

Cyclic adenosine 3',5'-monophosphate inhibits the availability of arachidonate to prostaglandin synthetase in human platelet suspensions.

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Research Article

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Cyclic Adenosine 3',5'-Monophosphate Inhibits the Availability of Arachidonate to Prostaglandin Synthetase in Human Platelet Suspensions

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ABSTRACT When thrombin is added to washed human platelets, one of its actions results in activation of a phospholipase that hydrolyzes arachidonic acid from phospholipids. The arachidonate is converted to the cyclic endoperoxides (prostaglandin G_2 and prostaglandin H_2) by fatty acid cyclo-oxygenase. These compounds are then converted to thromboxane A_2 , also called rabbit aorta-contracting substance, by thromboxane synthetase. These labile, pharmacologically active compounds then break down to inactive products including thromboxane B_2 and malonaldehyde. Incubation of platelets with either dibutyryl cyclic adenosine 3',5'-monophosphate (dBcAMP) or prostaglandin E_1 (PGE $_1$) before thrombin addition blocks the subsequent formation of oxygenated products of arachidonic acid including thromboxane A_2 , thromboxane B_2 , and malonaldehyde. In contrast, when arachidonic acid is added directly to platelets, prior incubation with dBcAMP or PGE $_1$ does not inhibit production of the prostaglandins or their metabolites. Thrombin treatment of platelets also blocks the acetylation of cyclo-oxygenase by aspirin since the hydrolyzed arachidonic acid competes with aspirin for the active site on cyclo-oxygenase. Prior treatment of platelets with dBcAMP or PGE $_1$ reverses the thrombin inhibition of the acetylation of cyclo-oxygenase. We conclude that agents which elevate platelet cAMP levels inhibit the hydrolysis of arachidonic acid from platelet phospholipids. We also find that prostaglandin synthesis can be dissociated, in part, from platelet aggregation and release, and that cAMP has separate actions on these processes. Higher thrombin concentrations are required to stimu-

late prostaglandin synthesis (0.05–2 U/ml) than are required to induce [^{14}C]serotonin release (0.02–0.1 U/ml). Furthermore, dBcAMP and PGE $_1$ both inhibit platelet aggregation induced by either arachidonic acid or prostaglandin H_2 without affecting the production of prostaglandin metabolites from these compounds.

INTRODUCTION

Recently, it has been postulated that the platelet release reaction induced by thrombin and other agents may be mediated by prostaglandins (1–6). When thrombin or collagen are added to intact platelets, arachidonic acid is rapidly hydrolyzed from platelet phospholipids (7, 8) and metabolized to cyclic endoperoxide compounds by a particulate enzyme fatty acid cyclo-oxygenase. Subsequently, the cyclic endoperoxides prostaglandin G_2 (PGG $_2$)¹ and prostaglandin H_2 (PGH $_2$) are converted to thromboxane A_2 (TA $_2$) which is broken down to nonprostanoid metabolites including malonaldehyde and thromboxane B_2 (TB $_2$). Both cyclic endoperoxides and TA $_2$, the latter being much more potent, induce platelet aggregation and release (5).

Aspirin inhibits platelet function by preventing formation of cyclic endoperoxides (9). Aspirin inactivates platelet cyclo-oxygenase, and we have recently shown that aspirin acetylates the enzyme's active site (10, 11).

¹ *Abbreviations used in this paper:* cAMP, cyclic adenosine 3',5'-monophosphate; dBcAMP, N⁶-O^{2'}-dibutyryl cyclic adenosine 3',5'-monophosphate; MDA, malonaldehyde; PGE $_1$, PGE $_2$, PGF $_{2\alpha}$, PGA $_2$, prostaglandin E $_1$, E $_2$, F $_{2\alpha}$, A $_2$; PGG $_2$, prostaglandin G $_2$; PGH $_2$, prostaglandin H $_2$; RCS, rabbit aorta-contracting substance; TA $_2$, thromboxane A $_2$; TB $_2$, thromboxane B $_2$.

