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# EFFECT OF GPIIb-IIIa COMPLEX LIGANDS ON CALCIUM ION MOVEMENT AND CYTOSKELETON ORGANIZATION IN ACTIVATED PLATELETS

Fabiola SINIGAGLIA, Antonella BISIO, Mauro TORTI,*Carlo L. BALDUINI, *Giampiera BERTOLINO and Cesare BALDUINI

Department of Biochemistry and *Department of Internal Medicine, University of Pavia, Italy

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We studied the influence of the occupancy of the fibrinogen receptor (GP IIb-IIIa complex)on two early aspects of agonist induced platelet activation: the increase of the intracellular $Ca^{2+}$ concentration and the cytoskeleton reorganization. A monoclonal antibody, a peptide containing the RGD sequence and fibrinogen purified from human plasma were used as GP IIb-IIIa ligands . The obtained results demonstrated that fibrinogen receptor occupancy inhibits $Ca^{2+}$ movement and cytoskeleton reorganization caused by low thrombin concentration and ADP. 

GP IIb-IIIa is a platelet-surface receptor for Fg as well as for Fn and vWf. These adhesive proteins contain at least one RGD sequence which is specifically recognized by purified platelet membrane GP IIb-IIIa(1). Moreover RGD containing peptides inhibit binding of Fg, Fn, and vWf to activated platelets(2-6) with a concomitant inhibition of platelet functions(4-7), mainly aggregation. Monoclonal antibodies directed against GP IIb-IIIa show similar effects(8-9),and moreover many Glanzmann thrombastenia-like syndromes have been demonstrated to be imputable to circulating autoantibodies directed against these membrane glycoproteins(10-11). The correlation between the binding of adhesive proteins, mostly Fg, to GP IIb-IIIa complex and platelet function has been further complicated by some recent observations which suggest that occupancy of specific sites on GP IIb-IIIa affects its structure, function and distribution on the platelet surface. Parise et al.(12) demonstrated that the RGDS peptide from the Fg $\alpha$-chain as well as the COOH-terminal decapeptide of the Fg $\gamma$-chain induces profound and specific changes in the conformation of purified GP IIb-IIIa which could be similar to the conformational changes caused by Fg binding to activated platelet surface. Fg receptor occupancy induces clustering of platelet associated GP IIb-IIIa(13), and is necessary to maintain Na+/H+ exchange in epinephrine-stimulated platelets(14). Furthemore Fg receptor associates with CS components during platelet aggregation(15) under conditions where Fg

Abbreviations: GP, Glycoprotein; Fg, Fibrinogen; Fn, Fibronectin; vWf, von Willebrand factor; MA, monoclonal antibody; CS, Cytoskeleton; BCA, Bicinchoninic acid; BSA, bovine serum albumin; PRP, Platelet rich plasma.

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is secreted from platelets and bound to their surfaces. It could thus be hypothesized that these, as well as other events, are signalled by different conformational states of GP IIb-IIIa related to the binding of specific ligands, and that they are regulated by the extent to which receptor sites are occupied. In the present study we examined the effect of Fg receptor occupancy on some aspects of platelet function. In particular we have taken two early events into account, the intracellular increase in $Ca^{2+}$ concentration and CS modification, induced in human platelets by different agonists. Experiments were performed by using as ligands an anti GP IIb-IIIa MA , PBM 6.4 (16) , that, as previously demonstrated (9), greatly enables platelet aggregation and ATP release, the pentapeptide Gly-Arg-Gly-Asp-Ser(GRGDS) and fibrinogen.

Our results demonstrated that Fg receptor occupancy, that is probably associated with GP IIb-IIIa conformational changes, modulates $Ca^{2+}$ intracellular increase as well as CS reorganization induced by thrombin and ADP.

## EXPERIMENTAL PROCEDURES

Reagents:Fura 2 was purchased from Molecular Probes; Sepharose 2B, gelatin- Sepharose, AFFI-Gel 731 and Sephadex G-25 were from Pharmacia; BCA was obtained from Pierce; $Na^{125}I$ was from Amersham International; BSA, human thrombin, ADP, GRGDS peptide, polyethylene glycol 6000 and hirudin were purchased from Sigma; human IgG were from Ismunit. All other reagents were analytical grade.

Platelet preparation:Blood was drawn from healthy volunteers who were drug free for at least 10 days before venipuncture. It was anticoagulated with ACD (acid-citrate -dextrose).PRP was obtained by centrifugation of blood at room temperature for 15 min at 120xg. In some experiments PRP was treated with 1 mM acetyl-salicilic acid (ASA) for 30' at 37°C. For measurement of intracellular calcium, PRP was incubated for 40 min at 37°C with 3 μM Fura 2 AM. Platelets as well as Fura 2-loaded platelets were gel filtered on Sepharose 2B, eluting with Hepes-Tyrode buffer, pH 7.4 (10mM Hepes, 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3) containing 0.5% BSA (w:v).Platelet count was adjusted to $2x10^8$/ml with the same buffer.

