EFFECTS OF ADENOSINE 3′:5′-MONOPHOSPHATE AND PLATELET AGGREGATION ON THROMBOXANE BIOSYNTHESIS IN HUMAN PLATELETS

Jan Åke LINDGREN, Hans-Erik CLAESSON, Hans KINDAHL and Sven HAMMARSTRÖM
Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden

Received 17 November 1978

1. Introduction

The prostaglandin (PG) endoperoxides, PGG_2 and PGH_2 [1], and thromboxane (TX)A_2 [2] cause rapid aggregation and induce the release reaction in human platelets. Aggregating agents, such as collagen and thrombin, stimulate release of arachidonic acid from platelet phospholipids. This leads to synthesis of endoperoxides, thromboxanes, prostaglandins and hydroxy acids [3–5].

Elevation of adenosine 3′:5′-monophosphate (cyclic AMP) levels in platelets prevents aggregation [6–8] and the simultaneous production of TXB_2 [9–12]. Two mechanisms for the inhibition of thromboxane formation have been proposed:

(1) Inhibition of prostaglandin endoperoxide synthase (EC 1.14.99.1) [9];
(2) Inhibition of arachidonic acid release from platelet phospholipids [10–12].

In the latter reports no inhibition of prostaglandin endoperoxide synthase was observed [10–12]. We have therefore reinvestigated the effects of cyclic AMP on arachidonic acid release and on the conversion of arachidonic acid to TXB_2. The results suggest that elevation of cyclic AMP levels inhibits both of these reactions in platelets and that the inhibitory effect of cyclic AMP on TXB_2 formation from arachidonic acid is not direct but secondary to inhibition of platelet aggregation.

2. Materials and methods

Blood, withdrawn from healthy donors who had not taken drugs for at least 10 days, was immediately mixed with 0.13 vol. 0.1 M trisodiumcitrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as in [13]. Collagen was obtained from Stago Laboratoire, Asnières, arachidonic acid from Nu Check Prep. Inc., Elysian, MN; colchicine and indomethacin from Sigma Chemical Co., St Louis, MO. PGI_2 was supplied by the Upjohn Co., Kalamazoo, MI. Thromboxane B_2 was determined by radioimmunoassay [14], and arachidonic acid by quantitative mass spectrometry [15]. Cyclic AMP was analysed by the protein-binding assay in [16]. Before assay the samples were purified as in [13]. Platelet aggregation was monitored by the continuous recording of light transmission in a Payton dual channel aggregometer (Payton Associates Ltd., Ontario).

3. Results

3.1. *Effects of PGI_2 on cyclic AMP levels and collagen-induced arachidonic acid release in PRP*

PRP (4 ml) was preincubated for 2 min at 37°C with indomethacin and $CaCl_2$ (final conc. 0.01 mM and 2.5 mM, respectively) followed by 15 s with PGI_2 (0.5–5 ng/ml). Collagen (10 μg/ml) was added and the samples were incubated for another 5 min. Two aliquots of 0.5 ml PRP were removed, each mixed with 0.5 ml 10% (w/v) trichloroacetic acid and rapidly frozen for analyses of cyclic AMP content. The remaining 3 ml of the samples were mixed with 4 vol. ethanol containing [5,6,8,9,11,12,14,15-$^2H_8$]arachidonic acid (10 μg) and frozen

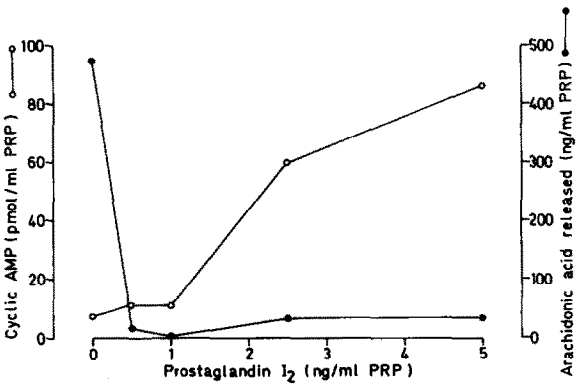


Fig.1. Effects of PGI_2 on cyclic AMP levels and collagen-induced arachidonic acid release. PRP (4 ml) was preincubated for 2 min at 37°C with 10 μM indomethacin and 2.5 mM $CaCl_2$ and then for 15 s with PGI_2 (0–5 ng/ml). After addition of collagen (10 μg/ml), 2 × 0.5 ml aliquots were removed and mixed with 0.5 ml 10% (w/v) trichloroacetic acid for cyclic AMP determinations. The remaining 3 ml of the samples were mixed with 12 ml ethanol containing [5,6,8,9,11,12,14,15-$^2H_8$]arachidonic acid (10 μg) for analyses of arachidonic acid.

