Biochem. J. (1971) **121**, 185–196
Printed in Great Britain

The Influence on Platelet Aggregation of Drugs that Affect the Accumulation of Adenosine 3′:5′-Cyclic Monophosphate in Platelets

BY D. C. B. MILLS AND J. B. SMITH
Medical Research Council Thrombosis Research Group, Department of Pharmacology, Royal College of Surgeons, Lincoln's Inn Fields, London W.C.2, U.K.

(*Received* 21 *July* 1970)

1. The involvement of intracellular 3′:5′-cyclic AMP in the inhibition of platelet aggregation by prostaglandin E_1, isoprenaline and adenosine has been examined by a radiochemical technique. Platelet-rich plasma was incubated with radioactive adenine to incorporate ^{14}C radioactivity into platelet nucleotides. Pairs of identically treated samples were taken, one for the photometric measurement of platelet aggregation induced by ADP, the other for estimation of the radioactivity of 3′:5′-cyclic AMP. 2. Theophylline, papaverine, dipyridamole and 2,6-bis-(diethanolamino)-4-piperidinopyrimido[5,4*d*]pyrimidine (compound RA233) were found to inhibit 3′:5′-cyclic AMP phosphodiesterase from platelets. At concentrations of 3′:5′-cyclic AMP greater than 50 μM the most active inhibitor was dipyridamole; at 3′:5′-cyclic AMP concentrations less than 19 μM, papaverine and compound RA233 were more active than dipyridamole. 3. In the presence of compound RA233 (50 μM), the effectiveness of prostaglandin E_1 as an inhibitor of platelet aggregation was increased tenfold. Compound RA233 also increased the stimulation by prostaglandin E_1 of the incorporation of radioactivity into 3′:5′-cyclic AMP. 4. Compound RA233 (50 μM) increased the effectiveness of both adenosine and 2-chloroadenosine as inhibitors of aggregation by 70–100-fold, and in the presence of compound RA233 both adenosine and 2-chloroadenosine stimulated the incorporation of radioactivity into 3′:5′-cyclic AMP; the extent of the stimulation was proportional to the logarithm of the nucleoside concentration. 5. Compound RA233 (100–500 μM) inhibited platelet aggregation by itself and caused small increases in the radioactivity of 3′:5′-cyclic AMP. Partial positive correlations were found between the radioactivity of 3′:5′-cyclic AMP in platelets measured at the time of addition of the aggregating agent (ADP) and the extent to which the aggregation was inhibited. 6. The results are interpreted as indicating that adenosine, 2-chloroadenosine, isoprenaline, prostaglandin E_1 and drugs that inhibit platelet 3′:5′-cyclic AMP phosphodiesterase all inhibit aggregation by a common mechanism involving intracellular 3′:5′-cyclic AMP.

Human blood platelets in citrated platelet-rich plasma aggregate on the addition of ADP, and the effect of ADP is inhibited by the structurally similar compounds adenosine (Born, 1962; Born & Cross, 1963), AMP (Born, 1962; Packham, Ardlie & Mustard, 1969) and by 2-chloroadenosine (Born, 1964; Maguire & Michal, 1968). The kinetics of this inhibition suggest a degree of competition between the inhibitors and ADP for a binding site at the platelet membrane (Skoza, Zucker, Jerushalmy & Grant, 1967; Maguire & Michal, 1968), although this does not explain the fact that inhibition by adenosine and 2-chloroadenosine increases with time (Born & Cross, 1963; Born, 1964). Also, AMP is less effective an inhibitor than adenosine, though more similar in structure to ADP. In human platelet-rich plasma the inhibitory effect of AMP can largely be attributed to breakdown to adenosine, and is greatly diminished by the addition of adenosine deaminase to the plasma (Rozenberg & Holmsen, 1968).

Platelet aggregation is also inhibited by PGE_1* (Kloeze, 1967; Emmons, Hampton, Harrison, Honour & Mitchell, 1967) which stimulates adenylate cyclase in particulate fractions of platelet homogenates (Wolfe & Shulman, 1969; Zieve & Greenough, 1969; Marquis, Vigdahl & Tavormina, 1969; Abdulla, 1969). In the presence of theophylline, PGE_1 increases platelet 3′:5′-cyclic AMP content

* Abbreviation: PGE_1, prostaglandin E_1.

(Robison, Arnold & Hartmann, 1969) and increases the radioactivity of 3′:5′-cyclic AMP in platelets with nucleotides labelled by the incorporation of radioactive adenosine (Vigdahl, Marquis & Tavormina, 1969) or adenine (Mills, Smith & Born, 1970). Inhibition of aggregation by both PGE_1 and by isopropylnoradrenaline (isoprenaline) is potentiated by theophylline, an inhibitor of 3′:5′-cyclic AMP phosphodiesterase, at concentrations of theophylline that themselves do not inhibit (Mills *et al.* 1970). Ardlie, Glew & Schwartz (1967) found that higher concentrations of methylxanthines (theophylline and caffeine) inhibit aggregation and suggested that inhibition might involve 3′:5′-cyclic AMP. A variable increase in the concentration of 3′:5′-cyclic AMP in platelets treated with caffeine has been shown (Salzman & Neri, 1969).

The drugs dipyridamole and papaverine, which block the incorporation of adenosine into platelets (Markwardt, Barthel, Glusa & Hoffman, 1967; Born & Mills, 1969; Mills *et al.* 1970) potentiate the inhibition of aggregation that adenosine causes. Adenosine and adenine nucleotides cause increases in 3′:5′-cyclic AMP in guinea-pig brain (Sattin & Rall, 1970) though subcellular preparations of adenylate cyclase from rat liver are inhibited by adenosine and by nucleotides (Moriwaki & Foa, 1970). Dipyridamole and papaverine inhibit phosphodiesterase from ox heart (Poch, Juan & Kukovetz, 1969) and this suggests a possible alternative explanation for their action on platelets.

We have investigated the effects of papaverine and of dipyridamole and its analogues 2,6-bis(diethanolamino) - 4 - piperidinopyrimido[5, 4*d*]-pyrimidine (compound RA233) and 2,4,6-trimorpholinopyrimido[5, 4*d*]pyrimidine (compound RA433) on platelet phosphodiesterase activity and on the inhibition of aggregation produced by PGE_1 and by adenosine and 2-chloroadenosine to determine the extent to which the formation of 3′:5′-cyclic AMP within the platelets is implicated in the inhibition of aggregation.

METHODS

Preparation of labelled platelets. Citrated platelet-rich plasma was prepared from normal healthy donors by the method of Mills & Roberts (1967) and the platelets were labelled with [U-^{14}C]adenine (sp. radioactivity 231 mCi/mmol) by the technique described by Ball, Fulwood, Ireland & Yates (1969). [U-^{14}C]Adenine (1–1.5 μM) was added to platelet-rich plasma and incubated for 45–80 min. Samples were taken during this period and centrifuged to determine the extent of incorporation of ^{14}C by counting the radioactivity of the supernatant plasma. A maximum of 85–90% of the added radioactivity was taken up and thereafter the plasma radioactivity increased by 5–10% of the total over the next 2 h.

