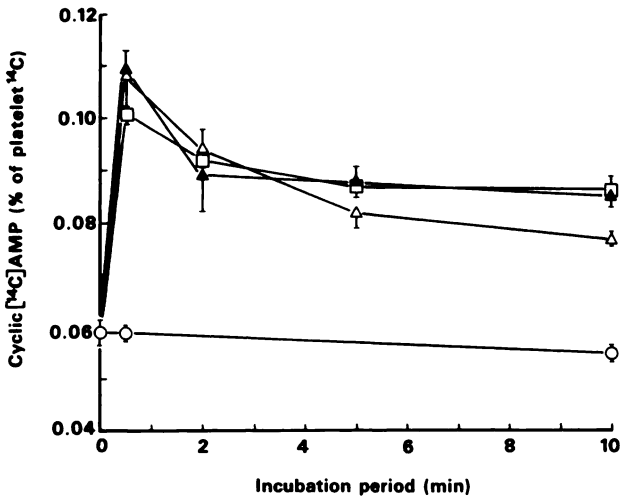

FIG. 2. *Time course of effects of adenosine (with and without NBTGR) and 2-chloroadenosine on levels of cyclic [^{14}C]AMP in platelets*

Incubation mixtures contained 0.9 ml of labeled platelet-rich plasma (400,000 platelets/mm^3), 0.098 ml of 0.154 M NaCl with or without adenosine or 2-chloroadenosine (final concentration, 10 μM), and 0.002 ml of DMSO with or without NBTGR (final concentration, 10 μM). Cyclic [^{14}C]AMP was isolated by method 1. Mean values ± standard errors from three identical incubation mixtures are given. ○, controls without any nucleoside; △, adenosine; ▲, adenosine plus NBTGR; □, 2-chloroadenosine.

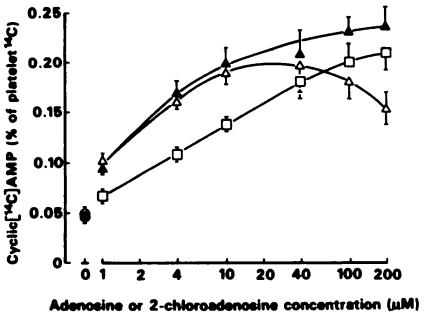

FIG. 3. *Effects of different concentrations of adenosine (with and without NBTGR) and 2-chloroadenosine on levels of cyclic [^{14}C]AMP in platelets*

Incubation mixtures contained 0.9 ml of labeled platelet-rich plasma (360,000 platelets/mm^3), 0.002 ml of DMSO with or without NBTGR (final concentration, 10 μM), and 0.098 ml of 0.154 M NaCl containing any other additions. Each was incubated for 0.5 min. After initial processing according to method 1, the resin eluates were divided in two; half was used for determination of cyclic [^{14}C]AMP by method 1, and half for measurement of total cyclic AMP (Table 3). Values for cyclic [^{14}C]AMP are means ± standard errors from three identical incubation mixtures. ○, no additions; ●, NBTGR; △, adenosine; ▲, adenosine plus NBTGR; □, 2-chloroadenosine.

[^{14}C]AMP levels occurred as the adenosine concentration was increased above 40 μM in the presence of NBTGR. The general pattern of results obtained by measurement of platelet cyclic [^{14}C]AMP shown in Fig. 3 was confirmed by measurement of total cyclic AMP levels in platelet-rich plasma from the same experiment (Table 3). However, the increases in total cyclic AMP with adenosine relative to the controls, although highly significant ($2p < 0.01$), were proportionately smaller than observed with the labeling technique, and the differences between the effects of adenosine and 2-chloroadenosine and of 200 μM adenosine with and without NBTGR did not reach statistical significance. These differences in the results obtained may have been due to the background of unlabeled cyclic AMP in plasma.

The effects of increasing adenosine concentrations were also studied in the presence of 0.1 μM PGE_1, which by itself increased the platelet cyclic [^{14}C]AMP levels about 8-fold in 0.5-min incubations. Figure 4 shows one of three such experiments, in each of which the same effects were observed. Addition of 4 μM adenosine with PGE_1 caused a further increase in cyclic [^{14}C]AMP, which was about twice as large as that caused by adenosine alone. With concentrations of adenosine above 20 μM this slight synergism disappeared, and adenosine caused progressively smaller increases in cyclic [^{14}C]AMP, until at 400 μM it began to inhibit the effect of the PGE_1. Thus the biphasic nature of the adenosine effect was more pronounced and appeared at lower concentrations of adenosine when PGE_1 was present. NBTGR blocked the inhibitory component of the action of adenosine in the presence as well as in the absence of PGE_1, but did not affect the increase in cyclic [^{14}C]AMP with PGE_1 alone or with PGE_1 and 4 μM adenosine (Fig. 4).