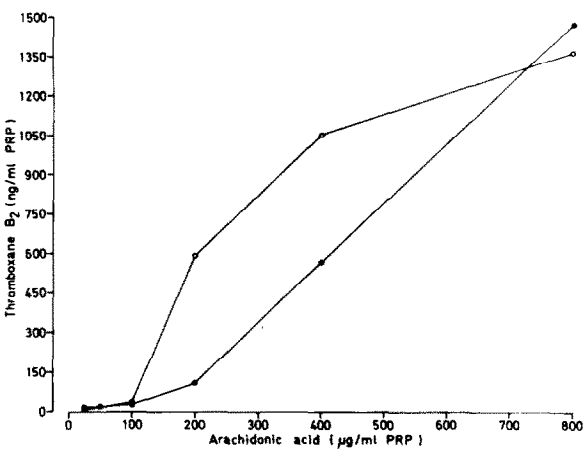


Fig.2. Arachidonic acid-induced TXB_2 synthesis in absence or presence of PGI_2. PRP (0.5 ml) was preincubated for 1 min at 37°C with $CaCl_2$ (2.5 mM) and for 15 s with or without PGI_2 (25 ng/ml). After 5 min incubation with arachidonic acid (25–800 μg/ml), indomethacin (10 μM) was added and the samples were rapidly frozen for determinations of TXB_2. Control (o——o); PGI_2 (•——•).

for analyses of free arachidonic acid. Controls without PGI_2 were treated in the same way. PGI_2 elevated the levels of cyclic AMP and caused inhibition of arachidonic acid release by >90% (fig.1). The arachidonic acid levels have been corrected for background levels found in PRP.

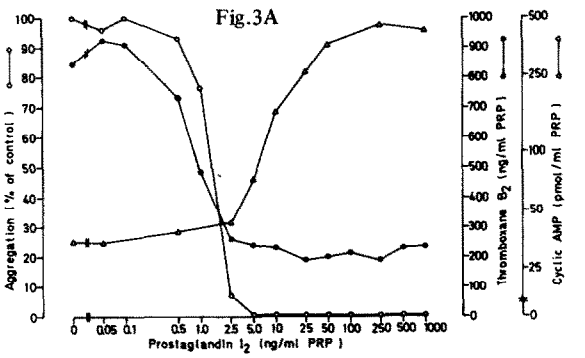
3.2. *Effects of PGI_2 on cyclic AMP levels, arachidonic acid-induced aggregation and TXB_2 synthesis in PRP*

PRP (0.5 ml) was preincubated for 1 min at 37°C with $CaCl_2$ (2.5 mM) and for 15 s with or without PGI_2 (25 ng/ml). Arachidonic acid (25–800 μg/ml) was then added. After 5 min incubation and subsequent addition of indomethacin (10 μM) the samples were rapidly frozen for analyses of TXB_2 (fig.2). At ⩽ 100 μg arachidonic acid/ml (which did not cause aggregation), TXB_2 levels were low and not influenced by PGI_2. At ⩾ 200 μg arachidonic acid/ml, aggregation was induced (not shown) and formation of TXB_2 stimulated. PGI_2 inhibited TXB_2 synthesis by 81% and 47% at 200 μg arachidonic acid/ml and 400 μg arachidonic acid/ml, respectively. At 800 μg arachidonic acid/ml, PGI_2 had no effect on the TXB_2 levels. PGI_2 prevented platelet aggregation at all concentrations of arachidonic acid.

In other experiments, PRP (0.5 ml) was incubated as above in an aggregometer with varying concentrations of PGI_2 (0–1000 ng/ml) and arachidonic acid (250 μg/ml or 1000 μg/ml). These samples were used for determinations of TXB_2 levels and platelet aggregation. Parallel samples were incubated in the same way and stopped with 0.5 ml 10% (w/v) trichloroacetic acid for measurements of cyclic AMP. Figure 3 shows the effects of PGI_2 on these parameters. At both concentrations of arachidonic acid, complete inhibition of the aggregation was observed, when ⩾ 5 ng PGI_2/ml was added. When 250 μg arachidonic acid/ml was added the formation of TXB_2 was partially inhibited by PGI_2 (⩾ 5 ng/ml caused ~75% inhibition). In contrast, no inhibition of TXB_2 formation was observed at the higher arachidonic acid concentration.

