Biochemical Pharmacology, Vol. 45, No. 9, pp. 1815–1820, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

REDUCTION BY ARACHIDONIC ACID OF PROSTAGLANDIN I_2-INDUCED CYCLIC AMP FORMATION

INVOLVEMENT OF PROSTAGLANDINS E_2 AND $F_{2\alpha}$

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(*Received* 18 *November* 1992; *accepted* 1 *February* 1993)

Abstract—Arachidonic acid reverses the increase in cyclic AMP levels of washed human platelets exposed to prostaglandin (PG)I_2, under conditions where the PGH_2 analogue U46619 is ineffective. This effect of arachidonic acid was inhibited by aspirin, a cyclooxygenase inhibitor, but not by the thromboxane (Tx) synthase inhibitor Ridogrel, which induces, by inhibiting the conversion of PGH_2 into TxA_2, an overproduction of PGE_2, PGD_2 and $PGF_{2\alpha}$. Addition of PGE_2 or $PGF_{2\alpha}$, which share a receptor with PGI_2, to washed human platelets also induced a decrease in cyclic AMP levels, but PGD_2, which interacts with a different receptor, had no effect. Thus neither PGD_2, PGG_2, PGH_2, TxA_2 nor TxB_2 formed from arachidonic acid via the cyclooxygenase pathway is involved in the decrease in cyclic AMP levels. These findings were confirmed using forskolin, a diterpene from the labdane family, which enhanced the formation of cyclic AMP synergistically with the PGs. Also, arachidonic acid, unlike U46619, is able to reverse the inhibition of platelet aggregation by PGI_2 after a lag phase of about 4 min. Our data indicate that arachidonic acid decreased cyclic AMP levels through its cyclooxygenase metabolites PGE_2 and $PGF_{2\alpha}$ probably interacting competitively with the receptor of PGI_2. In addition, intracellular cyclic AMP levels and the degree of aggregation of platelets by arachidonic acid seem to be inversely correlated.

Platelet activation by a wide variety of agents is inhibited in a concentration-dependent manner by substances that increase intracellular levels of cyclic AMP [1–5]. This important second messenger is synthesized by adenylate cyclase and hydrolysed by phosphodiesterases. Some prostaglandins (PGs†), such as PGI_2 (prostacyclin), PGE_2 and PGD_2, stimulate adenylate cyclase and, as a consequence, elevate the intracellular cyclic AMP content of various cells, including blood platelets. PGI_2, synthesized mainly by endothelial cells, is the most potent activator of adenylate cyclase and inhibitor of platelet aggregation, and probably the most relevant PG physiologically. In contrast, several aggregatory agents such as ADP, epinephrine, platelet-activating factor and thrombin have been shown to reduce basal and stimulated adenylate cyclase activity [6–9].

Even though increased levels of intracellular cyclic AMP are associated with suppression of platelet activation, there is no demonstration that a decrease in cyclic AMP content is sufficient to trigger platelet activation. In the present work, we took advantage of the chemical differences between arachidonic acid and the stable PGH_2 analogue U46619 to investigate whether in all instances platelet activation correlates with the decreased levels of intraplatelet cyclic AMP. More precisely, we studied whether the increased levels of cyclic AMP following platelet exposure to PGI_2 are reversed by arachidonic acid or U46619 and whether this effect correlates with aggregation. Further, we examined the nature of the arachidonic acid derivatives involved in this process.

MATERIALS AND METHODS

Reagents. [^{125}I] cyclic AMP was obtained from Pasteur Diagnostics (Lyon, France) and cyclic AMP antiserum from Instiitut Pasteur Foundation (Paris, France). Arachidonic acid, bovine serum albumin (Fraction V), unlabeled cyclic AMP and prostaglandins I_2, E_2, D_2 and $F_{2\alpha}$ were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Forskolin was from Calbiochem (France) and Heparin from Laboratoires Choay (Paris, France). The stable prostaglandin H_2 analogue 15(*S*)-hydroxy-11,9(epoxymethano)-prosta-5*Z*-13*E*-dienoic acid (U46619) was from the Upjohn Co. (Kalamazoo, MI, U.S.A.). Fibrinogen (Grade L) from Kabi (Stockholm, Sweden) was treated with diisopropyl fluorophosphate by standard procedures to inactivate coagulant contaminants. Ridogrel {(*E*)-5-[[[(3-pyridinyl)[3-(trifluoromethyl)phenyl]-methylen]amino] oxy] pentanoic acid} was from the Jansen Research Foundation (Belgium). Radioimmunoassay kits for thromboxane (Tx)B_2 and PGE_2 were purchased from Pasteur Diagnostics (France). Stock solutions of forskolin, PGE_2, PGD_2 and $PGF_{2\alpha}$ in ethanol and PGI_2 in Tris were diluted to final concentrations in saline.