Platelet aggregation. Aggregation was studied photometrically (Born, 1962) as described in detail by Mills & Roberts (1967), and measured as the maximum rate of change of optical transmission occurring 10–30 s after addition of ADP. High concentrations of ADP (100–500 μM) were used because at low concentrations of ADP the kinetics of adenosine inhibition suggest competition with ADP (Skoza *et al.* 1967) whereas at higher concentrations this is not the case (Salzman, Ashford, Chambers, Neri & Dempster, 1969). Preliminary experiments showed that when aggregation induced by 5 μM-ADP was just completely suppressed by combinations of PGE_1 and theophylline (Mills *et al.* 1970), increasing the ADP concentrations by 20–100-fold did not cause aggregation. Thus the inhibition was shown to be not competitive with respect to ADP. All experiments were carried out at 37°C.

Measurement of 3′:5′-cyclic AMP radioactivity. Samples (1 ml) of platelet-rich plasma were incubated with drugs in the aggregometer, and successive samples were used for measurement of platelet aggregation and for determination of radioactive 3′:5′-cyclic AMP. For the latter, the sample was removed from the aggregometer with a syringe and injected into 0.5 ml of 2 M-$HClO_4$ containing 0.12 μmol of 3′:5′-cyclic AMP and 0.05 μCi of 3′:5′-cyclic [^{3}H]AMP as an internal marker to control for variations in recovery at subsequent stages. Precipitated proteins were removed by centrifugation at 12000*g* for 2 min (Eppendorf Microfuge) and a portion (1.2 ml) of the supernatant was transferred to 1.0 ml columns of Dowex 50W (X4) resin packed in Pasteur pipettes. The columns were washed twice with 1.0 ml of water and eluted with two successive portions of 1.0 ml of water. The eluates were treated twice with $ZnSO_4$ and $Ba(OH)_2$ as described by Krishna, Weiss & Brodie (1968). The final supernatant (about 2.6 ml) was decanted into scintillation vials and the radioactivity was counted with 15 ml of dioxan–naphthalene–5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole scintillant (Scales, 1967) for 50–100 min in a Packard Tri-Carb scintillation counter. The radioactivity was corrected for channel crossover and for recovery of internal 3′:5′-cyclic [^{3}H]AMP standard, which varied from 25–35%, and was expressed as a percentage of the total counts in the platelets in 1.0 ml of plasma. In one experiment, ^{14}C-labelled platelets were isolated by adding EDTA (5 mM) to platelet-rich plasma, centrifuging at 2300*g* for 10 min at 4°C, and resuspending in 0.15 vol. of iso-osmotic saline (0.134 M-NaCl, 0.015 M-tris–HCl buffer, pH 7.4, 0.005 M-glucose). Radioactivity of 3′:5′-cyclic AMP was measured after isolation by paper chromatography (see Table 5 for details).

Preparation and assay of platelet 3′:5′-cyclic AMP phosphodiesterase. Washed human platelets from 80 ml of platelet-rich plasma were resuspended in 5 ml of 0.154 M-NaCl, sonicated twice for 10 s (Dawe Soniprobe, model 1130A) and the homogenate was centrifuged at 17500*g* for 20 min at 0°C. The supernatant, which contained essentially all the activity, was then dialysed overnight against 1 litre of 10 mM-tris–HCl buffer, pH 7.4, and the dialysed enzyme was stored at −15°C. Enzyme activity was retained on storage for at least 2 weeks, but was lost on successively thawing and refreezing. Phosphodiesterase activity was measured in 0.10 ml incubation mixtures containing 5 mM-$MgSO_4$, 20 mM-tris–HCl buffer, pH 7.4, and 35000 c.p.m. of 3′:5′-cyclic [^{3}H]AMP (13–130 μM). After 10 or 20 min at 37°C the reaction was stopped by the addition of 1.0 ml of 0.5 M-$HClO_4$ con-

taining 1000 c.p.m. of 3′:5′-cyclic [^{14}C]AMP as internal standard. Unchanged 3′:5′-cyclic [^{3}H]AMP was determined by the procedure used for platelet extracts. By this method the reaction rate was linear with increasing enzyme concentration up to 80% conversion. With initial concentrations of 3′:5′-cyclic AMP of 3–100 μM, the apparent K_m of the platelet phosphodiesterase for 3′:5′-cyclic AMP was 71 and 83 μM in two experiments.

Protein. Protein content was measured by the biuret reaction (Gornall, Bardawill & David, 1949).

Special materials. [U-^{14}C]Adenine (sp. radioactivity 231 mCi/mmol) and [8-^{3}H]adenosine 3′:5′-cyclic monophosphate (sp. radioactivity 3000 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [8-^{14}C]Adenosine 3′:5′-cyclic monophosphate (sp. radioactivity 40.91 mCi/mmol) was from Schwartz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Non-radioactive 3′:5′-cyclic AMP and ox heart phosphodiesterase (0.1 unit/mg) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. PGE_1 was a gift from Dr J. E. Pike, The Upjohn Co., Kalamazoo, Mich., U.S.A.; 2-chloroadenosine was given by Dr J. A. Montgomery, Southern Research Institute, Birmingham, Ala., U.S.A.

AG50W(X4) resin was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole was from CIBA (ARL) Ltd., Duxford, Cambs., U.K. Dipyridamole [Persantin, 2, 6-bis-(diethanolamino)-4, 8-dipiperidinopyrimido[5,4*d*]-pyrimidine] and compound RA233 [2,6-bis-(diethanolamino) - 4 - piperidinopyrimido[5,4*d*]pyrimidine] and compound RA433 [2,4,6-trimorpholinopyrimido[5,4*d*]-pyrimidine] were kindly provided by Dr J. W. Bell of Boehringer Ingelheim Ltd., Isleworth, Middx., U.K.

RESULTS

Platelet phosphodiesterase. The activity of a number of compounds as inhibitors of platelet 3′:5′-cyclic AMP phosphodiesterase was assessed at different concentrations of the 3′:5′-cyclic AMP substrate (Tables 1 and 2). Papaverine, dipyridamole and the dipyridamole analogues were tested at a final concentration in the incubation mixture of 50 μM; theophylline was tested at 1 mM (Table 1). The order of activity of the inhibitors varied according to the concentration of substrate used. Dipyridamole, which was the most active of the inhibitors at a high 3′:5′-cyclic AMP concentration (130 μM) was less effective than papaverine, and compounds RA233 and RA433 at 3′:5′-cyclic AMP concentrations of 19 μM or less.

Adenosine, 5′-AMP and 2-chloroadenosine were also tested (Table 2). AMP at concentrations up to 3 mM did not inhibit the enzyme; adenosine and 2-chloroadenosine caused partial inhibition at 0.3–3 mM.

Platelet aggregation. In the experiment shown in Fig. 1, isoprenaline, PGE_1 and adenosine were tested as inhibitors of aggregation with and without the addition of compound RA233 (50 μM, a concentration that by itself had little effect on aggregation). Fig. 1 shows that there was a qualitatively similar synergism between compound RA233 and isoprenaline, PGE_1 and adenosine. Concentrations of these compounds that by themselves had no effect on aggregation caused considerable inhibition in the presence of compound RA233. The shape of the aggregation curve in the presence of these combinations of inhibitors was different from the shape seen with various concentrations of ADP alone; for a given final change in transmission, the rate of change was lower in the inhibited samples than that brought about by low concentrations of ADP.