Measurement of $Ca^{2+}$ with Fura 2 :Fura 2 fluorescence was monitored continuously by using settings of 340 nm (excitation) and 510 nm (emission) in a Perkin-Elmer LS-3 Fluorescence Spectrometer. MA(50μg/ml), 400 μM pentapeptide or Fg( 0.4-1.2 mg/ml), when requested, were added just before stimulation to a 37°C prewarmed platelet suspension ($100-150x10^6$ cell/ml). Fura 2 fluorescence signals were calibrated by the method of Pollack et al (17). Peptide, MA and Fg did not modify by themselves the fluorescence of Fura 2-loaded platelets.

Fibrinogen purification:Fg was isolated from fresh human plasma by the polyethylene glycol 6000 precipitation procedure of Vila et al.(18). It was further purified by two sequential affinity chromatographies on gelatin-Sepharose and AFFI-Gel 731 to remove contaminating Fn and plasminogen, respectively. The columns were equilibrated and eluted with PBS buffer, pH 7.4, containing $2x10^5$U/l trasylol, 3 mM benzamidine, 2 mM PMSF. The purified fibrinogen was dialyzed overnight at 4°C against the same buffer, concentrated to 2-3 mg/ml and stored at -20°C . Protein concentration was determined by the BCA assay(19). Electrophoretic analysis under reducing and unreducing conditions according to Laemmli (20) revealed a pure preparation and suggested that no degradation occurred during the purification procedure.

Labelling of PBM 6.4 monoclonal antibody and fibrinogen:$^{125}I$ -labelling of the MA and of purified Fg was performed as previously described(21).The electrophoretic patterns were not modified by this treatment as verified after SDS-PAGE under reducing and unreducing conditions and autoradiography (data not shown). Ninety-five per cent of the radioactivity precipitated in 10% trichloroacetic acid. Protein concentration after labelling was determined by BCA assay (19).

Fibrinogen binding to gel filtered human platelets.

Binding assays were performed in triplicate using a method that involved rapid and complete separation of the platelets from the medium. Polypropylene BSA-precoated tubes were used in all phases of the experiments. In order to obtain a total GP IIb-IIIa complex dissociation, a suspension of gel filtered platelets in Hepes-Tyrode buffer prepared as above described was preincubated with

5mM EDTA for 10 min at 37°C. Alternatively, the cell suspension was rendered 1 mM $CaCl_2$ and incubated like EDTA -treated platelets. In a typical experiment, when 10 μM ADP was the agonist, EDTA or $Ca^{2+}$ treated platelets (at a final concentration of $5x10^8$/ml) were mixed with varying amounts of cold Fg(10-400 μg/ml) and the same amount of [$^{125}$I]-Fg (200 $x10^3$cpm). Potential Fg binding inhibitors, i.e. MA(50 μg/ml final concentration) or peptide (400μM final concentration), were added before or simultaneously with Fg. Binding experiments were initiated by addition of 10μM ADP. Samples were incubated at 37°C without stirring for 30 sec or 10-20 min, then100 μl aliquots were quickly layered over 140 μl of Hepes-Tyrode buffer rendered 11% sucrose (w:v) and centrifuged for 5 min at room temperature in a Heraeus Biofuge A at 13OOOxg. After centrifugation the tip of the tubes containing pelleted platelets was sliced off with a razor and the radioactivity associated with the cells determined by a LKB-$\gamma$ counter.

When thrombin was the agonist, experimental procedure was modified as follows: gel filtered platelets were stimulated with 0.1 U/ml thrombin for 3 min at 37°C , then hirudin was added (1U/ml) in order to prevent the clotting activity of the enzyme. The reaction was initiated by the addition of [$^{125}$I] -Fg mixed with different amount of cold Fg preceded or simultaneous with the eventual inhibitor addition as above described. Total, specific and aspecific Fg binding were determined either as described by Baldassare et al.(22 ) and by Di Minno et al. (23), obtaining similar results.

PBM 6.4 monoclonal antibody binding to gel filtered human platelets. GP IIb-IIIa complex dissociation and binding assays were performed as above described. In some experiments, EDTA or $Ca^{2+}$ treated platelets at a final concentration of $5x10^8$/ml were activated with thrombin (0.1- 0.5 U/ml) for 3 min at 37°C. Resting and activated platelets were mixed with PBM 6.4 (5-50 μg/ml) and [$^{125}$I]-MA($100x10^3$cpm) and incubated at 37°C without stirring for periods ranging from 1 to 60 min. In some experiments human IgG(100-500 μg/ml final concentration) were also present. Bound radioactivity was determined as above described.

Triton-insoluble residue analysis.Platelet CS were prepared after thrombin or ADP stimulation in the presence and in the absence of PBM 6.4 or GRGDS, as previously described(24). Triton-insoluble residues were quantitated by protein assay(19) and analyzed by SDS-PAGE(20) under reducing conditions. Gels were scanned with a CAMAG TLC-Scanner II at 546 nm. Peptide and MA did not significantly modify by themselves CS protein pattern of resting platelets.

