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Inhibition of cyclic AMP phosphodiesterase activity of human blood platelet membrane by ADP

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Purified human blood platelet membrane showed the presence of one low K_m (1.1 μM) and one high K_m (5.0 μM) cyclic AMP phosphodiesterase(s). Incubation of platelet-rich plasma or gel-filtered platelets with ADP (4.0 μM), a well-known platelet aggregating agent, resulted in the inhibition of phosphodiesterase activity of the isolated membrane by 25% in 5 min at 23°C. A Lineweaver-Burk plot of the enzymic activity of the membrane preparation showed that ADP specifically inhibited the low K_m (1.1 μM) phosphodiesterase by reducing the V_{max} from 241 to 176 pmol/mg per min with concomitant lowering of K_m to 0.5 μM. In contrast, neither the high K_m (5.0 μM) enzymic activity of the membrane preparation nor the phosphodiesterase activities of the cytosolic fraction of the ADP-treated platelets was affected. This effect of ADP, which was independent of platelet aggregation, reached maximal level within 5 min of incubation. When platelet-rich plasma was incubated longer in the presence of the nucleotide, the inhibition of phosphodiesterase activity began to decrease, and after 20 min of incubation approx. 90% of the original enzymic activity was regained. The incubation of platelet-rich plasma with 4.0 μM ADP also increased the cyclic AMP level to twice the basal level. The effect of ADP on the phosphodiesterase activity could be demonstrated only by incubating the intact platelets with the nucleotide. The treatment of isolated membrane from platelets, previously unexposed to ADP, with the nucleotide did not inhibit the enzymic activity. The inhibition of phosphodiesterase by the nucleotide in the absence of stirring, as expected, resulted in the inhibition of platelet aggregation when these cells were subsequently stirred with 1-epinephrine or an increased concentration of ADP.

Introduction

Aggregation of human blood platelets by aggregating agents like ADP, 1-epinephrine, collagen or thrombin is believed to be a critically important event both in normal blood coagulation and in the development of thrombosis (see Ref. 1 for a review). The aggregation process is mediated, in part, through intracellular synthesis of prostaglandin endoperoxides and thromboxane A_2 [2]. The homeostasis is maintained through the inhibition of platelet aggregation by several extra humoral factors including prostacyclin and prostaglandin E_1 through intracellular increase of cyclic AMP level [3,4]. However, the inhibition of platelet aggregation by other extra humoral factors, most notably by factor Xa is also known to occur through a cyclic-AMP-independent pathway [5]. The increase of cellular concentration of cyclic AMP level, which leads to the inhibition of platelet aggregation, could be achieved either through the activation of adenylate cyclase or through the inhibition of cyclic AMP phosphodiesterase (hereafter referred to as phosphodiesterase; Ref. 6).

ADP is classically known as one of the physiologically important platelet aggregating agents [7,8]. Although the nucleotide has been shown to mediate its effect through its receptors on platelet surface, the biochemical mechanism of ADP-induced platelet aggregation is not well-understood (see Ref. 9 for review). As with the other aggregating agents, the platelet suspension must be vigorously stirred with ADP to aggregate these cells. The physiological relevance of such stirring is not apparent, however.

In this paper we report that the incubation of platelet-rich plasma with ADP, in the absence of stirring, results in the increase of cellular cyclic AMP level through the specific inhibition of a low K_m phosphodiesterase(s) in the platelet membrane and subsequent inhibition of platelet aggregation induced by the agonist itself.

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Materials and Methods

Materials. [2,8-^{3}H]Adenosine 3',5'-cyclic phosphate (spec. act., 38 Ci/mmol) were obtained from New England Nuclear, Wilmington, DE. Myokinase and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis MO. All other chemicals used were of analytical grade.

Preparation of platelet-rich plasma. Blood was collected from normal healthy donors who had not taken any medication for at least 2 weeks before the blood donation. The blood samples were collected in 13 mM trisodium citrate (9:1, v/v). Platelet-rich plasma was obtained by centrifuging blood at $200 \times g$ for 15 min at 23°C. Gel-filtered platelets were prepared from platelet-rich plasma by using a Sepharose 6B column equilibrated with Tyrode's buffer (without Ca^{2+}) (pH 7.0) as described before [5].