Effects of adenosine on platelet cyclic [^{14}C]AMP in the presence of inhibitors of cyclic AMP phosphodiesterase. Both 2 mM papaverine and 20 mM caffeine increased

TABLE 3

Effects of adenosine (with and without NBTGR) and 2-chloroadenosine on total cyclic AMP in platelet-rich plasma

This experiment was carried out as part of that described in Fig. 3. Incubation mixtures contained 0.9 ml of labeled platelet-rich plasma, 0.002 ml of DMSO with or without NBTGR, and 0.098 ml of 0.154 M NaCl containing any other additions, and were each incubated for 0.5 min. Resin eluates (method 1) were divided in two; half was used for determination of cyclic [^{14}C]AMP (Fig. 3), and the total cyclic AMP was measured in the other half. Values are means ± standard errors from three identical incubation mixtures.

Additions	Cyclic AMP in platelet-rich plasma
	pmoles/ml
None	13.1 ± 1.3
NBTGR (10 μM)	13.1 ± 0.7
Adenosine (10 μM)	32.2 ± 0.8
Adenosine (10 μM) + NBTGR (10 μM)	31.8 ± 2.1
Adenosine (200 μM)	27.7 ± 2.2
Adenosine (200 μM) + NBTGR (10 μM)	35.4 ± 2.3
2-Chloroadenosine (10 μM)	26.5 ± 2.4
2-Chloroadenosine (200 μM)	29.4 ± 4.9

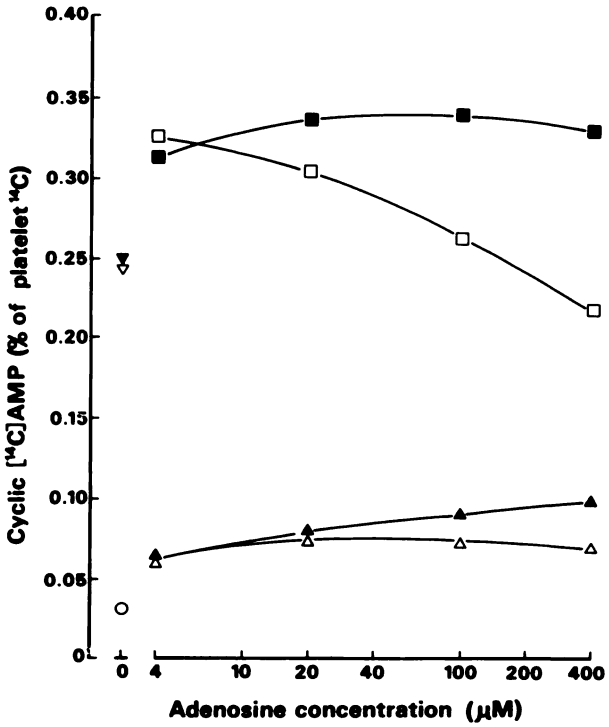

FIG. 4. *Effects of different concentrations of adenosine (with and without NBTGR) on platelet cyclic [^{14}C]AMP in the presence and absence of PGE$_1$*

Incubation mixtures contained 0.85 ml of labeled platelet-rich plasma (400,000 platelets/mm^3), 0.002 ml of DMSO with or without NBTGR (final concentration, 10 μM), and 0.148 ml of 0.154 M NaCl containing, in appropriate samples, PGE$_1$ (final concentration, 0.1 μM) and/or adenosine. Each was incubated for 0.5 min. Cyclic [^{14}C]AMP was isolated by method 2. ○, no additions; △, adenosine; ▲, adenosine plus NBTGR; ▽, PGE$_1$; ▼, PGE$_1$ plus NBTGR; □, PGE$_1$ plus adenosine; ■, PGE$_1$ plus adenosine and NBTGR.

platelet cyclic [^{14}C]AMP levels about 2-fold in 0.5 min (Table 4). When adenosine and papaverine were added together, a markedly synergistic rather than additive effect on platelet cyclic [^{14}C]AMP levels was observed with all concentrations of adenosine tested (Table 4). The decline in cyclic [^{14}C]AMP observed with high adenosine concentrations alone did not occur in the presence of papaverine. The increase in cyclic [^{14}C]AMP with papaverine and 400 μM adenosine relative to that with papaverine alone indicated an almost 10-fold activation of platelet adenylate cyclase by the adenosine. In contrast to these results, 1–4 μM adenosine did not increase platelet cyclic [^{14}C]AMP at all in the presence of 20 mM caffeine, which markedly potentiates the effects of PGE$_1$ (24). Higher adenosine concentrations did increase cyclic [^{14}C]AMP in the presence of caffeine to

TABLE 4

Effects of inhibitors of cyclic AMP phosphodiesterase on increases in platelet cyclic [^{14}C]AMP at different adenosine concentrations

Incubation mixtures contained 0.85 ml of labeled platelet-rich plasma (400,000 platelets/mm^3) and 0.15 ml of additions dissolved in 0.154 M NaCl. Each was incubated for 0.5 min. Cyclic [^{14}C]AMP was isolated by method 2.