3.3. *Effects of colchicine on cyclic AMP levels, arachidonic acid-induced aggregation and TXB_2 synthesis in PRP*

PRP (0.5 ml) was preincubated in an aggregometer



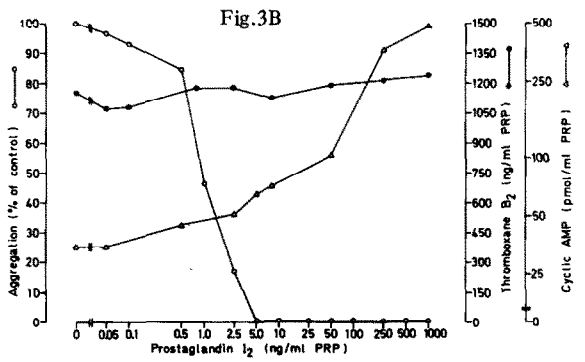
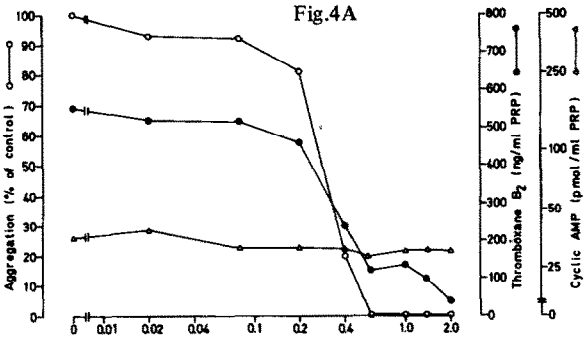


Fig.3. Effects of PGI_2 on cyclic AMP levels and arachidonic acid-induced aggregation and TXB_2 synthesis. PRP (0.5 ml) was preincubated for 1 min in an aggregometer at 37°C with $CaCl_2$ (2.5 mM) and for 15 s with PGI_2 (0–1000 ng/ml). The samples were then incubated for 5 min with: A (upper panel) 250 μg arachidonic acid/ml; B (lower panel) 1000 μg arachidonic acid/ml. Platelet aggregation was continuously recorded. After addition of indomethacin (10 μM), the samples were rapidly frozen for TXB_2 analysis. Identically incubated samples were stopped with 0.5 ml 10% (w/v) trichloroacetic acid for measurements of cyclic AMP.



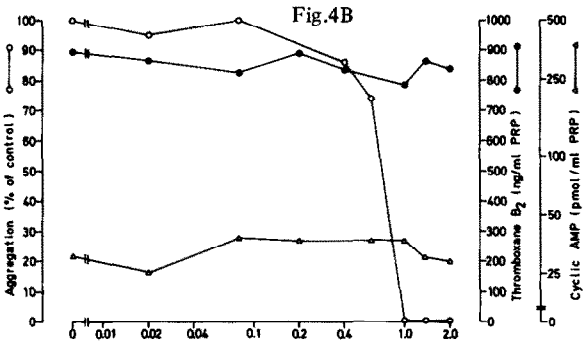


Fig.4. Effects of colchicine on cyclic AMP levels and arachidonic acid-induced aggregation and TXB_2 synthesis. PRP (0.5 ml) was preincubated for 1 min in an aggregometer at 37°C with $CaCl_2$ (2.5 mM) and colchicine (0–2 mM). The samples were then incubated for 5 min with: A (upper panel) 250 μg arachidonic acid/ml; B (lower panel) 1000 μg arachidonic acid/ml. Platelet aggregation was continuously recorded. After addition of indomethacin (10 μM), the samples were rapidly frozen for TXB_2 analyses. Identically incubated samples were stopped with 0.5 ml 10% (w/v) trichloroacetic acid for measurements of cyclic AMP.

for 1 min at 37°C with $CaCl_2$ (2.5 mM) and colchicine (0–2 mM) prior to incubation with arachidonic acid (250 μg/ml or 1000 μg/ml) for 5 min. Platelet aggregation was continuously monitored. Thereafter indomethacin (10 μM) was added and the samples were rapidly frozen for analyses of TXB_2. Parallel samples were identically incubated and stopped with 0.5 ml 10% (w/v) trichloroacetic acid for measurements of cyclic AMP. As shown in fig.4, colchicine inhibited platelet aggregation without elevating the cyclic AMP levels. Colchicine also decreased the formation of TXB_2 at the lower (250 μg/ml) but not at the higher (1000 μg/ml) concentration of arachidonic acid.

4. Discussion

PGI_2 (prostacyclin) was used to study the effects of elevated cyclic AMP levels [17,18] on platelet thromboxane formation. At low concentrations of PGI_2, the collagen-induced release of arachidonic acid was inhibited, as determined by direct measurements of arachidonic acid in PRP (fig.1). This is in agreement

with reports showing that cyclic AMP inhibits arachidonic acid release from platelet phospholipids [10–12].

