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EFFECTS OF α,β-METHYLENE-ADENOSINE-5′-DIPHOSPHATE ON BLOOD PLATELET AGGREGATION

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SUMMARY

The effects on platelet aggregation of α,β-methylene-adenosine-5′-diphosphate (Ado-PCP) have been investigated. Using human citrated platelet-rich plasma it has been shown that: (i) at concentrations of 10^{-3} M or higher Ado-PCP is able to induce platelet aggregation; (ii) the rate of Ado-PCP-induced aggregation increases on raising the pH of platelet-rich plasma above the pK_a for the secondary phosphonyl dissociation of Ado-PCP; (iii) at concentrations from $1 \cdot 10^{-4}$ to $5 \cdot 10^{-4}$ M Ado-PCP does not cause platelet aggregation itself, but it inhibits ADP-induced aggregation. This inhibition is also observed in washed platelet suspensions. The data suggest that Ado-PCP acts at the same site on the platelet membrane as does ADP and that ADP to AMP transformation is not a prerequisite for the process of aggregation. The observed effect of pH on the rate of Ado-PCP induced aggregation suggests that the ionization state of a nucleotide terminal acid group is important in the process of aggregation.

INTRODUCTION

Aggregation of blood platelets is an important early step in the formation of physiological hemostatic plugs and pathological thrombi. Adenosine diphosphate is one of the major inducers of platelet aggregation in vitro and from many in vivo investigations [1–5] it appears to be the principal nucleotide causing aggregation in physiological situations.

Although many conflicting theories to explain the action of ADP on blood platelets have been put forward [6–11], the precise mechanism by which ADP induces aggregation remains unknown. Possibly, the primary action of ADP is that of a regulator of certain platelet-membrane enzymes, such as ATPase [8] or adenyl cyclase [9]. Alternatively, ADP might serve as a substrate for enzymes in the platelet membrane. Both dephosphorylation [10] and phosphorylation [11, 12] of ADP have

Abbreviation: Ado-PCP, α,β-methylene-adenosine-5′-diphosphate.

been reported to take place during the process of ADP-induced platelet aggregation.

In this paper we present the results of experiments with α,β-methylene-adenosine-5′-diphosphate (Ado-PCP). This nucleotide cannot be dephosphorylated by enzymes present in plasma or platelets, as it possesses a methylene bridge in place of a pyrophosphate oxygen of ADP [13]. Therefore, investigation of the effects of Ado-PCP on platelet suspensions should provide an indication as to whether ADP to AMP dephosphorylation is or is not associated with the process of aggregation.

MATERIALS

Ado-PCP was obtained from Miles Laboratories, Kankakee, Ill.; ADP (di sodium salt, Grade I) from Sigma, St. Louis, Mo.; adenosine from Cyclo Chemical, Los Angeles, Calif. Adenosine deaminase (EC 3.5.4.4 from calf intestinal mucosa, Type I) and potato apyrase (Grade I, approximate activities per mg: 5′-ATPase, 1.34 units; 5′-ADPase, 0.4 unit; 5′-AMPase, 0.008 unit) were from Sigma, St. Louis, Mo.; bovine serum albumin (Fraction V, B grade) from Calbiochem, Los Angeles, Calif.; Prostaglandin E_1 from Upjohn Co., Kalamazoo, Mich. Cellulose plates for thin-layer chromatography were obtained from Eastman Kodak Company, Rochester, N.Y. Fibrinogen (95 % clottable) was prepared from bovine plasma by the procedure of Blombäck and Blombäck [14].

METHODS

Preparation of platelet-rich plasma

Human blood was anticoagulated with one-tenth vol. of 3.8 % sodium citrate. Platelet-rich plasma was prepared at room temperature by centrifuging the whole blood at $40 \times g$ for 20 min in an International PR-6 centrifuge with a No. 269 rotor. To obtain platelet-poor plasma the remainder of the blood after removal of platelet-rich plasma was centrifuged at room temperature at $2700 \times g$ for 12 min. Platelet counts were performed by phase-contrast microscopy in a Spencer Bright-Line hemocytometer using 1 % formaldehyde in 3.8 % sodium citrate as a diluting fluid [15]. For aggregation experiments, platelet count was adjusted to 300 000 platelets/mm^3 with platelet-poor plasma.

