

Inhibition and Subsequent Enhancement of Platelet Responsiveness by Prostacyclin in the Rabbit

Relationship to Platelet Adenosine 3',5'-Cyclic Monophosphate

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Abstract

Methods were developed for measuring changes in platelet sensitivity to a release-inducing stimulus and in platelet cyclic AMP in fresh whole blood samples from rabbits. These techniques permitted detection of the effects of exogenous and endogenous prostacyclin on circulating platelets. In these methods, rabbit platelets were labeled in vitro by incubation with [14 C]serotonin and [3 H]adenine and then transfused into other rabbits. Release of platelet [14 C]serotonin by a standard dose of synthetic platelet-activating factor (40 pmol/ml) and the platelet cyclic [3 H]AMP levels were then measured in citrated blood from the conscious animals within 2 min of arterial puncture. Bolus intravenous injections of prostacyclin (1–10 nmol/kg) caused concentration-dependent increases in platelet cyclic AMP after 2 min, which decreased \sim 75% by 5 min, and disappeared after 30 min. Significant inhibition of the platelet release reaction was detected 2 min but not 5 min after injection of 10 nmol of prostacyclin per kilogram. With lower doses, significant enhancement of the release of [14 C]serotonin was observed after 5 min. Similar changes in platelet responsiveness and cyclic [3 H]AMP were observed after release of endogenous prostacyclin by intravenous injection of angiotensin II (5 nmol/kg); inhibition of the release of [14 C]serotonin after 2 min was followed by potentiation after 5 min, though platelet cyclic [3 H]AMP remained above control values. In these experiments, the time course of the changes in platelet cyclic [3 H]AMP correlated closely with values for blood prostacyclin obtained previously (Haslam, R. J., and M. D. McClenaghan, 1981, *Nature [Lond.]*, 292:364–366). Prostacyclin also had a biphasic effect on the release of [14 C]serotonin when added to citrated blood in vitro, though both the increase in sensitivity to platelet-activating factor and the return of platelet cyclic [3 H]AMP towards control values took place more slowly. At all times, addition of platelet-activating factor decreased platelet cyclic [3 H]AMP towards but not below the control level observed in the absence of prostacyclin. Our results indicate that although transient increases in platelet cyclic AMP cause an immediate decrease in platelet responsiveness in vivo or in vitro, a period of enhanced platelet sensitivity follows as platelet cyclic AMP falls.

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Introduction

Prostacyclin (epoprostenol) (PGI_2)¹ is a potent inhibitor of platelet aggregation and a vasodilator that is synthesized predominantly in the vascular endothelium from the prostaglandin endoperoxide, PGH_2 (1, 2). Its action on platelets results from a receptor-mediated activation of adenylate cyclase and a consequent increase in platelet cyclic AMP (3–5). At physiological pH values, PGI_2 is spontaneously hydrolyzed to form the inactive compound, 6-keto- $\text{PGF}_{1\alpha}$ (2), and in vivo $>90\%$ of injected PGI_2 disappears from the circulation in ~ 2 min (6). Although it was initially suggested that PGI_2 is a circulating hormone that limits platelet aggregation in vivo (7, 8), studies using several different methods have shown that this is not the case (6, 9–12). These observations have led to the current hypothesis that, under physiological conditions, PGI_2 acts as a local inhibitor of platelet aggregation without affecting platelet function in the general circulation. However, certain pharmacological agonists, including angiotensin II (6, 13, 14), and some potential drugs that modify platelet function, such as nafazatrom (15, 16), can stimulate or enhance the release of PGI_2 into the circulation. In addition, inhibitors of thromboxane synthetase may re-orientate the metabolism of PGH_2 formed in platelets towards the synthesis of PGI_2 by endothelial cells (16–18). We have therefore attempted to develop methods that will permit the study, under physiological conditions, of the short-lived effects of PGI_2 on circulating platelets, both with respect to changes in their responsiveness to stimuli and cyclic AMP content.

Methods

Materials. [$2,8\text{-}^3\text{H}$]Adenine (27–35 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA), cyclic [$8\text{-}^{14}\text{C}$]AMP (50 mCi/mmol) from New England Nuclear Canada (Lachine, Quebec), and [^{14}C]serotonin (5-hydroxy-[side chain- $2\text{-}^{14}\text{C}$]tryptamine, 55 mCi/mmol) and Aqueous Counting Scintillant (ACS) were from Amersham Corp. (Oakville, Ontario). D-(–)-luciferin and luciferase, purified from *Photinus pyralis*, were obtained from Boehringer Mannheim Canada (Dorval, Quebec). Val²-angiotensin II, neutral alumina (WN-3), *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, and cyclic nucleotide phosphodiesterase were obtained from Sigma Chemical Co. (St. Louis, MO) and Dowex 50 resin (AG 50W-X8, 200–400 mesh) from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario). Synthetic 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine (synthetic platelet-activating factor [PAF]) was a gift from Dr. H. R. Baumgartner of F. Hoffman-La Roche and Co. (Basel, Switzerland). A stock solution was prepared by dissolving the compound in ethanol to give a 10-mM solution that

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1. *Abbreviations used in this paper:* ACD, acid citrate dextrose; ACS, Aqueous Counting Scintillant; EC_{50} , concentration causing 50% of the maximum effect; IC_{50} , concentration causing 50% inhibition; PAF, synthetic platelet-activating factor; PGI_2 , prostacyclin (epoprostenol).

was diluted to 0.1 mM with 0.154 M NaCl that contained 3.5 mg of crystallized bovine serum albumin/ml and 5 mM Hepes, pH 7.4. Further dilutions were made with 0.154 M NaCl that contained 3.5 mg of crystallized rabbit albumin/ml, just before their use. PGI₂ was a gift from Dr. J. Pike of Upjohn Co. (Kalamazoo, MI). Stock solutions of PGI₂ were prepared at a concentration of 1 mM in 9.4 mM Na₂CO₃, stored in 0.02-ml aliquots at -55°C, and diluted just before use in 0.139 M NaCl that contained 9.4 mM Na₂CO₃. Male New Zealand white rabbits (2.2–3.0 kg) were used in this study.

