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FURTHER CHARACTERIZATION OF HUMAN PLATELET ADP BINDING SITES USING 5'AMP. DEMONSTRATION OF A HIGHLY REACTIVE POPULATION OF SITES.

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Summary

The binding of AMP and ADP to specific sites on the human platelet membrane have been compared. Although the two nucleotides bound to the platelet membrane with similar affinities (Ka $\simeq$ 0.4 x $10^6 M^{-1}$), AMP binding was more rapid than ADP binding and involved a reduced number of binding sites. AMP competitively inhibited ADP binding by reacting with approximately half of its binding sites. Thus, the binding of AMP to specific sites in human platelet membrane provides a tool to differentiate two different types of ADP binding sites with similar affinities. AMP does not inhibit membrane nucleoside diphosphokinase, the enzyme which converts ADP into ATP when intact washed platelets or isolated platelet membranes are incubated with ADP in presence of divalent cations.

The concept of specific ADP receptor sites on the platelet membrane to trigger ADP-dependant aggregation has been in existance for many years (1). Binding of labelled ADP to platelets has been demonstrated in washed platelets aggregated after the addition of ADP to human platelet rich plasma (2, 3) or to isolated platelet membranes (4, 5, 6). In washed platelets, the results are complicated by the enzymatic degradation of ADP and subsequent incorporation of labelled adenosine (7). With isolated platelet membranes, the problem is to demonstrate that ADP binding occurs at physiological receptors. Previously (8), we demonstrated that AMP, - which is considered to be a competitive inhibitor of ADP effects on rabbit and human platelets (9) - acts as a competitive inhibitor of ADP binding to the platelet membrane. In the present study, the effect of AMP on both ADP binding and ADP metabolism was investigated further.

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MATERIAL AND METHODS

Chemicals

[U-^{14}C] ADP (550 mCi/mMole) and [U-^{14}C] AMP (538 mCi/mMole) were obtained from the Radiochemical Centre at Amersham, England. ADP (grade I, sodium salt), AMP (type III, sodium salt) and other chemicals from Sigma Chemical Company, St. Louis, Missouri. Sucrose (ultrapure-special enzyme grade) was obtained from Schwartz-Mann (division of Becton Dickinson, Orangeburg, N.Y.). 0.8 µm filters were supplied by Millipore corporation (ref. AA WP 02500). PEI cellulose TLC Ready Plastic Sheets F 1440 20 cm x 20 cm x 0.1 cm were obtained from Carl Schleicher & Schüll, Dassel, FRG. Unisolve (Koch Light Laboratories) was used as scintillation fluid.

Preparation of platelet plasma membranes

Human platelet plasma membranes were isolated from several units of fresh blood following the method of Barber and Jamieson (10) modified as follows : the lysate, obtained by glycerol lysis of washed platelets, was layered on top of 6 ml of a 30 % (w/w) sucrose solution and centrifuged overnight at 63 500 g at temperature of 4° C. The membranes were isolated from the sucrose interface and were diluted 1 : 10 with 0.01 M Tris 0.25 M sucrose pH 7.4 and centrifuged 1 hour at 100 000 g max at 4° C. The pellet was resuspended in 0.01 M Tris 0.15 M NaCl 0.001 M EDTA pH 7.4 (Tris-saline-EDTA buffer) and protein determination was performed by the method of Lowry et al (11).

AMP and ADP binding assays

For binding assays, the membrane suspension was diluted to a concentration of 0.2 mg/ml with Tris-saline-EDTA buffer and agitated continuously in a water bath at 37° C with [^{14}C] AMP or [^{14}C] ADP solutions. Incubation was stopped by rapid filtration of 1 ml samples on 0.8 µm filters. The filters were rinsed twice with 10 ml of buffer (the entire procedure took 10 seconds), transferred to scintillation vials, mixed with 10 ml of scintillation fluid, and counted in an Intertechnique SL 4000 liquid scintillation spectrometer.

The non specific binding was determined by incubating samples with the radioactive nucleotide and a 100 fold excess of unlabelled nucleotide.