The radioactivity of 3′:5′-cyclic AMP was determined in samples of the same plasma prelabelled with radioactive adenine, by using the same drug concentrations and incubation times as were used

Table 1. *Inhibition of platelet 3′:5′-cyclic AMP phosphodiesterase*

Results are expressed as percentage inhibition of the destruction of 3′:5′-cyclic [^{3}H]AMP during 20 min incubation at 37°C with platelet extract containing 0.06 mg of protein/tube. For details see the Methods section.

3′:5′-cyclic [^{3}H]AMP concn. (μM)	130	39	19	13
% conversion in controls	48.8	60.5	69.0	79.3
	% inhibition			
Drug				
Theophylline (1 mM)	47.1	45.6	42.4	44.6
Papaverine (50 μM)	59.0	56.1	59.5	62.5
Dipyridamole (50 μM)	65.3	55.3	43.0	38.6
Compound RA233 (50 μM)	49.6	53.4	48.5	50.3
Compound RA433 (50 μM)	63.1	50.7	47.6	52.8

Table 2. *Effects of adenosine, 2-chloroadenosine and 5′-AMP on platelet phosphodiesterase activity*

Enzyme activity was measured in 0.10 ml samples incubated for 10 min at 37°C with 20 μM-3′:5′-cyclic AMP containing 50000 c.p.m. of 3′:5′-cyclic [8-^{3}H]AMP and with 0.24 mg of platelet-extract protein/tube. In the control tubes containing no inhibitor the rate of 3′:5′-cyclic AMP disappearance was 1.23 nmol/0.1 ml in 10 min. Other details were as described for Table 1.

Concn. of inhibitor (μM) Inhibitor ...	% inhibition of 3′:5′-cyclic AMP destruction		
	Adenosine	2-Chloro-adenosine	5′-AMP
30	−1.9	−4.5	−10.6
300	2.0	11.7	−15.0
3000	27.1	57.6	−13.4

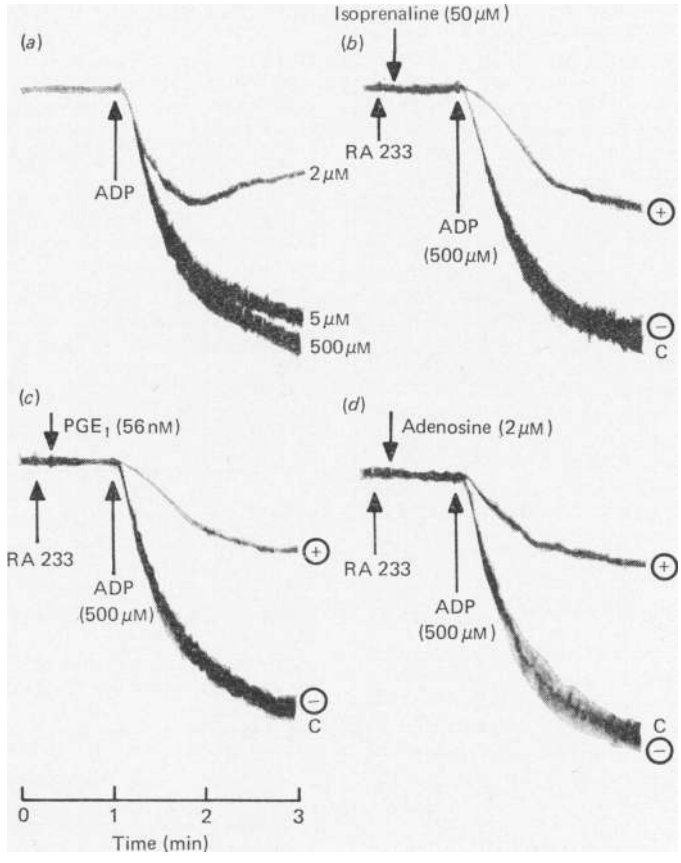

Fig. 1. Records of platelet aggregation in stirred human citrated platelet-rich plasma at 37°C, induced by ADP in the presence of an inhibitor of platelet 3′:5′-cyclic AMP phosphodiesterase (compound RA233) and of isoprenaline, PGE_1 and adenosine. Aggregation is represented by a downward deflexion of the trace. (*a*) ADP added alone to give final concentrations of 2, 5 and 500 μM (superimposed tracings). (*b*), (*c*) and (*d*) In each case aggregation was induced by the addition of ADP (final concentration 500 μM). C represents the control response in the presence of compound RA233 (50 μM). − represents the response in the presence of the drug (isoprenaline, PGE_1 or adenosine) without compound RA233. + represents the response in the presence of the drug plus compound RA233 (50 μM).

for investigating aggregation. The results are shown in Table 3. There was little effect on 3′:5′-cyclic AMP radioactivity with concentrations of isoprenaline, PGE_1 or adenosine that by themselves caused little or no inhibition of aggregation, though 3′:5′-cyclic AMP radioactivity was increased with higher concentrations of both PGE_1 and adenosine that did inhibit. In the presence of compound RA233, the effects of isoprenaline, PGE_1 and adenosine on 3′:5′-cyclic AMP radioactivity, were enhanced to an extent that was roughly proportional to the increased inhibition of aggregation.

Prostaglandin E_1. Inhibition of aggregation by a number of inhibitors of platelet phosphodiesterase in the presence and absence of PGE_1 is shown in Table 4. Comparison of Tables 1 and 4 shows that there was a direct relationship between the activities of the drugs as inhibitors of phosphodiesterase at low substrate concentrations and their ability to potentiate the inhibition of aggregation by PGE_1. Dipyridamole was only effective as a potentiator of the action of PGE_1 at high concentrations of the latter (see Table 5). In an experiment with platelets isolated after labelling and resuspended in buffered

Table 3. *Effect of inhibitors of platelet aggregation on the radioactivity of platelet 3′:5′-cyclic AMP*

Samples (1.0ml) of platelet-rich plasma, prelabelled with [^{14}C]adenine, were incubated for 20s with or without compound RA233 (50μM) before the addition of the second drug. Incubation was continued for a further 40s and the reaction was stopped by the addition of 50μl of 11.4M-$HClO_4$ and transferring the tubes to an ice-bath. Samples for measurement of aggregation were treated in a similar manner, but the incubations were performed in the aggregometer and ADP (final concentration 500μM) was added in place of $HClO_4$, and the rate of aggregation recorded (see Fig. 1).

	Without compound RA233		With compound RA233	
Second addition	%I*	% 3′:5′-cyclic [^{14}C]AMP	%I*	% 3′:5′-cyclic [^{14}C]AMP
None	0	0.090	11	0.096
Adenosine (2μM)	6	0.095	69	0.143
Adenosine (10μM)	17	0.113	95	0.175
PGE_1 (0.056μM)	1	0.116	78	0.173
PGE_1 (0.28μM)	49	0.320	98	0.324
Isoprenaline (50μM)	3	0.099	58	0.126

* %I = % inhibition of platelet aggregation measured as maximum rate of change of optical transmission.

