CYCLIC AMP INHIBITS SYNTHESIS OF PROSTAGLANDIN ENDOPEROXIDE (PGG_2) IN HUMAN PLATELETS

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Summary: Dibutyryl-cAMP but not dibutyryl-cGMP inhibited platelet aggregation and release of ^{14}C-serotonin and ADP when induced by collagen and arachidonate but not when induced by the endoperoxide PGG_2* The formation of thromboxane B_2 (TXB_2) induced by addition of collagen to platelet rich plasma (PRP) was decreased by dibutyryl-cAMP and agents known to increase the concentration of cAMP (PGE_1, PGD_2, theophylline and acetyl choline).

PGE_2 in concentrations known to decrease cAMP levels increased the formation of TXB_2 whereas concentrations of PGE_2 known to increase cAMP levels decreased the amount of TXB_2 formed. That this was due to an effect on the cyclo-oxygenase was indicated by inhibition of the transformation of arachidonic acid by DB-cAMP and by high concentrations of PGE_2. Additional support for regulation of the cyclo-oxygenase by cAMP and its relevance to platelet aggregation was obtained by demonstrating stimulation of PGG_2 induced aggregation by low concentrations of PGE_2 and the absence of this effect in the presence of a cyclo-oxygenase inhibitor.

Introduction

It was shown early that PGE_1 inhibits platelet aggregation (1) whereas PGE_2 although unable to induce aggregation by itself in small doses facilitates ADP-induced aggregation (2). Platelet adenyl cyclase is stimulated by PGE_1 (3) and both cAMP (4) and its dibutyryl derivative (5,6) inhibit aggregation. Since a number of inhibitors of platelet aggregation increased the level of cAMP whereas agents inducing or facilitating aggregation reduced cAMP levels it was proposed that platelet aggregation is regulated by cAMP (6).

The findings that PGE_2 reduces the level of cAMP in PRP and that inhibitors of prostaglandin synthesis inhibit the second phase of platelet aggregation led

* PG=prostaglandin

to the proposal that PGE_2 normally is involved in triggering the release reaction by reducing the level of cAMP (6).

Recent work has domonstrated that the endoperoxides PGG_2 and PGH_2 induce the release reaction (7) and aggregation (8,9). These compounds are further transformed into the thromboxanes TXA_2 and TXB_2 (10). TXA_2 is an unstable intermediate which causes release of ^{14}C-serotonin and aggregation (10). Its stable metabolite, TXB_2, is formed during aggregation induced by various aggregating agents as collagen or ADP. Deficiency of the cyclo-oxygenase, which catalyzes the formation of the endoperoxide PGG_2, leads to defect release mechanism and prolonged bleeding-time (7). These studies thus demonstrate a physiological role for the endoperoxides in platelet aggregation and release.

More recent work based on these findings has shown that the product of incubation of arachidonic acid with lysed platelets, and presumed to contain endoperoxides, causes reduction of cAMP in PRP (11). A unifying hypothesis in which aggregating agents cause formation of endoperoxides and PGE_2 which in turn induce the release reaction by decreasing the level of cAMP has been proposed (11).

The results from the present study on the mode of action of cAMP are at variance with this previously proposed sequence of events and role of cAMP.

Material and Methods

Blood from healthy donors, who had not taken any drugs for at least one week, was collected from the antecubital vein with 0.13 vol. of 0.1 M trisodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation at 200 x g at room temperature for 15 min. Calcium chloride (15 µl 0.25 M/ml PRP) was added before preincubation of each sample. Washed human platelets were prepared as previously described and suspended in Krebs-Henseleit medium without calcium (8).

Platelet aggregation in PRP was monitored by continuous recording of light transmission (Aggregometer, Chronolog. Crop., Broomall, Pa.) at 37°C.

^{14}C-serotonin release. The PRP was incubated for 45 min with 0.5 µM |2-^{14}C| serotonin creatine sulfate (58 mCi/mmol; The Radiochemical Center, Amersham, England). One ml samples were decanted into 0.2 ml icecold EDTA 0.1 M pH 7.4 with rapid mixing. After centrifugation aliquots were removed for determination of radioactivity.