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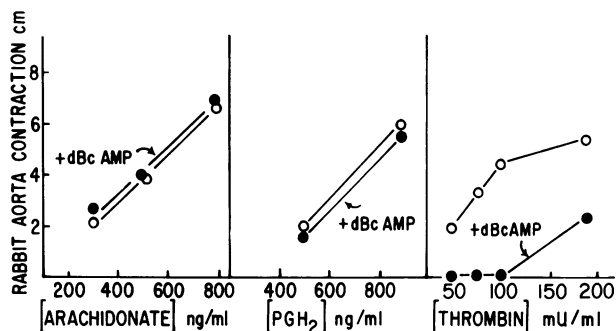


FIGURE 1 Generation of rabbit aorta-contracting substance (RCS). The generation of RCS by varying concentrations of arachidonate, PGH₂, and thrombin, ● with dBCAMP, and ○ alone. The platelet suspensions were incubated with 2 mM dBCAMP for 120 s before addition of the agonists. Because of the lability of RCS (TA₂) in aqueous media at 37°C, all samples were assayed at 30 s after the addition of arachidonate, PGH₂, or thrombin.

Arachidonic acid, the cyclo-oxygenase substrate, inhibits the aspirin-mediated acetylation by competing with the drug for the active site of the enzyme.

Agents which elevate platelet cyclic adenosine 3',5'-monophosphate (cAMP) levels (e.g. dibutyl (dB)cAMP or PGE₁) inhibit subsequent platelet release and aggregation induced by thrombin and a variety of aggregating agents (9, 12–16). Whether or not these aggregating agents depress the basal platelet cAMP level as the cause of platelet release and aggregation has been the subject of much controversy (9, 12–15). Increasing the platelet cAMP level has been reported to inhibit the activity of cyclo-oxygenase (16). This conclusion was based on results of experiments where platelets were incubated with dBcAMP before addition of collagen or arachidonate. This treatment resulted in a reduction in the formation of TB₂.

In contrast to this report we find that dBcAMP or PGE₁ appear to inhibit the hydrolysis of arachidonic acid from platelet phospholipids which occurs after thrombin addition to platelets. We find no evidence that high levels of cAMP affect platelet cyclo-oxygenase or thromboxane synthetase.

METHODS

Experiments were performed with washed human platelets prepared by modification of a previously described method (2). Fresh blood was withdrawn from healthy donors who had not taken any medication (including aspirin) for at least 2 wk before collection time. Blood withdrawn was collected directly into 7.5% (vol/vol) 77 mM EDTA and centrifuged in plastic tubes at 150 g for 10 min. The platelet-rich plasma was centrifuged at 1,800 g for 6 min and the platelet pellet was resuspended in 0.15 M NaCl:0.15 M Tris HCl (pH 7.4):77 mM EDTA (90:8:2, vol/vol/vol) and subsequently recentrifuged at 600 g for 5 min. The washed platelet pellet was resuspended in a calcium-free Krebs-Henseleit medium in a volume yielding 10⁹ platelets/ml.

The platelets were kept at 37°C throughout the experiments. A different washing technique was used for experiments in Fig. 7 and Table I (17). Platelet aggregation was monitored with a Payton single channel aggregometer. Arachidonate and thrombin were added to 0.4-ml platelet suspensions which were stirred at 1,100 rpm at 37°C. The endoperoxide, PGH₂, was added to the aggregometer cuvette and the acetone solvent was evaporated under a stream of nitrogen after which 0.4 ml of platelet suspension was immediately added. Either dBcAMP or PGE₁ was incubated (37°C) with the platelet suspensions for 2 or 1 min, respectively, before the addition of the aggregating agent. TA₂ content was determined by a bioassay which measures the contractile response of rabbit thoracic aorta strips as previously described (5). ADP release was determined utilizing an ADP-ATP enzymatic pyridine-nucleotide cycling method (18).² [¹⁴C]Serotonin release was measured as previously described (17).