Aggregation of platelets. Aggregation of platelets by ADP or epinephrine was studied at 37°C in an aggregometer (Chronolog, Broomal, PA) by stirring the platelet-rich plasma at 1200 rpm. The apparatus was calibrated so that the difference in light transmittance between platelet-rich plasma and platelet-poor plasma was defined as 100%. Complete aggregation was defined as an increase of more than 71% in light transmittance [10]. In certain phases of the work it was necessary to incubate platelets with aggregating agents without stirring and after incubation for a specified time, the aggregation was initiated by stirring.

Preparation of platelet membrane. Platelet-rich plasma was centrifuged at $3000 \times g$ for 15 min at 4°C. The pellet thus obtained was resuspended in equal vols of Tyrode's buffer (pH 7.0) containing 5 mM $MgCl_2$ and washed twice at 0°C by centrifugation. After the final washing, platelets were resuspended in the same buffer and disrupted by freezing and thawing using liquid N_2 [11]. The disrupted mass was centrifuged at $30000 \times g$ for 30 min in a Beckman ultracentrifuge (model L 350) at 0°C as described before [11]. The membrane pellet was resuspended in the same buffer and washed twice at 0°C as described above. After the final washing, the membrane was resuspended in the assay buffer (described below) at a protein concentration of 5–8 mg/ml.

Assay of cyclic AMP phosphodiesterase activity. Cyclic AMP phosphodiesterase activity of the platelet membrane preparations was determined by the rate of hydrolysis of cyclic AMP. A reaction mixture containing [^{3}H]cyclic AMP (0.1–10 μM) was incubated in 25 mM Tris-HCl buffer (pH 7.4), containing 5 mM $MgCl_2$ and approx. 70 μg of platelet membrane protein in a total vol of 200 μl at 23°C. The reaction was terminated by adding 0.2 ml of 0.2 M $ZnSO_4$, followed by 0.2 ml of 0.2 M $Ba(OH)_2$. After vigorous stirring for 1 min, the precipitate was separated by centrifugation at $8000 \times g$ for 4 min and the radioactivity of the supernatant was measured. The amount of cyclic AMP hydrolyzed was calculated from the rate of decrease in radioactivity in the supernatant [10]. The radioactivity was measured in a Searle scintillation counter (Isocap/300) with 60% efficiency for tritium. Protein was determined according to Lowry et al. [12] using bovine serum albumin as the standard. The K_m and V_{max} values of the membrane and cytosolic phosphodiesterase were determined by a Lineweaver-Burk plot using micro computer analysis (Enzfitter, Biosoft, Elsevier, Cambridge, U.K.).

Assay of adenylate cyclase. Adenylate cyclase activity of the platelet membrane preparation was determined by incubating 1.0 mM ATP containing [α-^{32}P]ATP ($1.2 \cdot 10^6$ dpm); 2.0 mM $MgCl_2$, 10 mM theophylline, 1 mM creatine phosphate, 1 unit of creatine phosphokinase and 25 mM Tris-HCl buffer (pH 7.5) in a total vol of 100 μl. Reactions were initiated by the addition of 20 μl of platelet membrane suspension containing 150 μg of protein and incubated at 23°C for 10 min. Reactions were terminated by the addition of 0.1 ml 1% SDS. The radioactive cyclic AMP was separated according to Salomon et al. [13]. Unlabelled cyclic AMP (1.0) mM) was added to the reaction mixture at termination to facilitate the recovery of the radionucleotide.

Determination of platelet cyclic AMP content. Platelet-rich plasma (5 ml) was incubated with 4.0 μM ADP for 5 min at 37°C without stirring. After incubation, the platelets were isolated by centrifugation at $8000 \times g$ at 23°C and washed twice with equal vols of Tyrode's buffer and the cyclic AMP contents were determined by the protein kinase method [14]. Control experiments were carried out in the absence of added ADP under identical conditions.

