CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE AND PROSTACYCLIN INHIBIT MEMBRANE PHOSPHOLIPASE ACTIVITY IN PLATELETS

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SUMMARY. [^{14}C]-Arachidonic acid is incorporated mainly into phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine of horse platelet membranes. Treatment of washed platelets with thrombin leads to a rapid loss of radioactivity from these phospholipids. The liberated [^{14}C]-arachidonate is immediately transformed into hydroxyacids and thromboxanes. Treatment with dibutyryl cyclic AMP, cyclic AMP phosphodiesterase inhibitors or prostacyclin, a newly discovered prostaglandin that stimulates platelet adenylate cyclase, prevents the action of thrombin on phospholipid break-down as well as on platelet aggregation. Dibutyryl cyclic AMP does not affect the metabolism of exogenous [^{14}C]-arachidonic acid. Cyclic AMP may thus play a crucial role in the regulation of platelet phospholipase acitivity, and this could explain at least in part the inhibition of aggregation caused by substances which, like prostacyclin, raise the levels of cyclic AMP.

Thrombin and collagen, which cause platelet aggregation, release arachidonic acid from phospholipids and induce the synthesis of endoperoxides, thromboxanes, prostaglandins and hydroxyacids from the liberated arachidonic acid (1,2). The available evidence suggests that endoperoxides either directly or after conversion to thromboxanes mediate platelet aggregation (3). The rate-limiting step in the formation of endoperoxides and other arachidonate metabolites seems to be the availability of free arachidonate, i.e. its generation from the esterified form by the action of phospholipase(s) A_2 (2).

Dibutyryl cyclic AMP prevents the platelet aggregation induced by different agents, and it has been proposed that this occurs by inhibition of the cycloxygenase (4). This report demonstrates that dibutyryl cyclic AMP, phosphodiesterase inhibitors and a very potent activator of adenylate cyclase, prostacyclin, block thrombin-induced phospholipid degradation in horse platelets. The results suggest that cyclic AMP regulates phospolipase(s) activity in platelets.

METHODS AND MATERIALS. [1-^{14}C]-Arachidonic acid was obtained from New England Nuclear, Boston, Massachusetts, thrombin (bovine plasma) from Sigma Chemical Co., St. Louis, Mo., and Kieselgel plates Sil G-25 from Brinkman Instruments, Inc., Westbury, N.Y. Prostacyclin was kindly provided by Norman Whittaker of The Wellcome Research Laboratories, Beckenham, U.K.

Preparation and Incubation of Labeled Platelets. Horse blood anticoagulated with ACD (Anticoagulant Citrate Dextrose) was centrifuged (300 x g, 15 min, 24°) and the platelet-rich plasma was removed and centrifuged again at 300 x g for 15 min. Samples (50 ml) of platelet- rich plasma were incubated with 1 μCi of

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[1-^{14}C] arachidonic acid at 37° for 120 min. EDTA was then added (1 mM) and platelets were sedimented at 3000 x g for 20 min at 4°. The platelets were gently resuspended in 100 ml of a Tris-saline-EDTA buffer (NaCl, 134 mM; 15 mM Tris-HCl, pH 7.4; 1 mM EDTA; 5 mM D-glucose), centrifuged again at 3000 x g for 20 min, and resuspended in 20 to 30 ml of the same buffer.

Samples of 0.5 ml of labeled platelets (1 to 3 mg protein) (5) were incubated at 37° with 10 µl of thrombin (50 units/ml) for 5 min.

Lipid Extractions. All incubations were carried out in duplicate. Incubations were stopped with 1.8 ml of chloroform:methanol, 1:2, v/v, and partitioned with 0.6 ml of chloroform and 0.6 ml of water (6). After centrifugation the lower phases were removed and the lipids were dried under nitrogen. The dried lipids were dissolved in 0.05 ml of chloroform and spotted on t.l.c. plates. One of the duplicate incubations was used for separation of phospholipids (chloroform: methanol:acetic acid:water, 50:30:8:4, v/v/v/v)(7), and the other for separation of arachidonic acid, hydroxyacids, thromboxane and prostaglandins (top phase of ethylacetate:2,2,4-trimethylpentane:acetic acid:water, 90:50:20:100, v/v/v/v)(8). Radioactive spots were localized by radioautography and radioactivity in each zone was determined by liquid scintillation.

