Biochem. J. (1970) **120**, 709–718
Printed in Great Britain

Effect of Prostaglandin E1 Alone and in Combination with Theophylline or Aspirin on Collagen-Induced Platelet Aggregation and on Platelet Nucleotides including Adenosine 3′:5′-Cyclic Monophosphate

By G. BALL, G. G. BRERETON, MARY FULWOOD, D. M. IRELAND and PATRICIA YATES
Glaxo Research Ltd., Greenford, Middlesex, U.K.

(*Received* 20 *July* 1970)

1. Human platelet nucleotides were labelled by incubating platelet-rich plasma with [U-^{14}C]adenine. With such platelets, the effects of prostaglandin E1, theophylline and aspirin were determined on collagen-induced platelet aggregation and release of platelet ATP and ADP. Intracellular changes of platelet radioactive nucleotides, particularly 3′:5′-cyclic AMP, were also determined both with and without collagen treatment. 2. Prostaglandin E1, theophylline and aspirin inhibited collagen-induced aggregation of platelets in a dose-dependent manner. Collagen-induced release of ATP and ADP and breakdown of radioactive ATP were also inhibited in a dose-dependent manner. 3. Prostaglandin E1 stimulated the formation of platelet radioactive 3′:5′-cyclic AMP in a dose-dependent manner. With a given dose of prostaglandin E1, maximum formation of radioactive 3′:5′-cyclic AMP occurred by 10–30s and thereafter the concentrations declined. The degree of inhibition of aggregation produced by prostaglandin E1, however, increased with its time of incubation in platelet-rich plasma before addition of collagen, so that there was an inverse relationship between the radioactive 3′:5′-cyclic AMP concentration measured at the time of collagen addition and the subsequent degree of inhibition of aggregation obtained. 4. Neither theophylline nor aspirin at a concentration in platelet-rich plasma of 1.7mM altered platelet radioactive 3′:5′-cyclic AMP contents. In the presence of prostaglandin E1, theophylline increased the concentration of radioactive 3′:5′-cyclic AMP over that noted with prostaglandin E1 alone, but aspirin did not. 5. Mixtures of prostaglandin E1 and theophylline had a synergistic effect on inhibition of platelet aggregation. The same was true to a lesser extent with mixtures of prostaglandin E1 and aspirin. Such mixtures also inhibited collagen-induced release of platelet ATP and ADP and breakdown of platelet radioactive ATP. 6. Certain concentrations of either theophylline or aspirin and mixtures of small concentrations of prostaglandin E1 with either theophylline or aspirin caused little or no increase of radioactive 3′:5′-cyclic AMP at the time of collagen addition, but inhibited aggregation to a marked degree, whereas higher concentrations of prostaglandin E1 alone caused a much greater increase of radioactive 3′:5′-cyclic AMP at the time of collagen addition but inhibited aggregation to a lesser extent. With these compounds there does not appear to be a correlation between these parameters.

PGE1* inhibits platelet aggregation induced by ADP, noradrenaline, thrombin and connective-tissue extract (Kloeze, 1967; Emmons, Hampton, Harrison, Honour & Mitchell, 1967) and also stimulates the formation of cyclic AMP in intact platelets (Robison, Arnold & Hartmann, 1969). At high concentrations, theophylline and caffeine inhibit ADP-induced aggregation (Ardlie, Glew & Schwartz, 1967). Caffeine raises the cyclic AMP concentrations of intact platelets (Salzman & Neri, 1969) and inhibits the phosphodiesterase activity of a supernatant fraction of homogenized platelets (Marquis, Vigdahl & Tavormina, 1969). These authors suggest that the formation of cyclic AMP in platelets may be involved in the control of platelet aggregation and this is supported by the finding by Mills, Smith & Born (1970) that PGE1 and theophylline mutually potentiate inhibition of ADP-induced aggregation, a result that might be expected if cyclic AMP formation is stimulated by

* Abbreviations: PGE1, prostaglandin E1; cyclic AMP, adenosine 3′:5′-cyclic monophosphate.

PGE1 and its breakdown is inhibited by theophylline.

The present paper compares the effects of PGE1, theophylline and aspirin alone and in combination on collagen-induced platelet aggregation, release of platelet nucleotides and breakdown of platelet radioactive ATP (see Ball, Fulwood, Ireland & Yates, 1969). The effect of these compounds on platelet radioactive cyclic AMP was also studied in an attempt to relate cyclic AMP formation in platelet-rich plasma with inhibition of collagen-induced platelet aggregation.

METHODS

Materials. 3′:5′-Cyclic GMP, 3′:5′-cyclic IMP, 3′:5′-cyclic CMP and 3′:5′-cyclic UMP were supplied by Boehringer Corp. (London) Ltd., London W.5, U.K.; 3′:5′-cyclic AMP was supplied by Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; 2′-AMP and 3′-AMP (mixed isomers) were supplied by Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. [U-^{14}C]Adenine (321mCi/mmol) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K., and 3′:5′-cyclic [8-^{14}C]AMP (40.9mCi/mmol) by Schwarz BioResearch Inc.

Theophylline hydrate was supplied by British Drug Houses Ltd., Poole, Dorset, U.K. PGE1, containing less than 0.3% of prostaglandin E2, was a gift from Professor D. A. Van Dorp, Unilever Research Laboratories Ltd., Vlaardingen, The Netherlands.

Chromatography paper was Whatman no. 54 (W. and R. Balston Ltd., Maidstone, Kent, U.K.). Laboratory chemicals were of A.R. grade and other chemicals and apparatus used have been described (Ireland & Mills, 1966; Ireland, 1967; Ball *et al.* 1969).