Preparation of labeled platelets for injection into rabbits. Blood was obtained by cannulation of the carotid artery from rabbits that had been anesthetized with pentobarbital and was mixed with 0.175 vol of acid citrate dextrose (ACD) anticoagulant (19). Platelet-rich plasma was prepared by centrifuging the blood twice at 250 g for 15 min. This material was centrifuged for 10 min at 3,500 g and the platelets from each rabbit were resuspended in 10 ml of ACD plasma. This procedure was repeated to remove residual red cells and the final suspension, containing 2.5×10^9 platelets/ml, was incubated for 90 min at room temperature with 4 μ M [³H]adenine. [¹⁴C]Serotonin (1 μ M) was added every 15 min during this incubation. About 65% of the [³H]adenine and 85% of the [¹⁴C]serotonin were taken up. After further centrifugation, the platelets were resuspended at 2.5×10^9 /ml in fresh ACD plasma that contained no pentobarbital. This platelet suspension, which contained $\sim 75 \mu$ Ci of ³H and $\sim 0.2 \mu$ Ci of ¹⁴C/ml, was injected into unanesthetized rabbits through the marginal ear veins. Each rabbit received, per kilogram, 2 ml of platelet-rich plasma containing 5×10^9 platelets which should increase the circulating platelet count by $<10^8$ /ml ($\sim 20\%$).

Studies on blood from rabbits injected with labeled platelets. In preliminary experiments, in which the effects of PAF and PGI₂ on platelet function in whole blood were investigated, samples of up to 20 ml of arterial blood were taken from the ears of conscious rabbits into 0.1 vol of 3.8% (wt/vol) Na₃ citrate.2H₂O. The release of [¹⁴C]serotonin or of ATP was measured, as described below, using 0.4–0.45 ml of blood. When the changes in platelet cyclic [³H]AMP were studied, 0.8 ml of citrated blood that contained labeled platelets was added to tubes that contained 0.04 ml of PGI₂ or vehicle. These samples were incubated at 37°C for various times and, in some cases, 0.04 ml of solution containing PAF was then added. The incubations were stopped by addition of 0.8 ml of 20% (wt/vol) TCA, and the cyclic [³H]AMP was determined, as described below. In some experiments, in which release of platelet [¹⁴C]serotonin was studied in native blood, all incubations were completed within 4 min of arterial puncture, during which time no blood coagulation occurred.

In experiments in which the effects of injection of PGI₂ or angiotensin II were studied, blood was taken at intervals from successively more proximal segments of the central arteries of the ears of conscious rabbits into 0.1 vol of 3.8% (wt/vol) Na₃ citrate.2H₂O (final volume usually 4.5 ml). These blood samples were subdivided as follows: two 0.4-ml samples were stirred without and two with PAF (40 pmol/ml) for 0.5 min before the addition of formaldehyde/EDTA and measurement of the [¹⁴C]serotonin released, as described below; three 0.1-ml samples were lysed in 0.6 ml of H₂O for determination of the total blood ¹⁴C; three 0.8-ml samples were each extracted with 0.8 ml of 20% (wt/vol) TCA for measurement of platelet cyclic [³H]AMP. All these procedures were carried out within 2 min of arterial puncture and the samples kept at 0°C until processed further.

Measurement of release of [¹⁴C]serotonin from platelets in whole blood. Samples of citrated blood (0.40–0.45 ml) containing labeled platelets were mixed with appropriate additions in siliconized glass aggregometer tubes and incubated at 37°C for various times. The release reaction was then induced by addition of up to 0.02 ml of PAF solution and the samples were stirred for 0.5 min in either an aggregation module (Payton Assoc. Ltd. [Scarborough, Ontario]) or, when release of ATP was also studied, in a Payton lumi-aggregation module; vehicle was added to control samples. All incubations were stopped by the addition of 0.5 ml of ice-cold 0.154 M NaCl containing paraformaldehyde and EDTA (final concentrations 1.5% [wt/vol] and 5 mM, respectively). These samples were centrifuged at 12,000 g for

0.5 min and the [¹⁴C]serotonin present in the supernatant was determined by counting in ACS scintillation fluid. To normalize the results, the [¹⁴C]serotonin released by PAF was expressed as a percentage of the total blood [¹⁴C]serotonin, which was extracted by a modification of the method of Anderson et al. (20). Three 0.1-ml blood samples were lysed in 0.6 ml of H₂O and mixed with 0.2 ml of 1 M ascorbic acid, followed by 0.2 ml of 5 M HClO₄. After 10 min at 0°C, these samples were centrifuged at 12,000 g for 2 min. The supernatant was partially neutralized by addition of 0.08 ml of 10 M KOH, and after a further 10 min at 0°C was centrifuged again to remove KClO₄. The ³H and ¹⁴C present in the supernatant were determined by liquid scintillation.