ADP-release was determined with the luciferase enzyme system. The release in one ml samples was stopped with 0.2 ml ice-cold 0.1 M EDTA pH 7.4 and rapid mixing. The luminescense reaction was performed in a glycyl-glycine buffer pH 7.4 in a liquid scintillation detector as described by Stanley et al. (12).

Transformation of ^{14}C-arachidonate. One ml samples of washed human platelets ($10^6/\mu l$) were incubated for 2 min at 37°C with 6.25 x 10^{-5} M [1-^{14}C]arachidonic (0.5 mCi/mmol; The Radiochemical Center, Amersham, England). Unlabeled arachidonic acid was purchased from Nu Chek Prep. Inc., Elysian, Minn. The reaction was stopped with 10 ml ice-cold 99.5% ethanol. After acid ether extraction and methylation with diazomethane thin-layer chromatography was carried out with the organic solvent system dioxane/benzene (40/50, v/v). In the experiments with PGE_2 the incubation time was 5 seconds.

Formation of TXB_2 assayed by radioimmunoassay. One ml samples of PRP were incubated for 5 min at 37°C with a collagen suspension (20 µg/ml PRP; Stago Laboratoire, Asnières, France). The reaction was stopped with 4 ml ice-cold 99.5% ethanol. The precipitate was removed by centrifugation and the amount of TXB_2 in the clear supernatant was determined by radioimmunoassay as described by Granström et al. (13).

N^6-O^2-Dibutyryl adenosine 3':5' cyclic monophosphoric acid, N^2-O^2-dibutyryl guanosine 3':5' cyclic monophosphoric acid, theophylline, acetylcholine chloride, adenosine 5'-diphosphate and indomethacin were all purchased form Sigma Chemical Company. PGE_1, PGE_2 and PGD_2 were kindly supplied by the Upjohn Company, Kalamazoo, Michigan.

The endoperoxides PGG_2 and PGH_2 were prepared as previously described (8).

Results

Aggregation. Aggregation of PRP induced by collagen (20 µg/ml) or arachidonate (200 µg/ml) was almost completely inhibited by 1.5 mM DB-cAMP, whereas the response to PGG_2 (500 ng/ml) was unaffected. In the case of PGG_2 1.4 x 10^{-5} M indomethacin was added in order to inhibit endogenous endoperoxide synthesis. DB-cGMP (1.5 mM) was without effect on aggregation induced by any of the agents (Fig. 1).

^{14}C-serotonin release. DB-cAMP (1 mM) almost completely inhibited the ^{14}C--serotonin release induced by ADP (10 µM), collagen (20 µg/ml) or arachidonate (200 µg/ml) but had no significant effect on PGG_2 induced release. The dose response (log.) relationship for the effect of DB-cAMP on ^{14}c-serotonin release induced by collagen was essentially linear. As in the case of aggregation DB--cGMP was without effect (Fig. 2). In the experiments with PGG_2, 1.4 x 10^{-5} M indomethacin was added.

ADP-release. In these experiments DB-cAMP was added or the cAMP level was increased by stimulation of adenyl cyclase with acetyl choline or by inhibition of phosphodiesterase with theophylline. There was a very pronounced effect of all these agents on collagen or arachidonate induced release of ADP whereas the response to PGG_2 was not significantly inhibited.