Chromatographic and electrophoretic solvents. The following chromatographic solvents were used as indicated in the text and Fig. 1: solvent 1, butan-1-ol–acetone–acetic acid–aq. 5% (v/v) NH_3 (sp.gr. 0.88)–water–0.1M-EDTA (45:15:10:10:19:1, by vol.) (Randerath & Struck, 1961); solvent 2, isobutyric acid–1M-NH_3–0.1M-EDTA (125:75:2, by vol.) (Krebs & Hems, 1953); solvent 3, 1M-ammonium acetate buffer (pH7.5)–ethanol–0.1M-EDTA (30:75:1, by vol.) (Humes, Roundbehler & Kuehl, 1968). Electrophoresis was performed as indicated in the text with: 0.02M-citric acid–sodium citrate buffer, pH3.5 (Thomson, 1960); 0.02M-sodium phosphate buffer, pH7; 1.5M-formic acid–2M-acetic acid (1:1, by vol.), pH1.9 (Efron, 1960).

Preparation of labelled platelet-rich plasma. Blood (45ml) was withdrawn from the arm veins of human volunteers of either sex, who were not taking any drugs, and mixed with 5ml of 3.8% (w/v) trisodium citrate in silicone-treated glass vials kept at 37°C. The blood was immediately centrifuged at 37°C for 4min at 560*g* and the resultant platelet-rich plasma was incubated at 37°C for 30min with 0.11nmol of [U-^{14}C]adenine/ml of platelet-rich plasma. After 30min incubation only trace amounts of radioactivity as adenine remained. The labelled platelet-rich plasma was kept at 37°C throughout the experiments, which lasted from 1 to 3h.

Aggregation of platelets. Platelets in plasma were aggregated by the addition of small amounts of bovine collagen suspension, prepared in 0.9% NaCl as described by Evans, Packham, Nishizawa, Mustard & Murphy (1968) and aggregation was followed optically in a modified absorptiometer at 37°C (Mills & Roberts, 1967*a,b*). The degree of aggregation was measured as the change in light transmittance (Δ%T) that had occurred 4min after the addition of the collagen suspension and inhibition of aggregation was expressed as $100\times(AC-AT)/AC$, where AC is the degree of aggregation obtained with a given amount of collagen suspension and AT is the degree of aggregation obtained in the presence of test compound and the same amount of collagen suspension. Test compounds were added to platelet-rich plasma at times before the collagen suspension as noted in text, Tables and Figures.

Platelets kept at 37°C in plasma tended to be more susceptible to the inhibitory effects of test compounds as the age of the platelet-rich plasma increased. For this reason when a comparison between the inhibitory activity of PGE1 plus theophylline or PGE1 plus aspirin was made with that of either PGE1, theophylline or aspirin, the single compounds were always tested after the mixtures.

Released nucleotides. Non-radioactive ATP and ADP released from platelets to the supernatant by the action of collagen were determined by a firefly bioluminescence method (Holmsen, Holmsen & Bernhardsen, 1966; Ball *et al.* 1969).

Radioactive nucleotides. Radioactive nucleotides, nucleosides and purines were determined by scintillation counting, after charcoal treatment and one-dimensional paper chromatography (Ireland & Mills, 1966; Ireland, 1967; Ball *et al.* 1969). The radioactive counting efficiency was about 65%; background count was about 20c.p.m. and total counts per sample were about 10^6.

The chromatographic solvent system used in this procedure (solvent 1) permitted the detection and measurement of platelet radioactive cyclic AMP (Fig. 1). The procedure was altered from that previously described by the inclusion of 30nmol of carrier cyclic AMP together with 30nmol of each of ATP, ADP, AMP, IMP, inosine and hypoxanthine.

Purified radioactive cyclic AMP added to platelet-rich plasma–trichloroacetic acid mixtures and subjected to charcoal treatment and chromatography was recovered in 90% yield, giving a charcoal recovery factor for cyclic AMP of 1.1. Recovery factors for the other substances assayed have been given (Ireland, 1967).

GTP and GDP were not well separated from ATP with solvent 1 (Fig. 1) and are included in the radioactivity attributed to ATP. When separated by using solvent 2 (Fig. 1) the amounts of radioactivity attributed to GTP and GDP in platelet-rich plasma, either with or without collagen treatment, were about 0.4% of the total recovered radioactivity as GTP and about 0.3% as GDP.

Untreated labelled platelet-rich plasma contained from 0.1% to 0.2% of the total recovered radioactivity as apparent cyclic AMP. These values were decreased by about 50% after purification of the radioactive adenine used for labelling platelet-rich plasma, but radioactivity other than cyclic AMP was possibly still present since samples chromatographed in a second dimension with solvent 1 or 2 showed a further loss of up to 50% of radioactivity attributed to cyclic AMP.

Labelled platelet-rich plasma to which PGE1 (0.56μM)

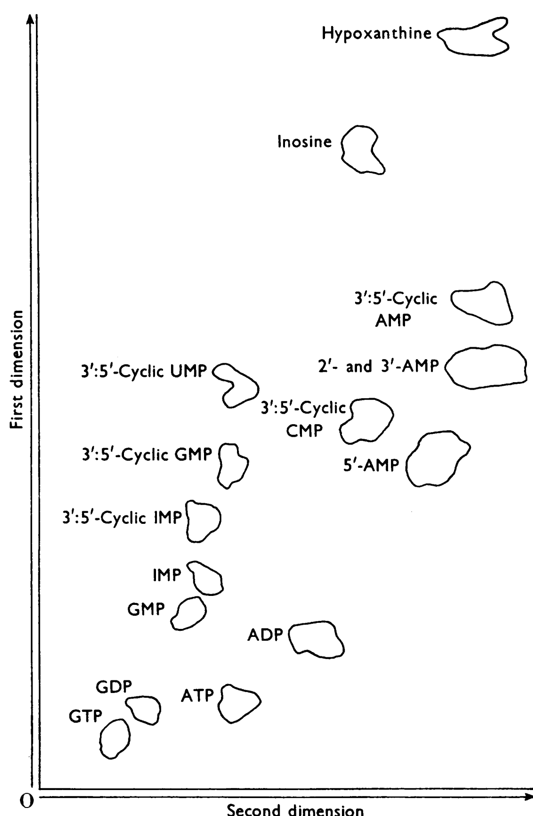

Fig. 1. Diagram showing descending paper-chromatographic separation of cyclic AMP from other nucleotides and from inosine and hypoxanthine. About 20nmol of each compound was applied to the origin, O. The first dimension was run with solvent 1 for 15h at room temperature. The paper was then dried at 100°C for 10min, left at room temperature for 8h and run in the second dimension with solvent 2 for 15h at room temperature. The compounds were detected under u.v. light.