PGI_2 prevents arachidonic acid-induced platelet aggregation [19]. With PGI_2 present, decreased conversion of exogenously added arachidonic acid to TXB_2 was observed at low but not at high concentrations of the fatty acid (fig.2,3). Figure 3A shows that PGI_2 caused parallel dose-dependent inhibition of platelet aggregation and TXB_2 synthesis when a low concentration of arachidonic acid was used. At a higher concentration of arachidonic acid, PGI_2 inhibited platelet aggregation without affecting the synthesis of TXB_2 (fig.3B). This suggests that cyclic AMP inhibits platelet aggregation at multiple steps, one of which is beyond the synthesis of prostaglandin endoperoxides and thromboxanes [10,13,20]. The latter effect is probably exerted by interference with contractile processes in platelets [12].

The inhibition of arachidonic acid-induced TXB_2 synthesis at low but not at higher substrate concentrations indicates a K_m change of the prostaglandin endoperoxide synthase. This could be a direct effect of cyclic AMP on the enzyme. Alternatively, the K_m might decrease during platelet aggregation and cyclic AMP counteract this effect by inhibiting aggregation. To distinguish between these two possibilities, colchicine was used as an agent which prevents aggregation without raising the levels of cyclic AMP (fig.4). The effects of colchicine on arachidonic acid-induced aggregation and TXB_2 synthesis were identical to those of PGI_2 (fig.4). This supports the second alternative described above, namely that the effect of cyclic AMP on this enzyme is secondary to inhibition of the aggregation. Further experiments regarding the influence of aggregation on the platelet endoperoxide synthase are in progress.

Acknowledgements

We thank Miss Margareta Hovgard and Miss Eva Ohlson for their excellent technical assistance. This work was supported by a grant from the Swedish Medical Research Council (project 03X-217).

References

[1] Hamberg, M., Svensson, J., Wakabayashi, T. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 345–349.
[2] Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Proc. Natl. Acad. Sci. USA 72, 2994–2998.
[3] Hamberg, M., Svensson, J. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 3824–3828.
[4] Bills, T. K., Smith, J. B. and Silver, M. J. (1976) Biochim. Biophys. Acta 424, 303–314.
[5] Blackwell, G. L., Duncome, W. G., Flower, R. J., Parsons, M. F. and Vane, J. R. (1977) Brit. J. Pharmacol. 59, 353–366.
[6] Marquis, N. R., Vigdahl, R. L. and Tavormina, P. A. (1969) Biochem. Biophys. Res. Commun. 36, 965–972.
[7] Salzman, E. W. and Levine, L. (1971) J. Clin. Invest. 50, 131–141.
[8] Mills, D. C. B. and Smith, J. B. (1971) Biochem. J. 121, 185–196.
[9] Malmsten, C., Granström, E. and Samuelsson, B. (1976) Biochem. Biophys. Res. Commun. 68, 569–576.
[10] Minkes, M., Stanford, N., Chi, M. M.-Y., Roth, G. J., Raz, A., Needleman, P. and Majerus, P. W. (1977) J. Clin. Invest. 59, 449–454.
[11] Lapetina, E. G., Schmitges, C. J., Chandrabose, K. and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 76, 828–835.
[12] Gerrard, J. M., Peller, J. D., Krick, T. P. and White, J. G. (1977) Prostaglandins 14, 39–50.
[13] Claesson, H.-E. and Malmsten, C. (1977) Eur. J. Biochem. 76, 277–284.
[14] Granström, E., Kindahl, H. and Samuelsson, B. (1976) Anal. Lett. 9, 611–627.
[15] Hammarström, S., Hamberg, M., Samuelsson, B., Duell, E. A., Stawiski, M. and Voorhees, J. J. (1975) Proc. Natl. Acad. Sci. USA 72, 5130–5134.
[16] Gilman, A. G. (1970) Proc. Natl. Acad. Sci. USA 67, 305–312.
[17] Gorman, R. R., Bunting, S. and Miller, O. V. (1977) Prostaglandins 13, 377–388.
[18] Tateson, J. E., Moncada, S. and Vane, J. R. (1977) Prostaglandins 13, 389–397.
[19] Moncada, S., Gryglewski, R., Bunting, S. and Vane, J. R. (1976) Nature 263, 663–665.
[20] Miller, O. V. and Gorman, R. R. (1976) J. Cyclic Nucl. Res. 2, 79–87.