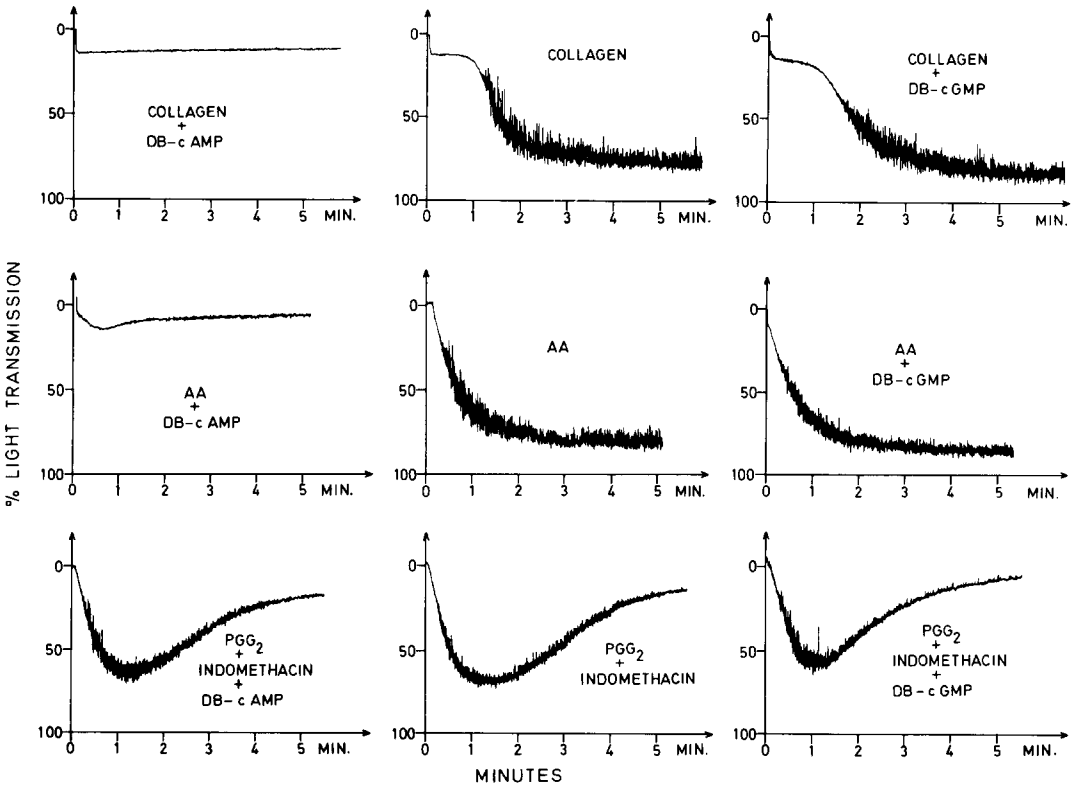
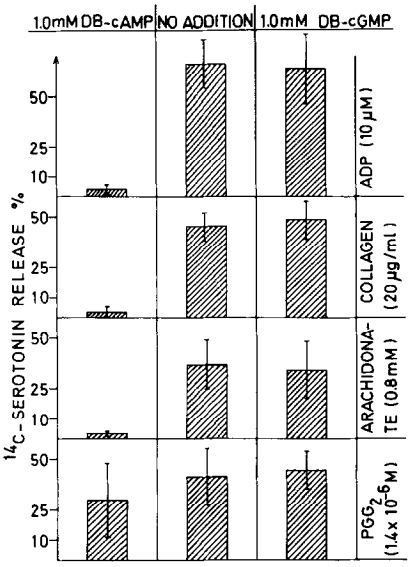


Fig. 1 Aggregating effects of collagen (20 µg/ml), arachidonate (0.5 mM) and PGG_2 (1.0×10^{-6} M). The PRP was preincubated without or in the presence of 1.5 mM DB-cAMP or 1.5 mM DB-cGMP. With PGG_2 as aggregating agent indomethacin (1.4×10^{-5} M) was added.

Formation of TXB_2 assayed by radioimmunoassay. Addition of DB-cAMP, PGE_1, PGE_2 (high doses), PGD_2, acetyl choline or theophylline caused decreased formation of TXB_2 in PRP upon addition of collagen (Fig. 3). In low doses PGE_2 caused an increased formation of TXB_2 (Fig. 3).