Measurement of release of ATP from platelets in whole blood. A modification of the method of Ingeman et al. (21) was used. Samples of citrated rabbit blood (0.45 ml) were mixed with 0.01 ml of luciferin (final concentration 0.175 mM), 0.02 ml of luciferase (2 μ g), and 0.01 ml of PGI₂ or vehicle in siliconized glass aggregometer tubes and incubated at 37°C. The basal luminescence was recorded for 0.5 min before the addition of 0.01 ml of PAF solution or vehicle. The change in luminescence after a further 0.5 min was then measured. The ATP released by PAF was calculated from the change in luminescence caused by addition of 2 or 5 nmol of ATP to blood samples containing only luciferin and luciferase. Linear responses were observed with up to 10 nmol of ATP. Values are expressed as nanomoles of ATP released per 10^9 platelets. The platelet count in the blood was determined according to Brecher and Cronkite (22). When blood samples were preincubated for 30 min with PGI₂, luciferin and luciferase were added after 29 min.

Measurement of platelet cyclic [³H]AMP in whole blood. Citrated blood samples (0.8 ml) containing platelets labeled with [³H]adenine were rapidly mixed with 0.8 ml of 20% (wt/vol) TCA and 0.05 ml of cyclic [¹⁴C]AMP (600 dpm), which was used to monitor the recovery of platelet cyclic [³H]AMP. The acidified samples were centrifuged and 0.02 ml of the supernatant was counted by liquid scintillation to determine the total ³H present. Labeled cyclic AMP was isolated from the acid extracts by chromatography on alumina and Dowex 50 resin (6). The final phosphate buffer eluates were lyophilized and counted for ³H and ¹⁴C by liquid scintillation. The cyclic [³H]AMP values found were corrected for recovery of cyclic [¹⁴C]AMP and were expressed as percentages of the total blood ³H.

Measurement of the purity of cyclic [³H]AMP isolated from labeled platelets in whole blood. Samples of citrated blood were taken from a rabbit that had been transfused with labeled platelets. The ³H- and ¹⁴C-labeled cyclic nucleotides were isolated as described above, acidified with HCl, and re-chromatographed on Dowex 50 to remove phosphate. The labeled cyclic AMP was dissolved in 0.1 ml of 1 mM cyclic AMP; 5 μ l was counted for ³H and ¹⁴C, and 45- μ l samples were incubated for 30 min at 30°C with and without 0.2 U of cyclic nucleotide phosphodiesterase [in 5 μ l of 100 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Na⁺ salt, pH 7.5), 40 mM MgSO₄, and 2.5 mM EDTA]. These incubations were terminated by boiling, and cyclic AMP and AMP were separated by thin-layer chromatography on cellulose using *n*-butanol/acetone/acetic acid/14.8 M NH₃/H₂O (90:30:20:1:60, by volume). Cyclic AMP and AMP were eluted from the cellulose with H₂O and counted for ³H and ¹⁴C. The purity of the material isolated as cyclic [³H]AMP by the standard method was calculated from the decrease (if any) in the ratio of ³H to ¹⁴C after its further purification and conversion to AMP.

Determination of the cellular distribution of ³H and ¹⁴C in blood from rabbits injected with labeled platelets. Blood samples (10 ml) from rabbits that had been injected with labeled platelets were taken into ACD anticoagulant and diluted with 3 vol of solution containing 13 mM Na₃ citrate, 5 mM dextrose, and 135 mM NaCl (pH 6.5). Platelets and lymphocytes were isolated from red cells and granulocytes by Ficoll-Hypaque density centrifugation (23) and separated by centrifugation through a discontinuous sucrose gradient (24). The platelets present in the red cell-granulocyte, lymphocyte, and platelet fractions were counted in a hemacytometer after dilution with 1% ammonium oxalate (22). The ³H and ¹⁴C present in these fractions were determined;

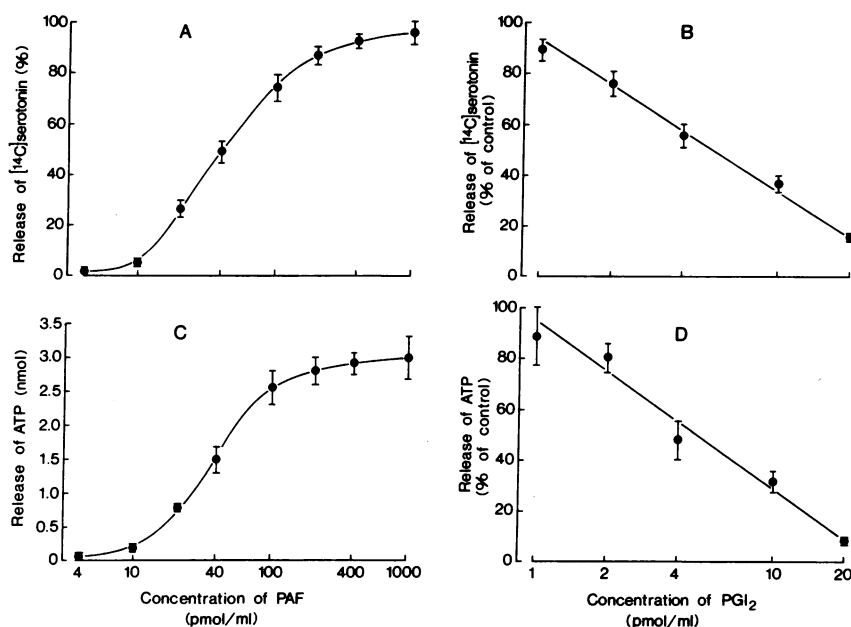


Figure 1. Release by PAF of [¹⁴C]serotonin and ATP from platelets in citrated rabbit blood; inhibitory action of PGI₂. The effects of different concentrations of PAF were determined in citrated blood samples from rabbits transfused with platelets that had been labeled with [¹⁴C]serotonin. The release of [¹⁴C]serotonin (A) and of ATP (C) were measured 0.5 min after addition of PAF. Other blood samples were incubated for 0.5 min with different concentrations of PGI₂ before determination of the release of [¹⁴C]serotonin (B) and ATP (D) by 40 pmol of PAF/ml. Values are means±SE from four estimations on separate blood samples from the same rabbit.

that containing red cells was first extracted as described for [¹⁴C]serotonin.