AMP and ADP metabolism

For enzymatic studies, the membranes were suspended in Tris buffer (pH 7.4) containing 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ at a concentration of 0.2 mg/ml. Incubation at 37° C with [^{14}C] AMP or [^{14}C] ADP was stopped by 15 minutes centrifugation in an Eppendorf centrifuge (12 000 g max, 4° C). The supernatants were then centrifuged for 30 minutes at 100 000 g max and 4° C in a Beckman ultracentrifuge. The radioactivity associated with the pellet was extracted by 30 minutes incubation at 37° C in 1 N HCl (98 % of the radioactivity was thus eluted).

The radioactive metabolites were analysed by thin-layer chromatography on PEI cellulose plates (12). The separated constituents were identified by autoradiography and counted in 1 ml 1 N HCl + 10 ml Unisolve.

RESULTS

A slight modification of Barber and Jamieson's technique allowed us to increase (approximately 1.5 x) the yield of the membrane fraction isolated

on the sucrose gradient without alteration of the degree of purity of the preparation. Electron microscopy revealed no contamination by intracellular organelles. The Ca^{++} ATPase and the bis(p-nitrophenyl)phosphate phosphodiesterase were purified 4- and 7-fold respectively whereas β-glycosidases and lactic dehydrogenase were present only at very low levels (ratio to lysate : 0.4 and 0.1 respectively). These results which are similar to those originally published (10), indicate that the membrane preparation was principally enriched by plasma membranes.

AMP and ADP binding assays were performed in Tris-saline-EDTA buffer since this buffer allowed binding of ADP to the platelet membrane without its enzymatic degradation (5). With or without the presence of divalent cations, approximately 20 % of AMP was metabolized during the time of the ex-

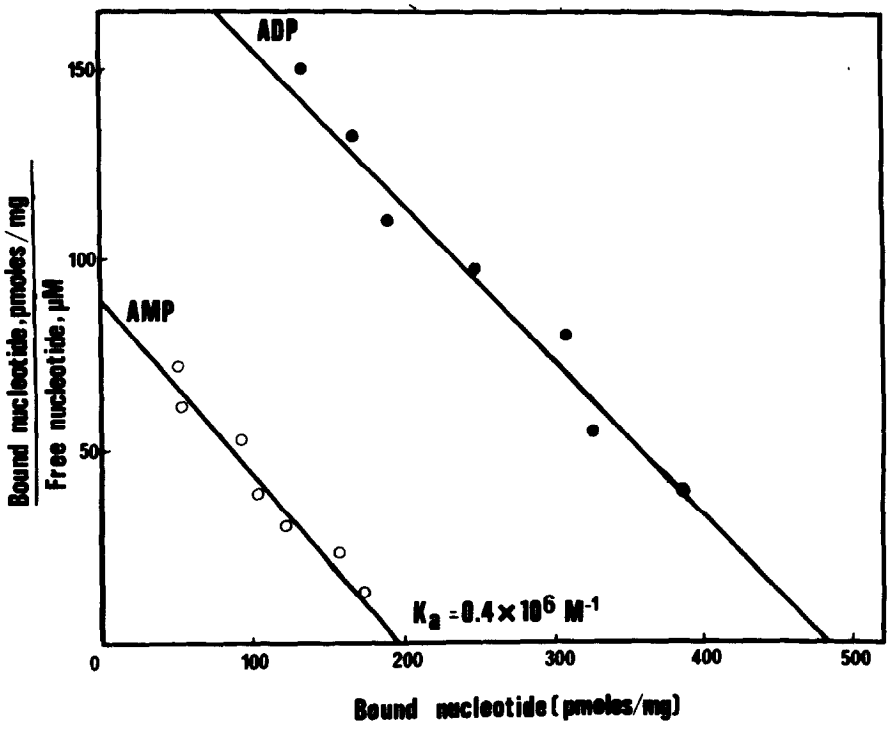

Fig. 1. Binding of [^{14}C] ADP (•) and [^{14}C] AMP (o) to human platelet membranes. Membrane samples (200 μg protein/ml) were incubated at 37° C for 30 minutes with various concentrations (from 0.5 to 10 μM) of each nucleotide. The amount of membrane-bound nucleotide was determined by Millipore filtration as described in the experimental section. Scatchard plot (13) was used to express the results. Each value is the mean of four experiments.

periment. The addition of divalent cations had no significant influence on ADP and AMP binding.