was added for 20s contained about 0.7% of the total recovered radioactivity as cyclic AMP. Such material after charcoal treatment and chromatography with solvent 1 was eluted from the paper with water, concentrated, pooled and subjected to further chromatography or electrophoresis. Over 90% of the radioactivity attributed to cyclic AMP ran with carrier cyclic AMP in each of the three chromatographic solvents given above and also by electrophoresis in the citrate and the phosphate buffers.

About the same number of counts was lost after the second dimension of chromatography irrespective of the radioactive cyclic AMP concentration recorded after the first dimension.

Individual values for radioactive cyclic AMP from duplicate assays of untreated platelet-rich plasma containing about 0.1% of the total recovered radioactivity as cyclic AMP varied by about ±10% of the mean value, those from duplicate assays of platelet-rich plasma treated with PGE1 and containing about 0.5% of the total recovered radioactivity as cyclic AMP varied by about ±5% of the mean value.

Radioactive cyclic AMP was also determined, as noted, by the method of Krishna, Weiss & Brodie (1968) as modified by D. C. B. Mills & J. B. Smith (unpublished work).

Purification of radioactive adenine and cyclic AMP. [U-^{14}C]Adenine was freed of contaminating radioactivity by electrophoresis on paper at room temperature with the formic acid–acetic acid mixture given above. In this system adenine moved towards the cathode (see Ireland & Mills, 1966). 3′:5′-Cyclic [8-^{14}C]AMP was also purified on paper by electrophoresis with 0.02M-phosphate buffer, pH7.0, in which system cyclic AMP moved towards the anode with a mobility less than that of AMP, ADP or ATP. For both substances 400V was applied for 2h and the dried areas of paper containing radioactivity were eluted with water. The areas containing radioactivity were located by running marker adenine or cyclic AMP beside the relevant radioactive substance and the position of the marker substances was detected under u.v. light.

RESULTS

Effect of age of platelet-rich plasma on aggregation and radioactive nucleotides. Since most experiments took a few hours to complete, the effect on aggregation and on radioactive nucleotides of keeping platelet-rich plasma at 37°C was studied. Results are shown in Table 1. From withdrawal of blood to the beginning of the experiment took about 45min. Over the next 3–4h the degree of aggregation obtained with a standard amount of collagen suspension declined in parallel with a loss of about 8–15% of platelet radioactive ATP. Radioactivity of ADP, AMP, cyclic AMP, inosine and hypoxanthine increased with time but there was little change in IMP.

The degree of inhibition of aggregation obtained in the presence of test compounds also tended to increase with the age of the platelet-rich plasma and the increase of inhibitory activity sometimes occurred without a decrease in the degree of aggregation induced by collagen alone.

Effects of PGE1 on aggregation, release of nucleotides and on radioactive nucleotides. PGE1 inhibited collagen-induced aggregation, release of ATP and ADP and breakdown of radioactive ATP in a dose-dependent manner (Table 2).

Maximum concentrations of radioactive cyclic AMP occurred 10–30s after the addition of PGE1 to platelet-rich plasma and thereafter declined at rates apparently proportional to the amount formed (Fig. 2). The amount of radioactive cyclic AMP formed, measured either 10, 20, 30 or 60s after the addition of PGE1, appeared to be proportional to the logarithm of the concentration of PGE1.

Inhibition of aggregation increased with the time

Table 1. *Effect on aggregation and radioactive nucleotides of keeping platelet-rich plasma at* 37°C

Blood was withdrawn and 15min later [U-^{14}C]adenine was added to platelet-rich plasma, prepared as described in the text. After 30min incubation at 37°C (zero time) two 1ml portions of labelled platelet-rich plasma were each stirred in an absorptiometer for 20s. Then to one portion 10μl of collagen suspension was added and the degree of aggregation determined 4min later, and to the other portion was added cold trichloroacetic acid containing carrier compounds for the determination of radioactivity (see the text). At each of the times indicated two 1ml portions of labelled platelet-rich plasma were similarly treated. Plasma contained A, 4.74×10^8 platelets/ml; B, 3.69×10^8 platelets/ml.

	Time (h)	Degree of aggregation (Δ%T)	Radioactivity of substance (% of total recovered counts)						
			ATP	ADP	AMP	Cyclic AMP	IMP	Inosine	Hypoxanthine
A	0	54	84.7	10.8	0.4	0.07	0.4	0.3	3.3
	1	50	81.9	10.3	0.7	0.09	0.4	0.5	6.0
	2	46	77.5	10.4	0.8	0.11	0.3	1.0	9.8
	3	36	72.3	11.4	1.0	0.15	0.2	1.4	13.5
B	0	51	86.9	10.9	0.4	0.08	0.2	0.2	1.3
	1	49	84.6	11.6	0.6	0.08	0.3	0.3	2.6
	2	47	83.6	11.1	0.7	0.08	0.4	0.4	3.7
	4	44	79.6	11.6	1.2	0.15	0.4	0.7	6.3