Table 4. *Potentiation of the effect of PGE_1 on platelet aggregation by inhibitors of platelet 3′:5′-cyclic AMP phosphodiesterase*

Tubes containing 1.0ml of platelet-rich plasma were warmed to 37°C for 5min before being transferred to the aggregometer. The plasma was stirred for 10s before the addition of the phosphodiesterase inhibitor, and PGE_1 (final concentration 0.014μM) was added 10s later. ADP (final concentration 100μM) was added 40s later to induce aggregation. Rates of aggregation of control samples measured at intervals through the experiment were 310, 270 and 236mm/min without PGE_1, and 278 and 276mm/min with PGE_1.

		% inhibition of aggregation	
Drug	Concentration (μM)	Without PGE_1	With PGE_1
Theophylline	500	0	11.0
	1000	0	52.6
Caffeine	1000	0.5	16.0
	2000	11.3	48.5
	4000	15.0	96.7
Papaverine	50	0	3.5
	100	0	37.5
	250	1.5	90.7
Dipyridamole	100	12.5	6.4
	250	18.3	13.7
	500	5.2	10.5
Compound RA233	50	16.0	22.2
	100	21.5	67.2
	150	38.5	93.9
Compound RA433	150	6.8	65.3
	250	28.2	88.1
	500	23.7	100

saline (see the Methods section), dipyridamole increased the effects of PGE_1 on the radioactivity of 3′:5′-cyclic AMP measured 4min after the addition of PGE_1. In this experiment 3′:5′-cyclic AMP was separated from other radioactive compounds by paper chromatography and higher control values were found than with the Dowex column procedure. For this reason, only increases in 3′:5′-cyclic AMP radioactivity are given. However, this procedure allowed a complete investigation of the distribution of radioactivity in the major fractions of platelet nucleotides as well as in 3′:5′-cyclic AMP. Neither compound RA233, theophylline nor PGE_1, alone or in combination,

Table 5. *Effects of theophylline, dipyridamole and compound RA233 on the radioactivity of 3′:5′-cyclic AMP in platelets resuspended in saline*

Samples of the platelet suspension containing 7.68×10^8 platelets in 0.5ml were incubated for 2min at 37°C with phosphodiesterase inhibitors before the addition of PGE_1. The incubation was continued for 4min and stopped by the addition of 1.0ml of ice-cold 0.139M-NaCl containing 10mM-EDTA and 2% (w/v) formaldehyde. The platelets were sedimented by centrifuging at 12000*g* for 30s (Eppendorf Microfuge) and after removal of the supernatant medium were homogenized with 20μl of 1.0M-$HClO_4$ containing adenine nucleotides and nucleosides (50nmol each of ATP, ADP, AMP, 3′:5′-cyclic AMP, IMP, adenosine, inosine and hypoxanthine). The mixture was adjusted to pH4 with 2M-K_2CO_3, and protein and $KClO_4$ were removed by centrifugation. Samples (10μl) of the extract were streaked on Whatman no. 541 paper and chromatographed by downward development in butan-1-ol–acetone–acetic acid–5% (v/v) aq. ammonia (sp.gr. 0.88)–water (9:3:2:2:4, by vol.) (Randerath & Strück, 1961). Spots corresponding to the various carriers were located under u.v. light, cut out and placed in a toluene-based scintillation mixture for liquid-scintillation counting (see the Methods section). Untreated platelets contained 0.136% radioactivity as 3′:5′-cyclic [^{14}C]AMP.

	Increase in % 3′:5′-cyclic [^{14}C]AMP		
Drug PGE_1 concn. (μM) ...	0	0.56	5.6
Theophylline (200μM)	—	0.014	0.061
Theophylline (2mM)	—	0.036	0.105
Dipyridamole (50μM)	—	0.065	0.145
Dipyridamole (500μM)	0.002	0.161	0.605
Compound RA233 (50μM)	—	0.064	0.145
Compound RA233 (500μM)	0.019	0.327	1.060

caused any consistent change in the pattern of radioactivity of ATP, ADP, AMP or IMP. Dipyridamole (500μM) caused an increase in the proportion of radioactivity recovered as IMP from 4.13 ± 0.16% to 6.72% (range 6.66–6.78%).

PGE_1 by itself, added 40s before ADP, caused inhibition of aggregation, with an S-shaped log (dose)–response curve at concentrations of 0.02–1μM (Fig. 2). In the presence of compound RA233 (50μM) the dose–response curve was unaltered in shape but was shifted to the left to an extent equivalent to tenfold increase in the effectiveness of PGE_1. Measurements of 3′:5′-cyclic AMP radioactivity made in the same experiment with labelled platelets are shown in Fig. 3. PGE_1 alone caused increases in the radioactivity of 3′:5′-cyclic AMP and in the presence of compound RA233 the increases were greater. However, compound RA233 caused a smaller potentiation of the effect of PGE_1 on radioactive 3′:5′-cyclic AMP concentrations than on inhibition of aggregation. When the radioactivity of platelet 3′:5′-cyclic AMP was plotted against percentage inhibition of aggregation (Fig. 4), three distinct curves were obtained, representing the effects of PGE_1 alone, PGE_1 together with compound RA233 (50μM) and high concentrations (100–250μM) of compound RA233 alone. Under each of these conditions there was a relationship between the increase in radioactive 3′:5′-cyclic AMP measured at the time of addition of ADP and the inhibition of aggregation, but the relationship was different in each case. An increase

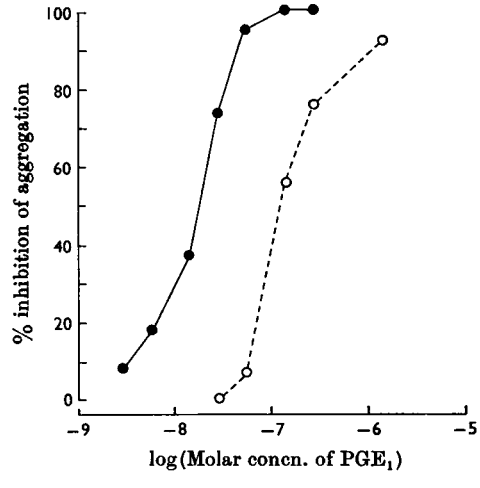

Fig. 2. Effect of PGE_1 in the presence (●) and absence (○) of an inhibitor of platelet phosphodiesterase (compound RA233, 50μM) on the aggregation of platelets induced by ADP (final concentration 100μM). Aggregation was measured as the maximum rate of change of optical transmission. Compound RA233 was added 10s before PGE_1 and ADP was added 40s after PGE_1.

of 20% in the radioactivity of 3′:5′-cyclic AMP brought about by PGE_1 in combination with compound RA233 was associated with 20% inhibition of aggregation. The same increase in 3′:5′-cyclic AMP radioactivity brought about by