Malonaldehyde (MDA) was measured as previously described (11). The molar extinction coefficient of the MDA-thiobarbituric acid product at 532 nm is 1.4×10^5 (11). The extent of platelet cyclo-oxygenase acetylation was determined by measuring [³H]protein (mol wt 85,000) after treatment with [³H]aspirin (10, 11). We measured TB₂ by thin-layer chromatography. After incubation of platelets with [1-¹⁴C]arachidonate, the platelet suspensions were acidified with 2.0 M citric acid (pH 3.0) and extracted twice with ice-cold ethyl acetate. The ethyl acetate extracts were then analyzed by thin-layer chromatography on silica gel plates with added standards of 10 μg each of PGE₂, PGF_{2α}, PGA₂, and arachidonic acid in chloroform: methanol:acetic acid:water::90:8:1:0.8 (19). The plates were then scanned in a Vanguard Strip Scanner (Vanguard Systems Inc., Dobbs Ferry, N. Y.) to determine radioactivity, and the standards were located by staining in iodine vapor.

Materials. Arachidonate was purchased from NuCheck Prep, Elysian, Minn. PGH₂ was prepared and purified as previously described (5). [1-¹⁴C]Arachidonate was purchased from Amersham/Searle Corp., Arlington Heights, Ill., and [¹⁴C]serotonin binoxalate was purchased from New England Nuclear, Boston, Mass. PGE₁ was kindly supplied by Dr. J. Pike, The Upjohn Co., Kalamazoo, Mich. N⁶-O^{2'}-dB cAMP-monophosphate was purchased from Sigma Chemical Co., St. Louis, Mo. Human α-thrombin was purified and assayed as previously described (20).

RESULTS

Effect of dBcAMP on formation of TA₂. The addition of increasing concentrations of arachidonate, PGH₂ or thrombin to platelet suspensions produced a dose-dependent increase in rabbit aorta-contracting substance (TA₂, Fig. 1). When the platelets were incubated with 2 mM dBcAMP before addition of arachidonate or PGH₂, there was no change in the production of the rabbit aorta-contracting substances (RCS). However, RCS formation induced by thrombin was markedly inhibited by dBcAMP (Fig. 1). The effect of varying the concentration of dBcAMP is shown in Fig. 2. Again, dBcAMP inhibited the formation of RCS after thrombin without affecting that produced

² The method measures the sum of ADP plus ATP, but separate experiments show that over 80% of the released nucleotide is ADP.

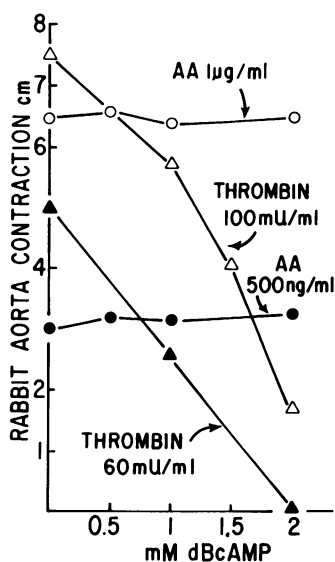


FIGURE 2 The effect of dBcAMP concentration on the generation of RCS induced by arachidonate and thrombin. The experiment was performed as in Fig. 1.

after arachidonic acid. The same results were obtained using PGE₁ (3 µM) where platelet cAMP levels were elevated 15-fold above base-line levels (data not shown).

When prostaglandin synthesis was measured by determining the formation of [¹⁴C]TB₂ from [1-¹⁴C]-arachidonate, similar results were obtained as shown in Fig. 3. Thus, incubation of platelets with 2 mM dBcAMP did not reduce the generation of TB₂. In other experiments using [1-¹⁴C]arachidonate at concentrations from 0.25 to 15 µg/ml (1–50 µM) and dBcAMP concentrations from 0.5 to 5 mM, similar results were obtained, i.e., dBcAMP did not affect the production of TB₂ from arachidonate.