Statistical analyses. Mean values±SE are given from replicates within experiments or from different experiments, as indicated. Statistical significance was usually established by either unpaired or paired *t* tests, as appropriate; *P* values were derived from two-sided *t* tests. In some experiments, analysis of variance was carried out (25). Concentrations causing 50% of the maximum effect (EC₅₀ values) and concentrations causing 50% inhibition (IC₅₀ values) were obtained by linear regression from Hill plots of results falling between 10 and 90% of the maximum effects observed.

Results

Methods were developed for the simultaneous measurement of platelet sensitivity to release-inducing stimuli and of changes in the platelet cyclic AMP content in fresh whole blood samples from rabbits. Platelets that had been labeled in vitro with [¹⁴C]serotonin and [³H]adenine were transfused into rabbits. Measurement of the release of [¹⁴C]serotonin induced by a standard dose of PAF was then used to determine platelet responsiveness, and measurement of blood cyclic [³H]AMP was used to monitor changes in platelet cyclic AMP levels.

Measurement of the platelet release reaction in whole blood; effects of PGI₂. PAF was selected as an appropriate physiological stimulus because of its ability to induce a rapid platelet secretory response in whole blood without causing blood coagulation. The release of [¹⁴C]serotonin by PAF in citrated blood samples from rabbits transfused with labeled platelets was dose-dependent (Fig. 1 A); half-maximal release was observed with 39±4 pmol of PAF/ml (four experiments). PGI₂ inhibited the release of [¹⁴C]serotonin caused by 40 pmol of PAF/ml with an IC₅₀ of 5.7±1.2 pmol/ml (four experiments; e.g., Fig. 1 B). Simultaneous measurement of the release of platelet ATP using firefly luciferin-luciferase (Fig. 1, C and D) showed that the behavior of the total platelet population in the blood samples was not significantly different from that of labeled platelets with respect to induction of the release reaction by PAF (EC₅₀ = 46±4 pmol/ml, four experiments; *P* > 0.7) or its inhibition by PGI₂ (IC₅₀ = 4.6±0.1 pmol/ml, four experiments; *P* > 0.5). Analysis of the variance of the results

shown in Fig. 1, B and D, indicated that measurement of the release of [¹⁴C]serotonin was less subject to error than measurement of the release of ATP (*P* < 0.025). Platelets in native blood showed the same sensitivity to PGI₂ with respect to inhibition of the release of [¹⁴C]serotonin by PAF (IC₅₀ = 4.6±0.9 pmol/ml, four experiments; *P* > 0.4). These results indicated that a PAF concentration of 40 pmol/ml was suitable for detection of the inhibitory action of PGI₂ on the release of [¹⁴C]serotonin in fresh whole blood samples.

When blood samples were taken 10 min after transfusion of labeled platelets into rabbits, the ¹⁴C present indicated that all the injected platelets were in the circulation (assuming a value of 65 ml of blood per kilogram). Moreover, blood ¹⁴C did not decrease significantly for at least 2 h (five rabbits). The release of platelet [¹⁴C]serotonin by PAF remained constant in blood samples taken throughout this period (Fig. 2 B).

Platelet cyclic [³H]AMP in whole blood. The ³H present in blood samples taken 10 min after transfusion of labeled platelets indicated that none had been lost from the circulation (as for ¹⁴C). However, a decrease in the platelet cyclic [³H]AMP level of 29±1% (27 rabbits), relative to that in the labeled

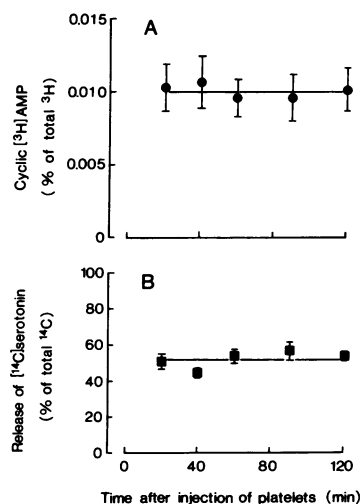


Figure 2. Platelet cyclic [³H]AMP (A) and release of platelet [¹⁴C]serotonin by 40 pmol of PAF/ml (B) in blood samples taken at various intervals after transfusion of rabbits with platelets labeled by preincubation with both [³H]adenine and [¹⁴C]serotonin. Values are means±SE from five to seven rabbits.

platelets before their injection, had occurred by this time. Dilution of labeled platelets into citrated blood or plasma *in vitro* also caused decreases in cyclic [^3H]AMP that were greater at 37°C (~20%) than at room temperature (~10%). Thus, most of the decrease in platelet cyclic [^3H]AMP *in vivo* was accounted for by dilution of the injected platelets and an increase in their temperature. Neither the total blood ^3H nor the platelet cyclic [^3H]AMP (Fig. 2 A) changed significantly from 10 min to 2 h after injection of the platelets. At the latter time, it proved possible to isolate $74 \pm 7\%$ of the platelets, which contained $74 \pm 7\%$ of blood ^{14}C and $72 \pm 6\%$ of blood ^3H (four experiments). Only $2.0 \pm 0.4\%$ of blood ^3H and $1.2 \pm 0.3\%$ of ^{14}C were found in platelet-poor plasma; the cyclic [^3H]AMP in this plasma was $1.1 \pm 1.8\%$ of the total blood cyclic [^3H]AMP (four experiments). These results established that the ^3H and ^{14}C in the transfused platelets remained within them during the course of the experiments, so that measurements made on whole blood samples could be assumed to reflect events associated with the platelets alone. When cyclic [^3H]AMP was isolated from whole blood in the presence of authentic cyclic [^{14}C]AMP and then treated with cyclic nucleotide phosphodiesterase, it was converted to AMP containing the same ratio of $^3\text{H}/^{14}\text{C}$. Thus, the identity of the compound isolated as cyclic [^3H]AMP was confirmed.