Preparation of platelet suspensions. Venous blood was obtained from healthy human volunteers who had taken no drugs for at least 10 days before venipuncture. Blood was anticoagulated with 1/6th

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† Abbreviations: PG, prostaglandin; Tx, thromboxane.

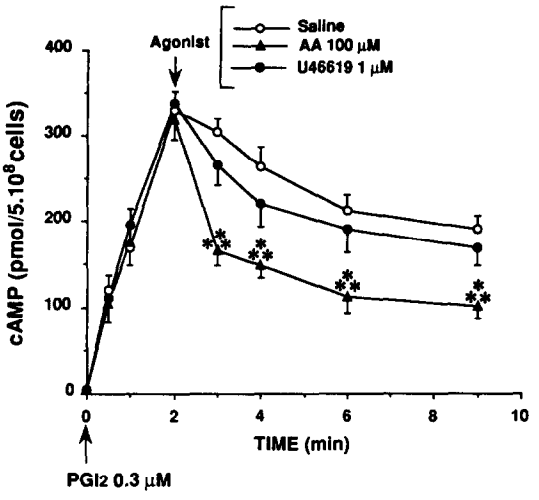

Fig. 1. Time-course of cyclic AMP (cAMP) formation by PGI_2-stimulated platelets: effect of arachidonic acid (AA) and of compound U46619. Platelet suspensions were incubated for 2 min at 37° with PGI_2 (0.3 μM) and then exposed to AA (100 μM), U46619 (1 μM) or saline. Samples were taken at various time intervals indicated in the abscissa and analysed for cAMP content. Results are expressed as the means ± SEM (N = 5). Significantly different (***$P < 0.001$) when compared to control platelets.

of its final volume of citric acid–citrate–dextrose (7, 93 and 139 mM, respectively, pH 6.4) containing heparin (20 U/mL). Platelet-rich plasma was obtained by standard differential centrifugation (200 *g*, 20 min). In order to wash the platelets, platelet-rich plasma was centrifuged (1700 *g*, 15 min, 37°) and the platelet pellet was resuspended and washed twice with a modified Tyrode's buffer containing bovine serum albumin (0.35%), according to Mustard *et al.* [10], in which apyrase was omitted and PGI_2 (10 nM) added for the first two steps of washing. The platelets were resuspended in the same buffer and their final number was adjusted to 5×10^8 cells/mL.

Measurement of platelet aggregation. Aggregation was measured turbidimetrically [11] at 37° in an Icare aggregometer (Marseille, France) on 0.4 mL samples of platelet suspension with stirring at 1100 rpm. Platelets were challenged with arachidonic acid (100 μM) or U46619 (1 μM) in the presence of fibrinogen (0.28 mg/mL).

Measurement of platelet cyclic AMP level. Cyclic AMP was measured as described by Cailla *et al.* [12]. Briefly, the samples were treated with 1 N perchloric acid and subsequently diluted as necessary 10–100-fold in a 0.1 M sodium citrate buffer at pH 6.2, acetylated and assayed. Cyclic AMP was expressed as pmol/5×10^8 cells.

Measurement of TxB_2 and PGE_2. TxB_2 and PGE_2 were measured by radioimmunoassay [13]. Briefly, platelet samples containing TxB_2 and PGE_2 were diluted in saline, together with antibody and radiolabeled antigen, and incubated overnight at 4°. Bound fractions were separated by precipitation with polyethylene glycol 6000 and after centrifugation the radioactivity contained in the pellets was counted.

Statistical analysis. Significance was evaluated by Student's *t*-test for unpaired data.