Preparation of washed platelet suspension

Human blood was collected into plastic centrifuge tubes containing acid citrate dextrose anticoagulant (1 part to 6 parts blood) [16]. The preparation of platelet suspensions was carried out at 0–4 °C because at higher temperatures the pellets of platelets were 'stickier' and more difficult to resuspend [17]. The anticoagulated blood was centrifuged at $120 \times g$ for 30 min at 4 °C in an International PR-6 centrifuge (No. 269 rotor) to obtain platelet-rich plasma. Platelet-rich plasma was then centrifuged in siliconized glass tubes (15-ml graduated conical centrifuge tubes) at $1000 \times g$ for 15 min at 4 °C. The platelet button was suspended in Ca^{2+}-free Tyrode's buffer [18] consisting of 8.0 g NaCl, 0.2 g KCl, 1.0 g $NaHCO_3$, 0.05 g $NaH_2PO_4 \cdot H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, 3.5 g bovine serum albumin, 1.0 g glucose and 0.076 g EGTA per liter (pH 6.2). In order to prevent the formation of platelet clumps on centrifugation, 50 ng/ml of prostaglandin E_1 [19] were added to the

suspension which was then centrifuged at $800 \times g$ for 10 min at 4 °C (the presence of prostaglandin E_1 in the first washing fluid had no effect on the response of the final platelet suspension to added ADP). The resulting platelet pellet was suspended once more in Ca^{2+}-free Tyrode's buffer (no EGTA, no prostaglandin E_1 included this time) and centrifuged at $800 \times g$ for 10 min at 4 °C. The final suspension (500 000 platelets/mm^3) was made in Tyrode's albumin buffer [18] consisting of 8.0 g NaCl, 0.2 g KCl, 1.0 g $NaHCO_3$, 0.05 g $NaH_2PO_4 \cdot H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.22 g $CaCl_2$, 1.0 g glucose and 3.5 g bovine serum albumin per liter (pH 7.35). In the experiments in which aggregation was followed as a function of pH of the platelet suspension, Tris buffer (1.45 g/l) was employed in place of $NaHCO_3$ in the Tyrode's albumin buffer [20]. The isolated platelets were kept on ice, as it was observed that platelet response to ADP declined more rapidly when they were kept at room temperature. The lowest concentration of ADP to which the platelets responded (in the presence of fibrinogen) was 10^{-6} M. ADP was added to platelet suspensions after 5 min preincubation of the suspension with 0.3 mg/ml of bovine fibrinogen.

Platelet aggregation

Platelet aggregation was studied by the method of Born [21] using either a Payton single-channel aggregation module with a Beckman 10″ flatbed recorder, or a Payton dual-channel aggregation module attached to two Beckman recorders. The reagents were all added in 0.9 % NaCl adjusted to neutrality. In the experiments in which Ado-PCP inhibition of ADP-induced aggregation was followed, the analog was incubated with platelet-rich plasma (or platelet suspension) for 1 min prior to ADP addition. The aggregation was characterized quantitatively by the "rate" and "extent" of the increase in light transmission (Fig. 1). The rate and extent in the control experiments in which ADP alone was used were each set equal to 100 %. The inhibition of ADP-induced platelet aggregation by Ado-PCP was then reported as percentage decrease in these parameters. It was observed that the responsiveness to ADP of both platelet-rich plasma and platelet suspensions decreased in the course of an experiment. Therefore, when the single-channel aggregometer was used, the inhibition by Ado-PCP was always assessed by interpolating an experiment in which Ado-PCP was present between two control experiments [22]. In later experiments, the dual-channel aggregometer allowed simultaneous observation of the tracings obtained from Ado-PCP-containing and control samples.