Determination of ADP. Degradation of ADP by platelets during the incubation of the nucleotide with platelet-rich plasma was assessed by measuring the amount of ADP in the plasma supernatant using the myokinase method according to the manufacturer instruction. Typically, platelet-rich plasma was incubated with 4 μM ADP at 37°C without stirring. After 5 min of incubation, plasma supernatant was collected by centrifugation at $8000 \times g$ at 23°C. The amount of ADP in the supernatant was measured by converting ADP into ATP by myokinase and subsequently measuring ATP by hexokinase and glucose-6-phosphate dehydrogenase-coupled reaction. Typically, 0.2 ml of the supernatant plasma was incubated with 2.3 mM β-NADP, 9.9 mM $MgCl_2$; 9.9 mM D glucose, 1 unit each of hexokinase and glucose-6-phosphate dehydrogenase in a total vol of 0.7 ml for 5 min at 37°C. The reaction was initiated by adding 10 units of myokinase. The amount of ADP was calculated from the increase of absorbance at 340 nm due to the production of NADPH.

Statistical analysis. Results, where appropriate, are shown as means ± standard deviation (S.D.), data were

analyzed by a paired Student's 't' test for significance (P).

Results

Effect of incubation of plate-rich plasma with ADP on phosphodiesterase activity

Incubation of platelet-rich plasma with ADP in the absence of stirring resulted in a gradual inhibition of phosphodiesterase activity in the isolated platelet membrane fraction, and after 5min of incubation, the enzymic activity was maximally inhibited by 25 ± 3%, $n = 15$) compared with the control ($p < 0.001$; Fig. 1). On further incubation, however, the enzymatic activity began to increase and after 20 min of incubation, the phosphosiesterase activity was restored by nearly 90 ± 3% compared to control.

The inhibitory effect of ADP on the platelet membrane phosphodiesterase was specific in that ATP, or AMP, or adenosine at similar concentration (4.0 μM) did not inhibit the enzymic activity. The extent of inhibition of the membrane phosphodiesterase activity was directly related to the amount of ADP added to the platelet-rich plasma and the enzymic activity was maximally inhibited by 4–6 μM of the nucleotide concentration (Fig. 2). This effect of ADP on the membrane phosphodiesterase activity was not mediated through the plasma components of the platelet preparation, since the nucleotide also inhibited the enzymic activity by 25% in 5 min at 37°C when gel-filtered platelets were used instead of platelet-rich plasma (data not shown). However, the inhibitory effect of ADP on the phosphodiesterase activity could only be demonstrated by incubating intact platelets with the nucleotide. Addition

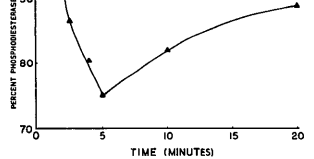

Fig. 1. Inhibition of phosphodiesterase activity of platelet membrane by ADP. Platelet-rich plasma was incubated with 4 μM of either ADP, ATP, AMP or adenosine for various times as indicated. After incubation, the platelets were isolated and the membrane fractions were prepared. The phosphodiesterase activities of the membrane fractions were determined as described in the Materials and Methods. ADP (▲), ATP (△), AMP (○), adenosine (●). Each point represents the mean of four experiments each in triplicate.

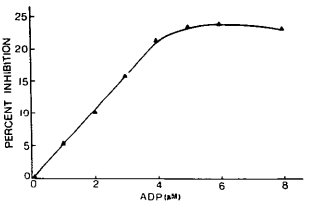

Fig. 2. Effect of increasing concentrations of ADP on the inhibition of platelets membrane phosphodiesterase. Platelet-rich plasma was incubated with various amounts of ADP as indicated for 5 min. After incubation, the phosphodiesterase activity of the membrane preparation was determined. Each point represents the mean six different experiments each in triplicate.

of ADP (4.0 μM) to the isolated membrane from platelets which had not been previously treated with the nucleotide produced no effect on the enzymic activity. While in the control membrane preparation the rate of cyclic AMP hydrolysis was 180 ± 25 pmol/min per mg, the same membrane preparation treated with 4.0 μM ADP hydrolysed 182 ± 30 pmol cyclic AMP/min per mg (P = non significant).