Effects of injection of PGI₂ on platelet cyclic [^3H]AMP and sensitivity to PAF. Rabbits that had been transfused with labeled platelets were injected with various amounts of PGI₂ (0, 1, 4, and 10 nmol/kg). Two control arterial blood samples were taken from each rabbit 10 and 30 min after injection of labeled platelets and PGI₂ was injected 5 min later. Fig. 3

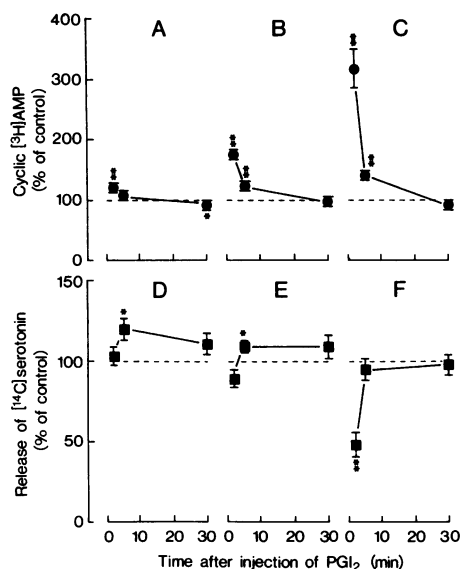


Figure 3. Effects of injection of PGI₂ on platelet cyclic [^3H]AMP and on the release of platelet [^{14}C]serotonin by PAF. Rabbits that had been transfused with labeled platelets received injections of PGI₂ as follows: 1 nmol/kg (A and D), 4 nmol/kg (B and E), and 10 nmol/kg (C and F). Arterial blood samples were taken 25 and 5 min before and 2, 5, and 30 min after injection of PGI₂. Blood cyclic [^3H]AMP levels (A, B, and C) and the release of [^{14}C]serotonin by 40 pmol of PAF/ml (D, E, and F) were measured immediately after arterial puncture. Values from each rabbit after injection of PGI₂ were expressed as percentages of the mean of the values obtained beforehand. Results (means \pm SE) from 5 to 10 rabbits are shown. Statistical significance is indicated by * ($P < 0.05$) and ** ($P < 0.01$).

shows the changes in platelet cyclic [^3H]AMP and in the [^{14}C]serotonin released by 40 pmol of PAF/ml at various times after injection of PGI₂. Significant dose-dependent increases in the cyclic [^3H]AMP content of blood samples were observed 2 min after injection of each dose of PGI₂ (Fig. 3, A, B, and C). These increases in cyclic [^3H]AMP were transient and had decreased by ~75% 5 min after injection of PGI₂. No increase in cyclic [^3H]AMP was detected after 30 min. Inhibition of platelet function was observed 2 min after injection of PGI₂ but only reached statistical significance with 10 nmol/kg (Fig. 3 F). This inhibition had completely disappeared by 5 min and, with lower doses of PGI₂, enhanced platelet responsiveness was observed at this time (Fig. 3, D and E). As with cyclic [^3H]AMP, the sensitivity of the platelets to PAF had returned to control levels by 30 min after injection of PGI₂. Injection of the PGI₂ vehicle did not significantly increase platelet cyclic [^3H]AMP or affect the release of [^{14}C]serotonin (not shown).

Effects of injection of angiotensin II on platelet cyclic [^3H]AMP and sensitivity to PAF. To determine whether PGI₂ released into the circulation affected platelet cyclic [^3H]AMP and function in the same way as injected PGI₂, rabbits that had been transfused with labeled platelets received single intravenous injections of 5 nmol of angiotensin II/kg. Two or three arterial blood samples were taken into citrate before injection of angiotensin II, and further blood samples were withdrawn 2, 5, 10, and 30 min afterwards. Fig. 4 shows a representative experiment in which the platelet cyclic [^3H]AMP and the [^{14}C]serotonin released by 40 pmol of PAF/ml were measured in citrated blood samples. Injection of angiotensin II, which markedly increased blood PGI₂ after 2 min (6), increased cyclic [^3H]AMP in the circulating platelets and inhibited the platelet release reaction induced by PAF added

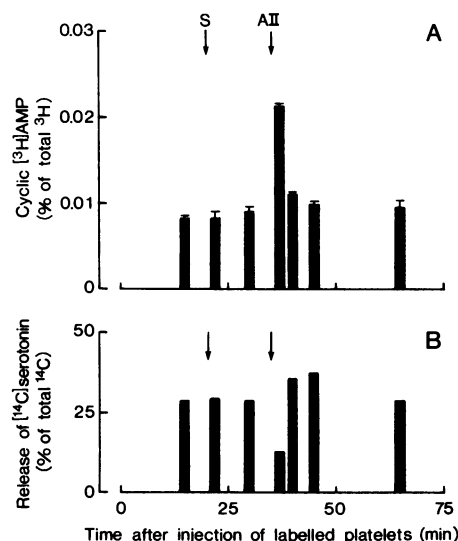


Figure 4. A representative experiment showing the effects of injection of angiotensin II on platelet cyclic [^3H]AMP and on the release of platelet [^{14}C]serotonin by PAF. A rabbit that had been transfused with labeled platelets received intravenous injections of 0.154 M NaCl (S) (0.25 ml/kg) and angiotensin II (AII) (5 nmol/kg) at the times shown by arrows. Arterial blood samples were taken at the indicated times and blood cyclic [^3H]AMP levels (A) and the release of [^{14}C]serotonin by 40 pmol of PAF/ml (B) were measured immediately. Cyclic [^3H]AMP values are means \pm SE from three determinations and release values are means from two determinations on each blood sample.