Equilibrium binding experiments were performed at 37° C with physiological concentrations (from 0.5 to 10 μM) of the two nucleotides. ADP and AMP were found to bind to a single population of sites on the platelet membrane with similar affinities : Ka = 0.4 (± 0.1) x $10^6 M^{-1}$ (figure 1). However, ADP possessed 2 to 3 times more binding sites than AMP as shown by the optimal binding capacities : 400 (± 80) pmoles/mg for ADP and less than 200 pmoles/mg for AMP.

In view of the rapid kinetics of ADP and AMP interactions with the platelet membrane (see below), equilibrium binding experiments were also conducted at 15° C to minimalize the eventual dissociation of membrane-bound nucleotide during filtration. Although the kinetics of the reactions were significantly reduced, no difference was found for determination of maximal ADP and AMP binding capacities.

The kinetics at 37° C of ADP and AMP binding to the platelet membranes were compared using 0.9 μM of each nucleotide. As shown in Figure 2, AMP binding was much faster than ADP binding, with 80 % of AMP binding occurring during the first 30 seconds of incubation. ADP binding, which involves more binding sites, usually lasted up to ten minutes with t 1/2 = 100 (±30) seconds (mean of eight experiments). Binding of ADP and AMP were fully reversible when a 100-fold dilution was applied to the sample after equilibrium had been reached. In addition, membrane-bound AMP dissociated much more rapidly than ADP since more than 80 % of the AMP molecules were released at 30 seconds while ADP molecules were released over a period of 20 minutes with t 1/2 = 120 seconds.

Preincubation of the platelet membranes during fifteen minutes at 37° C with 10 μM AMP (conditions which allow its maximal binding) led to subsequent inhibition of ADP binding. Since approximately 20 % of AMP was metabolized during the time of the experiment, we have confirmed that the meta-

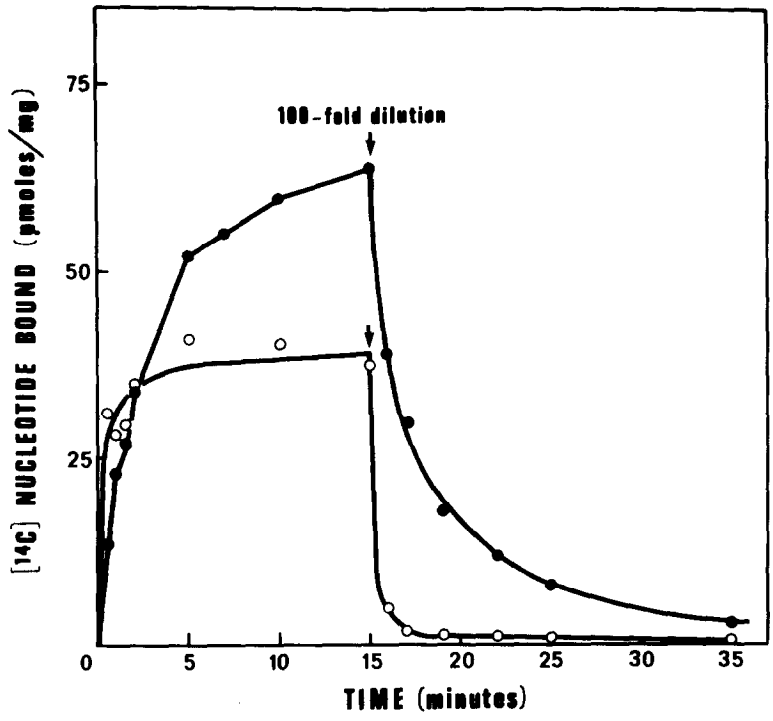

Fig. 2. Kinetics of the binding of [^{14}C] ADP (•) and [^{14}C] AMP (o) to human platelet membranes. Membrane samples (200 µg/ml) were incubated at 37°C with 0.9 µM of each nucleotide for various periods of time before Millipore filtration. Each value is the mean of eight (•) or four (o) experiments. At 15 minutes a 100-fold dilution was performed on each sample.

bolites formed (mainly IMP with a small amount of adenosine) did not bind to the membranes and had no effect on ADP binding (unpublished observations).