Adenosine concentration	Cyclic [^{14}C]AMP		
	No phosphodiesterase inhibitor	2 mM papaverine	20 mM caffeine
μM	*% platelet ^{14}C*		
0	0.031	0.063	0.065
1	0.047	0.188	0.062
4	0.067	0.252	0.068
20	0.075	0.290	0.072
100	0.072	0.322	0.092
400	0.070	0.330	0.154

some extent, but a synergistic effect was observed only with 400 μM adenosine (Table 4). These results suggest that caffeine competitively inhibits the action of adenosine on adenylate cyclase, in addition to inhibiting cyclic AMP phosphodiesterase.

The increase in cyclic [^{14}C]AMP in platelets in the presence of 2 mM papaverine was previously shown to be linear for about 1 min and was assumed to reflect endogenous adenylate cyclase activity (24). In the present study the accumulation of cyclic [^{14}C]AMP in the presence of 2 mM papaverine and adenosine (0.5 or 100 μM) was also found to be linear for 1 min. These results suggested that in the presence of 2 mM papaverine the residual cyclic AMP phosphodiesterase activity was sufficiently low to permit a kinetic study of the activation of adenylate cyclase by adenosine in intact platelets, provided that short incubation times were used. The adenosine-activated adenylate cyclase activity of platelets was calculated by subtraction of the cyclic [^{14}C]AMP formed in the presence of papaverine alone from that formed with both papaverine and adenosine present. Double-reciprocal plots of adenosine-activated adenylate cyclase activity against adenosine concentration were linear and gave K_A values for activation by adenosine of 0.9–1.3 μM (e.g., Figs. 5 and 7). Using this method, caffeine and theophylline were each found to be competitive inhibitors of the activation of adenylate cyclase by adenosine (Fig. 5). Theophylline was more effective than caffeine (K_i = 25 and 72 μM, respectively). Inosine and hypoxanthine at the concentrations tested (40 and 400 μM in each case) neither stimulated platelet adenylate cyclase themselves nor inhibited the action of adenosine.

To investigate whether or not adenosine stimulates the same adenylate cyclase as PGE_1, the effect of 10 μM adenosine in combination with different concentrations of PGE_1 was measured in the presence of papaverine (Table 5). The increase in cyclic [^{14}C]AMP with adenosine became progressively smaller as the concentration of PGE_1 was increased, and was insignificant with 4 μM PGE_1. Neither synergism between adenosine and PGE_1 nor inhibition of the action of PGE_1 by adenosine was observed in the presence of papaverine. These results indicate that the adenylate cyclase activated by adenosine is also activated by PGE_1.

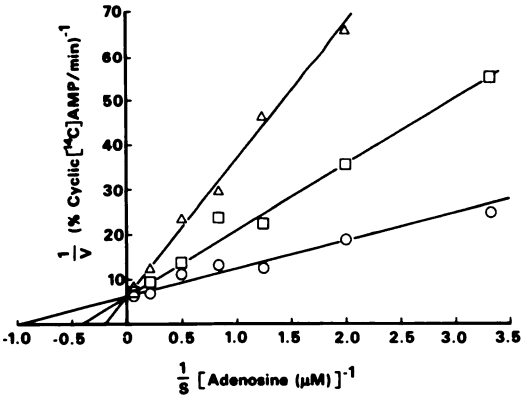

FIG. 5. *Kinetic analysis of activation of platelet adenylate cyclase by adenosine and of inhibition of this effect by methylxanthines*

Incubation mixtures contained 0.85 ml of labeled platelet-rich plasma (350,000 platelets/mm^3) and 0.15 ml of 0.154 M NaCl containing papaverine (final concentration, 2 mM) and a range of adenosine concentrations with or without a methylxanthine as indicated. Each was incubated for 0.25 min. Cyclic [^{14}C]AMP was isolated by method 2. The adenosine-activated formation of cyclic [^{14}C]AMP was calculated for each incubation mixture by subtracting the corresponding cyclic [^{14}C]AMP level found in the absence of adenosine and was expressed as cyclic [^{14}C]AMP (percentage of platelet ^{14}C) formed per minute. The results are plotted in reciprocal form against the reciprocal of the adenosine concentration. ○, adenosine; □, adenosine plus 100 μM caffeine; △, adenosine plus 100 μM theophylline. This experiment gave a K_A value for adenosine of 1.0 μM and K_i values for caffeine and theophylline of 72 and 25 μM, respectively.