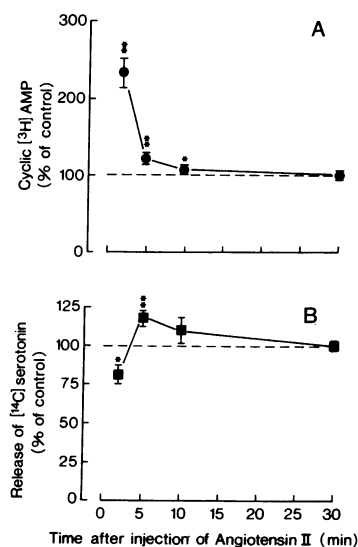


Figure 5. Summary of the effects of injection of angiotensin II on platelet cyclic [^3H]AMP and on the release of platelet [^{14}C]serotonin by PAF. Experiments were carried out essentially as shown in Fig. 4; blood cyclic [^3H]AMP levels (A) and the release of [^{14}C]serotonin by 40 pmol of PAF/ml (B) were measured before and after injection of angiotensin II (5 nmol/kg). Values obtained from each rabbit after injection of angiotensin II were expressed as percentages of the mean of the values obtained beforehand. Results (means \pm SE) from 6 to 10 rabbits are shown. Statistical significance is indicated by * ($P < 0.05$) and ** ($P < 0.01$).

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to blood taken at this time. However, after 5 min, the release of [^{14}C]serotonin by PAF was enhanced, although platelet cyclic [^3H]AMP was still above control levels, as observed after injection of PGI_2 . The results from 10 rabbits are summarized in Fig. 5. Both the increases in platelet cyclic [^3H]AMP and the inhibition and potentiation of platelet responsiveness after 2 and 5 min, respectively, were statistically significant. Control values for platelet cyclic [^3H]AMP and platelet responsiveness were re-established by 30 min, at which time blood PGI_2 had also returned to basal levels (6). None of these effects were observed after injection of saline into rabbits (e.g., Fig. 4).

Potentiation of the platelet release reaction by addition of PGI_2 to citrated whole blood; relationship to platelet cyclic [^3H]AMP. These experiments were undertaken to determine whether the stimulatory effects of PGI_2 on platelet responsiveness in vivo could be reproduced in vitro. The PAF concentration used to induce release of [^{14}C]serotonin was decreased to 20 pmol/ml to facilitate detection of any potentiation of the secretory response. When PAF was added after incubation of the blood with PGI_2 for 0.5 min, the release of platelet [^{14}C]serotonin was significantly inhibited by ≥ 2 pmol of PGI_2 /ml, whereas when PAF was added 30 min after addition of 0.5–4.0 pmol of PGI_2 /ml, the amount of [^{14}C]serotonin released was significantly greater than the controls (Fig. 6 A). At least 15 min incubation with PGI_2 was required for the development of enhanced sensitivity to PAF in whole blood in vitro. Thus, the results show that the enhanced platelet reactivity observed after injection of PGI_2 into rabbits is attributable to the action of PGI_2 on blood components only. When the release of both [^{14}C]serotonin and ATP by 15–30 pmol of PAF/ml was measured with and without the prior addition of 4 pmol of PGI_2 /ml (results not shown), potentiation of ATP secretion was difficult to demonstrate in individual experiments because of the error inherent in measuring ATP, but was significant overall ($P = 0.015$, analysis of variance in seven experiments). Moreover, no significant difference was observed between the enhancement of secretion detected by the two methods ($P = 0.58$, analysis of variance). This indicates that the potentiation of secretion by PGI_2 , like inhibition, reflects an effect of PGI_2 on the whole platelet population.

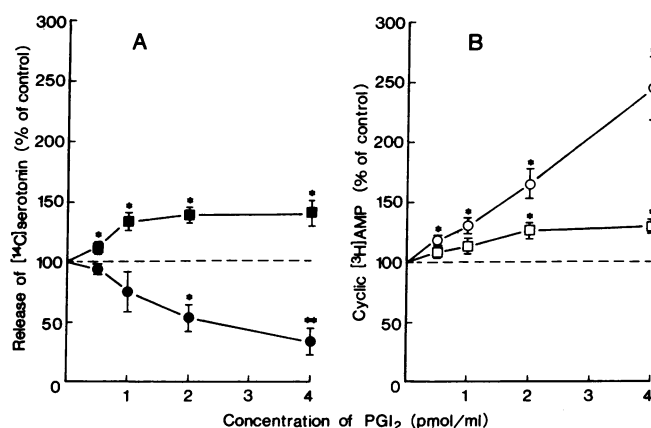


Figure 6. Effects of addition of PGI_2 to citrated rabbit blood containing labeled platelets on the release of platelet [^{14}C]serotonin by PAF and on platelet cyclic [^3H]AMP. Arterial blood was taken from rabbits that had been transfused with labeled platelets. The release of [^{14}C]serotonin 0.5 min after addition of 20 pmol of PAF/ml was measured in blood samples preincubated with the indicated concentrations of PGI_2 for 0.5 min (●) and 30 min (■) (A). Cyclic [^3H]AMP was also determined in blood samples incubated with the same concentrations of PGI_2 for 0.5 min (○) and 30 min (□) (B). These measurements were repeated on blood from three to four separate bleedings of each rabbit used. Average values, expressed as percentages of controls without PGI_2 , were determined for each rabbit and the results shown represent means \pm SE from four to six rabbits. Statistical significance is indicated by * ($P < 0.05$) and ** ($P < 0.01$).

