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ADENOSINE-3′,5′-DIPHOSPHATE AND COENZYME A—EFFECTS ON PLATELET FUNCTION*

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Abstract—The effects *in vitro* of adenosine-3′,5′-diphosphate and coenzyme A on human platelet aggregation and [^{14}C]hydroxytryptamine release were studied. Whereas coenzyme A, at concentrations between 0.06 and 0.24 mM, inhibited the aggregation of platelets induced by ADP, adenosine-3′,5′-disphosphate, which is part of the coenzyme A molecule, blocked both ADP- and thrombin-induced platelet aggregation. The ADP-induced platelet aggregation was inhibited at a lower adenosine-3′,5′-diphosphate (10–20 μM) concentration than was thrombin-induced aggregation (60–200 μM). Adenosine-3′,5′-diphosphate also inhibited [^{14}C]adenosine uptake by platelets in a concentration-dependent manner (20–200 μM), but only to a maximum of 40 per cent of total [^{14}C]adenosine radioactivity incorporated into the platelets. The inhibitory effect of adenosine-3′,5′-diphosphate and coenzyme A on the release reaction was further documented by the decrease in aggregation-induced release of [^{14}C]5-hydroxytryptamine from prelabeled platelets into the medium. The extent of inhibition caused by coenzyme A and adenosine-3′,5′-diphosphate was found to depend upon the concentration of inhibitor and incubation time. If these agents are indeed inhibitors of platelet aggregation, then they may serve as valuable tools to study platelet function.

The aggregation of platelets *in vitro* and *in vivo* by adenosine diphosphate (ADP) can be inhibited by compounds related to ADP, particularly adenosine and some of its analogues [1–9]. Although coenzyme A (Co A) and adenosine-3′,5′-diphosphate (3′,5′-ADP) contain adenosine as part of their structures [10], the effect of these agents on aggregation of platelets had not yet been investigated. In the present paper, we report the effect *in vitro* of Co A and 3′,5′-ADP on platelet aggregation and [^{14}C]5-hydroxytryptamine (5-HT) release from platelets induced by the addition of ADP, thrombin, collagen and epinephrine to platelet-rich plasma (PRP).

MATERIALS AND METHODS

Materials. Bovine collagen was obtained from Worthington Biochemicals, Freehold, NJ, and bovine thrombin from Parke Davis & Co., Detroit, MI. Coenzyme A, Li_3, 2 H_2O and 3′,5′-ADP Li_3, 3 H_2O were from P. L. Biochemicals Inc., Milwaukee, WI; N^6-2′-*O*-dibutyryl cyclic 3′,5′-AMP (dibutyryl cyclic 3′,5′-AMP) adenosine, ADP and *l*-epinephrine were from Sigma Chemical Co., St. Louis, MO; and 5-hydroxytryptamine-2[^{14}C] (57 mCi/m-mole) and uniformly labeled [^{14}C]adenosine (557 mCi/m-mole) were obtained from Amersham/Searle Corp., Arlington Heights, IL.

Methods. Venous blood from healthy volunteers who had not taken any medication during 2 weeks preceding the blood collection was drawn into 0.1 volume of 3.8 per cent by wt sodium citrate and centrifuged at 225 *g* for 10 min to yield platelet-rich plasma. The platelet release reaction was measured by the method of Jerushalmy and Zucker [11], monitoring the release of [^{14}C]5-HT from platelets prelabeled with this amine [12]. The PRP (40 ml) was incubated with 1 μM [^{14}C]5-HT for 15 min at 37°. Aliquots of 0.8 ml PRP were used to study the release reaction under different test conditions.

Platelet aggregation studies were performed according to the method of Born [1], using a dual channel platelet aggregometer (Payton Associates, Buffalo, NY). The aggregation response of platelets to ADP, collagen, epinephrine and thrombin was recorded on a Riken Denshi (Payton Associates) linear recorder. The sample of PRP (0.8 ml), in a final volume of 1.0 ml, was placed in a siliconized 1.5-ml cuvette containing a stirring bar and test reagent (Co A, 3′,5′-ADP, dibutyryl cyclic 3′,5′-AMP or adenosine) of a desired concentration. The cuvette was placed in the aggregometer, and ADP solution to give a final concentration of 10 μM was added to initiate the aggregation response. In other experiments, epinephrine (3 μg/ml), collagen (6.12 μg/ml) or thrombin (0.04–0.50 NIH units/ml) was added to PRP to induce a platelet aggregation response. The aggregation of platelets containing test reagents was compared to that observed in appropriate controls substituted with 0.85% NaCl.