Effects of agents that induce platelet aggregation on increases in platelet cyclic [^{14}C]AMP caused by adenosine. Concentrations of ADP and of epinephrine that can induce marked aggregation of platelets in stirred platelet-rich plasma almost completely blocked the increases in cyclic [^{14}C]AMP caused by 1–400 μM adenosine in the absence of papaverine, while a concentration of [8-arginine]vasopressin at least as effective in inducing aggregation in the absence of adenosine had no effect on cyclic [^{14}C]AMP levels (Fig. 6). As reported previously (24, 32), these aggregat-

TABLE 5

Effect of 10 μM adenosine on platelet cyclic [^{14}C]AMP levels in the presence of papaverine and varying concentrations of PGE$_1$

Incubation mixtures contained 0.85 ml of labeled platelet-rich plasma (360,000 platelets/mm^3) and 0.15 ml of additions dissolved in 0.154 M NaCl and were incubated for 0.5 min. All contained 2 mM papaverine. Cyclic [^{14}C]AMP was isolated by method 2. Values for cyclic [^{14}C]AMP are means ± standard errors from three identical incubation mixtures.

PGE$_1$ concentration	Cyclic [^{14}C]AMP		
	Adenosine absent	Adenosine present	Increase
μM		% platelet ^{14}C	
0	0.042 ± 0.000	0.170 ± 0.004	0.128 ± 0.004
0.02	0.076 ± 0.003	0.199 ± 0.004	0.122 ± 0.005
0.2	0.364 ± 0.004	0.454 ± 0.004	0.090 ± 0.006
1	1.004 ± 0.011	1.046 ± 0.015	0.042 ± 0.019
4	1.504 ± 0.015	1.509 ± 0.020	0.005 ± 0.025

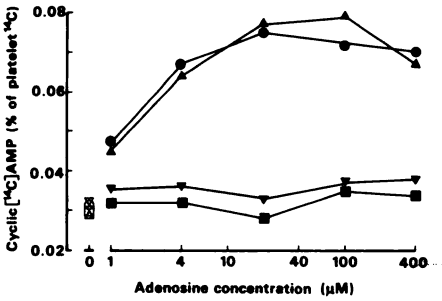

FIG. 6. *Effects of inducers of platelet aggregation on increases in platelet cyclic [^{14}C]AMP caused by adenosine*

Incubation mixtures contained 0.85 ml of labeled platelet-rich plasma (400,00 platelets/mm^3) and 0.15 ml of 0.154 M NaCl containing the additions specified (final concentrations given). Each was incubated for 0.5 min. Cyclic [^{14}C]AMP was isolated by method 2. ○, no additions; ●, adenosine; □, 4 μM ADP; ■, adenosine plus 4 μM ADP;▽, 10 μM epinephrine;▼, adenosine plus 10 μM epinephrine;△, [8-arginine]vasopressin (100 milliunits/ml);▲, adenosine plus [8-arginine]vasopressin (100 milliunits/ml).

ing agents did not affect the cyclic [^{14}C]AMP level in the absence of adenosine. The mechanism of action of ADP was investigated by kinetic studies in the presence of papaverine. Unlike the methylxanthines, ADP caused mainly noncompetitive inhibition (K_i approximately 0.9 μM) of the activation of adenylate cyclase by adenosine (Fig. 7). With adenosine concentrations of 100 μM and above, there was some evidence in several experiments of an additional, possibly competitive, element to the inhibition (e.g., Fig. 7).

If cyclic AMP mediates the inhibition of ADP-induced platelet aggregation by adenosine, there should be some correlation between levels of platelet cyclic [^{14}C]AMP and the inhibtion of aggregation. This was investigated in platelet-rich plasma to which 0.5 μM ADP and various concentrations of adenosine with and without papaverine were added simultaneously. The increase in cyclic [^{14}C]AMP and the associated inhibition of platelet aggregation were measured after 0.5 min in labeled and unlabeled samples of the same platelet-rich plasma. The results (Fig. 8) show that under these conditions there was an ordinal relationship between increasing cyclic [^{14}C]AMP levels and increasing inhibitions of aggregation in both the presence and absence of papaverine. Papaverine acted synergistically with adenosine with respect to both the increases in cyclic [^{14}C]AMP and the inhibitions of platelet aggregation. Very small increases in cyclic [^{14}C]AMP (to less than 160% of the level in the absence of adenosine) were associated with substantial (up to 40%) inhibition of aggregation in the absence of papaverine, while much larger increases in cyclic [^{14}C]AMP were associated with marked inhibition in the presence of papaverine.