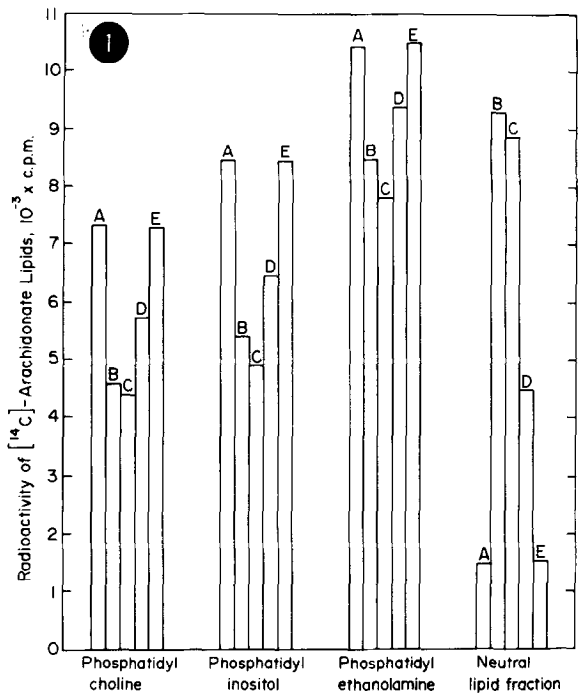
Platelet Aggregation. This was measured by continuous recording of light transmission (aggregometer, Chronolog Corp., Broomall, Pa.) through 1.5 ml of washed platelets resuspended in Tris-saline-EDTA buffer at 37°. $CaCl_2$ (6.6 mM) was always added.

RESULTS. Thrombin-Induced Phospholipid Degradation. After two hours of incubation with [^{14}C]-arachidonic acid more than 95% of the incorporated radioactivity in horse platelets is found in phospholipids. Phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine are labeled almost equally (Fig. 1, Table 2) while very little radioactivity is found in phosphatidylserine. Lysophosphatidylcholine and sphingomyelin are not labeled. Phosphatidic acid and the neutral lipid fraction (which includes free fatty acids, hydroxyacids, cholesterol and mono, di and triglycerides) are present in a single spot moving with the solvent front and contain less than 5% of the total radioactivity.

When washed platelets are incubated for 5 min with thrombin (1 unit/ml) the radioactivity in the three major labeled phospholipids falls substantially (Fig. 1, Table 2). This decrease in phospholipids is accompanied by a corresponding increase in the "neutral lipid fraction" in the same t.l.c. (Fig. 1). Examination of the oxygenated products of arachidonic acid in the same labeled lipid extracts reveals that thrombin causes the appearance of radioactive spots which have been tentatively identified as a 20-C hydroxyacid, which is a lipoxygenase product named HETE, and a 17-C hydroxy acid, HHT, derived from endoperoxides and thromboxanes. A small amount of free arachidonic acid is also present (Figs. 2,4 and Table 2). Only upon long exposure is it possible to observe a faint band related to thromboxane B_2.

Similar effects of thrombin have recently been reported using human (1) and rabbit (2) platelets.

Effects of Dibutyryl Cyclic AMP on Thrombin-Induced Phospholipid Degradation. Fig. 1 shows one of five separate, essentially similar experiments in which



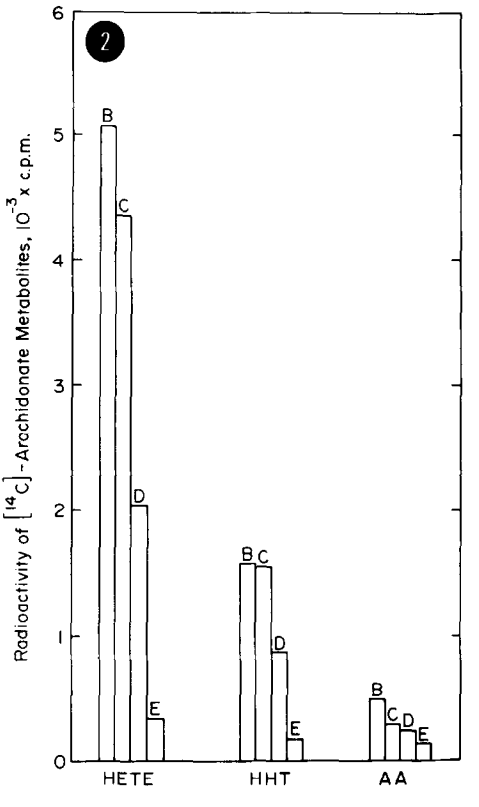


Fig. 1. Effect of dibutyryl cyclic AMP on thrombin-induced phospholipid degradation. All incubations had identical amounts (1.3 mg protein) of [^{14}C]-arachidonate-labeled platelets. Preincubations ± dibutyryl cyclic AMP were for 10 min followed by 5 min incubation ± thrombin (1 unit/ml). A, control without additions; B, action of thrombin; C, preincubation with 10^{-5} M dibutyryl cyclic AMP followed by thrombin; D, the same as in C but with 10^{-4} M dibutyryl cyclic AMP; E, the same as in C but with 10^{-3} M dibutyryl cyclic AMP. Controls with dibutyryl cyclic AMP alone have no effect on phospholipid degradation.