The adenosine uptake by platelets was determined with 0.8 ml PRP containing 0.1 μM [^{14}C]adenosine in a final volume of 1.0 ml. The samples were incubated for 15 min at 37° and centrifuged at 12000 *g* for 2 min at 27°. The total radioactivity in the platelet pellet and supernatant solution of each sample was determined and the per cent of total radioactivity in the platelet pellet was

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calculated. Similar adenosine uptake measurements were made with PRP samples incubated with varying concentrations of Co A and 3′,5′-ADP. The radioactivity determinations were made using an Intertechnique scintillation spectrometer, model SL40. The counting efficiency was 70 per cent.

RESULTS

Effect of coenzyme A on platelet aggregation. The inhibitory effect *in vitro* of Co A upon ADP-induced platelet aggregation is shown in Fig. 1. The inhibitory potency of Co A increased with increasing concentration. Co A (0.06 to 0.24 mM) inhibited the "secondary" as well as "primary" aggregation of platelets by ADP. At higher concentrations (≥ 0.50 mM), the aggregation of platelets by ADP was completely abolished. However, Co A did not affect aggregation of platelets induced by aggregating agents such as epinephrine, collagen and thrombin.

Effect of 3′,5′-ADP on platelet function. The aggregation of platelets induced by ADP and thrombin in control and 3′,5′-ADP-treated PRP is shown in Fig. 2. The inhibitory potency of 3′,5′-ADP was found to depend upon the concentration of the inhibitor and the incubation time. At a low concentration of 10 μM, 3′,5′-ADP inhibited both the "secondary" and "primary" aggregation of platelets, whereas at concentrations above 20 μM, aggregation of platelets by ADP was almost completely blocked. Adenosine at a concentration of 10 μM inhibited ADP-induced platelet aggregation by about 55 per cent after 5 min of incubation and the inhibitory effect was almost abolished when the incubation was extended up to 30 min. However, incubation of PRP with 3′,5′-ADP (10 μM) for 30 min before the addition of ADP produced about 70 per cent inhibition of aggregation, which is in striking contrast to the loss of inhibition seen with adenosine. The 3′,5′-ADP, at concentrations of

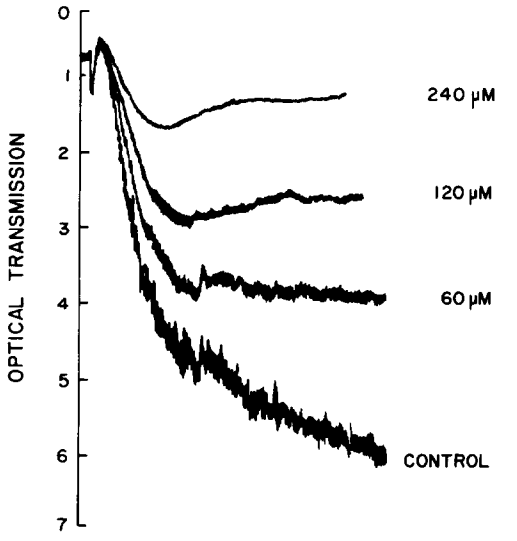

Fig. 1. Inhibition by coenzyme A of platelet aggregation induced by 10 μM ADP. Aggregation is indicated by increased light transmission through stirred (900 rev/min) platelet-rich plasma (PRP) at 37°. Normal saline (control) or coenzyme A of varying concentrations was incubated with PRP (0.80 ml) at 37° for 20 min before the addition of ADP.

≥ 60 μM, completely inhibited thrombin-induced platelet aggregation. The concentration of 3′,5′-ADP required to completely abolish the thrombin-induced aggregation varied (60–200 μM) depending upon the individual donor's platelets. Unlike Co A, 3′,5′-ADP inhibited both ADP and thrombin-induced platelet aggregation of human platelets. However, 3′,5′-ADP did not show any effect on the aggregation of platelets by epinephrine and collagen. The effect of 3′,5′-ADP on the release of [^{14}C]5-HT from platelets treated with aggregating agents was also tested. A concentration-dependent inhibition of