Studies were carried out to see whether some of the unexpected findings with respect to changes in cyclic [^{14}C]AMP levels would be reflected in effects on platelet

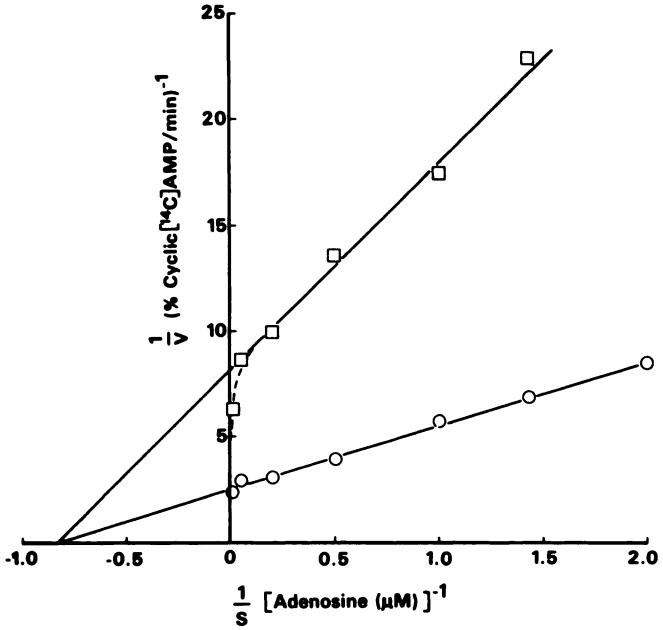

FIG. 7. *Kinetic analysis of inhibition by ADP of activation of platelet adenylate cyclase by adenosine*

Incubation mixtures contained 0.85 ml of labeled platelet-rich plasma (400,000 platelets/mm³) and 0.15 ml of 0.154 M NaCl containing papaverine (final concentration, 2 mM) and a range of adenosine concentrations with or without ADP (final concentration, 2.0 μM). Each was incubated for 0.5 min. Cyclic [^{14}C]AMP was isolated by method 2. The rate of adenosine-activated formation of cyclic [^{14}C]AMP was calculated as for Fig. 5 and plotted in reciprocal form against the reciprocal of the adenosine concentration. ○, adenosine; □, adenosine plus ADP. This experiment gave a K_A value for adenosine of 1.2 μM and a K_i value for ADP of 0.88 μM.

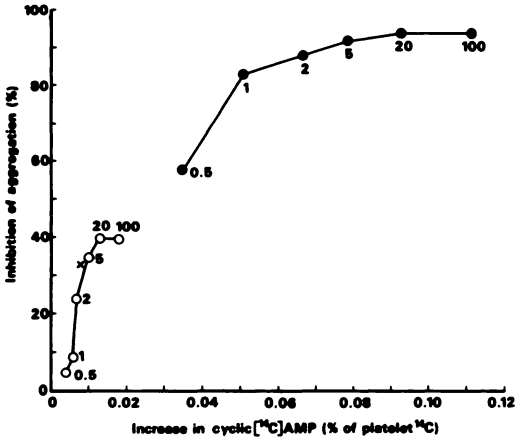

FIG. 8. *Relationships between inhibitions of ADP-induced platelet aggregation by adenosine with and without papaverine and associated increases in platelet cyclic* [^{14}C]*AMP*

For measurement of the inhibition of platelet aggregation, 0.85 ml of unlabeled platelet-rich plasma (395,000 platelets/mm³) was stirred in an aggregation module with simultaneous additions of ADP (final concentration, 0.5 μM) and aggregation inhibitors giving a final volume of 1 ml. Aggregation was recorded for up to 1 min, and the change in extinction 0.5 min after addition of ADP was measured. The percentage inhibition of aggregation was calculated relative to frequent controls without inhibitors. For measurement of the increases in platelet cyclic [^{14}C]AMP, incubation mixtures contained 0.85 ml of the same platelet-rich plasma, but labeled with [^{14}C]adenine, and identical additions. Each was incubated for 0.5 min but without continuous stirring. Cyclic [^{14}C]AMP was isolated by method 2. The cyclic [^{14}C]AMP present in controls without inhibitors was subtracted from that in the incubations with inhibitors to give the increase associated with inhibition of platelet aggregation. ×, 0.4 mM papaverine; ○, adenosine; ●, adenosine plus 0.4 mM papaverine. The final micromolar concentrations of adenosine used are indicated.

aggregation induced by ADP. As predicted, low concentrations (4–10 μM) of adenosine were found to inhibit aggregation more powerfully than the same con-

centrations of 2-chloroadenosine, provided that they were added with or only 0.5 min before ADP, while with high concentrations of the two compounds (200–400 μM) 2-chloroadenosine was the more effective (Fig. 9). However, decreasing levels of inhibition on increasing the adenosine concentration to 100 μM or above were not observed. NBTGR (10 μM) had no effect on aggregation by itself or on the inhibition of aggregation by 4–10 μM adenosine but did slightly potentiate the action of 200 μM adenosine added 0.5 min before 4 μM ADP. Finally, 0.4 mM caffeine, which alone had no effect on aggregation induced by 1 μM ADP, partially blocked inhibition of the aggregation by 10 μM adenosine (Fig. 10).