In the experiments in which platelet aggregation was followed as a function of pH of platelet-rich plasma, it was particularly important to prevent CO_2 diffusion from the plasma. Therefore, after adjusting the pH (which was done with 0.1 M HCl or 0.1 M NaOH during continuous stirring), the plasma aliquots were pipetted into cuvets, which were capped immediately. The reagents were then added with Hamilton microsyringes by piercing the rubber cap and injecting the reagent into the platelet-rich plasma.

Glassware

All glassware used in the preparation and storage of platelets was previously siliconized using 10 % v/v solution of SC-87 Dri Film siliconizing fluid (General Electric, Waterford, N.Y.) in CCl_4.

Thin-layer chromatography of Ado-PCP

Ado-PCP was purified by thin-layer chromatography on cellulose plates using isobutyric acid–1 M ammonia (5 : 3, VH) [13] or propanol–conc. ammonia–water (11 : 7 : 2, by vol.) [23] solvent systems. The following R_F values were found for ADP, Ado-PCP and adenosine, respectively: 0.54, 0.60 and 0.83 (isobutyric acid–ammonia); 0.31, 0.25 and 0.72 (propanol–conc. ammonia–water). The spots were visualized under a short-wave ultraviolet lamp, the area corresponding to Ado-PCP scraped off and the nucleotide eluted with 0.01 M HCl. The sample was then lyophilized and the residue dissolved in 0.9 % NaCl to a desired concentration.

Apyrase activity

Apyrase (ATP diphosphohydrolase, EC 3.6.1.5) activity was assayed as described earlier [24] except that ADP (1 mM) was used as a substrate. The final concentration of Ado-PCP, when present, was 1 or 5 mM.

RESULTS

Inhibition of ADP-induced aggregation by Ado-PCP

When $1 \cdot 10^{-4}$ to $5 \cdot 10^{-4}$ M Ado-PCP was added to platelet-rich plasma no change in percentage transmission was observed. However, if ADP was subsequently added to the samples, then ADP-induced aggregation was greatly reduced (Table I). In the experiments reported in the table Ado-PCP purified by thin-layer chromatography was used. This was done in order to remove any trace of adenosine which might have been present in the commercial sample and which is a potent inhibitor of aggregation [25].

Further evidence that adenosine contamination was not responsible for the observed inhibition comes from experiments in which the use was made of adenosine deaminase. This enzyme converts adenosine to inosine [26] and thereby can abolish the inhibitory effect of adenosine on platelet aggregation. Platelet-rich plasma was incubated for 1 min with Ado-PCP (non-chromatographed) in the presence of adenosine deaminase (5 μg/ml) and subsequently challenged with ADP. The same percentage inhibition of ADP-induced aggregation as that reported in Table I was obtained. It should be pointed out that under the same conditions, adenosine deaminase completely abolished the inhibition caused by $2 \cdot 10^{-5}$ M adenosine. The

TABLE I

INHIBITION OF ADP-INDUCED AGGREGATION BY CHROMATOGRAPHED Ado-PCP IN PLATELET-RICH PLASMA

Platelet aggregation was induced by $2 \cdot 10^{-6}$ M ADP. Values represent averages of ten ($2 \cdot 10^{-4}$ M Ado-PCP) or five ($5 \cdot 10^{-4}$ M Ado-PCP) measurements.

Ado-PCP concentration (M)	Percentage inhibition	
	Rate	Extent
$2 \cdot 10^{-4}$	25	37
$5 \cdot 10^{-4}$	45	63

TABLE II

INHIBITION OF ADP-INDUCED AGGREGATION BY Ado-PCP IN WASHED PLATELET SUSPENSIONS

Platelet suspension was incubated for 1 min with Ado-PCP in the presence of 5 μg/ml of adenosine deaminase before the aggregation was induced by $4 \cdot 10^{-6}$ M ADP. Values represent averages of two measurements.