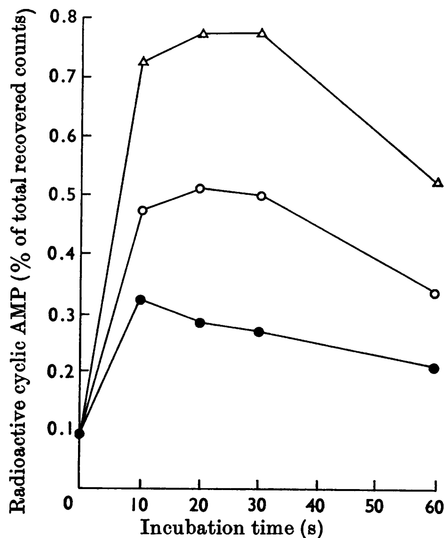

Fig. 2. Effect of PGE1 on platelet radioactive cyclic AMP at different incubation times. Various amounts of PGE1 and 0.9% NaCl were added to 1ml portions of labelled platelet-rich plasma to give a final volume of 1.02ml. The platelet-rich plasma was stirred at 37°C in the absorptiometer for the times indicated and incubations were terminated by the addition of cold trichloroacetic acid containing carrier compounds. Radioactive cyclic AMP was determined by scintillation counting after charcoal treatment and chromatography as described in the text. Samples to which only 0.9% NaCl was added were run at the beginning and end of the experiment with a 10s stirring time. The values of radioactive cyclic AMP obtained for these samples were 0.096 and 0.090% of the total recovered counts. Plasma contained 4.25×10^8 platelets/ml. ●, 0.14μM-PGE1; ○, 0.28μM-PGE1; △, 0.56μM-PGE1.

of incubation of PGE1 with platelet-rich plasma, before the addition of collagen, up to at least 5min. Table 3 shows the degree of aggregation obtained and the amounts of radioactive cyclic AMP formed when PGE1 was incubated with platelet-rich plasma for 1 and 4min before the addition of collagen. Inhibition of aggregation was greater after 4min than after 1min incubation whereas radioactive cyclic AMP concentrations were less.

Incubation of platelet-rich plasma with PGE1 (final concentration 1μM) for 20s gave a value for radioactive cyclic AMP of 1.35% of the total recovered radioactivity and 4min later the value obtained was 0.94% of the total recovered radioactivity. If collagen was added 20s after the PGE1, the radioactive cyclic AMP concentration obtained 4min later was 0.79% of the total recovered radioactivity. Collagen added to platelet-rich plasma containing 0.18% of the radioactivity as cyclic AMP did not cause a significant variation from this value when measured 20, 40, 60, 120 and 240s after addition.

Effects of theophylline and of aspirin. Aspirin inhibits collagen-induced aggregation, release of ATP and ADP and breakdown of radioactive ATP (Ball *et al.* 1969). Theophylline at high concentration also inhibited these effects of collagen treatment (Table 2). Theophylline at a concentration of 2.6mM appeared to cause some decay of radioactive ATP with the formation of ADP, but recovery of total radioactivity with this concentration of theophylline was about 12% lower than that obtained with lower concentrations of theophylline or without it; this result might be explained by failure of ATP to be absorbed on the charcoal in the presence of the theophylline.

Table 2. *Effect of inhibitors of collagen-induced aggregation on platelet radioactive ATP and release of ATP and ADP*

Portions (1 ml) of labelled plasma containing 3.3–4.7 × 10^8 platelets were stirred in the absorptiometer at 37°C either with or without added test compound. Test compounds were added in aqueous solution or 0.9% NaCl except for aspirin, which was added in 0.1% (w/v) $NaHCO_3$ solution. Collagen suspension (10 μl) was added as noted 1 min after addition of test compound. Incubations were stopped (see Ball *et al.* 1969) 4 min later and assays for radioactivity and released nucleotide were as described in the text. The degree of aggregation was measured as the change in light-transmittance (Δ%T) that had occurred and the results are the mean of two determinations. Samples to which no addition was made were incubated in the absorptiometer at the beginning and end of each experiment and the results given are the mean values obtained. Samples to which only collagen suspension was added were incubated in the absorptiometer half-way through the experiment. Radioactivity as inosine varied randomly from 0.3 to 0.7% of the total recovered counts. Where very little nucleotide was released the ATP/ADP ratios would not be reliable and they are therefore not included.