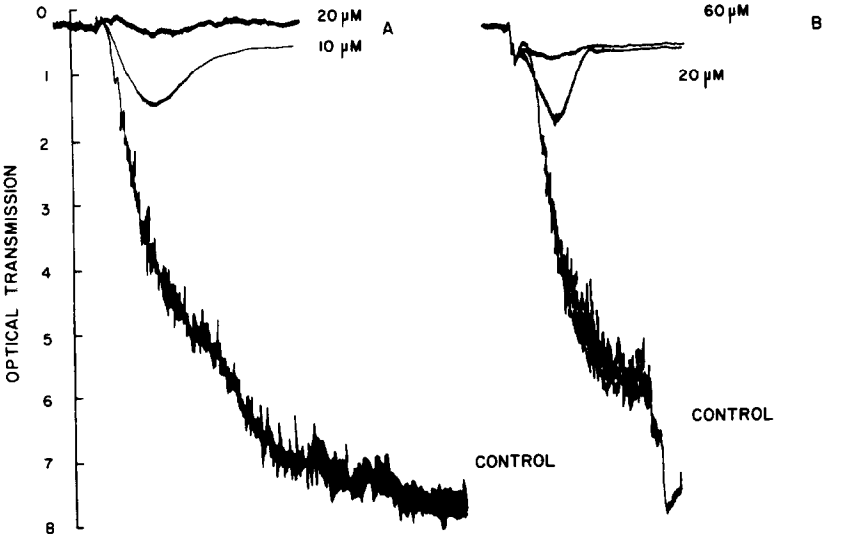

Fig. 2. Inhibition by 3′,5′-ADP of platelet aggregation induced by 10 μM ADP (A) and 0.5 units of thrombin (B). Aggregation is indicated by an increase in optical transmission. Portions of 0.8 ml PRP were incubated with varying concentrations of 3′,5′-ADP for 30 min at 37° before the addition of the aggregating agent.

Table 1. Effect of 3′,5′-ADP concentration on the release of [^{14}C]5-HT from platelets*

Aggregating agent	3′,5′-ADP concn. (μM)	[^{14}C]5-HT released (%)
ADP (5 μM)	0	35 ± 6.0
	4	15
	40	0
Thrombin (0.3 units/ml)	0	54 ± 5.0
	4	50
	40	9

* Platelet suspensions prelabeled with [^{14}C]5-HT were treated with 3′,5′-ADP for 30 min at 37°. The results are the means of three experiments for the 3′,5′-ADP-containing platelet suspension and the means of five experiments ± 1 S. E. for controls not treated with 3′,5′-ADP.

[^{14}C]5-HT release process from platelets by 3′,5′-ADP was observed (Table 1). Complete inhibition of [^{14}C]5-HT release was obtained at 3′,5′-ADP level of 40 μM.

The per cent of total [^{14}C]adenosine radioactivity incorporated into platelets was determined and compared with samples incubated with varying concentrations of Co A and 3′,5′-ADP. Increasing concentrations of Co A slightly increased the incorporation of [^{14}C]adenosine radioactivity into platelets. The 3′,5′-ADP at concentrations between 20 and 200 μM, on the other hand, progressively inhibited the incorporation of adenosine radioactivity into platelets in a concentration-dependent manner (Fig. 3). However, at all chosen concentrations of ⩾ 200 μM, 3′,5′-ADP exerted a maximum of 40 per cent inhibition of [^{14}C]adenosine uptake by platelets.

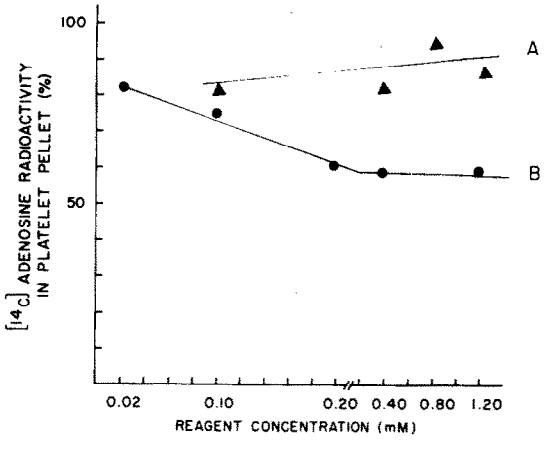

Fig. 3. Effect of varying concentrations of coenzyme A and 3′,5′-ADP on [^{14}C]adenosine uptake by the platelets in platelet-rich plasma. Platelets were incubated with the reagents for 30 min prior to the addition of [^{14}C]adenosine to PRP. The effect of coenzyme (▲) and 3′,5′-ADP (●) on the incorporation of [^{14}C]adenosine radioactivity into platelets is shown. Each point is an average value of five experimental determinations.