Effect of platelet aggregation on the inhibition of membrane phosphodiesterase by ADP

In the above experiments, the platelets in the absence of stirring, were not aggregated when these cells were incubated with ADP. Since the aggregation of platelets during the incubation with ADP might modify the effect of the nucleotide on the phosphodiesterase activity, in a different experiment platelet-rich plasma was stirred (1200 rpm) with 4.0 μM ADP for 5 min at 37°C. The phosphodiesterase activity of the 'membrane' fraction of the aggregated platelets showed similar degree of inhibition (23 ± 4%) when compared with that of the membrane fraction of the platelets incubated with the similar concentration of ADP in the absence of stirring (25 ± 3%).

Specificity of the inhibitory effect of ADP on various forms of phosphodiesterase(s) in platelets

Since phosphodiesterases are known to occur in multiple forms with different K_m values [10], kinetic studies were carried out to determine the specificity of the inhibitory effect of ADP on the different phosphodiesterase(s) in platelets. A Lineweaver-Burk plot of the hydrolysis of cyclic AMP by the membrane and cytosolic fractions from platelets showed the existence of one low K_m (1.1 μM) and one high K_m (5.0 μM) phosphodiesterase in both of these preparations (n = 10). The same analysis showed that the enzymic activity

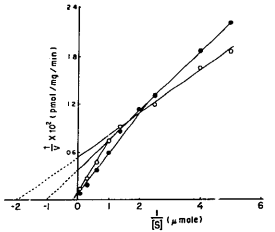

Fig. 3. Lineweaver-Burk plots of the phosphodiesterase activity of the membrane and cytosolic fractions of platelets incubated with ADP. Platelet-rich plasma was incubated with 4 μM ADP for 5 min as described in the Materials and Methods. The K_m and V_{max} values of the phosphodiesterase activities of the membrane and soluble fractions were determined by Lineweaver-Burk plots. ADP-treated platelets (○); control platelets (●). Each point represents the mean of three experiments which are comparable to seven other experiments.

of the membrane preparation from the platelets incubated with ADP was specifically inhibited in the case of low K_m phosphodiesterase without affecting the high K_m enzymic form (Fig. 3). It was also found that the inhibition of low K_m phosphodiesterase by ADP was noncompetitive in nature, indicating that the residual ADP in the incubation mixture, through its competition with the substrate, was not responsible for the observed inhibition of the enzymatic activity. As a result of the incubation of platelet-rich plasma with ADP (4.0 μM), the K_m value of the low K_m phosphodiesterase decreased from 1.1 to 0.5 μM with simultaneous decrease of V_{max} from 241 to 176 pmol cyclic AMP hydrolyzed/min per mg (Table I). In contrast, neither the low K_m or the high K_m phosphodiesterase from the cytosolic fraction was affected by the treatment of platelets with ADP.

Effect of ADP on cellular cyclic AMP level and on the activity of adenylate cyclase in platelets

Since the inhibition of phosphodiesterase would be expected to increase cyclic AMP level in platelets, the nucleotide content of the platelets incubated in the presence of ADP was determined and compared with the control. It was found that the incubation of platelet-rich plasma with 4.0 μM ADP doubled the cyclic nucleotide content from basal level of 1.5 ± 02.0 to 3.0 ± 0.10 pmol/10^8 cells in 5 min. Since adenosine, a breakdown product of ADP, is known to increase cyclic AMP level in platelets [15], in a different experiment platelet-rich plasma was incubated with adenosine (4.0 μM) instead of ADP under identical condition. Adenosine at this concentration did not increase the cyclic AMP level ($1.7 \pm 0.020/10^8$ cells) when compared to control. The increase of cyclic AMP level in the ADP-treated platelets was not due to the stimulation of the adenylate cyclase activity in these cells. It was found that the adenylate cyclase activity of the control membrane preparation which produced 2.18 pmol cyclic AMP/mg protein per 10 min was similar to that produced by the membrane prepared from the ADP-treated platelets (2.11 pmol cyclic AMP/mg protein per 10 min).