Ado-PCP concentration (M)	Percentage inhibition	
	Rate	Extent
$2 \cdot 10^{-4}$	38	42
$5 \cdot 10^{-4}$	79	85

analogous experiments were carried out with washed platelet suspensions, where approx. 80 % inhibition of ADP-induced aggregation was obtained by $5 \cdot 10^{-4}$ M Ado-PCP (Table II).

It has also been observed that preincubation of platelet-rich plasma with Ado-PCP caused an inhibition of the decrease of percentage transmission which immediately follows ADP addition and which represents change of shape of the platelets [27]. However, this inhibition could not be evaluated quantitatively so far, because of the overlapping effect of aggregation on percentage transmission.

Ado-PCP-induced aggregation

On increasing the concentration of Ado-PCP added to platelet-rich plasma, it was observed that the nucleotide at a level of 1 mM or higher was able to induce platelet aggregation (Fig. 1B). Using the procedure of Born et al. [28] the relative activity of Ado-PCP was expressed as:

$$\frac{\text{concentration of ADP required to produce a given extent of aggregation}}{\text{concentration of Ado-PCP required to produce the same extent of aggregation}} \times 100\,\%$$

Under these conditions, Ado-PCP exhibited only 0.05 % of the ADP activity. In contrast to ADP, no disaggregation occurred with Ado-PCP (Fig. 1) even after 10 min of incubation. It seemed possible that the aggregating effect of Ado-PCP could be a result of trace contamination of the Ado-PCP sample by ADP. An attempt was made to exclude this possibility by inclusion of apyrase in the incubation mixture containing platelet-rich plasma and Ado-PCP. However, this approach was found to be invalid since it was observed separately that 5-fold excess of Ado-PCP over ADP inhibited apyrase activity (dephosphorylation of ADP) by approx. 40 %. In a second attempt to exclude the possibility of contamination, Ado-PCP was purified by thin-layer chromatography. Comparable rates and extents of aggregation were obtained with the chromatographed and non-chromatographed Ado-PCP (Table III), suggesting that the observed aggregation cannot be a result of ADP contamination.

The effect of pH was then investigated. The pH of platelet-rich plasma used in the experiments was 7.7. At this pH all phosphate groups of ADP should be predominantly ionized, while Ado-PCP would still possess one unionised phosphate hydroxy-group [29]. Possible differences in the ionization states of the two nu-