In eight experiments, the binding of 0.9 µM [^{14}C] ADP to platelet membranes was compared in the presence or absence of 10 µM AMP. Three experiment were also conducted using 10 µM [^{14}C] AMP and 0.9 µM unlabelled ADP to follow the fate of membrane-bound AMP molecules during subsequent ADP binding. Results are shown on table I and figure 3.

In table I the inhibitory effect of AMP was calculated at various times during ADP binding. During the first two minutes of incubation, it was observed that AMP inhibition remained constant (= 63 %) despite the release of approximately 20 % of the membrane-bound AMP molecules which may be considered as released from sites not involved in ADP binding. In contrast, the release of a further 10 % of AMP molecules between two and ten minutes was

TABLE I. EFFECT OF AMP ON ADP BINDING+

Time after addition of [^{14}C]ADP (minutes)	ADP binding (a) (pmoles/mg) Control	+ 10 μM AMP	AMP inhibition of ADP binding (%)	Fate of (b) membrane-bound AMP (pmoles/mg)
0	0	0	0	160
1	23	8.5	63	142
2	33.5	12.5	63	124
5	52	20.5	61	117
7	55	23	58	115
10	60	27	55	112
15	64	29	55	112

+ Mean of eight experiments.

a Membrane samples (200 μg/ml) were preincubated at 37°C for 15 minutes with buffer or with 10 μM AMP before the addition of 0.9 μM [^{14}C]ADP (mean of eight experiments)

b Membrane samples were preincubated at 37°C for 15 minutes with 10 μM [^{14}C]AMP before the addition of 1 μM ADP (mean of three experiments).

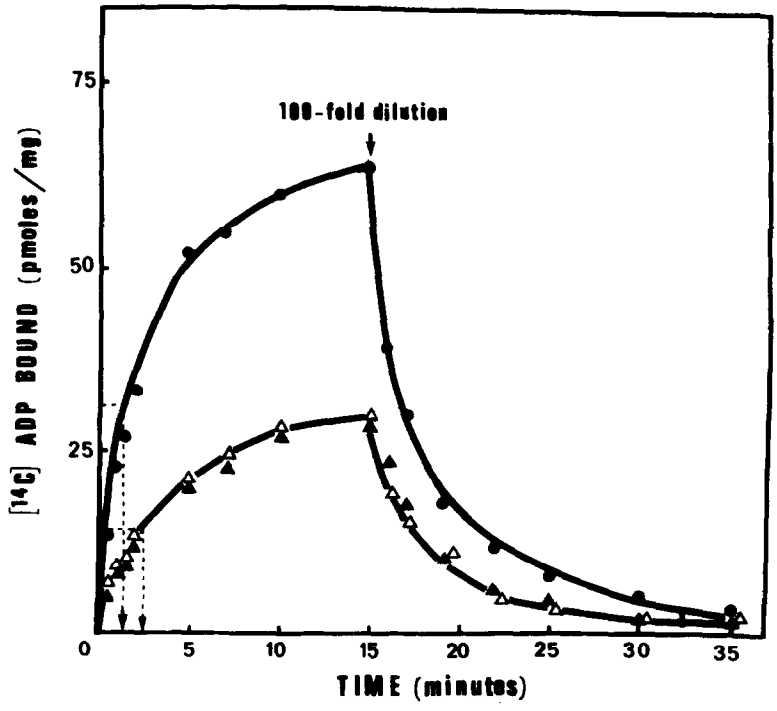

Fig. 3. Effect of AMP on [^{14}C] ADP binding. The binding of 0.9 μM of [^{14}C]ADP in presence of 10 μM AMP (△) was compared to the binding of 0.9 μM (●) and 0.4 μM (▲) of [^{14}C] ADP. Membrane samples (200 μg/ml) were preincubated at 37° C for 15 minutes with 10 μM AMP (△) or with buffer (●, ▲) before the addition of 0.9 μM (● , △) or 0.4 μM (▲) of [^{14}C] ADP. The kinetics of ADP binding was then measured. At 15 minutes, a 100-fold dilution was performed on each sample. Each value is the mean of three experiments.