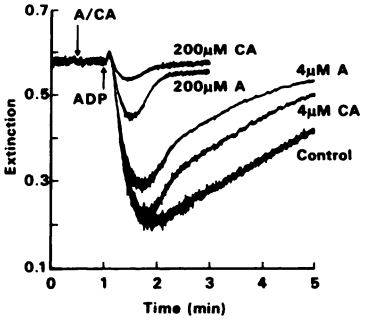

FIG. 9. *Inhibition of ADP-induced platelet aggregation by low and high concentrations of adenosine and 2-chloroadenosine*

Incubation mixtures containing 0.85 ml of platelet-rich plasma (450,000 platelets/mm³) and a final total of 0.15 ml of additions in 0.154 M NaCl were stirred in an aggregation module. Adenosine (A) or 2-chloroadenosine (CA) (final concentrations as indicated) was added after 0.5 min (except in the control), and ADP (final concentration, 0.5 μM) was added after 1 min.

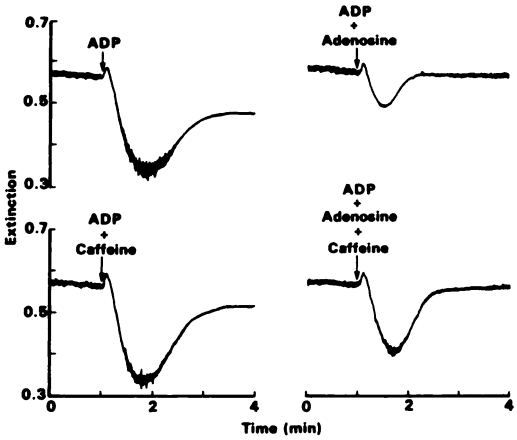

FIG. 10. *Antagonism by caffeine of inhibition of ADP-induced platelet aggregation by adenosine*

Incubation mixtures contained initially 0.92 ml of platelet-rich plasma (400,000 platelets/mm³) and 0.03–0.07 ml of 0.154 M NaCl and were stirred in an aggregation module for 1 min before simultaneous addition in 0.154 M NaCl of final concentrations of 1 μM ADP, 10 μM adenosine, and 400 μM caffeine as indicated (final volume, 1 ml).

DISCUSSION

Measurement of small changes in cyclic AMP levels in functionally intact platelets in plasma is difficult, as the proportion of the total volume occupied by platelets is small (less than 0.5%) while plasma itself contains relatively large and variable amounts of cyclic AMP (33). Isolation of platelets by centrifugation tends to stimulate them and may release cyclic AMP (34). We therefore used the method of first labeling cells with [^{14}C]adenine, followed by isolation of cyclic [^{14}C]AMP after addition of various stimuli (35). In the earliest attempts to apply this method to platelets (13, 36) approximately 0.1% of the platelet ^{14}C was found in presumptive cyclic [^{14}C]AMP in the control samples of platelet-rich plasma. No increase in platelet cyclic [^{14}C]AMP was detected in the presence of theophylline alone (13, 36), and only small, variable increases were observed with adenosine alone (13). Our results suggest that these difficulties were probably due to the presence of large, variable proportions of impurities in the cyclic [^{14}C]AMP isolated. Thus, using purified [*U*-^{14}C]adenine and improved methods for isolation of cyclic [^{14}C]AMP, we found highly reproducible control levels of cyclic [^{14}C]AMP in individual experiments, the values of which lay in the range of 0.028–0.059% of the platelet ^{14}C in all the experiments on which this paper is based. Although, as determined by susceptibility to 3′,5′-cyclic nucleotide phosphodiesterase, the radiochemical purity of this material was still only 80–90%, the measured increases in cyclic [^{14}C]AMP induced by adenosine or PGE_1 were entirely due to formation of authentic cyclic [^{14}C]AMP.