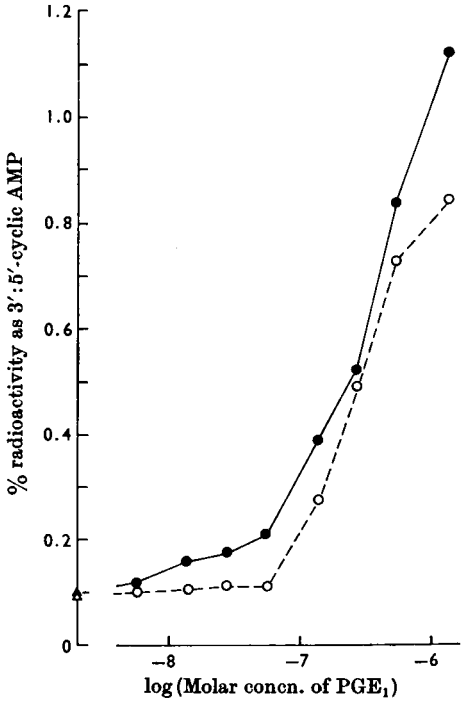

Fig. 3. Effects of PGE_1 on the incorporation of radioactivity into 3':5'-cyclic AMP in platelets prelabelled with [^{14}C]adenine. PGE_1 alone (○) and PGE_1 in the presence of compound RA233, (50 μM) (●). Compound RA233 was added 10s after PGE_1 and the reaction was stopped 40s later by the addition of $HClO_4$ (see Fig. 2). Control values in samples to which no PGE_1 was added are also shown: ▲, with compound RA233; △, without compound RA233.

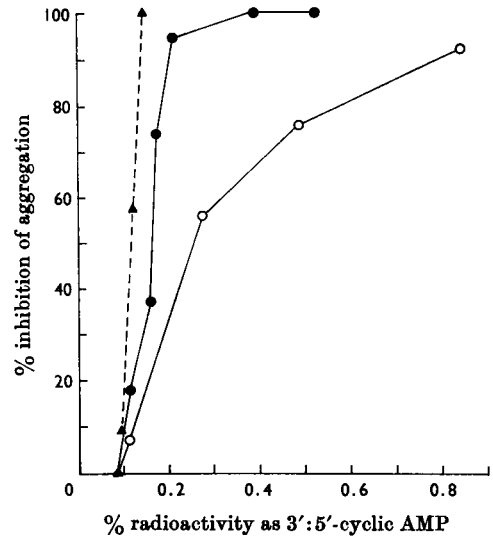

Fig. 4. Relation between incorporation of radioactivity into 3':5'-cyclic AMP and inhibition of aggregation with PGE_1 alone (○), PGE_1 in the presence of compound RA233 (50 μM) (●) and compound RA233 (50–500 μM) alone (▲). Results from the experiment of Figs. 2 and 3.

PGE_1 alone and by compound RA233 alone was associated with 10 and 60% inhibition of aggregation respectively.

Adenosine and 2-chloroadenosine. When added 40s before ADP, adenosine and 2-chloroadenosine inhibited aggregation to an extent that depended on concentration (Fig. 5). Inhibition by 2-chloroadenosine increased linearly with the logarithm of the concentration and approached 100%; with adenosine inhibition increased up to 50% and then remained almost constant as the adenosine concentration was increased. In the presence of compound RA233 (50 μM) the dose–response curves were displaced to the left to an extent indicating about a 100-fold increase in effectiveness. In addition, the dose–response curve for adenosine in the presence of compound RA233 approached 100% inhibition.

Adenosine incubated for 40s with labelled platelets caused a small and variable increase in the

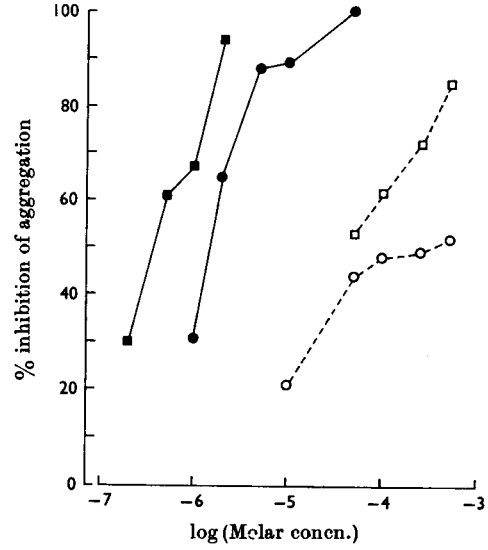

Fig. 5. Potentiation of the inhibition of platelet aggregation caused by adenosine and by 2-chloroadenosine by compound RA233. ○, Adenosine alone; ●, adenosine added 10s after compound RA233 (50 μM); □, 2-chloroadenosine alone; ■, 2-chloroadenosine added 10s after compound RA233 (50 μM). Platelet-rich plasma was incubated for 40s with the nucleoside before the initiation of aggregation by ADP (100 μM).

Table 6. *Specificity of the isolation procedure*

Nucleotides were extracted from samples of labelled platelets containing 6.2×10^9 cells at 0°C with 0.10ml of 1.5M-$HClO_4$ containing 0.1μmol each of AMP, adenosine and inosine. 3′:5′-Cyclic [^{14}C]AMP (0.02μCi) was added to an extract of unlabelled platelets. The extracts were neutralized and 50μl portions were incubated for 10min at 37°C with 20μl of a reaction mixture containing 11mM-$MgCl_2$, 68mM-tris–HCl buffer, pH7.4, and 50μg of ox heart phosphodiesterase. Another 50μl sample was incubated with the same reaction mixture without phosphodiesterase. The reaction was stopped by boiling for 2min and diluting with 1.0ml of water containing 3′:5′-cyclic [^{3}H]AMP (0.05μCi). Samples (0.10ml) were taken for measurement of total radioactivity and the remainder used for the measurement of 3′:5′-cyclic AMP radioactivity (see the Methods section). Sample 1, labelled platelets without addition. Sample 2, labelled platelets incubated for 40s with compound RA233 (50μM). Sample 3, labelled platelets incubated for 40s with compound RA233 (50μM) and adenosine (100μM). Sample 4, unlabelled platelets with added 3′:5′-cyclic [^{14}C]AMP.

Sample	Total [^{14}C] (c.p.m.)	Phosphodiesterase	3′:5′-Cyclic [^{14}C]AMP c.p.m. −	c.p.m. +	% −	% +	% hydrolysed
1. Control	363853		185.7	39.7	0.051	0.011	78
2. Compound RA233	340950		285.0	22.0	0.084	0.006	93
3. Compound RA233 + adenosine	318929		427.9	78.7	0.134	0.025	81
4. Standard	8667		7573.0	710.0	87.4	8.2	91