RESULTS

Effect of arachidonic acid and U46619 on PGI_2-induced cyclic AMP formation in washed platelets

As shown in Fig. 1, the effect of arachidonic acid (100 μM) and compound U46619 (1 μM) on cyclic AMP formation by intact platelets pretreated with PGI_2 (0.3 μM) was examined. PGI_2 was used at a concentration of 0.3 μM, which inhibits completely aggregation induced by both stimulating agents. When platelets were preincubated with PGI_2, cyclic AMP levels increased rapidly to a maximum level within 1–2 min and then gradually declined, because of the absence of a cyclic AMP phosphodiesterase inhibitor in the medium. The addition of arachidonic acid at 2 min resulted in a marked and rapid decrease in the amounts of cyclic AMP synthesized. In contrast, compound U46619 (1 μM), added under the same conditions, had no significant effect on stimulated levels of platelet cyclic AMP (Fig. 1). Furthermore, U46619 (0.01–1 μM) did not modify significantly the elevated levels of cyclic AMP induced by prior exposure to PGI_2 (results not shown).

Reversal by arachidonic acid of PGI_2-induced platelet inhibition

Figure 2 shows representative tracings of platelet

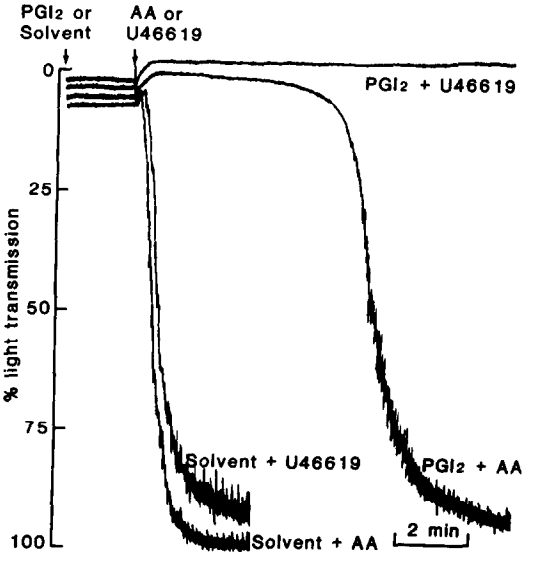

Fig. 2. Reversal by arachidonic acid (AA) of PGI_2-induced platelet inhibition. Representative tracings of platelet aggregation by AA and by the PGH_2 analogue U46619 with and without PGI_2. Washed platelets were incubated with PGI_2 (30 nM) or with saline for 2 min before the addition of the agonists (AA, 100 μM or U46619, 1 μM). This result is representative of three experiments.

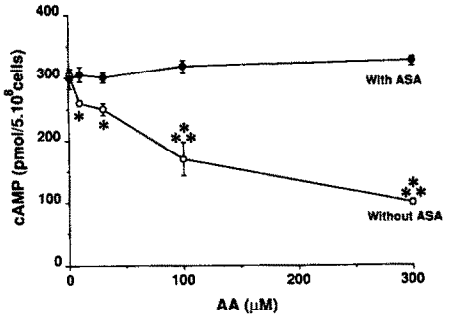

Fig. 3. Effect of a range of concentrations of arachidonic acid (AA) on platelet cyclic AMP (cAMP) levels elevated by PGI_2 in the presence or the absence of aspirin (ASA). Aliquots of washed platelets pretreated or not with ASA (100 μM, 10 min) were incubated with PGI_2 (0.3 μM) for 2 min. AA was added at the concentrations indicated on the abscissa. The reactions were stopped after 2 min and the cAMP content was measured as described in Materials and Methods. The results shown on the ordinate are expressed as pmoles of cAMP (pmol/5 × 10^8 cells). Each point represents the mean ± SEM from five separate experiments. The basal levels of cAMP were 6 ± 1 and 6 ± 2 pmol/5 × 10^8 cells with and without ASA, respectively. Significantly different (*$P < 0.05$, ***$P < 0.001$) when compared to ASA-treated platelets.