The increase in platelet cyclic [^3H]AMP measured 0.5 min after addition of 0.5–4.0 pmol of PGI_2 /ml to citrated blood was roughly proportional to the PGI_2 concentration added. After 30 min, the increases in cyclic [^3H]AMP in the blood samples were substantially lower but still significant with ≥ 2 pmol of PGI_2 /ml ($P < 0.05$) (Fig. 6 B). Thus, potentiation of the platelet release reaction by PGI_2 was associated with increased levels of cyclic AMP at the time of addition of PAF. Since PAF inhibits cyclic AMP formation in intact rabbit platelets (26), the effects of incubation of blood for 0.5 min

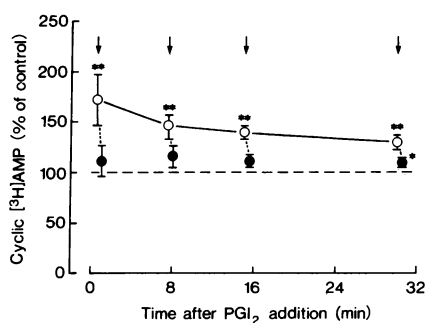


Figure 7. Effects of addition of PGI_2 and PAF to whole blood on platelet cyclic [^3H]AMP. Citrated arterial blood was taken from a rabbit that had been transfused with labeled platelets. Blood samples were incubated for the indicated periods with and without 2 pmol of PGI_2 /ml. PAF (20 pmol/ml) was added to some samples at the times indicated by arrows and cyclic [^3H]AMP was determined before (○) and 0.5 min after (●) addition of PAF. The amounts of platelet cyclic [^3H]AMP found in incubations with PGI_2 were expressed as percentages of the amounts found in corresponding incubations without additions. Values shown are means \pm SE from four identical incubations using blood from separate bleedings of the same rabbit. Statistical significance is indicated by * ($P < 0.05$) and ** ($P < 0.01$).

with PAF on the increases in cyclic AMP caused by prior addition of PGI₂ were also determined (Fig. 7). PAF decreased platelet cyclic [³H]AMP but never below the level observed in the absence of PGI₂.

Discussion

Methodological aspects. Our objective was to develop methods that would permit analysis of the relationship between the sensitivity of rabbit platelets to aggregating agents and the platelet cyclic AMP level under conditions as close to those pertaining in vivo as possible. Such methods could be used to study the actions of antiplatelet drugs that may increase platelet cyclic AMP either by direct effects on platelets or indirectly via the release of PGI₂ into the circulation. As PGI₂ has a *t*_{1/2} of ~9 min in citrated blood (6), preparation of platelet-rich plasma before measurement of either platelet responsiveness (e.g., 27–33) or platelet cyclic AMP (e.g., 18 and 30) could give misleading results. This consideration suggested that appropriate measurements should be made in whole blood samples as soon as they were removed from the animal. However, the presence of cyclic AMP in plasma and white cells precludes detection of small increases in platelet cyclic AMP by conventional assays using whole blood or even platelet-rich plasma. We therefore transfused rabbits with platelets labeled by preincubation with [³H]adenine. Previous studies have established the sensitivity of the prelabeling method of measuring changes in cyclic AMP in rabbit platelets (6), and have shown that in washed platelets from this species increases in cyclic [³H]AMP are proportional to the increases in total cyclic AMP measured by a radioimmunoassay (26). We found no evidence for removal of the transfused platelets from the circulation or of loss of ³H-labeled adenine nucleotides from them during the course of our experiments. Thus, changes in whole blood cyclic [³H]AMP levels could be assumed to reflect exclusively changes within the transfused platelets. Significant increases in platelet cyclic [³H]AMP were observed in blood containing >0.4 pmol of PGI₂/ml.

We initially attempted to measure platelet aggregation in whole blood by the impedance method (34), but could not accept the delay required for stabilization of the initial response and the relatively low sensitivity of the method to inhibition of aggregation by PGI₂ (35). We then adapted the technique of measuring the release of ¹⁴C from platelets containing [¹⁴C]serotonin to whole blood samples, using blood from rabbits transfused with platelets that had been labeled simultaneously with [³H]adenine and [¹⁴C]serotonin. PAF, which was particularly effective in releasing platelet [¹⁴C]serotonin in whole blood, was used as the stimulus. In some experiments, secretion of dense-granule ATP from platelets was measured in blood by the firefly luciferin-luciferase method (21) at the same time as the release of [¹⁴C]serotonin. These studies indicated that the transfused platelets responded to both PAF and PGI₂ in a manner indistinguishable from the total platelet population. The methods used were quite sensitive to the presence of PGI₂. Significant inhibition of the release of platelet [¹⁴C]serotonin was observed in blood with PGI₂ concentrations > 1–2 pmol/ml and 50% inhibition required ~5 pmol/ml. These values are comparable with those previously reported for inhibition of platelet aggregation and the release reaction in rabbit platelet-rich plasma (6, 36). However, a recent study with human blood (37) suggests that measurement of the free platelets remaining after addition of an aggregating agent might provide an even more sensitive method of detecting

inhibitory effects of PGI₂, perhaps comparable with the sensitivity of our measurements of increases in platelet cyclic [³H]AMP.