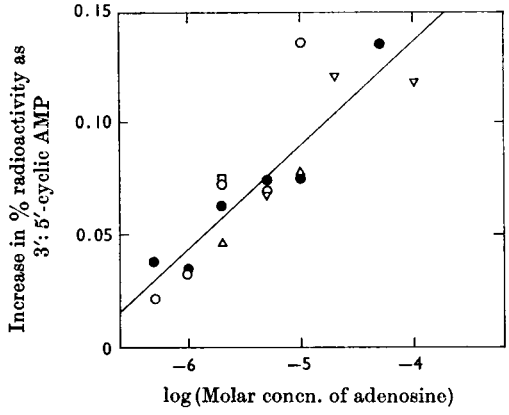

Fig. 6. Correlation between adenosine concentration and increased incorporation of radioactivity into 3′:5′-cyclic AMP in platelets prelabelled with [^{14}C]adenine. Results from five experiments are given. The diagonal line is the regression of increase in % radioactivity as 3′:5′-cyclic AMP on the logarithm of the adenosine concentration; the correlation coefficient was calculated as $r = 0.88$. Expt. 1, 3.96×10^8 platelets/ml; compound RA233, 40μM; 3′:5′-cyclic [^{14}C]AMP in controls, 0.071%; (▽). Expt. 2, 4.85×10^8 platelets/ml; compound RA233, 50μM; 3′:5′-cyclic [^{14}C]AMP in controls, 0.090%; (○). Expt. 3, 5.15×10^8 platelets/ml; compound RA233, 50μM; 3′:5′-cyclic [^{14}C]AMP in controls, 0.096%; (△). Expt. 4, 5.01×10^8 platelets/ml; compound RA233, 50μM; 3′:5′-cyclic [^{14}C]AMP in controls, 0.106%; (□). Expt. 5, 4.73×10^8 platelets/ml; compound RA233, 100μM; 3′:5′-cyclic [^{14}C]AMP in controls, 0.085%; (●).

radioactivity of platelet 3′:5′-cyclic AMP (Tables 3 and 7). In the presence of compound RA233 (20–100μM) these effects were magnified. To verify that the observed increases were due to radioactive 3′:5′-cyclic AMP, extracts of labelled platelets were incubated with ox heart phosphodiesterase before the isolation procedure; of the radioactivity isolated as 3′:5′-cyclic AMP, 78% in the untreated platelets and 81% in the stimulated platelets was susceptible to the action of phosphodiesterase (Table 6). Fig. 6 shows results from five experiments with blood from different donors in which increases in the radioactivity of platelet 3′:5′-cyclic AMP are plotted against adenosine concentration. The concentration of compound RA233 used in the different experiments was chosen to have little effect on aggregation but to potentiate the action of adenosine to about the same extent. A similar relationship between dose and increased 3′:5′-cyclic AMP radioactivity was found in three experiments with 2-chloroadenosine (Fig. 7). Aggregation was measured in the same three experiments and Fig. 8 shows the relationship between increases in the radioactivity of platelet 3′:5′-cyclic AMP and percentage inhibition of aggregation. For a given increase in 3′:5′-cyclic AMP radioactivity there was a greater degree of inhibition of aggregation with the higher concentration of compound RA233. With combinations of adenosine and compound RA233 there was a general correlation between increases in 3′:5′-cyclic AMP radioactivity and inhibition of aggregation ($r = 0.86, n = 11$ in five experiments).

Comparisons between PGE_1 and adenosine. The effects of theophylline and compound RA233 on inhibition of aggregation and on radioactive 3′:5′-cyclic AMP increases produced by PGE_1 and by adenosine were compared in the experiment summarized in Table 7. This shows that compound RA233 (20μM) was less effective than theophylline

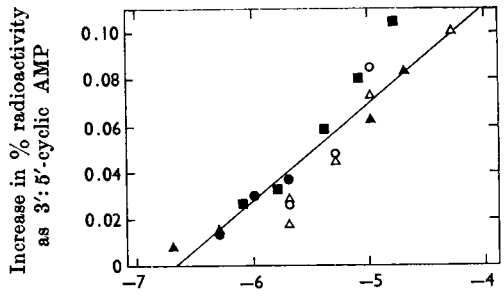

Fig. 7. Correlation between concentration of 2-chloroadenosine and increased incorporation of radioactivity into 3':5'-cyclic AMP in labelled platelets. Results from three experiments are given. The diagonal line represents the regression of increase in % radioactivity as 3':5'-cyclic AMP on the logarithm of the 2-chloroadenosine concentration. The correlation coefficient was calculated as $r = 0.93$. Expt. 1, 4.52×10^8 platelets/ml; compound RA233, 20 μM; control 3':5'-cyclic[^{14}C]AMP, 0.146%; (○): compound RA233, 50 μM; control 3':5'-cyclic [^{14}C]AMP, 0.170%; (●). Expt. 2, 5.36×10^8 platelets/ml; compound RA233, 20 μM; control 3':5'-cyclic [^{14}C]AMP, 0.050%; (△): compound RA233, 50 μM; control 3':5'-cyclic [^{14}C]AMP, 0.065%; (▲). Expt. 3, 4.58×10^8 platelets/ml; compound RA233, 50 μM; control 3':5'-cyclic [^{14}C]AMP, 0.090%; (■).

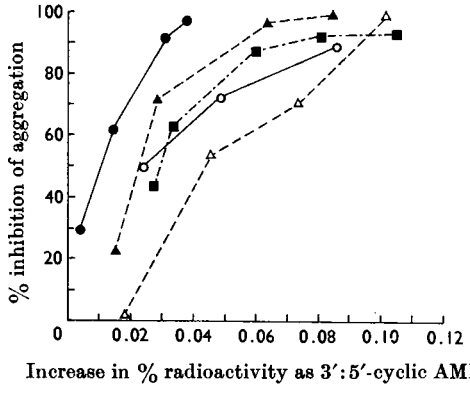

Fig. 8. Relation between increased incorporation of radioactivity into 3':5'-cyclic AMP and inhibition of aggregation with 2-chloroadenosine in the presence of compound RA233 in three experiments. The symbols refer to the caption of Fig. 7.

(1.5 mM) in potentiating PGE_1, but was more effective in combination with adenosine. In most experiments theophylline caused a slight inhibition of the adenosine effect. Both compound RA433 and papaverine behaved in a similar way to compound RA233. These differences suggest that some action other than inhibition of platelet phosphodiesterase is involved in the effects of either methyl xanthines or of the other compounds. The known abilities of papaverine and dipyridamole to inhibit the incorporation of adenosine by platelets, and the structural similarity between the RA compounds and dipyridamole, suggested the investigation of the effects of phosphodiesterase inhibitors on adenosine uptake.

Inhibition of adenosine uptake. Dipyridamole, compound RA233 and theophylline were compared as inhibitors of the uptake of radioactive adenosine by platelets (Table 8). Dipyridamole inhibited adenosine uptake at low concentrations (0.5–5 μM) and compound RA233 also caused inhibition of uptake but at slightly higher concentrations; theophylline did not inhibit at up to 800 times the effective concentration of dipyridamole. Dipyridamole, the most active of the uptake inhibitors, did not cause any major potentiation of inhibition of aggregation produced by adenosine, nor was a combination of dipyridamole with theophylline more effective than theophylline alone. This indicates that the differences between these compounds as potentiators of adenosine and PGE_1 cannot be attributed to their effects on adenosine uptake.

DISCUSSION

A controlling function for intracellular 3':5'-cyclic AMP on platelet aggregation has been put forward to explain the effects of drugs that inhibit 3':5'-cyclic AMP phosphodiesterase (Ardlie *et al.* 1967) and of drugs that stimulate adenylate cyclase (Wolfe & Shulman, 1969; Zieve & Greenough, 1969; Marquis *et al.* 1969; Abdulla, 1969). It has also been claimed that there is an association between the degree of inhibition of aggregation and the increased incorporation of radioactivity into 3':5'-cyclic AMP in platelets containing radioactive ATP (Vigdahl *et al.* 1969). In these latter experiments radioactive 3':5'-cyclic AMP formation in the presence of 50 mM-caffeine was compared with aggregation in the absence of caffeine. This concentration of caffeine strongly inhibits platelet aggregation by itself (Ardlie *et al.* 1967).

