Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 693-700

EVIDENCE THAT CYCLIC AMP MAY REGULATE Ca^{2+}-MOBILIZATION AND PHOSPHOLIPASES IN THROMBIN-STIMULATED HUMAN PLATELETS

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Received February 23, 1983

SUMMARY: The regulation of human platelet responses by cyclic AMP (cAMP) has been investigated by measuring thrombin-stimulated serotonin release, Ca^{2+} uptake and phospholipase activity. Thrombin-induced 1,2-diacylglycerol (DG) formation as a result of phospholipase C activation was inhibited by pretreatment with dibutyryl cAMP (dbcAMP) in a dose-dependent manner. Subsequent failure to produce phosphatidic acid (PA), which is converted from 1,2-DG by phosphorylation and would serve as intracellular Ca^{2+} ionophore, appeared to parallel the decrease in Ca^{2+} uptake activity. Phospholipase A_2 activity, monitored by the production of [^{3}H]lysophosphatidylcholine and [^{3}H]lysophosphatidylethanolamine, was also suppressed by dbcAMP. These data indicate that the intracellular cAMP level may be closely associated with Ca^{2+} uptake and phospholipases activation. In addition, it is suggested that alteration of intracellular cAMP regulates phospholipase activation and consequently platelet responses, perhaps by controlling available Ca^{2+} content.

Adenosine 3',5'-cyclic monophosphate (cAMP) and Ca^{2+} have been presented to be interrelated intracellular messengers which mediate excitation-contraction and stimulus-secretion coupling in contractile and secretory cells respectively (1). The functional activity of the platelet is also closely related to the intracellular concentration of these two intracellular messengers (2). It is well established that the mobilization of intracellular Ca^{2+} plays a decisive role in platelet activation (3,4). On the other hand, it has been shown that activation is prevented or reversed by all measures which result in an increased level of cAMP within cell (5,6). This oppositing effect is obviously due to the existence in platelet of a calcium pump regulated by cAMP (7). Recent studies (8,9) have shown that cAMP enhances phosphorylation of a 22K protein (called

Abbreviations: cAMP, adenosine 3',5'-cyclic monophosphate, dbcAMP; dibutyryl cAMP, PI; phosphatidylinositol, PA; phosphatidic acid, DG; diacylglycerol, PC; phosphatidylcholine, PE; phosphatidylethanolamine, PRP; platelet-rich plasma.

0006-291X/83 $1.50

phospholamban) resulting in sequestration of free available calcium into storage sites. Thus the relationship between Ca^{2+} and cAMP in platelet can be considered as a bidirectional contol system.

It is now generally believed that phospholipid metabolism is one of the earliest and most crucial steps in the sequence of the activation responses of the platelet (10). Especially phosphatidylinositol (PI) metabolism (PI-cycle), initiated by the activation of a specific phospholipase C, has been reported to be a primary event of receptor-linked calcium mobilization (11). We (12) have recently observed that Ca^{2+} influx due to thrombin-activation is associated with production of phosphatidic acid (PA), a key intermediate of PI-cycle, and triggers the intracellular responses (e.g. phospholipase A_2 activation, arachidonic acid liberation and aggregation). In contrast to Ca^{2+} mobilization, to our knowledge, relatively little is known about the relationship between the level of another messenger cAMP and the activity of phospholipases, although many investigators (13- 15) reported that dbcAMP inhibited production of thromboxane A_2, B_2 and arachidonic acid liberation from phospholipids in platelet. In order to gain some insight into the role of cAMP in regulation of these phospholipases; phospholipase C specific for PI (16) and A_2 (17) in thrombin-stimulated human platelets, we have now investigated the effect of cAMP on activities of phospholipases by measuring their radioactive products, 1,2-DG and lysophospholipids.

MATERIALS AND METHODS

Preparation of labeled human platelets: Fresh blood from healthy volunteers was drawn into plastic tubes containing 1/6 vol of acid-citrate-dextrose (ACD) and then centrifuged at room temperature at 164xg for 10 min (12,18). The resulting supernatant,platelet-rich plasma(PRP), was incubated with either [^{14}C]5-hydroxytryptamine (serotonin) (0.2 μCi/100ml PRP), [^{3}H]arachidonic acid (50 μCi/100ml PRP) or [^{3}H]glycerol (500 μCi/100ml PRP) for 1h at 37°C. After labeling, platelets were washed two times with Tris-citrate-bicarbonate buffer (19)(containing 1mM EGTA, pH 7.4) as previously described (12,18). The washed labeled platelets were finally resuspended in Tris-citrate-bicarbonate buffer (containing 1mM $CaCl_2$, pH 7.4) to a final concentration of 2 x 10^9/ml. Suspension was at least 98% pure.