Treatment	Degree of aggregation (Δ%T)	Nucleotide released (nmol/10^8 platelets)			Radioactivity of substance (% of total recovered counts)					
		ATP	ADP	ATP/ADP ratio	ATP	ADP	AMP	IMP	Inosine+ hypoxanthine	ATP/ADP ratio
No addition	0	0.02	0.02	—	80.2	12.9	0.7	0.5	5.7	6.3
PGE1 (0.56 μM)	0	0.02	0.02	—	79.5	11.9	0.7	0.4	6.9	6.7
Collagen	43.7	0.72	0.96	0.8	63.9	17.1	1.7	3.6	13.4	3.7
PGE1 (0.11 μM) + collagen	31.6	0.46	0.52	0.9	68.8	16.5	1.1	3.8	9.7	4.2
PGE1 (0.17 μM) + collagen	12.0	0.16	0.28	0.6	71.9	15.1	1.0	2.9	8.8	4.8
PGE1 (0.28 μM) + collagen	2.6	0.07	0.14	0.5	77.0	12.9	0.9	1.7	7.3	6.0
PGE1 (0.56 μM) + collagen	1.4	0.03	0.06	—	78.3	12.0	0.9	1.1	7.2	6.5
No addition	0	0.02	0.01	—	80.7	12.3	0.6	0.4	5.8	6.6
Theophylline (2.6 mM)	0	0.02	0.02	—	78.2	15.3	1.1	0.5	4.8	5.1
Collagen	45.8	0.75	0.93	0.8	59.8	20.1	2.4	3.2	14.3	3.0
Theophylline (1.1 mM) + collagen	37.6	0.48	0.68	0.7	65.8	17.4	1.3	3.8	11.5	3.8
Theophylline (1.8 mM) + collagen	14.6	0.16	0.35	0.5	72.4	15.4	1.0	3.1	7.9	4.7
Theophylline (2.6 mM) + collagen	0	0.05	0.11	0.5	77.1	15.3	1.2	1.2	5.0	5.0
No addition	0	0.01	0.03	—	83.7	10.8	0.6	0.5	4.5	7.8
PGE1 (0.028 μM) + theophylline (0.6 mM)	0	0.01	0.03	—	83.3	10.7	0.6	0.4	4.7	7.8
Collagen	52.7	0.94	1.31	0.7	67.0	16.6	1.3	2.4	12.6	4.0
PGE1 (0.028 μM) + theophylline (0.3 mM) + collagen	38.4	0.33	0.42	0.8	72.5	15.3	0.8	2.1	9.1	4.7
PGE1 (0.028 μM) + theophylline (0.4 mM) + collagen	11.9	0.10	0.27	0.4	76.7	14.1	0.7	1.5	6.8	5.4
PGE1 (0.028 μM) + theophylline (0.6 mM) + collagen	1.4	0.06	0.08	0.8	81.5	11.4	0.6	0.9	5.3	7.1
Theophylline (0.6 mM) + collagen	48.9	0.80	1.01	0.8	67.3	16.7	1.3	2.5	12.1	4.0
PGE1 (0.028 μM) + collagen	44.6	0.85	0.98	0.9	69.1	15.8	1.2	2.0	12.3	4.4
No addition	0	0.02	0.02	—	83.3	11.8	0.6	0.4	4.0	7.1
PGE1 (0.11 μM) + aspirin (0.25 mM)	0	0.02	0.02	—	82.4	11.5	0.6	0.4	4.9	7.2
Collagen	58.4	0.99	1.19	0.8	64.6	18.2	1.6	1.8	13.8	3.6
PGE1 (0.11 μM) + aspirin (0.1 mM) + collagen	50.2	0.44	0.62	0.7	70.0	17.4	1.3	1.5	9.8	4.0
PGE1 (0.11 μM) + aspirin (0.2 mM) + collagen	9.1	0.10	0.15	0.7	78.6	14.1	0.7	0.8	5.7	5.6
PGE1 (0.11 μM) + aspirin (0.25 mM) + collagen	4.3	0.07	0.14	0.5	78.8	13.4	0.9	0.8	5.9	5.8
Aspirin (0.25 mM) + collagen	29.1	0.12	0.19	0.6	72.1	16.0	1.1	1.3	9.3	4.5
PGE1 (0.11 μM) + collagen	52.2	0.60	0.70	0.9	68.2	17.0	1.3	1.3	12.0	4.0

Table 3. *Effect of incubation time of PGE1 with platelet-rich plasma on inhibition of aggregation and on radioactive cyclic AMP*

Two 1 ml portions of labelled platelet-rich plasma were each simultaneously stirred at 37°C in an absorptiometer. The same amount of PGE1 was added to each and after the incubation times noted cold trichloroacetic acid containing carrier compounds was added to one portion, which was processed for the determination of radioactivity, and to the other portion was added 10 μl of collagen suspension for the determination of platelet aggregation 4 min later. Samples that were incubated for 4 min were run before the corresponding samples incubated for 1 min. Samples to which no addition was made were run at the end of the experiment. Plasma contained 3.73×10^8 platelets/ml.

Concn. of PGE1 in platelet-rich plasma (μM)	1 min incubation		4 min incubation	
	Radioactive cyclic AMP (% of total recovered counts)	Inhibition of aggregation (%)	Radioactive cyclic AMP (% of total recovered counts)	Inhibition of aggregation (%)
0	0.17	0	0.20	0
0.28	0.31	28	0.29	42
0.42	0.44	36	0.33	66
0.80	0.56	82	0.42	97

Table 4. *Effect of theophylline and of aspirin on platelet radioactive cyclic AMP at various incubation times*

Portions of labelled platelet-rich plasma (1 ml) to which 0.2 ml of a solution containing either theophylline, aspirin or 0.9% NaCl was added, were stirred at 37°C in the absorptiometer. The final concentration of theophylline or aspirin in the platelet-rich plasma was 1.7 mM. After the times noted incubations were terminated by the addition of cold trichloroacetic acid containing carrier compounds and radioactive cyclic AMP was determined as described in the text. Control samples to which NaCl was added were incubated in the absorptiometer at the beginning, half-way through and at the end of the experiment. Individual results are given with the mean value in parentheses. Plasma contained 2.08×10^8 platelets/ml final volume.

	Radioactive cyclic AMP (% of total recovered counts)		
Incubation time (s) ...	20	60	120
Addition			
NaCl		0.09, 0.08, 0.10 (0.09)	
Theophylline	0.10, 0.10 (0.10)	0.11, 0.10 (0.11)	0.09, 0.11 (0.10)
Aspirin	0.10, 0.09 (0.10)	0.08, 0.10 (0.09)	0.08, 0.08 (0.08)

Neither theophylline nor aspirin at a concentration of 1.7 mM significantly altered radioactive cyclic AMP concentrations 20, 60 or 120 s after addition of either compound (Table 4).

Concentrations of theophylline and aspirin that did not cause a significant increase of radioactive cyclic AMP concentrations at the time of collagen addition inhibited aggregation (Tables 5 and 6).

Effects of combinations of PGE1 and theophylline. PGE1 and theophylline at concentrations in platelet-rich plasma that separately had little effect on collagen-induced aggregation, release of ATP or ADP or breakdown of radioactive ATP, together inhibited these effects of collagen (Table 2).

Theophylline added together with PGE1 to platelet-rich plasma increased the radioactive cyclic AMP concentration over that obtained with PGE1 alone, both at 20 s and 120 s after addition of the compounds (Fig. 3).