Fig. 2. Effect of dibutyryl cyclic AMP on thrombin-induced production of oxygenated metabolites from [^{14}C]-arachidonate-labeled platelets. Chromatography of duplicate incubations from the experiment in Fig. 1. All details as in Fig. 1. HETE, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12L-hydroxy-5,8,10, Heptadecatrienoic acid; AA, arachidonic acid.

^{14}C-labeled platelets are preincubated for 10 to 15 min with dibutyryl cAMP followed by thrombin stimulation for 5 min. Thrombin-induced phospholipid breakdown is prevented in a concentration-dependent manner by cyclic AMP. The thrombin-induced formation of oxygenated products from arachidonic acid decreases in a manner which parallels the degree of inhibition which dibutyryl cyclic AMP causes on thrombin-induced phospholipid degradation (Fig. 2). Cyclic AMP phospho-

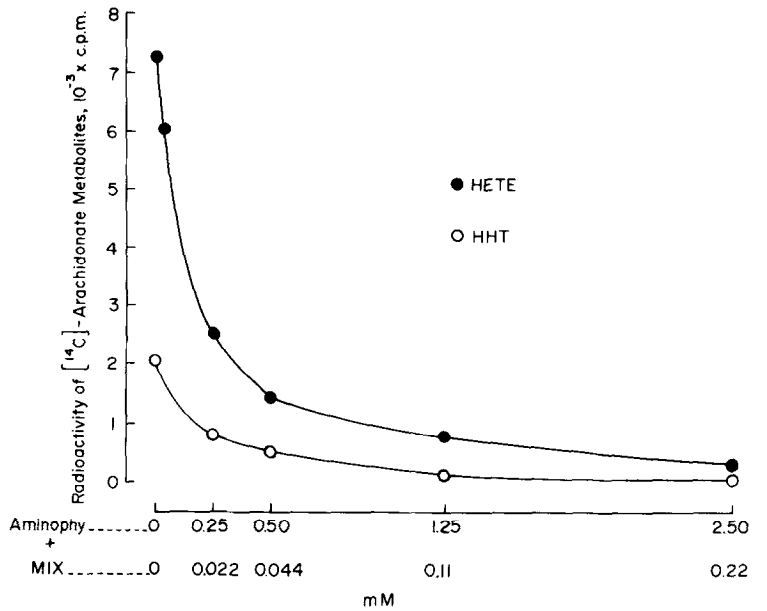


Fig. 3. Effect of cyclic AMP phosphodiesterase inhibitors on thrombin-induced production of oxygenated products from [^{14}C]-arachidonate-labeled platelets. All assays contained identical amounts of protein (3.15 mg protein). Preincubations with aminophylline (Aminophy.) and methylisobutylxanthine (MIX) were for 10 min followed by thrombin (1 unit/ml) for 5 min. Control incubations with the maximal amounts of aminophylline and MIX alone had no effect.

TABLE 1. Stimulation of horse platelet adenylate cyclase by prostacyclin and PGE_1

Additions		Cyclase Activity, pmoles cAMP/min
Control		2.2
Prostacyclin	(2.8×10^{-10} M)	2.5
"	(2.8×10^{-9} M)	5.7
"	(2.8×10^{-8} M)	11.2
"	(2.8×10^{-7} M)	13.4
PGE_1	(2.2×10^{-8} M)	4.9
"	(2.2×10^{-7} M)	10.4
"	(2.2×10^{-6} M)	12.4
"	(2.2×10^{-5} M)	12.4

Washed horse platelets were broken by hypotonic lysis (30 min at 0° in 5 mM Tris-HCl, pH 7.6, 1 mM EDTA) followed by Polytron homogenization (30 sec). The whole homogenate was used for adenylate cyclase determinations (15). The assay mixtures contained 100 μM GTP; the assays were run for 5 min at 30°.