Effect of incubation time on the inhibition of platelet aggregation by ADP

To determine the effect of exposure of platelet to ADP on the subsequent aggregation of these cells by the agonist itself, platelet-rich plasma was incubated with 4.0 μM ADP for various times without stirring at 37° C. After incubation, stirring (1200 rpm) was begun to initiate platelet aggregation. It was found that with the increase of incubation time, the aggregation of platelets by the agonist itself was increasingly inhibited (Fig. 4). This inhibition of platelet aggregation was not due to the breakdown of ADP in the platelet suspension. As determined by the myokinase method, 88% (3.52 μM) of the added ADP (4 μM) was present in the platelet-rich plasma after 5 min of incubation at 37° C. Furthermore, the addition of more ADP (4.0 μM) to the platelet suspension previously incubated with the nucleotide failed to aggregate these cells when stirring was begun (Fig. 4). It was also found that the incubation of platelets with ADP in the absence of stirring not only made these cells resistant to the aggregatory effect of the

TABLE I

Effect of incubation of platelet-rich plasma with ADP on phosphodiesterase(s) activity of membrane and cytosolic fractions

Platelet-rich plasma ($n = 10$) was incubated with 4.0 μM ADP for 5 min at 23°C. After incubation, the platelets were separated by centrifugation. The isolated platelets were homogenized and the membrane and cytosolic fractions were separated by ultra centrifugation. The phosphodiesterase activity of these fractions were analyzed by Lineweaver-Burk plot as described in Materials and Methods.

Cellular fraction	K_m (μM) (mean ± S.D.)		V_{max} (pmol cyclic AMP hydrolyzed/mg per min) (mean ± S.D.)	
	low	high	low	high
Membrane				
Untreated platelets	1.1 ± 0.16	5.0 ± 0.32 [a]	241 ± 10 [a]	1255 ± 55 [b]
ADP-treated platelets	0.5 ± 0.12	5.0 ± 0.42	176 ± 8	1186 ± 65
Cytosol				
Untreated platelets	1.1 ± 0.20	5.0 ± 0.33 [b]	313 ± 20 [b]	3333 ± 112 [b]
ADP-treated platelets	1.1 ± 0.18	5.0 ± 0.30	340 ± 22	3320 ± 120

[a] $P < 0.001$.
[b] P = non significant.

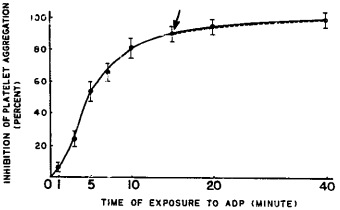

Fig. 4. Time-course of inhibition of platelet aggregation by ADP. Platelet-rich plasma was incubated with 4 μM ADP for various times as indicated, without stirring. After incubation for the specified time, stirring was begun to initiate platelet aggregation. The arrow indicates the time when either additional amount of ADP (4.0 μM, solid line) or 1-epinephrine (2–10 μM; broken line) was added to the platelet-rich plasma and the stirring was begun.

agonist itself, but platelets even failed to aggregate when the platelet suspension was challenged with 1-epinephrine (2–10 μM; Fig. 4).