Effect of dBcAMP on formation of MDA. MDA is a product of arachidonate metabolism which may arise both from breakdown of cyclic endoperoxides and TA₂ (Fig. 4). The effect of increasing thrombin concentration on MDA production is shown in Fig. 5. The formation of MDA is dependent on several enzymes (Fig. 4) but the limiting step is arachidonate hydrolysis from phospholipids. This was demonstrated by measuring the inhibition of cyclo-oxygenase acetylation after thrombin addition (Fig. 5). It has been shown previously that aspirin acetylates the active site of cyclo-oxygenase and that the acetylation is competitively inhibited by the substrate arachidonate (11). The inhibition by arachidonate correlates with its activity as a cyclo-oxygenase substrate (i.e., K_m as substrate = K_i as inhibitor). Furthermore, other unsaturated fatty acids are less effective as inhibitors of acetylation in parallel with their function as cyclo-oxygenase substrates (i.e., better substrates are better

inhibitors [11]). We suggest that the degree of inhibition of aspirin acetylation of cyclo-oxygenase after thrombin treatment is a function of the arachidonate concentration presented to the enzyme. As shown in Fig. 5, the inhibition of acetylation by thrombin parallels malonaldehyde production. That the inhibition of acetylation is due to arachidonic acid is suggested by the finding that when aspirin was added 5 min after thrombin, no inhibition of acetylation was observed (data not shown). The arachidonate hydrolyzed after thrombin is all metabolized in 5 min. Assuming that inhibition of acetylation of cyclo-oxygenase can be used to measure arachidonic acid

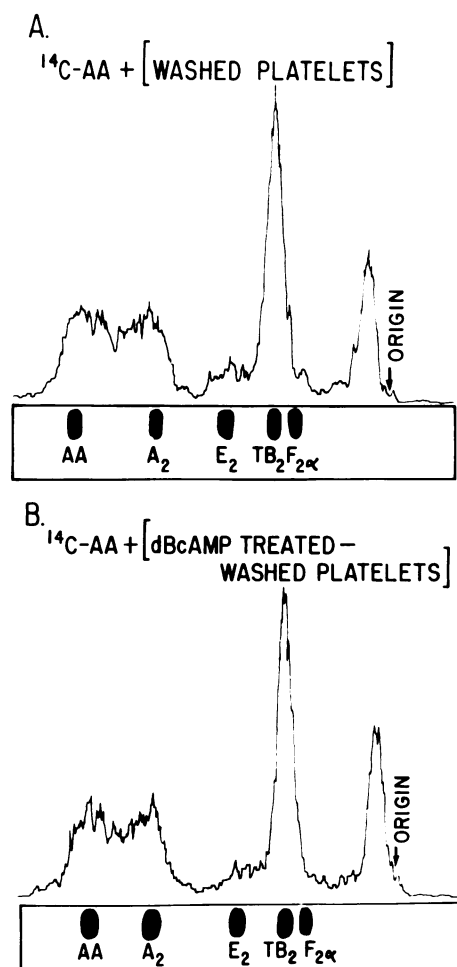


FIGURE 3 Thin-layer chromatogram radioactivity scan of reactions of [¹⁴C]arachidonate with platelets. [¹⁴C]Arachidonate, 1.5 µg (5×10^5 cpm), was added to platelet suspensions in an aggregometer cuvette stirred at 1,100 rpm at 37°C, alone, or after a 2-min incubation with 2 mM dBcAMP. After 3 min the reaction mixtures were acidified and extracted. The products were fractionated by thin-layer chromatography and scanned as described in Methods. On the radiochromatograms shown, the TB₂ peak radioactivity was 3,000 counts.