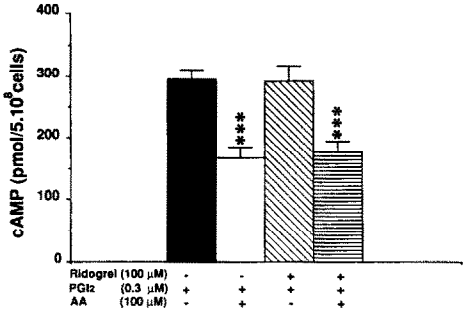

Fig. 4. Effect of Ridogrel on the arachidonic acid (AA)-induced decrease in cyclic AMP (cAMP) elevated by PGI_2. Washed platelets preincubated with Ridogrel (100 μM) for 2 min as indicated (+), were exposed to PGI_2 (0.3 μM). The platelets were then challenged when indicated (+) with AA (100 μM) for 2 min and cAMP levels were determined. The basal levels of cAMP were 6 ± 2 and 7 ± 3 pmol/5 × 10^8 cells in the absence and presence of Ridogrel, respectively. Each point represents the mean from six separate experiments. Significantly different (***$P < 0.001$) when compared to platelets treated with PGI_2 alone.

aggregation induced by arachidonic acid (100 μM) and U46619 (1 μM), and their effects on platelet inhibition by PGI_2 (30 nM). As expected, PGI_2 inhibited platelet aggregation caused by both arachidonic acid and U46619. Arachidonic acid, unlike U46619, reversed the inhibition of platelet aggregation by PGI_2 after a lag phase of about 4 min. The cyclic AMP values determined at the same time points were, 63 ± 6, 54 ± 2 and 41 ± 4 pmol/5.10^8 cells for PGI_2 alone (2, 4 and 6 min after PGI_2 addition, respectively); 21 ± 3 and 6 ± 3 pmol/5.10^8 cells for PGI_2 plus arachidonic acid (2 and 4 min after arachidonic acid addition, respectively); 47 ± 2 and 34 ± 3 pmol/5.10^8 cells for PGI_2 plus U46619 (2 and 4 min after U46619 addition, respectively).

Effect of arachidonic acid on PGI_2-induced cyclic AMP synthesis in aspirin (acetyl salicylic acid) pretreated platelets

To determine if the decrease in cyclic AMP by arachidonic acid was mediated by cyclooxygenase metabolite(s), the platelet suspension was incubated with aspirin. As seen in Fig. 3, aspirin (100 μM, 10 min), which blocks the cyclooxygenase pathway, suppressed the ability of arachidonic acid to decrease cyclic AMP levels elevated by PGI_2. Aspirin was also effective when higher concentrations of arachidonic acid were used.

Effect of Ridogrel on the arachidonic acid-induced decrease in cyclic AMP synthesis elevated by PGI_2

Ridogrel is a combined specific TxA_2 synthetase and TxA_2 PG endoperoxide receptor antagonist in one molecule [14]. When platelets were incubated with Ridogrel (100 μM), the effect of arachidonic acid on cyclic AMP formation persisted (Fig. 4). As expected, TxB_2 synthesis was suppressed by Ridogrel, whereas PGE_2 synthesis was enhanced (Table 1).

Table 1. Effect of Ridogrel and PGI_2 on arachidonic acid-induced PGE_2 and TxB_2 formation by platelets

	Solvent	Ridogrel	PGI_2
PGE_2 (ng/mL)	75 ± 8	123 ± 13*	70 ± 5
TxB_2 (ng/mL)	610 ± 65	10 ± 4†	610 ± 130

Platelet suspensions were incubated with PGI_2 (0.3 μM), Ridogrel (100 μM) or its solvent for 2 min and then exposed to arachidonic acid (100 μM).

The amounts of TxB_2 and PGE_2 formed 3 min after stimulation are indicated.

The results are the means ± SEM of five to seven separate experiments.

Significantly different (*$P < 0.01$ and †$P < 0.001$) when compared to the solvent-treated platelets stimulated with arachidonic acid.

Effects of direct additions of PGE_2, PGD_2 and $PGF_{2\alpha}$ on the PGI_2-induced increase in cyclic AMP formation

Next, we examined the effects of direct additions of the arachidonic acid derivatives PGE_2, PGD_2 and $PGF_{2\alpha}$ on cyclic AMP synthesis by platelets. The addition of PGE_2 (0.01–10 μM) or $PGF_{2\alpha}$ (0.03–10 μM) to the platelets reduced concentration dependently cyclic AMP levels (Fig. 5A and B), whereas PGD_2 (0.01–10 μM) was inactive (Fig. 5C).

