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CHANGES IN GUANYLATE CYCLASE ACTIVITY OF HUMAN PLATELETS IN ADP-INDUCED AGGREGATION

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The role of the cyclic GMP (cGMP) system in the regulation of platelet aggregation and disaggregation in mammals has aroused increased interest during the last few decades. Whereas it was previously held that cGMP mediates initiation of aggregation [11], subsequent studies have suggested that the cGMP system exercises negative control over platelet aggregation and transmits the signal for their disaggregation [5, 9-11, 14]. It is assumed in this case that the process of platelet aggregation itself can induce activation of the cGMP system, which exerts its regulatory action by a "negative feedback" principle. Experimental data so far available in support of this view are based mainly on two groups of facts: 1) guanylate cyclase (GC) activators, which raise the cGMP level in platelets, exhibit an antiaggregative action [5, 11, 14]; 2) during aggregation, the cAMP concentration in platelets is increased [7, 10]. Under these circumstances, no information is available on the state of GC in aggregating platelets or on the effect of the aggregation process on GC activity.

We showed previously that activity of GC and its sensitivity to the NO-containing activator sodium nitroprusside (SNP) are depressed in platelets with increased ability to aggregate [2, 3]. This observation assumes a functional connection between the aggregation process and GC. In the investigation described below a more detailed study was made of changes in activity of GC and its ability to undergo activation in human platelets during their aggregation. Platelet aggregation was induced by ADP in concentrations enabling only the reversible phase of aggregation to be recorded.

EXPERIMENTAL METHOD

Blood was taken from donors before breakfast, from the cubital vein, and a 3.8% solution of trisodium citrate was used as the anticoagulant, in the ratio of 1:9 by volume with blood. The blood was centrifuged for 10 min at 450g, and the resulting platelet-rich plasma was diluted to the concentration of $2.5 \cdot 10^8$ platelets/ml, necessary to study reversible aggregation [3]. Platelet-deprived plasma was obtained by centrifugation of platelet-rich plasma for 30 min at 650g. ADP was used as the inducer of aggregation and the course of aggregation was monitored by measuring the increase in the transmittance of light (540 nm) in the test plasma during continuous mixing (1000 rpm) [3].

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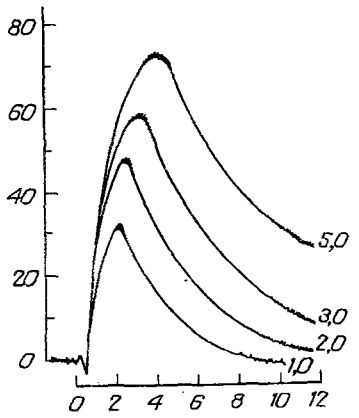

Fig. 1

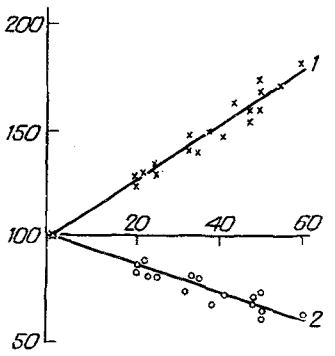

Fig. 2

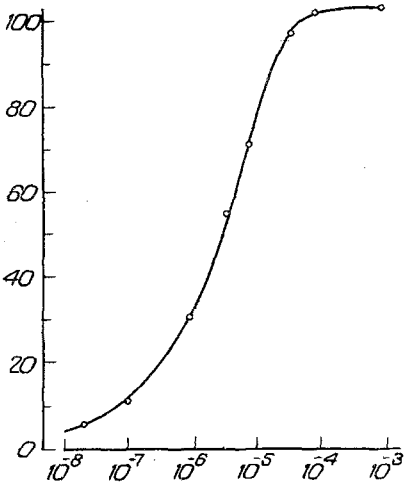

Fig. 3

Fig. 1. Dependence of degree of platelet aggregation on plasma ADP concentration. Abscissa, time (min) after addition of ADP, final ADP concentration (in μM) indicated next to corresponding aggregometer traces. Ordinate, platelet aggregation (per cent).

Fig. 2. Changes in basal activity and SNP-activated GC activity depending on degree of platelet activation. 1) SNP-activation of GC, coefficient of regression 0.90, 2) basal GC activity, coefficient of regression 0.89. Abscissa, degree of platelet aggregation (per cent). Ordinate, changes in parameters of GC (per cent of control).

Fig. 3. Dependence of antiaggregative action of SNP on concentration during its preincubation with platelets for 3 min. Abscissa, SNP concentration in plasma (in M). Ordinate, inhibition of ADP-induced aggregation (per cent).

For the enzymologic tests platelets were isolated from the blood by the method described previously [1], using Ficoll–Paque ("Pharmacia," Sweden). A suspension of washed platelets in 50 mM Tris-HCl (pH 7.6), containing 0.2 mM dithiothreitol, was sonicated at 0°C for 20 sec, then centrifuged for 1 h at 105,000g. The supernatant was used as the GC preparation. GC activity was determined by the method described previously [1] in the presence of 1 mM GTP and 4 mM $MgCl_2$. The quantity of cGMP formed in the course of the enzymic reaction (10 min at 37°C) was determined by radioimmunoassay, using the "cGMP RIA Kit" (Amersham, Great Britain). To determine the cGMP concentration in the platelets, the plasma was centrifuged at 1500g for 3 min, 50 mM Tris-HCl (pH 7.5), containing 4 mM EDTA was added to the residue of platelets, and the sample was sonicated, kept in a boiling water bath for 5 min, and centrifuged for 10 min at 1500g. The cGMP content was determined in the supernatant.