Dose–response curves for the inhibition of aggregation with various amounts of PGE1, theophylline and PGE1 plus theophylline are shown in Fig. 4. These results and those given in Table 5 indicate that small concentrations of PGE1 plus theophylline have a synergistic effect on inhibition of aggregation. Theophylline (0.2–0.6 mM) and PGE1 (0.014–0.028 μM) caused little or no inhibition of aggregation by themselves. The results given in Table 5 show that mixtures of PGE1 and theophylline at such concentrations caused little or no increase of radioactive cyclic AMP concentrations at the time of collagen addition (20 s after addition of the compounds) and almost completely inhibited aggregation. By contrast, PGE1 alone caused large increases of radioactive cyclic AMP concentrations at the time of collagen addition but less inhibition of aggregation.

Incubation of platelet-rich plasma with a mixture

Table 5. *Effect of PGE*1, *theophylline and PGE*1 *plus theophylline on inhibition of aggregation and on radioactive cyclic AMP*

Two 1 ml portions of labelled platelet-rich plasma were simultaneously stirred at 37°C, each in a separate absorptiometer. Identical additions of 0.9% NaCl alone or with either PGE1, theophylline or PGE1 plus theophylline were made to each portion of platelet-rich plasma (final volume, 1.06 ml) and after 20 s incubation one portion was treated for the determination of platelet aggregation and the other for the determination of radioactive cyclic AMP. The platelet-rich plasma used for experiment A was labelled as given in the text and radioactive cyclic AMP was determined by the method given in Table 3 and the text. The platelet-rich plasma used for experiment B was labelled with 1.25 μM-[U-^{14}C]adenine for 1 h at 37°C and radioactive cyclic AMP was determined by the method of Krishna *et al.* (1968) as modified by D. C. B. Mills & J. B. Smith (unpublished work). The degree of aggregation and radioactive cyclic AMP content of platelet-rich plasma to which only NaCl was added were determined at the beginning and end of each experiment. Plasma contained: A, 6.21×10^8 platelets/ml; B, 10.57×10^8 platelets/ml.

	Treatment	Inhibition of aggregation (%)	Radioactive cyclic AMP (% of total recovered counts)
A	NaCl	0	0.11, 0.10
	PGE1 (0.028 μM) + theophylline (0.3 mM)	22	0.17
	PGE1 (0.028 μM) + theophylline (0.5 mM)	94	0.18
	Theophylline (1.0 mM)	0	0.12
	PGE1 (0.14 μM)	7	0.39
	PGE1 (0.28 μM)	55	0.51
B	NaCl	0	0.08, 0.11
	PGE1 (0.028 μM) + theophylline (0.25 mM)	0	0.12
	PGE1 (0.028 μM) + theophylline (0.35 mM)	12	0.13
	PGE1 (0.028 μM) + theophylline (0.5 mM)	94	0.13
	PGE1 (0.028 μM) + theophylline (0.8 mM)	98	0.14
	PGE1 (0.028 μM) + theophylline (1.0 mM)	99	0.16
	Theophylline (1.0 mM)	9	0.10
	Theophylline (1.5 mM)	93	0.12
	Theophylline (2.0 mM)	100	0.12
	PGE1 (0.028 μM)	0	0.12
	PGE1 (0.14 μM)	52	0.23
	PGE1 (0.42 μM)	99	0.49

Table 6. *Effect of PGE*1, *aspirin and PGE*1 *plus aspirin on inhibition of aggregation and on radioactive cyclic AMP*

Two 1 ml portions of labelled platelet-rich plasma were simultaneously stirred at 37°C, each in a separate absorptiometer. Identical additions of 0.9% NaCl alone or with either PGE1, aspirin or PGE1 plus aspirin were made to each portion of platelet-rich plasma (final volume, 1.04 ml) and after 1 min incubation one portion was treated for the determination of platelet aggregation and the other for the determination of radioactive cyclic AMP (Table 3 and text); other details are given in Table 5. Plasma contained 4.66×10^8 platelets/ml.

Treatment	Inhibition of aggregation (%)	Radioactive cyclic AMP (% of total recovered counts)
NaCl	0	0.11, 0.18
PGE1 (0.11 μM) + aspirin (0.10 mM)	27	0.18
PGE1 (0.11 μM) + aspirin (0.19 mM)	96	0.18
Aspirin (0.19 mM)	12	0.13
Aspirin (0.47 mM)	88	0.12
PGE1 (0.11 μM)	16	0.17
PGE1 (0.22 μM)	30	0.37

of PGE1 (final concentration 1 μM) and theophylline (final concentration 0.9 mM) for 20 s gave a value for radioactive cyclic AMP of 1.44% of the total recovered radioactivity and 4 min later the value obtained was 0.89% of the total recovered radioactivity. If collagen was added 20 s after the mixture of PGE1 and theophylline the radioactive cyclic AMP concentration obtained 4 min later was 0.72% of the total recovered radioactivity.