We have attempted to correlate increases in radioactive 3':5'-cyclic AMP formation with changes in platelet responsiveness to the aggregating effect of ADP under identical conditions. Such a correlation was observed with PGE_1 over the concentration range 0.05–1 μM (Fig. 4). The synergistic action of mixtures of PGE_1 and theophylline on platelet aggregation (Mills *et al.* 1970) has been shown to be related to the co-operative effects of these drugs on the incorporation of radioactivity into platelet 3':5'-cyclic AMP (Table 7). This

Table 7. *Potentiation of the effects of PGE_1 and adenosine on platelet aggregation and platelet 3′:5′-cyclic AMP radioactivity by phosphodiesterase inhibitors*

Samples of platelet-rich plasma containing 5.30×10^8 platelets in 1.0ml prelabelled with [^{14}C]adenine were incubated at 37°C in the aggregometer for 10s after addition of the phosphodiesterase inhibitor (first addition). The second addition was then made and the incubation continued for a further 40s before transferring the contents of the aggregometer tube into a centrifuge tube containing 0.5ml of 2M-$HClO_4$ and 3′:5′-cyclic [^{3}H]AMP as recovery standard. A 1.0ml plastic syringe with a wide-bore needle with the bevel removed by grinding was used for the transfer. Aggregation was studied under identical conditions with samples of platelet-rich plasma preincubated with non-radioactive adenine. The basal 3′:5′-cyclic AMP radioactivity was taken as the mean of three determinations made at the beginning, middle and end of the experiment (0.074, 0.064 and 0.085% as 3′:5′-cyclic AMP respectively).

	First addition					
	None		Compound RA233 (20μM)		Theophylline (1.5mM)	
Second addition	%I*	% 3′:5′-cyclic [^{14}C]AMP	%I*	% 3′:5′-cyclic [^{14}C]AMP	%I*	% 3′:5′-cyclic [^{14}C]AMP
None	0	0.074	6	0.079	6	0.070
Adenosine (2μM)	24	0.096	86	0.122	1	0.095
(5μM)	37	0.091	94	0.136	19	0.076
PGE_1 (14nM)	−10	0.099	15	0.077	74	0.125
(56nM)	20	0.081	64	0.130	100	0.189

* %I = % inhibition of aggregation (induced by 100μM-ADP).

Table 8. *Inhibition of incorporation of adenosine into platelets by phosphodiesterase inhibitors*

Samples of platelet-rich plasma (5.0ml) were incubated at 37°C with [8-^{14}C]adenosine (15mCi/mmol) at a final concentration of 1.0μM. Subsamples of 1.0ml were taken at 5, 10, 20 and 30min, centrifuged for 30s at 12000*g* and after removal of the plasma by suction the platelet pellet was mopped up with 0.1ml of 1% (v/v) Triton X-100 and a small piece of glass-fibre filter paper and transferred to a scintillation-counting vial together with one 0.1ml wash for the determination of radioactivity by liquid-scintillation counting (see the Methods section). In the absence of inhibitors the control rate of uptake was linear up to 30min, when 33.7% of the radioactivity (equivalent to 81.2pmol of adenosine/10^8 platelets) had been taken up.

Dipyridamole		Compound RA233		Theophylline	
Concn. (μM)	%I*	Concn. (μM)	%I*	Concn. (μM)	%I*
0.5	34	1	1.5	50	−5
1.0	48	5	48	100	6
5.0	89	20	77	400	6

* %I = % inhibition of uptake of radioactivity.

supports the involvement of 3′:5′-cyclic AMP in the control of platelet aggregation.

A number of drugs have been found that are more powerful than theophylline as inhibitors of the 3′:5′-cyclic AMP phosphodiesterase of platelets. These include papaverine and dipyridamole and derivatives of the latter known as compounds RA233 and RA433 (Table 1). The drugs that were most active as inhibitors of platelet phosphodiesterase at low substrate concentrations were also the most powerful potentiators of the inhibitory effects of low concentrations of PGE_1 on platelet aggregation (Table 4). Dipyridamole potentiated the effects of high concentrations of PGE_1 (Table 5) though not of low concentrations (Table 4). This is consistent with the greater inhibition of platelet phosphodiesterase by dipyridamole at high, as compared with low, substrate concentrations (Table 1).

The effects of compound RA233 were investigated in detail. The increased activity of PGE_1 as an inhibitor of aggregation caused by compound RA233 cannot be attributed to additive effects of the two drugs, but is a true potentiation or synergistic effect (Fig. 2). As with different concentrations of PGE_1 alone, different concentrations of PGE_1 in the presence of compound RA233 caused a dose-

dependent increase in the degree of inhibition of aggregation that was correlated with increased accumulation of radioactive 3′:5′-cyclic AMP. This was also observed with increasing concentrations of compound RA233 alone. However, the slope of the correlation was different in the three cases (Fig. 4), and the extent of the inhibition of aggregation associated with a given radioactive 3′:5′-cyclic AMP concentration was increased by the presence of compound RA233. This implies that the rate of aggregation is not directly related to the 3′:5′-cyclic AMP concentration measured at the time of addition of the aggregating agent. During aggregation induced by ADP there is a fall in the concentration (Salzman & Neri, 1969) and radioactivity (D. C. B. Mills & J. B. Smith, unpublished work) of platelet 3′:5′-cyclic AMP. If aggregation is controlled by the concentration of 3′:5′-cyclic AMP present during the course of, rather than immediately before, aggregation, an apparent increase in the effectiveness of 3′:5′-cyclic AMP by inhibitors of phosphodiesterase would be expected.

When low concentrations of ADP are used to induce aggregation, both dipyridamole and papaverine potentiate the inhibition of aggregation produced by adenosine and also block the incorporation of radioactive adenosine into the platelets (Born & Mills, 1969). This was interpreted as evidence that adenosine acts at the platelet membrane rather than during or after its penetration into the cells. Since the inhibition of aggregation by adenosine is, like that produced by PGE_1 and by isoprenaline, potentiated by inhibitors of platelet 3′:5′-cyclic AMP phosphodiesterase, it could be explained as an activation of platelet membrane adenylate cyclase. Platelets incubated with adenosine and with 2-chloroadenosine showed small increases in radioactive 3′:5′-cyclic AMP formation over controls, but without any demonstrable dose-dependence; this could be because the increases are transient, as they are with PGE_1 (Ball, Brereton, Fulwood, Ireland & Yates, 1970). In the presence of the phosphodiesterase inhibitor compound RA233, the increases observed were larger and clearly correlated with the concentration of adenosine (Fig. 6) or 2-chloroadenosine (Fig. 7) and with the degree of inhibition of aggregation produced (Fig. 8).