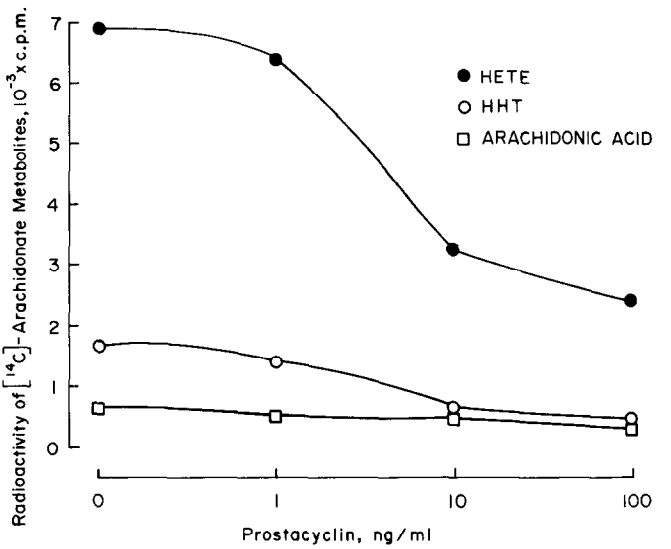


Fig. 4. Effect of prostacyclin on thrombin-induced production of oxygenated products from [^{14}C]-arachidonate-labeled platelets. All assays contained identical amounts of protein (3.2 mg protein). Preincubation with prostacyclin was for 2 min followed by 5 min with thrombin (1 unit/ml).

TABLE 2. Prior incubation with prostacyclin on thrombin action on [^{14}C]-arachidonate-labeled platelets.

Preinc. (min)	PGX	THR	PC	PI	PE	HETE	HHT	AA
50	-	-	32626	22789	28405	-	-	-
50	-	+	17321	14819	25797	18590	4215	3278
2	+	+	32571	23870	27296	3103	866	1383
10	+	+	19930	18421	28373	7967	2305	2902
50	+	+	17638	14979	26382	18035	3893	2522

3 mg of protein of washed horse platelets were used per assay. Prostacyclin was added during the indicated preincubation time and thrombin was then added for an incubation time of 5 min. Prostacyclin (PGX) was used at 1 µg/ml and thrombin (THR) at 1 unit/ml; PC, PI and PE refer to phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine, respectively. For other abbreviations see text and Fig. 2.

diesterase inhibitors, such as aminophylline and methylisobutylxanthine, which increase the endogenous concentration of cyclic AMP, also inhibit the thrombin-induced phospholipid degradation as well as decrease the formation of oxygenated products derived from arachidonic acid (Fig. 3).

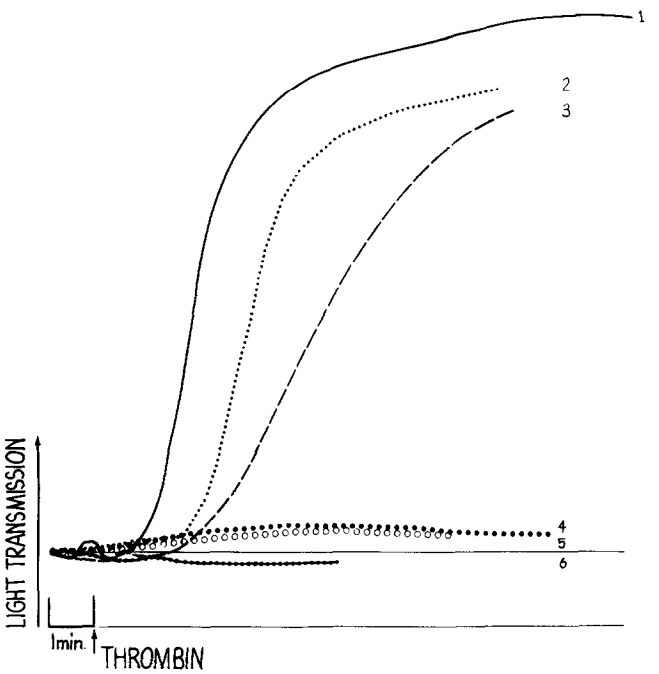


Fig. 5. Aggregometer tracings showing the action of dibutyryl cyclic AMP and cyclic AMP phosphodiesterase inhibitors on thrombin-induced aggregation. 1, thrombin, 0.67 unit/ml; 2, 0.33 mM dibutyryl cyclic AMP; 3, 0.25 mM aminophylline and 0.015 mM methylisobutylxanthine; 4, 0.66 mM dibutyryl cyclic AMP; 5, 0.5 mM aminophylline and 0.03 mM methylisobutylxanthine; 6, 0.33 mM dibutyryl cyclic AMP, 0.25 mM aminophylline, and 0.015 mM methylisobutylxanthine. Platelets were preincubated for 1 min with the compounds and then thrombin (0.67 unit/ml) and $CaCl_2$ (6.6 mM) were added.