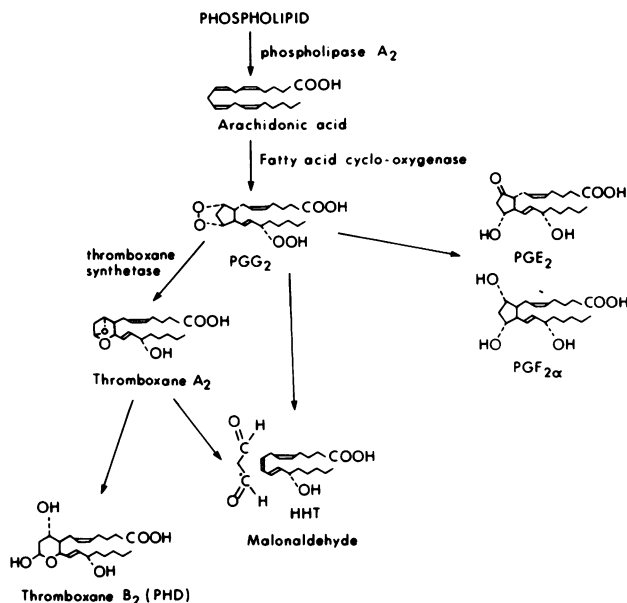


FIGURE 4 Metabolism of arachidonate by platelets. HHT is L-12-hydroxy-5,8,10-heptadecatrienoic acid. It has not been clearly established whether malonaldehyde and HHT are metabolic products of TA₂, PGG₂, or both.

hydrolysis, we tested the effect of dBcAMP on the inhibition of acetylation of cyclo-oxygenase by thrombin. As shown in Table I, dBcAMP treatment of platelets alone had no effect on cyclo-oxygenase acetylation whereas the inhibition of acetylation by

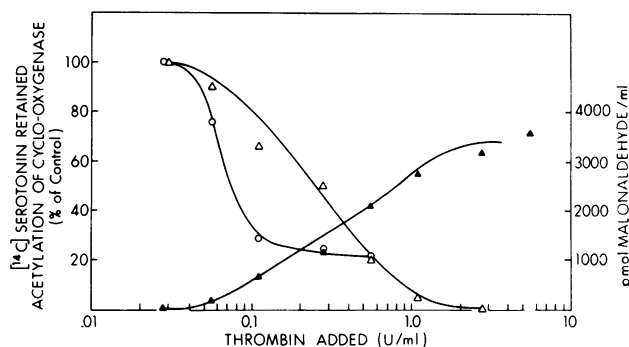


FIGURE 5 Effect of thrombin on malonaldehyde production, cyclo-oxygenase acetylation, and serotonin release in platelets. Platelet suspensions containing [¹⁴C]serotonin were prepared in Tris-buffered saline (17). [¹⁴C]Serotonin release in 5 min was measured as described (17) except the volume was 0.1 ml and the final platelet concentration was 2×10^9 /ml. Malonaldehyde formation in 5 min induced by thrombin was assayed as described in Methods. Acetylation by [³H]acetyl-³H]aspirin of cyclo-oxygenase was performed as described in the legend of Table I except the final thrombin concentration was varied. All incubations were performed in parallel and completed within 30 min of each other. ○, [¹⁴C]Serotonin retained; △, inhibition of acetylation by [³H]acetyl-³H]aspirin; ▲, malonaldehyde formed.

thrombin was reversed by prior dBcAMP incubation. Similar results were obtained using 3 μ M PGE₁. We conclude that these agents reverse the inhibition of acetylation by preventing arachidonic acid hydrolysis. In other experiments we showed that treatment of platelets with dBcAMP inhibited MDA formation induced by thrombin, but not that induced by arachidonate or PGH₂ (Fig. 6).

Dissociation of prostaglandin synthesis from platelet aggregation and release. Our experiments suggest that elevation of platelet cAMP levels blocks the arachidonate hydrolysis that follows thrombin addition without significantly affecting cyclo-oxygenase or thromboxane synthetase. There are other sites of cAMP action in platelets as shown in Figs. 7 and 8. Thus, platelet aggregation and ADP release induced by arachidonate, PGH₂, or thrombin were inhibited by prior treatment with dBcAMP even though there was no inhibition of prostaglandin synthesis from either arachidonate or PGH₂ as measured by RCS, TB₂ (data for PGH₂ not shown), or MDA formation. These results suggest that platelet aggregation and release are blocked by dBcAMP by a mechanism which is independent of its effects on arachidonate metabolism. The dissociation between prostaglandin synthesis and serotonin release is shown in Fig. 5 where the concentration of thrombin required to stimulate prostaglandin synthesis (0.05–2 U/ml) is greater than that required for serotonin release (0.02–0.1 U/ml).