Interference of forskolin with the effects of arachidonic acid, PGE_2, PGD_2 and $PGF_{2\alpha}$ on cyclic AMP levels

Forskolin, a diterpene from the labdane family,

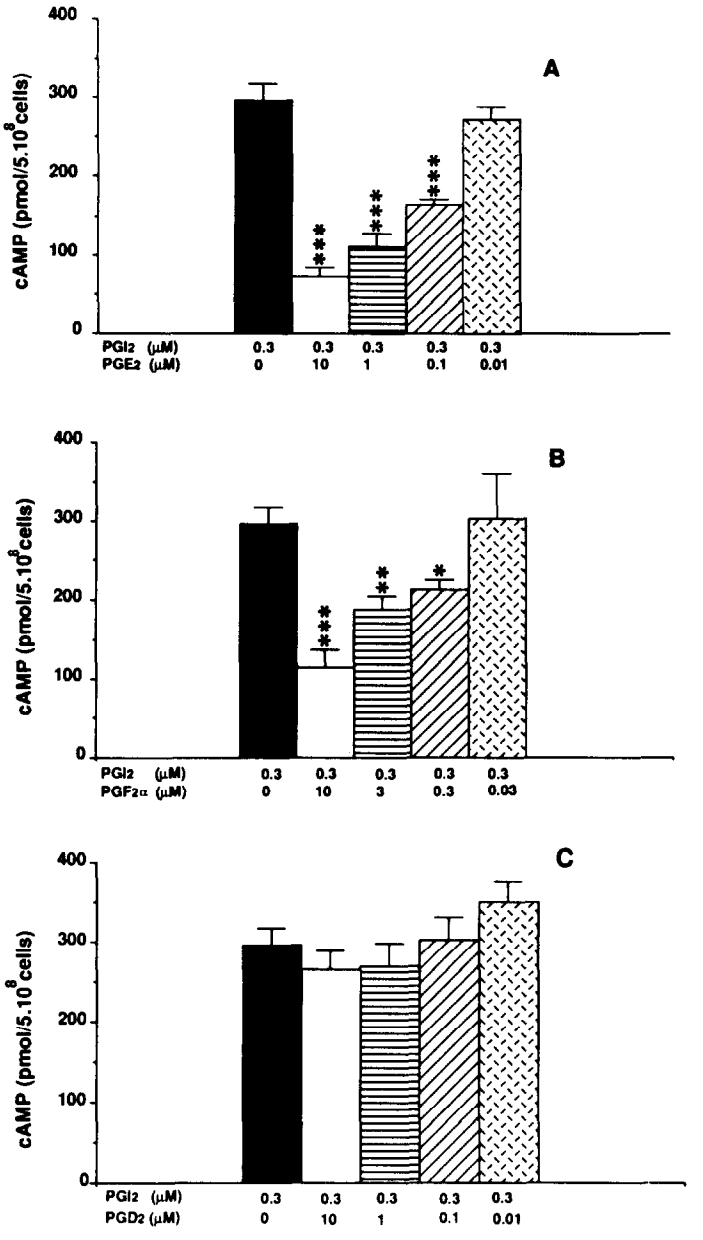

Fig. 5. Effect of PGE_2, $PGF_{2\alpha}$ on PGI_2-induced cyclic AMP (cAMP) formation. Washed platelets were preincubated for 2 min at 37° with 0.3 μM PGI_2 and then PGE_2 (A), $PGF_{2\alpha}$ (B) or PGD_2 (C) was added at the concentrations indicated in the figures. The basal level of cAMP was 6 ± 2 pmol/5×10^8 cells. Results are expressed as the means ± SEM from three separate experiments. Significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) when compared to platelets treated with PGI_2 alone.