*Effects of combinations of PGE*1 *and aspirin.*

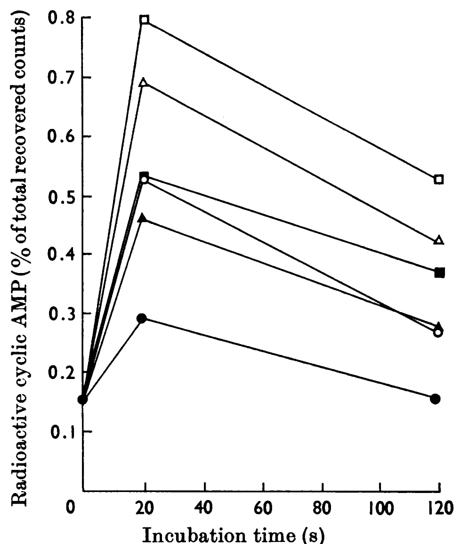

Fig. 3. Comparison of the amount of radioactive cyclic AMP formed after treatment of platelet-rich plasma either with PGE1 or with PGE1 plus theophylline. To 1 ml portions of labelled platelet-rich plasma was added 0.9% NaCl alone or with either PGE1 or PGE1 plus theophylline to give a final volume of 1.11 ml. The mixtures were stirred at 37°C in the absorptiometer for the times indicated. Other details were as given in Fig. 2 except that platelet-rich plasma to which only NaCl was added was stirred for 120 s (beginning of experiment) and 20 s (end of experiment). The values of radioactive cyclic AMP obtained for these samples were 0.13 and 0.17% of the total recovered counts. Plasma contained 3.62×10^8 platelets/ml. ●, 0.13 μM-PGE1; ▲, 0.13 μM-PGE1 + 0.9 mM-theophylline; ■, 0.13 μM-PGE1 + 1.8 mM-theophylline; ○, 0.26 μM-PGE1; △, 0.26 μM-PGE1 + 0.9 mM-theophylline; □, 0.26 μM-PGE1 + 1.8 mM-theophylline.

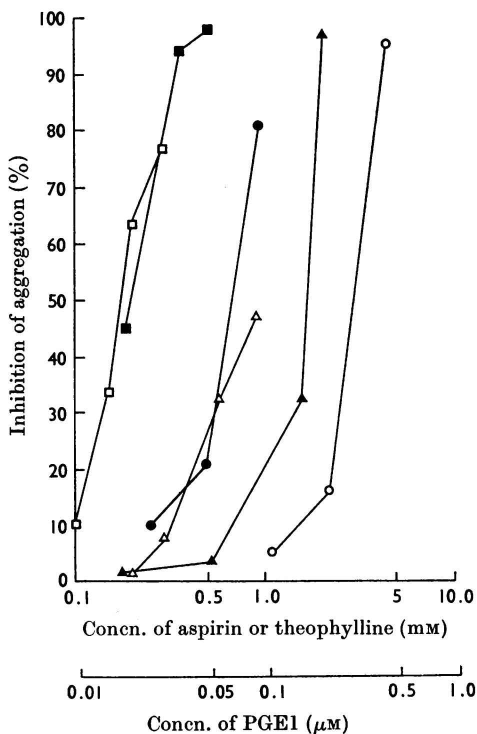

Fig. 4. Inhibition of aggregation obtained with various amounts of PGE1, theophylline and PGE1 plus theophylline or with PGE1, aspirin and PGE1 plus aspirin. To 1 ml amounts of platelet-rich plasma in the absorptiometer were added various amounts of either PGE1, theophylline or a mixture of various amounts of theophylline with a constant amount of PGE1. After 20 s collagen suspension was added and the degree of aggregation determined 4 min later. In another experiment various amounts of either PGE1, aspirin or a mixture of various amounts of aspirin with a constant amount of PGE1 were added to platelet-rich plasma and after 1 min collagen was added and aggregation assayed as above. First expt.: ●, PGE1; ▲, theophylline; ■, PGE1 (0.025 μM) + amounts of theophylline as indicated; second expt.: ○, PGE1; △, aspirin; □, PGE1 (0.11 μM) + amounts of aspirin as indicated. The final incubation volume for the first experiment was 1.11 ml and for the second experiment 1.10 ml.

Concentrations of PGE1 and aspirin in platelet-rich plasma that separately had little effect on collagen-induced aggregation, release of ATP and ADP or breakdown of radioactive ATP, together inhibited these effects of collagen (Table 2).

Aspirin added together with PGE1 to platelet-rich plasma did not significantly affect the concentrations of radioactive cyclic AMP obtained with PGE1 alone, whether at 20 s or 120 s after addition of the compounds (Fig. 5).

Dose–response curves for the inhibition of aggregation with various amounts of PGE1, aspirin and PGE1 plus aspirin indicate that small concentrations of PGE1 plus aspirin have a synergistic effect on inhibition of aggregation (Fig. 4).

The results given in Table 6 show that mixtures of PGE1 and aspirin at low concentrations of each caused little or no increase of the radioactive cyclic AMP concentration at the time of collagen addition (1 min after addition of the compounds) and almost completely inhibited aggregation. By contrast, PGE1 alone caused a large increase of the radioactive cyclic AMP concentration at the time of collagen addition but less inhibition of aggregation.

DISCUSSION

Collagen induces platelets in plasma to release ATP, ADP and other substances to the supernatant

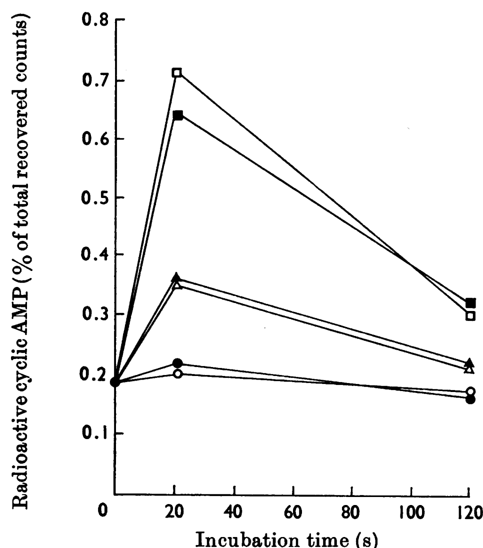

Fig. 5. Comparison of the amount of radioactive cyclic AMP formed after treatment of platelet-rich plasma with either PGE1 or PGE1 plus aspirin. To 1 ml portions of labelled platelet-rich plasma was added 0.9% NaCl alone or with either PGE1 or PGE1 plus aspirin to give a final volume of 1.21 ml. The mixtures were stirred at 37°C in the absorptiometer for the times indicated. Other details were as given in Figs. 2 and 3. The values of radioactive cyclic AMP obtained for control samples were 0.20% (beginning of experiment) and 0.18% (end of experiment) of the total recovered counts. Plasma contained 2.93×10^8 platelets/ml. ●, 0.05μM-PGE1; ○, 0.05μM-PGE1+ 1.65mM-aspirin; ▲, 0.1μM-PGE1; △, 0.1μM-PGE1+ 1.65mM-aspirin; ■, 0.2μM-PGE1; □, 0.2μM-PGE1+ 1.65mM-aspirin.

and the released ADP in the presence of Ca^{2+} causes platelet aggregation (Haslam, 1967). Inhibitors of collagen-induced aggregation might act therefore either by inhibiting the release of platelet ADP or by interfering with the reactions of the released ADP that cause platelets to adhere to one another or by a combination of both mechanisms.