Transformation of ^{14}C-arachidonate. The effect of DB-cAMP on the transformation of ^{14}C-arachidonate in washed platelets is shown in Fig. 4. The formation of TXB_2 was almost abolished with concentrations required to inhibit aggregation or release of ^{14}C-serotonin induced by e.g. collagen. The absence of other polar products as PGE_2 and $PGF_{2\alpha}$ indicated that the cyclo-oxygenase was inhibited



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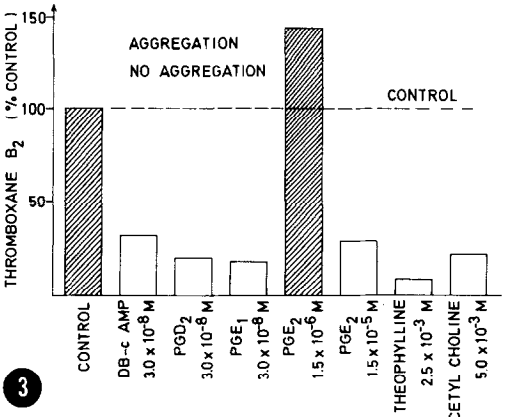

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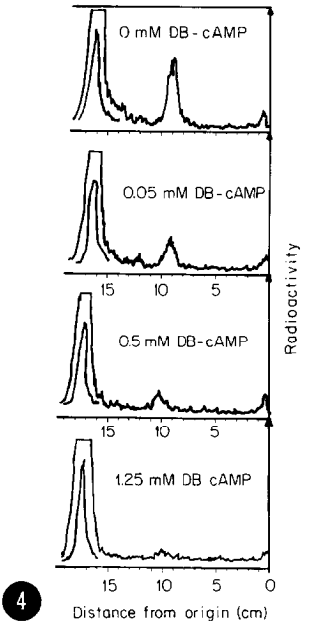
Fig. 2 ^{14}C-serotonin release ($\bar{X}$ + S.D., n=5) with ADP (10 µM), collagen (20 µg/ml), arachidonate (0.8 mM) or PGG_2 (1.4 x 10^{-6} M). The PRP was preincubated without or in the presence of 1.0 mM DB-cAMP or 1.0 mM DB-cGMP. With PGG_2 as releasing agent indomethacin (1.4 x 10^{-5} M) was added.

Fig. 3 Formation of thromboxane B_2 assayed by radioimmunoassay 5 min after addition of collagen (20 µg/ml). DB-cAMP, PGD_2, PGE_1, PGE_2, theophylline or acetyl choline were added 15 min before addition of collagen.

(Fig. 4). Similar results were obtained with theophylline and acetyl choline. DB-cGMP did not inhibit the formation of TXB_2.

The effect of PGE_2 on transformation of ^{14}C-arachidonic acid is shown in Fig. 5. A marked increase of thromboxane B_2 is seen with low concentrations of PGE_2 (10-1500 ng/ml) whereas higher concentrations rather caused a decreased formation.

Stimulation of aggregation by PGE_2. The submaximal reversible aggregation induced by PGG_2 (200 ng/ml) was markedly increased by PGE_2 (1 µg/ml). However, in the presence of 1.4 x 10^{-5} M indomethacin no such potentiation was observed with PGE_2 (Fig. 6).



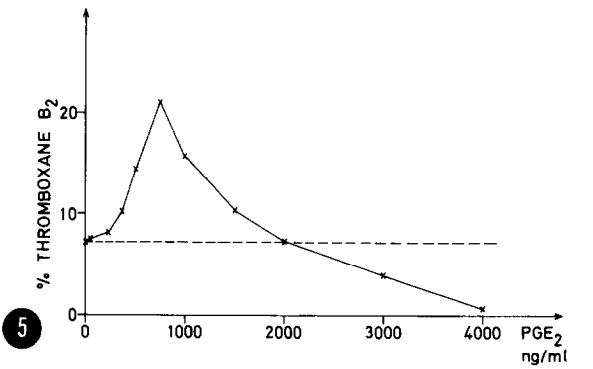


Fig. 4 Thin layer chromatogram of products formed after incubation of washed human platelets with ^{14}C-arachidonic acid (20 µg/10^6 platelets) at 37°C for 2 min. DB-cAMP was added in increasing concentrations.