DISCUSSION

Previous investigations have suggested that platelet-aggregating agents stimulate prostaglandin synthesis

TABLE I
Incorporation of ³H from [acetyl-³H]Aspirin into Cyclo-Oxygenase in Intact Platelets

Additions	Control	+2.5 mM dBcAMP
cpm incorporated/ 2×10^8 platelets		
None	135	124
+0.5 U/ml thrombin	35	129
+1 U/ml thrombin	19	123

Suspensions of platelets were incubated at 37°C for 15 min either alone or with dBcAMP. [acetyl-³H]Aspirin was added to a final concentration of 10 μ M and the incubation was continued for an additional 5 min. Thrombin, if present, was added 15 s after the aspirin. At the end of the incubation, samples were boiled in sodium dodecyl sulfate, electrophoresed, and radioactivity in cyclo-oxygenase assessed (10, 11). Each gel contained material from 2×10^8 platelets. The values presented are averages of duplicate experiments. The radioactivity incorporated without thrombin corresponds to one-half maximal acetylation under these conditions.

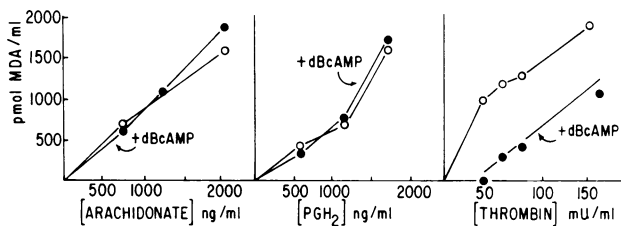


FIGURE 6 Arachidonate-, PGH₂-, and thrombin-induced generation of MDA. Platelet suspensions were incubated 3 min with the indicated concentrations of arachidonate, PGH₂, or thrombin with or without prior incubation with 1 mM dBcAMP as in Fig. 1, and then assayed for MDA as described in Methods.

in platelets by activating a phospholipase which hydrolyzes arachidonic acid from platelet phospholipids (1, 3, 7, 8). When platelets lipids were labeled with radioactive arachidonic acid and the platelets then treated with thrombin, free arachidonic acid appeared with subsequent formation of prostaglandins, endoperoxides, and thromboxanes (7, 8). It is also suggested by our experiments that the hydrolysis of arachidonic acid from platelet lipids is the limiting step in platelet prostaglandin synthesis. Aspirin acetylates the active site of cyclo-oxygenase, and the substrate for the enzyme, arachidonic acid, serves as a competitive inhibitor of the acetylation reaction. We have shown that thrombin inhibits the acetylation of cyclo-oxygenase by aspirin with a dose-response curve which parallels MDA production. This result suggests that availability of arachidonic acid to cyclo-oxygenase controls the production of endoperoxides and the other metabolites. We also conclude that agents which elevate cAMP levels inhibit MDA production by diminishing arachidonic acid hydrolysis rather than by inhibiting cyclo-oxygenase directly. This conclusion is suggested from the finding that dBcAMP blocks thrombin-induced RCS (TA₂), TB₂, and MDA production but does not reduce formation of these arachidonate metabolites when arachidonate is added directly to platelets. That arachidonic acid hydrolysis is inhibited by cAMP is also suggested by the findings that the thrombin-induced inhibition of acetylation of cyclo-oxygenase is also blocked by dBcAMP (Table I). The mechanism by which dBcAMP inhibits arachidonic acid hydrolysis remains to be elucidated. It is tempting to speculate that the arachidonic acid phospholipase may be regulated by cAMP. cAMP modulates the phospholipase A₂ of rat epididymal fat cells although increasing cAMP stimulates the enzyme in that case (21). It is also possible that cAMP regulates calcium flux thereby affecting both platelet aggregation and phospholipase activity.