DISCUSSION

These findings demonstrate that 3′,5′-ADP *in vitro* inhibits the "secondary" as well as the "primary" wave of ADP- and thrombin-induced platelet aggregation and the release of prelabeled [^{14}C]5-HT from platelets. Whereas the secondary aggregation is thought to be due to expulsion from platelets of a portion of their content of adenine nucleotides, especially ADP, the primary phase of platelet aggregation appears to be independent of such a "release reaction" [13]. These phenomena are probably inhibited by 3′,5′-ADP as a result of its binding to platelets. It has been shown that the aggregation of platelets by ADP is partially prevented by adenosine triphosphate (ATP) and by adenosine monophosphate (AMP) and by adenosine, the last being the most effective [1, 2]. Preincubation of human PRP with 3′,5′-ADP, up to 30 min before the addition of ADP, did not reduce the inhibitory potency of 3′,5′-ADP, which is in contrast to the results of preincubating adenosine in human PRP. The ability of adenosine to inhibit ADP-induced platelet aggregation decreased with increasing periods of incubation, while that of 3′,5′-ADP increased. The most probable explanation of this difference between adenosine and 3′,5′-ADP is that the adenosine was increasingly inactivated by plasma deaminase [8], whereas the 3′,5′-ADP was not. It was further shown that the inhibitory effect of AMP on ADP-induced platelet aggregation is lost during prolonged incubation [4], presumably as a consequence of the hydrolysis of AMP by plasma 5′-nucleotidase to adenosine and a concomitant deamination of adenosine to inosine [5]. These findings indicate that 3′,5′-ADP was probably not acted upon either by plasma adenosine deaminase or 5′-nucleotidase. Whether the increased inhibitory potency of 3′,5′-ADP with incubation time was due to conversion of 3′,5′-ADP in plasma into more active form than the parent compound or because of other factors, such as a lag in the uptake and interaction of 3′,5′-ADP with an intracellular site of action on the platelets, are the aspects that remain to be determined. A similar explanation may pertain with regard to the action of Co A on platelet aggregation.

We found that the inhibitory potency of 3′,5′-ADP on ADP-induced platelet aggregation was about 10-fold higher than that of dibutyryl cyclic AMP and Co A [14]. The possibility that the lithium ions of 3′,5′-ADP inhibited ADP-induced platelet aggregation was ruled out, since we found in support of the observations made by others that these ions can inhibit platelet aggregation at concentrations above 5 mM [15]. It appears that the effect of ADP in bringing about platelet aggregation and the effect of 3′,5′-ADP in inhibiting it are highly specific events. This conclusion is supported by the very low concentration at which 3′,5′-ADP acts to inhibit platelet aggregation. The differences in the inhibitory potency of 3′,5′-ADP on thrombin-induced platelet aggregation suggest that the mechanism whereby 3′,5′-ADP inhibits ADP-induced platelet aggregation is different from the mechanism of inhibition of thrombin-induced aggregation. Perhaps, 3′,5′-ADP directly inhibits the activity of thrombin or the

binding of thrombin to platelets or the release of ADP from platelets.

While no definite statements can be made at the present time about the inability of 3′,5′-ADP to block the epinephrine- and collagen-induced platelet aggregation, at least it can be suggested that this may be because 3′,5′-ADP fails to inhibit the alternate mechanism of platelet aggregation involving the liberation of archidonate from platelet phosphoglycerides during platelet response to these agents and the subsequent conversion of arachidonate to prostaglandin endoperoxides [16–18], which by themselves can cause aggregation of platelets even in the absence of releasable ADP from platelets [19, 20].

Platelets appear to possess extracellular membrane receptor for adenosine which is distinct from that for ADP, and it regulates the inhibition of platelet function by adenosine by activating platelet adenylate cyclase [21]. It is possible that the partial inhibitory potency of 3′,5′-ADP on adenosine uptake by platelets can result in the accumulation of extracellular adenosine which in turn can activate platelet adenylate cyclase. A recent report suggests that the uptake of adenosine by platelets involves two pathways, one with a low (major) and another a high (minor) K_m system [22]. Since 3′,5′-ADP contains an adenosine part in its structure, it is possible that 3′,5′-ADP also interacts with the adenosine receptor sites of the cells and thereby affects the uptake of adenosine by platelets.

The fact that 3′,5′-ADP inhibits both ADP- and thrombin-induced platelet aggregation and also the adenosine uptake by platelets, while Co A acts only on ADP-induced platelet aggregation, not only indicates a distinction between the modes of action of these inhibitors on platelet aggregation, but also suggests that the nature of the phosphate group at the 5-position of the ribose moiety probably determines the specificity of the inhibitor as a modifier of platelet function.

Since recent investigations have indicated an important role for the cyclic nucleotides in the functioning of platelets [23–26], it will be of great interest to learn how 3′,5′-ADP can alter the level of cyclic nucleotides in the platelets. Whether the effect of 3′,5′-ADP on aggregation of platelets is due to the increased level of platelet cyclic AMP, with a concomitant conversion of cyclic AMP to 3′,5′-ADP by a platelet phosphorylating enzyme (if any), or is due to simple accumulation of the inhibitor, with a subsequent conversion of 3′,5′-ADP to cyclic AMP, are the aspects that remain to be determined.

Since 3′,5′-ADP and Co A can strongly inhibit platelet function, they can probably serve as useful tools to understand the mechanisms of ADP- and thrombin-induced platelet aggregation.

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