If PGE_1 and adenosine were acting solely through stimulation of adenylate cyclase, and theophylline and compound RA233 solely through inhibition of phosphodiesterase, then the relative extent of potentiation of PGE_1 and of adenosine by compound RA233 should match that produced by theophylline. Table 7 shows that compound RA233 was more active than theophylline as a potentiator of the effects of adenosine at concentrations that caused similar degrees of potentiation of PGE_1. Compound RA233, but not theophylline, inhibited the uptake of adenosine by platelets (Table 8). However, dipyridamole, an even more effective inhibitor of adenosine uptake than compound RA233, caused little or no potentiation of adenosine inhibition under these conditions (with aggregation induced by 100 μM-ADP) and a combination of dipyridamole with theophylline was no more effective than theophylline alone. This appears to rule out the possibility that the ability of compound RA233 to block the uptake of adenosine by platelets contributes to its activity as an adenosine potentiator. Theophylline antagonizes the effects of adenosine on 3′:5′-cyclic AMP concentration in guinea-pig cerebral-cortex slices (Sattin & Rall, 1970). A similar effect in platelets would explain our results.

Adenosine and 2-chloroadenosine inhibit 3′:5′-cyclic AMP phosphodiesterase from ox heart (Horlington & Watson, 1970). We found that 2-chloroadenosine and, to a lesser extent, adenosine, inhibited phosphodiesterase from human platelets, but only when present at relatively high concentrations (Table 2). As adenosine is phosphorylated to nucleotides as rapidly as it is incorporated into platelets (Ireland & Mills, 1966), it is unlikely that the concentration of adenosine available to interact with intracellular phosphodiesterase can reach the high concentrations that cause inhibition of the enzyme. Uptake and metabolism of 2-chloroadenosine by platelets have not been studied and inhibition of phosphodiesterase in intact cells by this compound cannot be excluded. The similarity in the effects of adenosine and 2-chloroadenosine suggest that this is not the case.

Assuming 3′:5′-cyclic AMP to be in equilibrium with the rapidly labelled fraction of platelet nucleotides (6 μmol/10^{11} platelets; Mills & Thomas, 1969) the radioactivity of 3′:5′-cyclic AMP in untreated platelets corresponds to 3–7 nmol/10^{11} platelets, increasing to 48 nmol 40s after addition of 1 μM-PGE_1. This can be compared with the results of Robison *et al.* (1969), who found a basal concentration of 2 nmol/10^{11} platelets, increasing to 14 nmol 20 min after addition of 1 μM-PGE_1.

The similarities in the effects on platelet aggregation and on platelet 3′:5′-cyclic AMP radioactivity of low concentrations of PGE_1 with those of adenosine and of 2-chloroadenosine suggest that these nucleosides inhibit aggregation by the same mechanism as does PGE_1. The correlations obtained between increased radioactivity of 3′:5′-cyclic AMP and inhibition of aggregation indicate that this mechanism may involve the activation of platelet adenylate cyclase.

We are grateful to Professor G. V. R. Born for support and encouragement and to Mr D. M. Ireland for helpful discussions. Blood was kindly donated by employees of

the Equity and Law Insurance Company and of the Land Registry, both of Lincoln's Inn Fields, London W.C.2, U.K. J.B.S. gratefully acknowledges financial support from the Medical Research Council.

REFERENCES

Abdulla, Y. H. (1969). *J. Atheroscler. Res.* **9**, 171.

Ardlie, N. G., Glew, G. & Schwartz, C. J. (1967). *Thromb. Diath. haemorrh.* **18**, 670.

Ball, G., Brereton, G. G., Fulwood, M., Ireland, D. M. & Yates, P. (1970). *Biochem. J.* **120**, 709.

Ball, G., Fulwood, M., Ireland, D. M. & Yates, P. (1969). *Biochem. J.* **114**, 669.

Born, G. V. R. (1962). *Nature, Lond.*, **194**, 927.

Born, G. V. R. (1964). *Nature, Lond.*, **202**, 95.

Born, G. V. R. & Cross, M. J. (1963). *J. Physiol., Lond.*, **167**, 178.

Born, G. V. R. & Mills, D. C. B. (1969). *J. Physiol., Lond.*, **202**, 41 *P*.

Emmons, P. R., Hampton, J. R., Harrison, M. J., Honour, A. J. & Mitchell, J. R. A. (1967). *Br. med. J.* ii, 468.

Gornall, A. G., Bardawill, G. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Horlington, M. & Watson, P. A. (1970). *Biochem. Pharmac.* **19**, 955.

Ireland, D. M. & Mills, D. C. B. (1966). *Biochem. J.* **99**, 283.

Kloeze, J. (1967). In *Prostaglandins*, p. 241. Ed. by Bergstrom, S. & Samuelsson, S. A. London: Interscience Publishers.

Krishna, G., Weiss, B. & Brodie, B. B. (1968). *J. Pharmac. exp. Ther.* **163**, 379.

Maguire, H. M. & Michal, F. (1968). *Nature, Lond.*, **217**, 571.

Markwardt, F., Barthel, W., Glusa, E. & Hoffman, A. (1967). *Arch. exp. Path. Pharmak.* **257**, 420.

Marquis, N. R., Vigdahl, R. L. & Tavormina, P. A. (1969). *Biochem. biophys. Res. Commun.* **36**, 965.

Mills, D. C. B. & Roberts, G. C. K. (1967). *J. Physiol., Lond.*, **193**, 443.

Mills, D. C. B., Smith, J. B. & Born, G. V. R. (1970). *Proc. 18th Annual Symp. on Blood, Detroit* (in the Press).

Mills, D. C. B. & Thomas, D. P. (1969). *Nature, Lond.*, **222**, 991.

Moriwaki, K. & Foa, P. P. (1970). *Experientia*, **26**, 22.

Packham, M. A., Ardlie, N. G. & Mustard, J. F. (1969). *Am. J. Physiol.* **217**, 1009.

Poch, G., Juan, H. & Kukovetz, W. R. (1969). *Arch. exp. Path. Pharmak.* **264**, 293.

Randerath, K. & Struck, H. (1961). *J. Chromat.* **6**, 365.

Robison, G. A., Arnold, A. & Hartmann, R. C. (1969). *Pharmac. Res. Commun.* **1**, 325.

Rozenberg, M. C. & Holmsen, H. (1968). *Biochim. biophys. Acta*, **155**, 342.

Salzman, E. W., Ashford, T. P., Chambers, D. A., Neri, L. L. & Dempster, A. P. (1969). *Am. J. Physiol.* **217**, 1330.

Salzman, E. W. & Neri, L. L. (1969). *Nature, Lond.*, **224**, 609.

Sattin, A. & Rall, T. W. (1970). *Molec. Pharmac.* **6**, 13.

Scales, B. (1967). *Int. J. appl. Radiat. Isotopes*, **18**, 1.

Skoza, L., Zucker, M. B., Jerushalmy, Z. & Grant, R. (1967). *Thromb. Diath. haemorrh.* **18**, 713.

Vigdahl, R. L., Marquis, N. R. & Tavormina, P. A. (1969). *Biochem. biophys. Res. Commun.* **37**, 409.

Wolfe, S. M. & Shulman, N. R. (1969). *Biochem. biophys. Res. Commun.* **35**, 265.

Zieve, P. D. & Greenough, W. B. (1969). *Biochem. biophys. Res. Commun.* **35**, 462.