To observe the effect of dibutyryl cAMP (dbcAMP), platelets were preincubated with dbcAMP in desired concentrations at 37°C for 5min. Other additions are indicated in figures.

Release reaction: [^{14}C]Serotonin-labeled platelet suspension (0.2 ml) containing 4 x 10^8 cells was activated by different concentrations of thrombin at 37°C for appropriate incubation time. The reactions were terminated by the addition

of 0.8 ml of ice-cold 5 mM EDTA-buffer and then spun at 1,645 xg for 2 min at 4°C (12). The radioactivities in the supernatant and pellet were counted with toluene/Triton X-100/2,2'-p-phenylene-bis-(5-phenyloxazole)/2,5-diphenyloxazole (1000 ml : 500 ml : 0.2 g : 4 g) (scintilation cocktail A) (12). The percentage of serotonin release was expressed as :

[(dpm in supernatant)/(dpm in supernatant + dpm in pellet)] x 100

Measurement of $^{45}Ca^{2+}$ uptake: The $^{45}Ca^{2+}$ incorporation into platelets was observed as described previously (12). Briefly, non-labeled platelets (4 x 10^8 cells) suspended in Ca^{2+}-free buffer (0.2 ml) were stimulated with thrombin in the presence of 800 nCi of $^{45}Ca^{2+}$ and reactions were stopped as described above. After centrifugation at 4°C for 2 min, the resulting pellets were washed two times with cold 5mM EDTA. The radioactivity of cells (pellets) was determined using scintilation cocktail A.

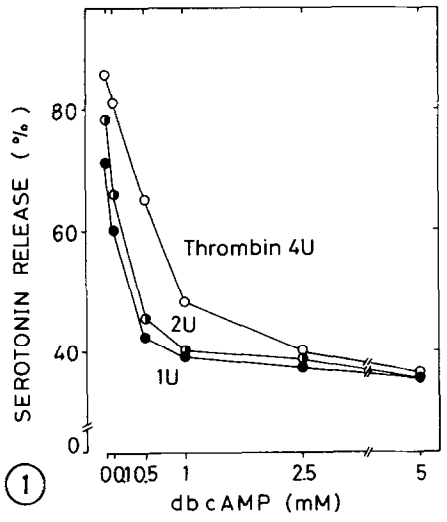
Lipid metabolism: One milliliter of [^{3}H]arachidonic acid- or [^{3}H]glycerol-labeled platelet suspension (containing 2 x 10^9 cells) was incubated with thrombin at 37°C for indicated time. The reactions were terminated by adding 4 ml of chloroform/methanol (1 : 2, v/v), and lipid extraction was carried out by a modification (18) of the procedure of Bligh & Dyer (20). The phospholipids were separated by two dimensional chromatography on silica gel 60 plates impregnated with magnesium acetate (2.5 %), using chloroform/methanol/13.5 N ammonia water (65 : 35 : 6, v/v) in the 1st dimension, and chloroform/acetone/methanol/acetic acid/water (3 : 4 : 1 : 1 : 0.5, v/v) in the 2nd dimension (21). The neutral lipids were analyzed on borate (0.4M)-impregnated silica gel 60 plates by a solvent system of chloroform/aceton (96 : 4, v/v)(22). Spots were identified by comigration with authentic standards. The areas corresponding to individual lipids were scraped into vials and the radioactivity was determined with toluen/Triton X-100/water/2,2'-p-phenylen-bis-(5-phenyloxazole)/2,5-diphenyloxazole (800 ml : 200 ml : 50 ml : 0.24 g : 3.3 g)(18).

Materials: [2-^{3}H]Glycerol (200 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (78.2 Ci/mmol) , [^{14}C]5-hydroxytriptamine (serotonin) (58.5 mCi/mmol) and $^{45}CaCl_2$ were purchased from New England Nuclear. Silica gel 60 plates were obtained from Merck. Dibutyryl adenosine cyclic-3',5'- monophosphate and thrombin were products of Sigma and Mochida Pharmaceutical Co., respectively. All other chemicals were of reagent grade.

RESULTS

Inhibition by cAMP of thrombin-induced serotonin release : [^{14}C]Serotonin-labeled platelets (4 x 10^8 cells) were preincubated with various concentrations of dbcAMP for 5 min, and then activated by thrombin for 60 sec. As shown in Fig.1, dbcAMP produced a concentration-dependent inhibition of the thrombin-induced serotonin release. This inhibitory effect of dbcAMP on release reaction was observed at all thrombin concentrations tested (4, 2 and 1 units per 2 x 10^9 platelets).