It has been shown that amitriptyline, *N*-ethylmaleimide, *p*-chloromercuribenzenesulphonate and aspirin inhibit aggregation in proportion to the degree that they inhibit the release of ADP (Ball *et al.* 1969). PGE1 and theophylline acted similarly (Table 2). Further, PGE1 and theophylline both inhibited collagen-induced breakdown of radioactive ATP in proportion to the degree of inhibition of ADP release and inhibition of aggregation that they produced, which was also characteristic of the compounds mentioned above. Thus inhibition of collagen-induced aggregation by these compounds appears to be caused by their inhibitory action on ADP release, although, with the exception of aspirin, which does not inhibit reversible ADP-induced aggregation (O'Brien, 1968), they may to some extent also inhibit the action of such ADP as is released.

PGE1 and theophylline mutually potentiate the inhibition of ADP-induced aggregation (Mills *et al.* 1970), a result that might be explained by PGE1 stimulating platelet cyclic AMP formation (Robison *et al.* 1969) and theophylline, which inhibits platelet cyclic AMP phosphodiesterase (D. C. B. Mills & J. B. Smith, unpublished work), inhibiting its breakdown. The question arose whether the release reaction of platelets might be controlled by cyclic AMP concentrations since PGE1 and theophylline had a synergistic effect on inhibition of collagen-induced aggregation (Fig. 4) and since combinations of PGE1 and theophylline inhibited ADP release with amounts of each compound that separately had no effect on ADP release (Table 2).

It was confirmed that PGE1 stimulated the formation of radioactive cyclic AMP in platelets containing radioactive ATP (see Vigdahl, Marquis & Tavormina, 1969; and Fig. 2), but theophylline at a concentration of 1.7mM did not significantly alter radioactive cyclic AMP concentrations (Table 4). Theophylline did, however, increase the amount of radioactive cyclic AMP formed with a given dose of PGE1, a result in keeping with its known inhibitory properties on platelet cyclic phosphodiesterase (D. C. B. Mills & J. B. Smith, unpublished work).

Maximum elevation of radioactive cyclic AMP concentrations with PGE1 occurred within 10–30s of adding the compound to platelet-rich plasma and thereafter declined (Fig. 2). Since the degree of inhibition of aggregation produced by PGE1 increased with its time of incubation in platelet-rich plasma, before addition of collagen, up to at least 4min, there was an inverse relationship between the radioactive cyclic AMP concentration obtained at the time of collagen addition and inhibition of aggregation (Table 3). Any relationship therefore between cyclic AMP and inhibition of aggregation cannot be dependent solely on the cyclic AMP concentration at the time of collagen addition.

Theophylline at concentrations that caused little or no increase of radioactive cyclic AMP concentrations at the time of collagen addition almost completely inhibited aggregation (Table 5). Mixtures of PGE1 and theophylline, in amounts that had a synergistic effect on the inhibition of aggregation, also caused little or no increase of radioactive cyclic AMP concentrations at the time of collagen addition and almost completely inhibited aggregation, but PGE1 alone caused considerable increases of radioactive cyclic AMP concentrations at the time of collagen addition and less inhibition of aggregation (Table 5). So with these compounds there

would not appear to be a correlation between the radioactive cyclic AMP concentrations at the time of collagen addition and the degree of inhibition of aggregation.

Aspirin had no effect on platelet radioactive cyclic AMP (Table 4) neither did it alter the effect of PGE1 on radioactive cyclic AMP (Fig. 5). There is therefore no evidence that aspirin stimulates cyclic AMP formation or that it inhibits cyclic AMP breakdown, as theophylline does. Nevertheless, PGE1 and aspirin exerted a synergistic effect on inhibition of collagen-induced aggregation (Fig. 4) and such mixtures inhibited release of ATP and ADP and breakdown of radioactive ATP during collagen treatment of platelet-rich plasma (Table 2). Aspirin alone and mixtures of PGE1 and aspirin having a synergistic effect on inhibition of aggregation caused little or no increase of radioactive cyclic AMP concentrations at the time of collagen addition and almost completely inhibited aggregation, whereas PGE1 alone caused considerable increases in radioactive cyclic AMP concentrations at the time of collagen addition but less inhibition of aggregation (Table 6).

High concentrations of radioactive cyclic AMP, induced by either PGE1 or a mixture of PGE1 and theophylline, decayed to lower concentrations after the addition of collagen than in its absence (see the text), indicating an effect of collagen on cyclic AMP breakdown. However, collagen had no determinable effect on the very small concentrations of radioactivity present as cyclic AMP in platelets not treated with these compounds. The relevance of these findings to the relation between cyclic AMP concentrations during aggregation, as opposed to before aggregation, and the degree of inhibition of aggregation remains to be determined, but as mentioned above it seems possible to inhibit aggregation with drugs or combinations of drugs that cause very little or no increase of cyclic AMP concentrations.

We are most grateful to Professor D. A. Van Dorp for his gift of PGE1, and to Mr C. D. B. Mills and Mr J. B. Smith for their interest and advice.

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