Fig. 5 Effect of PGE_2 on the transformation of ^{14}C-arachidonic acid by washed platelets (10^6/ml) at 37°C for 5 sec.

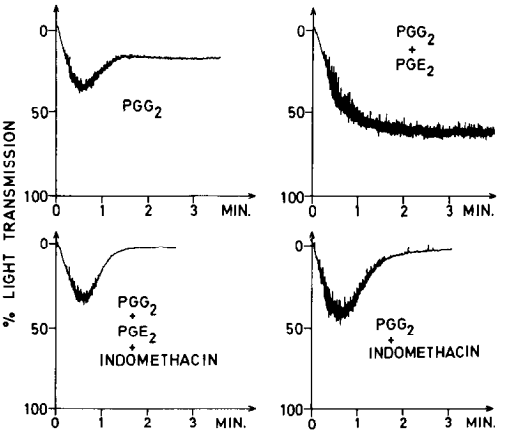


Fig. 6 Platelet aggregation induced by PGG_2 (0.6×10^{-6}M) and the effect of PGE_2 (1 µg/ml PRP) in the presence or absence of indomethacin (1.4×10^{-5}).

Discussion

Dibutyryl cAMP but not dibutyryl cGMP inhibited platelet aggregation and the release of ^{14}C-serotonin and ADP when induced by collagen or arachidonate but not when induced by the endoperoxide PGG_2. Agents (PGE_1 and PGD_2, high doses of PGE_2, theophylline and acetyl choline) which increase the level of cAMP had similar effects as DB-cAMP on these parameters.

The finding that the effect of the endoperoxide PGG_2 on aggregation and release was not abolished by DB-cAMP indicated that the synthesis of PGG_2 might be inhibited by the cyclic nucleotide derivative. In order to test this hypothesis the formation of TXB_2, which is the main stable metabolite of PGG_2 in platelets, was determined after addition of collagen to PRP. The PRP was preincubated with DB-cAMP or agents (PGE_1, PGD_2, theophylline and acetyl choline) known to increase the level of cAMP. With all of these compounds there was a substantial inhibition of the formation of TXB_2 and inhibition of aggregation. PGE_2 was tested at different concentrations, one which resulted in a decreased level of cAMP and one which had the opposite effect. The low concentration gave increased formation of TXB_2 whereas the higher concentration reduced the formation of TXB_2.

Additional experiments were carried out to test whether the effect of cAMP was on the cyclo-oxygenase or whether it was due to inhibition of the release of arachidonic acid or inhibition of the enzyme(s) catalyzing the formation of thromboxanes. ^{14}C-arachidonate was therefore incubated with platelets in the presence of various concentrations of DB-cAMP. This experiment showed that the formation of labeled TXB_2 from arachidonate was inhibited by DB-cAMP apparently by inhibition of the cyclo-oxygenase since no additional transformation products of PGG_2 were observed. Similar experiments with PGE_2 indicated that the changes in formation of TXB_2 by this compound mentioned above was due to cAMP mediated regulation of cyclo-oxygenase activity.

Additional support for the inhibition of the cyclo-xoygenase by cAMP and its relevance to the mechanism of platelet aggregation was obtained by studying the effect of PGE_2 on PGG_2 induced aggregation. PGE_2 enhanced the aggregating effect

of PGG_2. However, when a cyclo-oxygenase inhibitor was added there was no effect of PGE_2, since the reduction in the level of cAMP induced by PGE_2 could not cause activation of the cyclo-oxygenase.

The finding that cAMP can regulate platelet cyclo-oxygenase, which catalyzes formation of the endoperoxide PGG_2, opens new possibilities of studying the control of the release-reaction and aggregation of human platelets. Further work is in progress to study the occurrence of this regulatory mechanism in other systems.

Acknowledgement

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