EXPERIMENTAL RESULTS

The degree of aggregation obtained by the use of ADP in final concentrations in the plasma of 0.5 to 5 μM is shown in Fig. 1. The degree of aggregation of the platelets increased with an increase in concentration of the inducer, but in each case it was reversible in character, i.e., when aggregation reached a maximum, disaggregation started. To determine GC activity we used platelets at the peak of aggregability (in accordance with Fig. 1), and compared the results with those obtained with control platelets, not treated with ADP. The results relating to changes in basal activity of GC and its ability to undergo SNP activation, depending on the degree of activation of the platelets, are given in Fig. 2. The degree of SNP-activation was calculated as the ratio of GC activity in the presence of 0.1 mM SNP to its activity in the absence of SNP. It will be evident that, with an increase in aggregation, the basal activity of platelet GC decreased, and the sensitivity of GC to the activator increased; both these relationships are linear in character.

TABLE 1. Platelet GC Activity During ADP-Induced Aggregation and Its Prevention by SNP (M ± m)

Experimental conditions	GC activity, pmoles cGMP/mg protein/min	Degree of SNP-induced activation
Control	181±14	9,9±0,7
Aggregation	119±9*	16,2±1,1*
Prevention of aggregation	196±15	9,4±0,4

Legend. Aggregation (50%) induced by 2 μM ADP. SNP (0.1 mM) added 3 min before addition of ADP, which completely prevented aggregation. Results of five independent experiments on platelets from blood of different donors are given. *$p < 0.05$ compared with control.

The results indicate that the development of aggregation is reflected in the enzymic properties of platelet GC. This is evidence in support of the view that the development of the aggregation process is a signal for the cGMP system, which is responsible for negative control over aggregation [5, 9-11].

It must be pointed out that the reduction of the basal GC activity which we found during platelet aggregation does not agree, at first glance, with facts in the literature indicating an increase in the intrathrombocytic cGMP level during this period [7, 11]. An increase in the cGMP concentration does in fact take place during aggregation, and we observed it experimentally. In intact platelets the cGMP level was 1.45 ± 0.11 pmole/10^9 cells, but by the time of appearance of peak aggregation (5 μM ADP) it had increased to 2.20 ± 0.13 pmoles/10^9 cells. It must be recalled that the intrathrombocytic cGMP concentration reflects GC activity in vivo, due to the action of endogenous activators and inhibitors on the enzyme [4, 7]. When GC activity is recorded in vitro in an enzyme preparation from the cytosol of washed and disintegrated platelets, the "true" activity of the enzyme is manifested, and this value can be used to judge ability of the platelets to aggregate. The increase in sensitivity of GC to the activator which we found evidently determines the increase in GC activity during aggregation, and this leads ultimately to elevation of the intrathrombocytic cGMP level.

The changes we recorded in the properties of GC may be due to differences in the degree of saturation of GC with heme. It is known [4, 8, 11] that GC contains heme as its prosthetic group; heme transmits the activating action of NO and NO-containing compounds on GC. Loss of heme by the enzyme leads to loss of sensitivity to NO-activators, whereas the addition of heme-containing protein to heme-deficient GC restores the ability of GC to be activated by NO. It has been suggested [4, 8] that a change in heme-deficiency of GC is the mechanism of regulation of its activity in the cell. Another regulating factor may be endogenous NO, found in various tissues of the body, and whose physiological role is linked with its action on GC [12]. The increase in sensitivity of platelet GC to SNP which we found could be evidence of an increase in saturation of GC by heme, as was verified by further experiments. Addition of hemoglobin in a concentration of 12 μM [1] to a preparation of GC from intact platelets led to an increase in the activating action of SNP by 1.5 times (158 ± 10% relative to normal), which we also recorded previously [1], and which indicates partial heme-deficiency of platelet GC. On the addition of hemoglobin to GC obtained from aggregated platelets, increased sensitivity of the enzyme to SNP was no longer observed (101 ± 7% of normal, pooled results of six independent experiments). It can be tentatively suggested that a decrease in heme-deficiency of GC is one of the factors acting on the platelet cGMP system during aggregation.

If platelets are incubated in SNP before ADP is added to them, this leads to weakening of aggregation or even its complete prevention (Fig. 3). Our preliminary experiments showed that the optimal duration of preincubation is 3-5 min. The antiaggregating effect of SNP also depended on its concentration and reached 100% at 10^{-4} M (Fig. 3). If GC activity was determined in platelets whose aggregation was completely prevented by SNP, the parameters of platelet guanylate cyclase were virtually indistinguishable from the control (Table 1).

The regulatory role of the cGMP system in platelet aggregation can thus be defined as an increase in the sensitivity of GC to an endogenous activator, conjecturally to NO. The results can explain the antiaggregating effect produced in vivo and in vitro by preparations containing NO (SNP) or giving rise to the formation of NO with the participation of tissue thiols (trinitroglycerin and its analogs) [7, 9, 12, 13]. The increase in sensitivity of platelet GC to NO-activators during aggregation makes it

possible to act on the enzyme pharmacologically with a view to preventing spontaneous platelet aggregation, which occurs in diabetes, atherosclerosis, angina, and other diseases [3, 13].

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