associated with a decrease of the AMP inhibitory effect (from 63 to 55 %) showing that those AMP molecules are probably released from ADP binding sites. On figure 3, it can be observed that the rate of binding of 0.9 μM [^{14}C] ADP was slowest in presence of 10 μM AMP : t 1/2 = 160 seconds instead of 100 seconds. This effect resulted from the binding of 55 % less ADP molecules to the platelet membrane in presence of AMP. Thus, the binding of 0.9 μM [^{14}C]ADP in presence of AMP was similar to the binding of 0.4 μM [^{14}C] ADP (figure 3).

The effect of AMP on ADP metabolism was measured in Tris-saline buffer (pH 7.4) containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$. In these conditions, 20 % of [^{14}C] ADP was transformed into [^{14}C] ATP in less than thirty seconds. In table II, the effects of increasing concentrations of AMP on ADP binding and ADP transformation into ATP are compared. It can be seen that AMP inhibits ADP binding with little or no effect on its transformation into ATP. Though the results of ADP and AMP binding are similar to those measured in Tris-saline-EDTA buffer, the inhibitory effect of AMP is less pronounced in medium containing divalent cations. Using 0.9 μM [^{14}C] ADP, 10^{-5}M AMP only inhibits 25 % of ADP binding compared to 55 % in Tris-saline-EDTA buf-

TABLE II. EFFECT OF AMP ON ADP METABOLISM[+]

Concentration of AMP (M)	Inhibition of ADP binding (in %)	ADP metabolism $\frac{[^{14}C]ATP}{[^{14}C]ADP}$
0	0	0.42
10^{-5}	25	0.44
10^{-4}	70	0.40
10^{-3}	70	0.34

[+] Mean of three experiments.
Membrane samples (0.2 mg/ml) were preincubated at 37°C for 15 minutes with buffer or with unlabelled AMP (10^{-5} to 10^{-3}M) before the addition of 0.9 μM [^{14}C] ADP. ADP binding and ADP metabolism were measured 15 minutes after the addition of [^{14}C]ADP.

fer (Table I). This effect does not appear to be correlated to increased degradation of AMP in presence of divalent cations (as shown by chromatographic analyses).

DISCUSSION

We have previously demonstrated that ADP binds to specific receptor sites on the human platelet membrane (5, 6) ; this binding is partially antagonized by AMP which behaves as a competitive inhibitor (8). In the present work, AMP was used as a tool to differentiate two populations of ADP binding sites with similar affinities.

When ADP and AMP binding to the human platelet membrane were compared, it was observed that the two reactions occur with the same affinity but different velocities. Binding of AMP occurs in less than 30 seconds while binding of ADP lasts up to 10 minutes with t 1/2 = 100 seconds. Following a 100-fold dilution of the membrane suspension, 80 % of the membrane-bound AMP molecules were released at 30 seconds while ADP molecules were released during 20 minutes with a t 1/2 = 120 seconds. Although AMP competitively inhibits ADP binding to a fraction of its receptor sites, it does not appear to modify the nucleoside diphosphokinase activity (NDPK) of the platelet membrane i.e. the enzyme which converts ADP into ATP in presence of divalent cations (14). This result may be taken to indicate that the ADP receptor sites recognized by AMP on the platelet membrane are not involved in ADP metabolism. A similar result was recently obtained by Adler and Handin (15) who separated ADP receptor sites from ADP metabolic sites on a sucrose gradient.

In conclusion, AMP has been shown to rapidly interact with a population of ADP receptor sites present on the human platelet membrane. The fact that AMP inhibits ADP induced platelet shape change and aggregation (9) led us to assume that this population may represent the physiological receptors of ADP on the platelet surface.

Further studies are needed to better characterize the different types of ADP binding sites present on the platelet membrane which may not be recognized either from equilibrium or from kinetic studies.

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