Our methods have several advantages over those previously available for use with experimental animals. Thus, measurements of both platelet responsiveness and cyclic AMP could be made in the same whole blood sample before significant breakdown of PGI₂ could occur. The inhibitory activity of PGI₂ on platelets could be detected with a sensitivity similar to that achievable in platelet-rich plasma, and increases in platelet cyclic [³H]AMP could be observed with PGI₂ concentrations well below those causing inhibition of [¹⁴C]serotonin release. Finally, these methods could be applied to conscious rabbits using blood samples obtained without cannulation or heparinization of the animals; under these conditions, control levels of circulating PGI₂ are negligible (6).

Relationship between blood PGI₂ and platelet cyclic AMP. The pattern of changes in platelet cyclic [³H]AMP in blood samples taken at various times after injection of either PGI₂ or angiotensin II correlated quite well with the corresponding changes in blood PGI₂, which were measured at the same times in a previous study (6). For example, after injection of 5 nmol of angiotensin II/kg, the average circulating blood PGI₂ amounted to 7.55, 1.53, and 0.26 pmol/ml after 2, 5, and 10 min, respectively, whereas the corresponding increases in platelet cyclic [³H]AMP in the present experiments were 131, 21, and 7%. Moreover, both the blood PGI₂ and increase in platelet cyclic [³H]AMP 2 min after injection of 1 nmol of PGI₂/kg were almost identical to those seen 5 min after injection of angiotensin II. However, all these increases in cyclic [³H]AMP were about half as large as might have been predicted from the effects of addition of PGI₂ to citrated blood containing labeled platelets (e.g., Fig. 6). The reason for this discrepancy is unclear, but possibilities include unidentified differences between the experimental conditions used in the two studies or the release in vivo of factors that diminish the effect of PGI₂ on platelet cyclic [³H]AMP levels. Angiotensin II does not itself affect platelet cyclic AMP (6). In any case, the parallel decreases in blood PGI₂ and platelet cyclic [³H]AMP after injection of PGI₂ or angiotensin II indicate both that activation of platelet adenylate cyclase does not significantly outlast the presence of PGI₂ in the circulation and that PGI₂ receptors do not become desensitized under these conditions. The latter conclusion is supported by preliminary experiments indicating that a second bolus injection of 10 nmol of PGI₂/kg, given 35 min after the first, causes an identical increase in platelet cyclic [³H]AMP. Increases in platelet cyclic AMP have been detected in platelet-rich plasma from human subjects (30) and dogs (18) receiving intravenous infusions of PGI₂ and, in the former, platelet cyclic AMP usually returned to control levels within 30 min of the end of the infusion. However, the latter studies did not permit any detailed correlation of platelet cyclic AMP levels with blood PGI₂.

Relationship between platelet cyclic AMP levels and the release of platelet [¹⁴C]serotonin by PAF in whole blood. The role of cyclic AMP in mediating the inhibition of platelet aggregation and the release reaction by a variety of agents, including PGI₂, has been well-documented (for reviews, see references 5 and 38). However, studies on this subject have almost all been carried out in platelet-rich plasma or with washed platelet preparations, in which the increases in platelet cyclic AMP induced by various compounds were maintained. In the present study, the expected transient inhibition of

platelet responsiveness by high concentrations of injected or endogenously released PGI₂ was readily observed, but was lost well before the blood PGI₂ and platelet cyclic AMP returned to control levels. Moreover, significant enhancement of platelet responsiveness to PAF was detected during the period when platelet cyclic AMP was still slightly elevated. The same phenomenon was observed on addition of PGI₂ to whole blood in vitro, though enhanced platelet reactivity developed less rapidly under these conditions, presumably because of the slower removal of PGI₂. We cannot at present exclude the possibility that unknown actions of PGI₂ on platelets or on other blood components play a role in this effect, but our results are consistent with the idea that increases in platelet cyclic AMP may reset the sensitivity of platelets to aggregating agents, so that enhanced responses are observed when the cyclic AMP decreases below inhibitory levels. The ability of PAF (26) and of some other aggregating agents, such as ADP (38), to inhibit platelet adenylate cyclase and so further reduce platelet cyclic AMP towards control levels, could facilitate the development of potentiated responses. At present, we can only speculate as to the mechanisms by which cyclic AMP could reset platelet sensitivity. However, both the accumulation of Ca²⁺ ions by the platelet dense-tubular membranes (39, 40) and the synthesis of platelet polyphosphoinositides (41) are probably enhanced by cyclic AMP and so could be involved.

Possible significance of the enhancement of the platelet release reaction by PGI₂ in the rabbit. Although enhanced platelet sensitivity to aggregating agents does not so far appear to have been observed during or after continuous infusion of PGI₂ into rabbits (27), there have been several reports of a rebound in platelet responsiveness associated with prolonged infusions of PGI₂ into man (29, 31, 32). As the latter studies were carried out with platelet-rich plasma prepared at various times after venepuncture, they cannot be directly compared with ours, but the long delay before the appearance of potentiated responses suggests that the mechanism responsible may differ from that operating in our experiments. More obviously relevant to our results are reports that addition of very low concentrations of PGI₂ can stimulate aggregation of human platelets (42), and that the functional viability of washed platelets is greatly prolonged when they are washed in the presence of PGI₂ (43). All these effects of PGI₂ on the responsiveness of platelets to aggregating agents can be distinguished from desensitization of platelets to the inhibitory action of PGI₂, which also occurred during prolonged infusion of the compound into man (28, 31, 32), but which appears to be relatively receptor-specific (31). However, no evidence for desensitization of the cyclic AMP response to PGI₂ was obtained in the present study. It is possible that the enhanced platelet responsiveness after exposure to PGI₂, which we have detected for the first time in an experimental animal, has a physiological role. Thus, the local production of PGI₂ could not only limit platelet deposition at sites of vascular injury, but could also help to maintain or enhance the subsequent responsiveness of platelets that are not irreversibly lost at such sites.

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