is a powerful stimulant of adenylate cyclase activity [15, 16], which interacts directly with the catalytic subunit of adenylate cyclase [17]. The increase in cyclic AMP content in the presence of 50 μM forskolin was comparable to that obtained with 0.3 μM of PGI_2 (Fig. 6A). When forskolin (50 μM) was added 2 min before or after PGE_2 (0.3 μM), cyclic AMP levels were increased 3–4-fold compared to forskolin or PGI_2 used alone (Figs 6A and 7A). In addition, PGD_2 (Fig. 7A), PGE_2, $PGF_{2\alpha}$ (Fig. 7B) and arachidonic acid itself (Fig. 6B) synergized with forskolin, though less markedly than did PGI_2 (Fig. 7A). In no case did PGE_2 or $PGF_{2\alpha}$ decrease cyclic AMP levels.

DISCUSSION

In the present study, we have shown that the addition of arachidonic acid to platelets reverses rapidly the increase in cyclic AMP levels due to

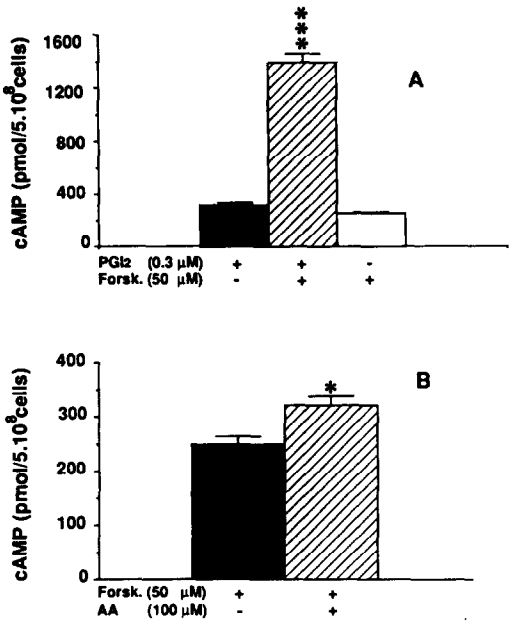

Fig. 6. Effect of forskolin (Forsk.) and its interference with arachidonic acid (AA) on PGI_2-induced cyclic AMP (cAMP) formation. Washed platelets were preincubated for 2 min at 37° with 0.3 μM PGI_2 and then Forsk. was added at the concentrations indicated in (A). Forsk. preceded AA addition (B). The basal levels of cAMP were 6 ± 3 pmol/5×10^8 cells. Results are expressed as the means ± SEM (N = 3). Significantly different (*$P < 0.05$, ***$P < 0.001$) when compared to platelets treated with PGI_2 (A) or Forsk. (B) alone.

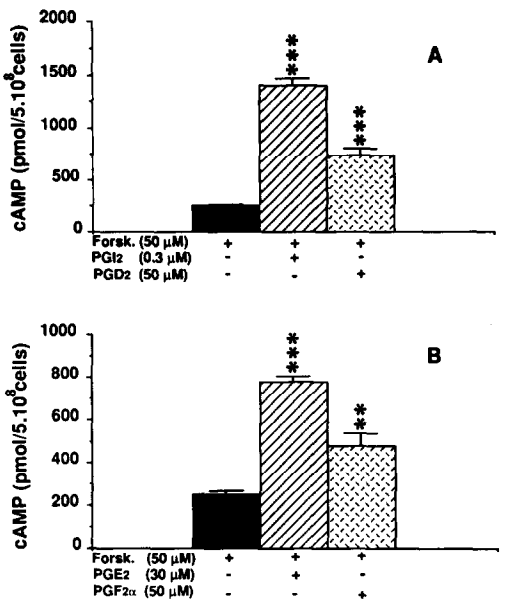

Fig. 7. Effect of PGD_2 PGE_2, $PGF_{2\alpha}$ and PGI_2 on forskolin (Forsk.) induced cyclic AMP (cAMP) formation. Washed platelets were preincubated with Forsk. (50 μM) for 2 min and then PGI_2, PGD_2 [0.3, 50 μM, respectively (A)] PGE_2 or $PGF_{2\alpha}$ (30, 50 μM, respectively (B)] was added. The basal and PGD_2, PGE_2 and $PGF_{2\alpha}$-stimulated levels of cAMP were 6 ± 2, 110 ± 17, 80 ± 23 and 46 ± 13 pmol/5×10^8 cells, respectively. Results are expressed as the means ± SEM from three separate experiments. Significantly different (**$P < 0.01$, ***$P < 0.001$) when compared to Forsk.-pretreated platelets.