Effects of Prostacyclin. Prostacyclin is a recently discovered prostaglandin which inhibits platelet aggregation very effectively (9). Prostacyclin is also a very potent stimulator of platelet adenylate cyclase (Table 1 and Ref. 10, 11,12). At very low concentrations prostacyclin is substantially more potent than PGE_1. When ^{14}C-labeled platelets are pretreated for one or two minutes with prostacyclin before exposure to thrombin, or when prostacyclin and thrombin are added together, there is marked inhibition of the action of thrombin on phospholipid degradation.

Prostacyclin at 0.01 to 0.1 μg/ml very effectively inhibits the thrombin-induced production of oxygenated products of arachidonic acid (Fig. 4). The effects of prior incubation with prostacyclin on phospholipid breakdown are shown on Table 2. Preincubation for 2 min is very effective but after 10 min the effects are greatly decreased and after 50 min they are completely abolished.

TABLE 3. Metabolism of exogenous [^{14}C]-arachidonic acid by horse platelets.

Additions	TXB_2	HHT	HETE
-	6672	73065	58412
Thrombin	7900	78634	61302
Dibutyryl cAMP	6896	70024	61186
Prostacyclin	6657	74844	55076

Washed horse platelets were preincubated for 10 min at 37° with or without 1 mM dibutyryl cyclic AMP and then incubated for 2.5 min at 37° with 2.0 x 10^5 c.p.m. of [1-^{14}C]-arachidonic acid (61 mCi/nmol). When thrombin (1 unit/ml) or prostacyclin (1 µg/ml) were added, these were present during the 2.5 min incubation period. Radioactivity is expressed as c.p.m. TXB_2 refers to thromboxane B_2. For HHT and HETE see legend Fig. 2.

These results are consistent with the lability of prostacyclin (9). The kinetics of stimulation of adenylate cyclase activity of platelet homogenates preincubated with prostacyclin show a time course very similar to that described in Table 2 for inhibition of phospholipase activity (11).

Effects of Dibutyryl Cyclic AMP and Cyclic AMP Phosphodiesterase Inhibitors on Platelet Aggregation. Aggregation of washed platelets by thrombin (0.67 units/ml) is completely inhibited by 0.66 mM dibutyryl cyclic AMP and also by 0.5 mM aminophylline plus 0.03 mM methylisobutylxanthine (Fig. 5). Dibutyryl cyclic AMP at 0.33 mM or 0.25 mM aminophylline with 0.015 mM methylisobutylxanthine are only partially effective, but in combination they completely abolish platelet aggregation. Prostacyclin is known to be a potent inhibitor of platelet aggregation (9).

DISCUSSION. It is well known that cyclic AMP prevents platelet aggregation (4,10,13). Malmsten et al. (4) recently proposed that the site of action of dibutyryl cyclic AMP in inhibiting platelet aggregation is on the cyclooxygenase. We have been unable to confirm these results with the systems used here. Preincubation of washed horse platelets with dibutyryl cyclic AMP (1.0 mM for 10 min) or with prostacyclin (1 µg/ml) does not show any inhibition of the cyclooxygenase products observed following a 2.5 min period of incubation with [1-^{14}C]-arachidonate (Table 3).

Dibutyryl cyclic AMP and agents which increase the concentrations of cyclic AMP, such as phosphodiesterase inhibitors and prostacyclin, inhibit platelet aggregation. The same concentrations of these compounds also prevent the thrombin-induced deacylation of arachidonate-labeled phospholipids. Since arachidonic acid is almost exclusively esterified at the 2'-acyl position of phospholipids (2,14), this deacylation might reflect a membrane-bound phospholipase A_2 activity.

It is therefore reasonable to conclude that the intracellular concentration of cyclic AMP may regulate the thrombin-induced stimulation of phospholipase activity. This action of cyclic AMP could at least partially explain the inhibitory effect of prostacyclin on platelet aggregation since the availability of arachidonic acid necessary for the formation of endoperoxides and thromboxanes would be restricted. These data of course do not exclude the possibility that cyclic AMP may have effects other than that on phospholipase activity which would independently alter platelet aggregation.

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