Our results conflict with recent findings of Malmsten et al. (16) who report that dBcAMP inhibits the for-

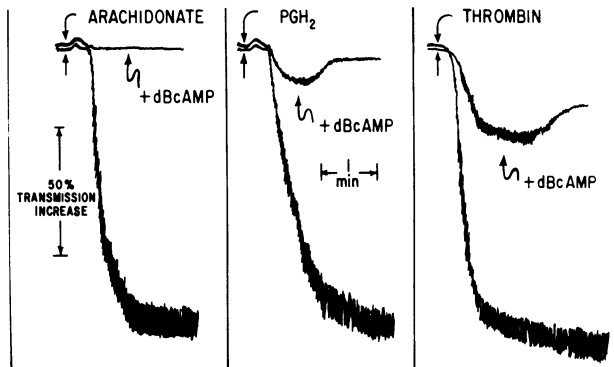


FIGURE 7 Platelet aggregation. Aggregation of human platelet suspensions by arachidonate (400 ng/ml), PGH₂ (500 ng/ml), and thrombin (150 mU/ml). dBcAMP (1 mM) was preincubated with platelet suspensions as described in Fig. 1.

mation of TB₂ from arachidonic acid. These authors incubated intact platelets with [¹⁴C]arachidonate in the presence and absence of dBcAMP, and after acid-ether extraction of the platelets, determined TB₂ formation by thin-layer chromatography. We have measured TB₂ in the same manner that they did at widely varying concentrations of both arachidonic acid and dBcAMP and find no inhibition. There is an additional unexplained difference between our results. Malmsten found that dBcAMP inhibited arachidonate but not PGH₂-induced platelet aggregation whereas we find that aggregation is inhibited in both cases without evident effect on prostaglandin synthesis. We are unable to explain the reason for the differences in experimental results. We conclude that dBcAMP has no direct inhibitory effect on cyclo-oxygenase.

How cyclic endoperoxides or thromboxanes function in promoting platelet aggregation or release in

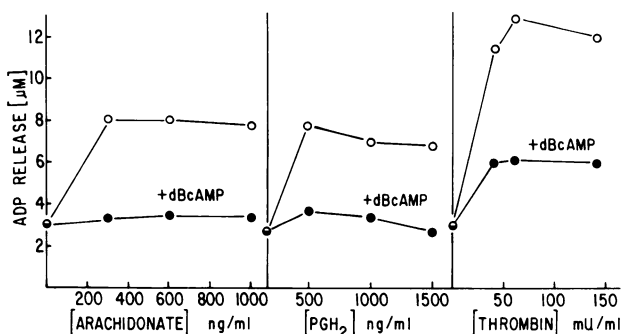


FIGURE 8 Arachidonate-, PGH₂-, and thrombin-induced ADP release. ADP released during aggregation of platelet suspensions was assayed as described in Methods by removing a 20-μl sample of the reaction mixture 3 min after the addition of the aggregating agent. 1 mM dBcAMP was preincubated with platelet suspensions as described in Fig. 1.

vivo remains to be elucidated. That prostaglandin metabolites may not be obligate intermediates in the release reaction induced by thrombin is suggested by several lines of evidence. As shown in Fig. 5, the concentration of thrombin required to induce serotonin release from platelets is significantly less than that required to stimulate arachidonic acid hydrolysis. Further, aspirin-treated platelets or platelets from donors who have ingested aspirin respond normally to thrombin in vitro (22–24). Even using collagen, where aspirin treatment does inhibit the release reaction, higher concentrations of collagen overcome aspirin inhibition (23). However, prostaglandins are important in vivo since aspirin induces a mild hemostatic defect in normal individuals, and in patients with defects in intrinsic coagulation, a much more severe defect is produced (25). If not essential for aggregation and release, prostaglandins may serve as modifiers of platelet function, the effects of which could be important in pathological states such as myocardial infarction (26).

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