No relationship between the incorpora-

tion of adenosine and the accumulation of cyclic [^{14}C]AMP in platelets was found. In the presence of 10 μM NBTGR, incorporation of 1–10 μM adenosine over 0.5 min was reduced to unmeasurable levels and, from results with 10-min incubations, was inhibited by at least 96%. Despite this, the accumulation of cyclic [^{14}C]AMP was unaffected by NBTGR in short incubations with these low adenosine concentrations. Moreover, NBTGR did not increase cyclic [^{14}C]AMP levels by itself, nor did it increase cyclic [^{14}C]AMP levels further in the presence of PGE_1, indicating that the compound did not inhibit cyclic AMP phosphodiesterase at the concentration used. Thus it can be concluded that incorporation of adenosine is not required for an increase in cyclic [^{14}C]AMP to occur and that the site of action of adenosine is probably at an extracellular membrane receptor. With inhibitors of adenosine transport such as papaverine or pyrimidopyrimidines, which also inhibit platelet cyclic AMP phosphodiesterase and potentiate the inhibition of aggregation by adenosine (13, 37), such a firm conclusion is not possible. Related results have recently been reported by Huang and Daly (38), who found that NBTGR and other inhibitors of the incorporation of adenosine into brain slices did increase the cyclic AMP response of the slices to adenosine. These authors suggested that the drugs acted by increasing the effective concentration of adenosine at extracellular receptor sites. The differences between the effects of NBTGR in the platelet and brain systems can be explained by the absence of a diffusion barrier limiting access of adenosine to the cell membrane in the former. The conclusion that adenosine acts on a membrane receptor at the outer surface of the platelet is supported by the finding that adenosine itself activates adenylate cyclase in broken cell preparations from platelets (14) and by our failure to detect unmetabolized adenosine within the platelets.

On addition of adenosine with or without NBTGR, platelet cyclic [^{14}C]AMP levels reached a maximum after about 0.5 min and then declined. The results show that this decline cannot be accounted for by dilution of [^{14}C]ATP by unlabeled ATP formed from the adenosine. In any case, a similar early maximum level of cyclic [^{14}C]AMP has been seen after addition of PGE_1 (24, 36) or isoproterenol (24) to platelets. As formation of cyclic [^{14}C]AMP was linear in the presence of adenosine and papaverine for at least 1 min, an increased cyclic AMP phosphodiesterase activity appears to be a possible explanation. In 0.5-min incubations the optimal activity of adenosine in increasing platelet cyclic [^{14}C]AMP levels was observed in the concentration range 10–40 μM, above which less cyclic [^{14}C]AMP accumulated. A similar maximum, but around 200 μM, was observed in the effect of adenosine on cyclic AMP levels in brain slices (16, 38). This apparent difference in optimal concentration could reflect the diffusion gradient of adenosine within brain slices. The inhibitory effect of high adenosine concentrations on the accumulation of platelet cyclic [^{14}C]AMP was more pronounced in the presence of PGE_1. NBTGR blocked the decline in cyclic [^{14}C]AMP levels with high adenosine concentrations in both the presence and absence of PGE_1, suggesting that adenosine caused this effect at a site distinct from that involved in increasing cyclic AMP levels. This biphasic action of adenosine in intact platelets closely parallels the two independent and opposing effects of the compound on adenylate cyclase previously observed in a platelet particulate fraction (14). Because of this, we consider that inhibition of adenylate cyclase is more likely to account for the second action of adenosine in intact platelets than a potentiation of cyclic AMP phosphodiesterase. Although papaverine blocked the inhibitory component of the action of adenosine, this could be due to its NBTGR-like effect on adenosine transport rather than to inhibition of phosphodiesterase. Although NBTGR did not significantly inhibit incorporation of adenosine above 200 μM into platelets in 10-min incubations, it might do so in 0.5-min incubations, such as were used in the studies on cyclic [^{14}C]AMP. Thus the anomalous decrease in adenosine incorporation at concentrations above 10 μM in the absence of NBTGR, which has also been observed with tissue culture cells (39), could in prin-

ciple be due to the progressive extracellular accumulation of inhibitory metabolites such as inosine and hypoxanthine (40). If so, inhibition of adenosine incorporation by NBTGR could still account for the effect of the latter in blocking the inhibitory action of adenosine on cyclic [^{14}C]AMP formation in 0.5-min incubations. Although no adenosine was found intracellularly with the extracellular concentrations examined (up to 40 μM), significant amounts of intracellular adenosine might be found with higher extracellular adenosine concentrations, as reported for erythrocyte ghosts (41). Support for an intracellular site for the inhibitory action of adenosine is provided by the observations that adenosine inhibited platelet adenylate cyclase in a broken cell preparation in the presence of papaverine (14), whereas in intact platelets papaverine prevented this effect. Moreover, NBTGR did not block the inhibition of adenylate cyclase by adenosine in broken cell preparations.[2] Adenosine has also been shown to inhibit adenylate cyclase in broken cell preparations from a variety of other tissues (42–44).