PGI_2. By contrast, under similar conditions, the stable PGH_2 analogue U46619 failed to decrease significantly these levels, in agreement with Mills and Macfarlane [18] and Brass *et al.* [19]. Nevertheless, our findings disagree with those of Bonne *et al.* [20] and Avdonin *et al.* [21]. The latter reported that U46619 at 3–100 μM causes a decrease in PGE_1-stimulated cyclic AMP levels. This effect of U46619 is probably due to the fact that, at high concentrations, the stable PGH_2 analogues bind non-specifically to the receptor of the adenylate cyclase activator PGE_1, leading to a decrease in cyclic AMP. The ability of arachidonic acid to decrease cyclic AMP formation is corroborated by its ability to reverse progressively the inhibitory effect of PGI_2 on platelet aggregation. Under the same conditions, the restoration of aggregation was not observed with U46619, in agreement with its failure to reduce the cyclic AMP levels elevated by PGI_2 (Fig. 2).

Since most pro-aggregatory agents reduce adenylate cyclase activity [6–9, 22], we hypothesized that arachidonic acid might decrease cyclic AMP levels via its pro-aggregatory derivatives PGG_2, PGH_2 and TxA_2. Indeed, inhibition by aspirin of the effect of arachidonic acid (Fig. 3) indicates that cyclooxygenase metabolites are involved in the increase in cyclic AMP levels and that non-metabolized arachidonic acid and lipoxygenase products are not involved. In addition, TxA_2 and TxB_2, and the cyclic endoperoxides PGG_2 and PGH_2 are also not involved, since Ridogrel, which inhibits Tx synthesis and blocks the cyclic endoperoxide PG/TxA_2 receptor [14], failed to prevent the decrease in cyclic AMP levels caused by arachidonic acid. It should be noted that the concentration of Ridogrel used here (100 μM) combines both properties of the molecule. In contrast, the direct addition of PGE_2 and $PGF_{2\alpha}$ to washed human platelets caused a decrease in cyclic AMP levels, whereas that of PGD_2 had no effect.

These differences between PGD_2 on one hand and PGE_2 and $PGF_{2\alpha}$ on the other may result from different affinities for the PGI_2 receptor. In agreement, exposure of platelets to PGI_2 desensitizes them to further challenges with PGE_2 or $PGF_{2\alpha}$, but not with PGD_2. Reciprocally, treating platelets with PGD_2 desensitizes them to a second challenge with PGD_2, but does not affect the subsequent cyclic AMP formation in response to PGI_2 [23]. Besides, the order of efficacy of PGs in increasing the intraplatelet cyclic AMP content is: $PGI_2 > PGD_2 > PGE_2 > PGF_{2\alpha}$ [24]. Thus PGE_2 and $PGF_{2\alpha}$, which share a common receptor with PGI_2, probably inhibited competitively the persistent stimulation of cyclic AMP synthesis by the latter, whereas PGD_2, which possesses a distinct receptor, only slightly potentiated the PGI_2 effect. Accordingly,

forskolin, another agent that stimulates adenylate cyclase directly by interacting with the catalytic subunit of the enzyme [17], enhanced the formation of cyclic AMP synergistically with PG, particularly PGI_2.

Our data indicate that arachidonic acid causes a decrease in cyclic AMP levels via its cyclooxygenase metabolites PGE_2 and PGE_{2a}, probably following a competitive interaction with the PGI_2 receptors. This indicates that the decrease in cyclic AMP levels caused by arachidonic acid accounts for its reversing effect on PGI_2-induced platelet inhibition. Our results identify a mechanism by which arachidonic acid down-regulated the PGI_2-induced increase in cyclic AMP levels in human platelets. This process would attenuate an excessive increase in platelet cyclic AMP levels in response to the overproduction of PGI_2 by endothelial cells.

Acknowledgements—We thank Drs M. Bachelet and S. Mamas for their helpful discussion and we are grateful to Janssen Research Foundation (Belgium) for the gift of Ridogrel.

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