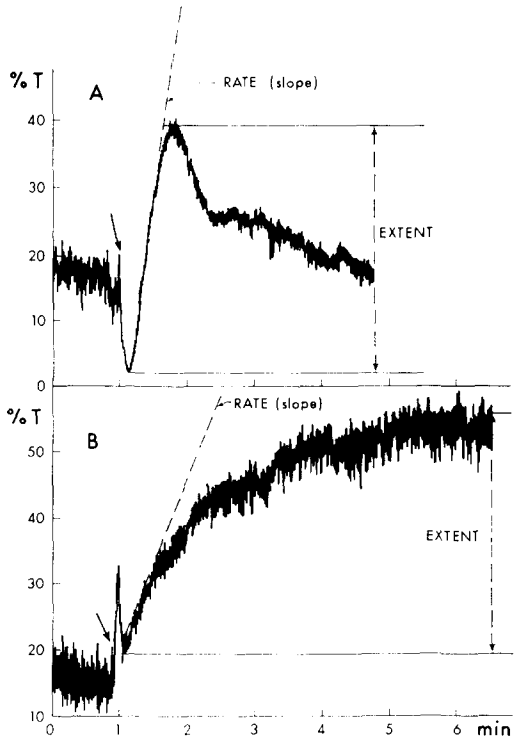

Fig. 1. Aggregation curves obtained by addition of nucleotides to platelet-rich plasma. Platelet samples (0.5 or 1.0 ml) were warmed to 37 °C for 3 min in siliconized standard-sized cuvets before placing them in the aggregometer cell compartment (also maintained at 37 °C), where they were stirred at 1000 rev./min for an additional 2 min. The appropriate reagents were added from microsyringes at points indicated by the arrows. Data were recorded as plots of the percent transmission (% T) versus time. In this experiment the full scale deflection of the recorder corresponded to 1 mV. (A) 8.5 μl of $1 \cdot 10^{-4}$ M ADP added (final concentration $8.5 \cdot 10^{-7}$ M). (B) 100 μl of $2 \cdot 10^{-2}$ M Ado-PCP added (final concentration $1.8 \cdot 10^{-3}$ M).

TABLE III

Ado-PCP INDUCED PLATELET AGGREGATION IN PLATELET-RICH PLASMA

Values represent averages of two measurements.

Ado-PCP concentration (M)	Aggregation	
	Rate (cm/min)	Extent (cm)
$1.8 \cdot 10^{-3}$*	6.7	6.8
$1.8 \cdot 10^{-3}$	7.7	6.0

* Chromatographed Ado-PCP.

TABLE IV

EFFECT OF pH OF PLATELET-RICH PLASMA ON Ado-PCP-INDUCED AND ADP-INDUCED PLATELET AGGREGATION

Values represent averages of two measurements.

pH of platelet-rich plasma	Aggregation rate (cm/min)	
	$1.8 \cdot 10^{-3}$ M Ado-PCP	$1 \cdot 10^{-6}$ M ADP
7.3	1.9	52.0
7.6	9.0	110.5
8.0	39.4	90.0
8.3	41.9	82.5
8.6	43.2	73.7

cleotides could then account for the much lower aggregating ability of Ado-PCP. Table IV shows the effect of pH of platelet-rich plasma on Ado-PCP induced aggregation. Raising the pH from 7.3 to 8.0 greatly stimulated the aggregation rate (20-fold increase). It was not possible to evaluate the effect of pH on aggregation extent, because at pH values above 8.0 this parameter decreased rapidly in the course of experiments. For comparison, the table shows the effect of pH on ADP-induced aggregation. The optimal aggregation rate was obtained at pH 7.6; no further stimulation was observed at higher pH values.

So far we have obtained Ado-PCP induced aggregation only in platelet-rich plasma; no detectable aggregation occurred with washed platelet suspensions on addition of 1 or 2 mM Ado-PCP even when the pH of the suspending Tyrode's albumin was raised above 8.0 through use of Tris in place of bicarbonate. Although washed platelets responded to 10^{-6} M ADP in the presence of fibrinogen, they were less sensitive to ADP than were platelets in platelet-rich plasma. Thus, 2 to 4 times higher ADP concentration had to be added to the washed platelet suspensions to obtain the same extent of aggregation as in platelet-rich plasma. This reduced sensitivity accounts probably for the inability of Ado-PCP to induce aggregation of washed platelets.

DISCUSSION

In this paper we have demonstrated that an ADP analog, α,β-methyleneadenosine-5′-diphosphate, is able to inhibit ADP-induced aggregation (Tables I, II) and, at high concentrations, to induce platelet aggregation itself (Tables III, IV).

The possibility that the inhibitory activity of Ado-PCP was a result of adenosine contamination of the sample was eliminated by purification of Ado-PCP by thin-layer chromatography as well as by incubation of the nucleotide with adenosine deaminase. Similarly, purification of Ado-PCP by thin-layer chromatography did not alter the aggregating ability of the nucleotide. Therefore, it is difficult to escape the conclusion that the component responsible for both the inhibitory and aggregating activities is Ado-PCP itself. However, these phenomena (i.e. the inhibition of ADP-induced aggregation at certain Ado-PCP concentration and Ado-PCP-induced

aggregation at higher concentrations of the nucleotide) cannot be explained on the basis of data obtained so far.