Effect of dbcAMP on $^{45}Ca^{2+}$ influx: Fig.2 demonstrates thrombin-induced Ca^{2+} uptake activity into dbcAMP-loaded platelets. When thrombin was added to platelets in the presence of $^{45}Ca^{2+}$, an accumulation of cellular $^{45}Ca^{2+}$ took place and reached a maximum with further incubation, as observed in our earlier study



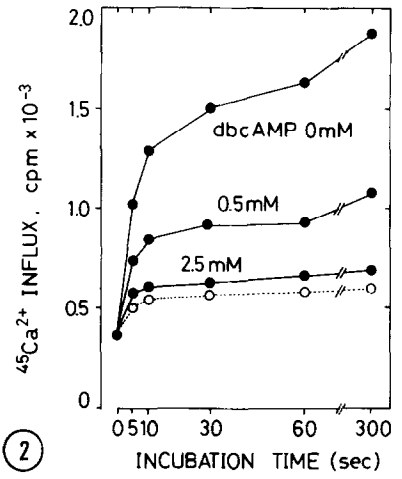


Fig.1 Effects of dbcAMP on thrombin-induced serotonin release. [^{14}C]Serotonin-labeled platelets (4 x 10^8 platelets) were pretreated with different concentrations of dbcAMP for 5 min at 37°C, and then stimulated with thrombin 4(o), 2 (⊙) and 1 (●) units/2 x 10^9 platelets for 60 sec at 37°C. Each value represents mean of triplicate determinations.

Fig.2 Effects of dbcAMP on thrombin-stimulated $^{45}Ca^{2+}$ uptake. Washed platelets (4 x 10^8 platelets) were preincubated with dbcAMP (0 to 2.5 mM) for 5 min at 37°C. Thrombin (2 units/2 x 10^9 platelets) plus $^{45}Ca^{2+}$ (800 nCi) were then added for indicated time at 37°C. In control experiments (o····o), thrombin and dbcAMP were not added. Each value represents mean of triplicate determinations.

(12). Preincubation with dbcAMP, however, suppressed the $^{45}Ca^{2+}$ uptake. At the concentration of dbcAMP 2.5 mM the progressive enhancement of $^{45}Ca^{2+}$ uptake was almost completely abolished, indicating the equal $^{45}Ca^{2+}$ uptake to the unstimulated control platelets. In this case serotonin release was also reduced by about 35% (Fig.1). Essentially similar results were seen even by washing to eliminate extracellular dbcAMP after preincubation (data not shown).

Effect of dbcAMP on thrombin-stimulated phospholipases activities: After preincubation with dbcAMP, [^{3}H]arachidonic acid-labeled platelets were activated by thrombin for 10 sec and the release of [^{3}H]arachidonic acid was determined. [^{3}H]Arachidonic acid liberation in response to thrombin was decreased in dbcAMP-treated platelets in a dose-dependent manner (data not shown), suggesting inhibitory action of dbcAMP on phospholipases.

The production of 1,2-DG in platelets prelabeled with [^{3}H]arachidonic acid was used to detect and measure phospholipase C activity. Rittenhouse-Simmons

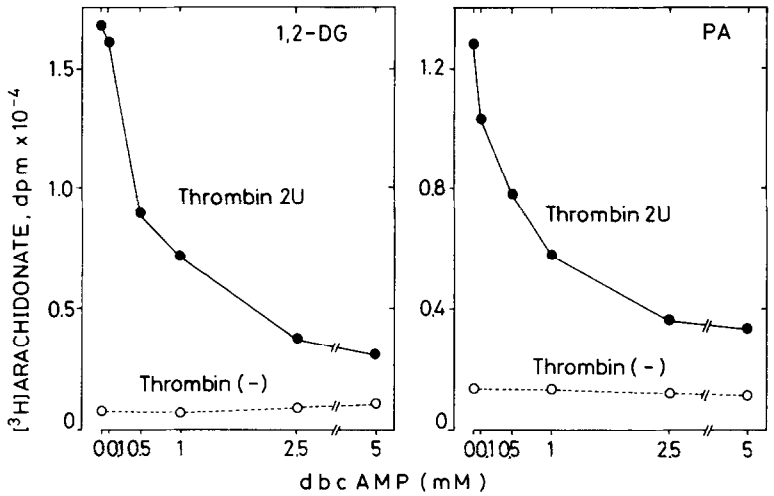


Fig.3 Effects of dbcAMP on thombin-induced production of 1,2-diacylglycerl (DG) (left) and phosphatidic acid (PA) (right). [^{3}H]Arachidonic acid-labeled platelets (2 x 10^9 platelets) were preincubated with different concentrations of dbcAMP for 5 min at 37°C. They were then stimulated with thrombin (2 units/2 x 10^9 platelets) (●) at 37°C for 10 sec. o---o, controls in which thrombin was not added Each value is the mean of two different experiments performed in duplicate.