The marked synergism in increasing cyclic [^{14}C]AMP levels between adenosine and papaverine, a potent inhibitor of platelet and other cyclic AMP phosphodiesterases (13, 45), relative to the very weak synergism with PGE_1, an activator of platelet adenylate cyclase (46), indicates that the principal action of adenosine in platelets is to stimulate adenylate cyclase rather than to inhibit cyclic AMP phosphodiesterase. Our failure to obtain synergism between caffeine and adenosine, except at very high concentrations of the latter, is consistent with the evidence that methylxanthines block the increase in cyclic AMP levels with adenosine in brain slices (16, 38). In the presence of 2 mM papaverine the increase in cyclic [^{14}C]AMP in both the presence and absence of adenosine was linear for up to 1 min and thus provided a method of measuring the activation of adenylate cyclase by adenosine in intact platelets. Adenosine increased platelet adenylate cyclase activity by a maximum of 8–10-fold. Double-reciprocal plots of the effects of different adenosine concentrations of platelet adenylate cyclase were linear, indicating that adenosine interacts with a saturable site. Half-maximal activation of adenylate cyclase was achieved with 0.9–1.3 μM adenosine, and both caffeine and theophylline were shown to be competitive inhibitors of the action of adenosine, with K_i values of 72 and 25 μM, respectively. No comparable quantitative data are yet available from other tissues, but the evidence clearly indicates that brain, at least, possesses specific adenosine receptors with similar if not identical properties (16, 38).

It has been shown in several laboratories that ADP and epinephrine, which are powerful inducers of platelet aggregation, each markedly inhibits the increase in platelet cyclic AMP caused by PGE_1 (32, 47, 48). On the other hand, vasopressin, which causes as pronounced a platelet aggregation as ADP, does not have this effect (32). These three aggregating agents had effects identical with those seen in the presence of PGE_1, when cyclic [^{14}C]AMP levels in platelets were elevated with adenosine. The difference between the effects of ADP and of vasopressin suggests that the action of ADP depends upon a specific receptor–adenylate cyclase interaction and cannot be secondary to aggregation or to those aspects of the aggregation mechanism that are common to the actions of both aggregating agents. The effect of ADP was analyzed in detail in the presence of papaverine, and it was found to cause noncompetitive inhibition (K_i approximately 0.9 μM) of the activation of adenylate cyclase by adenosine concentrations below 100 μM. This establishes that the adenosine receptor is distinct from the ADP receptor and that adenosine concentrations below 100 μM do not inhibit platelet aggregation by direct antagonism of the action of ADP, as was initially believed (6, 7). Noncompetitive inhibition by ADP with a very similar K_i value has also been reported for the effect of ADP on the activation of adenylate cyclase by PGE_1 (24), which suggests that ADP acts by the same mechanism in both cases. In this connection, our evidence also suggests that adenosine and PGE_1 activate the same adenylate cyclase.

[2] Unpublished observations.

With concentrations of adenosine above 100 μM some evidence of an additional, possibly competitive interaction with ADP was obtained. This could represent a weak affinity of adenosine for the ADP receptor.

Our results strongly support the view that the effects on platelet aggregation of adenosine concentrations below 100 μM are mediated by cyclic AMP (13, 24), both by demonstrating increases in platelet cyclic AMP levels with adenosine more clearly than has been done before and by providing evidence of correlations between cyclic AMP levels and the inhibition of aggregation under a variety of conditions. First, low concentrations of 2-chloroadenosine both increased cyclic [^{14}C]AMP and inhibited aggregation by ADP less than adenosine, provided that short incubation times were used, while high concentrations of 2-chloroadenosine both increased cyclic [^{14}C]AMP and inhibited aggregation more than adenosine. These different relative activities of the two nucleosides at different concentrations have not previously been recognized (2). Second, caffeine partially blocked the inhibition of ADP-induced aggregation by adenosine, which is in accord with its antagonism of the effect of adenosine on platelet adenylate cyclase. Third, the synergism between adenosine and papaverine with respect to the accumulation of cyclic [^{14}C]AMP was also expressed in the inhibition of platelet aggregation by the two compounds. Fourth, the previous observation that adenosine is a much more potent inhibitor of vasopressin-induced platelet aggregation than of ADP-induced aggregation (5) can now readily be explained by the different cyclic [^{14}C]AMP levels observed in the presence of adenosine and each of these two aggregating agents. This last observation also emphasizes that it is the concentration of cyclic AMP resulting from the interaction of adenosine and the aggregating agent, rather than that produced by adenosine alone, which determines the inhibition of aggregation. When cyclic [^{14}C]AMP levels were measured after the simultaneous addition of ADP and adenosine, the increases associated with a substantial inhibition of aggregation were small relative to the control, unstimulated level of cyclic [^{14}C]AMP. This suggests that much of the cyclic [^{14}C]AMP present in the absence of adenosine is not available for inhibition of platelet function, possibly as a result of being protein-bound. The over-all relationship between the increases in platelet cyclic [^{14}C]AMP and the inhibition of platelet aggregation by adenosine with and without papaverine was roughly hyperbolic, which is consistent with the view that cyclic AMP acts by binding to a saturable site on the regulatory subunit of a protein kinase (34).

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