Born [30] has suggested that the platelet membrane contains a specific ADP-receptor site and that the binding of ADP to this site initiates the sequence of events leading to aggregation. Based on the above data and on the structural similarities of ADP and Ado-PCP, we speculate that Ado-PCP also binds at this site on the platelet membrane. Unfortunately, it was not possible to determine by kinetic analysis whether Ado-PCP acts as a competitive inhibitor of ADP in platelet aggregation because of platelet instability during the course of such an experiment.

Results in Table IV show that Ado-PCP-induced aggregation is greatly stimulated on raising the pH of platelet-rich plasma above the pK_a of 8.0 [29] for the secondary phosphonyl dissocation of the nucleotide. No comparable effect of pH on ADP-induced aggregation could be observed. ADP is fully ionized above pH 7.0 [29]. Therefore, most probably, the observed stimulation of Ado-PCP-induced aggregation is due to the ionization of the terminal OH-group and not a result of other processes, e.g. effect of pH on enzymes involved in aggregation. In the latter case, the increase of pH would be expected to affect ADP- as well as Ado-PCP-induced aggregation in a comparable way. Thus, above pH 8 Ado-PCP possesses three negative charges and causes platelet aggregation much more readily than below pH 8 when it has only two negative charges. This result is in agreement with the observation of Gaarder and Laland [6], who noted that nucleotides known to bring about aggregation have an uneven number of negative charges at physiological pH, whereas the inhibitors either have no charge at all (e.g. adenosine) or have an even number of charges (e.g. AMP, ATP). However, even when fully ionized, Ado-PCP is still a much less potent aggregating agent than ADP (Table IV). Possibly this is due to conformational differences between the two nucleotides. As has been demonstrated for methylene diphosphonic acid and pyrophosphate [31], bond angles and distances of P–CH_2–P are different from that of P–O–P and therefore Ado-PCP may not be able to fit so well into the "ADP-binding site" on the platelet membrane. Recently, Gough et al. [32] reported that Ado-PCP did not initiate aggregation of sheep platelets in citrated platelet-rich plasma, nor did it potentiate or inhibit the effect of ADP in concentrations up to $2 \cdot 10^{-4}$ M. In contrast, we report that Ado-PCP at concentrations of $1 \cdot 10^{-4}$ M or higher is able to significantly inhibit aggregation of human blood platelets. Possibly, the discrepancies in data can be reconciled by taking into consideration the difference in the species of origin of the platelets used. The finding of Gough et al. [32] that Ado-PCP was devoid of aggregating ability appeared to support the proposal of Spaet and Lejnieks [10] that the breakdown of ADP to AMP and P_i is a key energy-providing reaction in platelet aggregation. However, we have shown that, providing Ado-PCP concentration is increased to $1 \cdot 10^{-3}$ M, the nucleotide is able to induce human platelet aggregation. Therefore, it is evident that ADP to AMP transformation is not a prerequisite for the process of aggregation.

Recently, Nachman and Ferris [33] demonstrated that isolated membranes from human platelets were capable of binding of [^{14}C]ADP. Exposure of the membranes to Ado-PCP did not significantly alter [^{14}C]ADP binding. This result is apparently not consistent with our finding that Ado-PCP is able to inhibit ADP-induced aggregation probably by binding to the "ADP-receptor sites" on the platelet membrane. On one hand, Nachman and Ferris were able to show that various agents

known to inhibit platelet aggregation (e.g. 2-chloroadenosine, AMP, ATP) significantly decreased [^{14}C]ADP binding by the platelet membranes. On the other hand, adenosine and prostaglandin E_1, also very potent inhibitors of platelet aggregation, did not interfere with ^{14}C-ADP binding. Concentrations of nucleoside diphosphates, CDP, UDP and IDP that had been shown to lead to platelet aggregation [34] failed to inhibit [^{14}C]ADP binding by platelet membranes. Thus, it is evidently very difficult to assess to what degree the ADP binding by platelet membranes is related to the phenomena of ADP-induced aggregation.

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