Discussion

ADP has been classified as a 'primary' aggregating agent [16] that 'produces secretion requiring platelet aggregation' [9]. Although, the ADP-induced platelet aggregation may not necessarily be always producing secretion [5], the nucleotide has been proposed to be the final common mediator of all aggregating agonists [9]. Our results, on the other hand, have shown that this agonist of platelet aggregation can also act as an inhibitor of platelet aggregation. The ADP-induced platelet aggregation which could be demonstrated only when the platelet suspension is stirred, a procedure of uncertain physiologic relevance, is, however, faster than the inhibitory effect of the nucleotide. While the ADP-induced primary platelet aggregation could be demonstrated within a minute, the inhibitory effect of the compound which is apparently mediated through the increase of cyclic AMP level via the inhibition of phosphodiesterase activity could be demonstrated only after 2–3 min of incubation (Fig. 4). However, while it remains to be established that the in vivo increase of ADP actually causes platelet aggregation in the circulation, these results indicate that the nucleotide might be an inhibitor of the aggregation process under physiological conditions. The inhibition of platelet membrane phosphodiesterase by ADP was independent of the platelet aggregation process, since the degree of inhibition of the enzymic activity in both aggregated (stirred) and non-aggregated (non-stirred) platelets was the same. These results show that the platelet aggregation induced by ADP once started before the increase of cyclic AMP level, continued to completion, even during the accumulation of the cyclic nucleotide in these cells.

Although the inhibitory effect of ADP on platelet phosphodiesterase activity was transient in nature, the inhibition of the enzymic activity could only be demonstrated by incubating these cells with ADP. Incubation of either the isolated membranes or the cell homogenate with ADP produced no inhibition of the enzymic activity. This failure of ADP to inhibit phosphodiesterase when added to the isolated membrane could not be due to the lack of cytosolic factor(s), since the nucleotide also failed to inhibit the enzyme in cell-free homogenate as well. However, the receptors of ADP and its coupling to phosphodiesterase might be impaired due to homogenization procedure, and, as such, this might account for the lack of effect of ADP on the enzymatic activity in the isolated membrane and platelet homogenate preparations. The effect of ADP on the inhibition of phosphodiesterase activity was highly specific. Only the membrane-bound, low K_m phosphodiesterase was inhibited. Neither the high K_m phosphodiesterase of the membrane and cytosolic fractions nor the low K_m enzymic activity of the soluble fractions was affected by the nucleotide treatment. Since the cellular concentration of cyclic AMP is usually very low (1–2 μM), the low K_m phosphodiesterases are thought to be physiologically more important than the high K_m enzyme. Although the physiological significance of the differential inhibition on low K_m phosphodiesterases in platelets (membrane-bound vs. soluble enzyme) by ADP is not known, the inhibition of the membrane-bound low K_m phosphodiesterase activity by the nucleotide in the absence of stirring, was sufficient to elicit its physiological function through the inhibition of platelet aggregation by increasing cyclic AMP level when these cells were subsequently stirred with the nucleotide or 1-epinephrine. It should be mentioned here that the increase of cyclic AMP level by ADP through the inhibition of phosphodiesterase activity in platelets by various pharmacological agents leads to the inhibition of aggregation of these cells, no increase in cyclic AMP level has ever been demonstrated [17,18]. However, in contrast to ADP, which is a physiologically important compound, the above pharmacological agents were various drugs, and as such, their effects on platelet phosphodiesterase(s) activity may not be comparable to that of ADP.

It has been discussed above that the inhibition of phosphodiesterase activity by ADP was transient in nature and the enzyme regained 90% of its original activity within about 20 min after the cells were treated with the nucleotide. Although the mechanism of this 'recuperation' remains unknown, the inhibition of platelet aggregation was continued even when no inhibition of the phosphodiesterase activity could be demonstrated (Figs. 1 and 4). These results indicate that the

continued inhibition of phosphodiesterase activity for the increase of cyclic AMP level was not necessary to inhibit platelet aggregation. Similar effect of prostaglandin E_1 which inhibits platelet aggregation through a transient increase of cellular cyclic AMP level through the activation of adenylate cyclase is also known [4,19].

It could be argued that the inhibition of platelet aggregation by ADP was due to the desensitization of the ADP receptors which occurred during the incubation of these platelets in the absence of stirring. However, the addition of l-epinephrine to the platelet suspension previously incubated with ADP in the absence of stirring also failed to aggregate these cells (Fig. 4). Since specific receptors are involved in the cases of these agonists of platelet aggregation [9], these results suggest the participation of cyclic AMP as the mediator of the inhibitory process, even if the ADP receptors were desensitized by the nucleotide.

Acknowledgements

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