(16) and we (23) have reported that upon human platelet activation by thrombin the level of 1,2-DG markedly and transiently increased within 10 sec. Fig.3 demonstrates that peincubation of platelets with dbcAMP results in a marked decrease in thrombin-induced 1,2-DG formation in a dose-associated fashion. In addition, phosphatidic acid (PA), converted from 1,2-DG by its kinase, also failed to increase by raised dbcAMP concentration. These findings are agreeable with the results of Billah et al.(24) who observed the inhibitory effect of dbcAMP on phospholipase C in deoxycholate-treated horse platelets.

The rise in radioactive lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) from [^{3}H]glycerol-labeled platelet phospholipids in response to thrombin was measured as phospholipase A_2 activity. As shown in Fig.4, dbcAMP-pretreatment inhibited formation of these two lysophospholipids. The reduced production of lysophospholipids due to decreased hydrolysis of PC and PE appeared to be dependent on the concentration of dbcAMP. At 2.5mM dbcAMP, production of these lysophospholipids was approximately two-thirds inhibited (Fig.4).

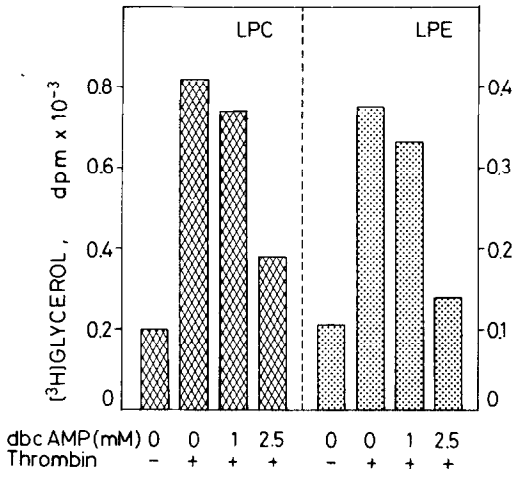


Fig.4 Effects of dbcAMP on thrombin-induced lysophospholipids formation. [^{3}H]Glycerol-labaled platelets (2 x 10^9 cells) were preincubated with concentrations dbcAMP from 0 to 2.5mM for 5 min at 37°C. they were then with stimulated with thrombin (2 units/2 x 10^9 cells) as indicated. Each value is the mean of two different experiments performed in duplicate. LPC; lysophosphatidylcholine, LPE; lysophosphatidylethanolamine.

DISCUSSION

Dibutyryl cAMP-preincubation inhibited many responses of human platelets to thrombin, and this effect was not affected even by removing extracellular dbcAMP. The data obtained here, showing concentration-dependent inhibition of lysophospholipids and 1,2-DG formation by cAMP, suggest that phospholipase A_2 and C in platelets may be regulated by the intracellular level of cAMP. The mechanism by which cAMP inhibits activation of phospholipases is unknown. However, both enzymes are known to require Ca^{2+} for activation (25,26). Käser-Glanzmann et al.(7, 8) have reported that increased intracellular cAMP accelerates the uptake of free Ca^{2+} into storage site(s) (e.g. dense tubular system) through the phosphorylation of 22K protein 'phospholamban' by cAMP-dependent protein kinase. It can be thus considered that the depletion of cytosolic available Ca^{2+} resulted in failure to activate phospholipase C (Fig.3). This consequently caused the decrease in production of PA (Fig.3), which is converted from PI by time-sequential action of phospholipase C and DG kinase (11,18, 21,24). It is reported by Serhan et al. (27) and Ohsako & Deguchi (28) that PA could play as intracellular Ca^{2+} ionophore. Our preceding report (12)

has described that upon platelet activation by thrombin, a marked enhancement in Ca^{2+} influx occurs in parallel with PA production as a result of phospholipase C activation, which commences prior to the onset of phospholipase A_2 activation and subsequent liberation of arachidonic acids. The reduced PA production may explain in part an inhibitory effect of dbcAMP on the repressed Ca^{2+} influx activity (Fig.2).

It has been shown that higher concentration of Ca^{2+} and production of PA are necessary for phospholipase A_2 activation (12,25,26,29), which suggests that phospholipase A_2 activity may be affected via dbcAMP by a different mechanism from that for phospholipase C activation. Hirata (30) has resently described a phospholipase A_2- inhibitory protein 'lipomodulin'. Phosphorylation by cAMP-dependent protein kinase of this protein causes loss of its inhibitory action against phospholipase A_2, which takes place in a Ca^{2+}-dependent manner. Thus, together with lack of PA, 'lipomodulin' and phospholamban(-like protein) may also play some role in phospholipase A_2 regulation.

ACKNOWLEDGEMENT

This study was supported in part by research grants from Ministry of Education, Culture and Science, and from Ministry of Health and Welfare of Japan, for the project of platelet preservation.

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