## RESULTS

Studies with monoclonal antibody PBM 6.4:The MA specificity towards platelet membrane GP IIb-IIIa complex was previously demonstrated by immunoprecipitation(16). MA binding to platelet surfaces largely resembled Fg binding to activated platelets, i.e. it was dependent from $Ca^{2+}$and platelet activation; moreover, it was very rapid and not inhibited by the presence of human IgG in the medium.(data not shown). The amount of [$^{125}$I]-Fg bound to thrombin (O.1 U/ml) or ADP (5-10 μM) activated platelets, when preincubated for 3 min at room temperature with 50 μg/ml of MA,was significantly decreased. The degree of inhibition depended on the type of agonist used, ranging from 46% to 53% when 10 μM ADP was the stimulus, and only reaching a 32% value when platelets were activated with 0.1 U/ml thrombin. 50 μg/ml MA added to Fura 2-loaded platelets ($100x10^6$ cells/ml) just before stimulation with 10 μM ADP or 0.1 U/ml of thrombin, determined a significant inhibition of the increase of free intracellular calcium induced by both the agonists (Table I). ASA-pretreated platelets behaved like untreated platelets with respect to their responsiveness to agonists and were equally inhibited by MA. Table II reports the effect of MA on the amount of proteins recovered in the Triton-insoluble extract from thrombin treated platelets. Thrombocytes were activated with 0.1 U/ml enzyme at 37°C for 1 min in the presence of 1 mM $Ca^{2+}$. 50 μg/ml MA, when present, was added to 0.5 ml of prewarmed platelet suspension($1x10^9$ cells/ml). Pretreatment with MA PBM 6.4, significantly decreased platelet ability to organize CS

Table I: Effect of GP IIb-IIIa ligands on cytoplasmic free $Ca^{2+}$ increase in human activated platelets. Ligands concentrations are reported in the text. Results are mean ± S.D of 4 to 6 experiments. The significances (p) of the differences between the experiments performed in the absence and in the presence of each ligand are reported.

| | Cytoplasmic free-$Ca^{2+}$ concentration (nM) | | |
|---|---|---|---|
| | Agonists | | |
| | None | Thrombin (0.1 U/ml) | ADP (10 μM) |
| Platelets | 98 ± 12 | 564 ± 21 | 400 ± 35 |
| Platelets+MA | 102 ± 10 | 390 ± 27 p< 0.005 | 270 ± 39 p< 0.01 |
| Platelets +GRGDS | 101 ± 13 | 279 ± 55 p< 0.005 | 278 ± 38 p< 0.01 |
| Platelets+Fibrinogen | 100 ± 14 | 281 ± 99 p< 0.005 | 243 ± 41 p< 0.005 |

proteins in response to thrombin stimulus. Electrophoretic analysis and densitometric scanning of the gels showed , in the presence of MA, an evident decrease of all major CS components (Fig.1).

Studies with the RGD sequence containing peptide. In our experimental conditions, RGD sequence containing peptide, in accordance with literature data(4,6), strongly inhibits specific Fg binding to thrombin or ADP activated platelets.

TableII: Effect of GP IIb-IIIa ligands on cytoskeleton protein increases in human activated platelets. Ligands concentrations are reported in the text. Results are mean ± S.D of 4 experiments. The significances (p) of the differences between the experiments performed in the absence and in the presence of each ligand are reported.

| | Cytoskeleton proteins (%of total platelet proteins) | |
|---|---|---|
| | Agonist | |
| | None | Thrombin (0.1 U/ml) |
| Platelets | 7.19 ± 1.8 | 16.28 ± 1.7 |
| Platelets+MA | 7.44 ± 1.8 | 9.50 ± 1.4 p< 0.01 |
| Platelets+GRGDS | 7.58 ± 2.0 | 10.46 ± 1.2 p< 0.01 |

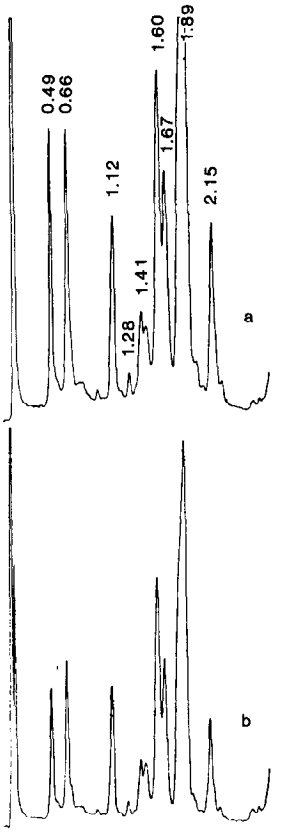


Figure1:Densitometric scanning of SDS-PAGE of Triton-insoluble residues from human platelets ; effect of Monoclonal Antibody . CS protein pattern obtained respectively from:(a), 0.1U/ml thrombin treated platelets; (b), like (a) in the presence of MA (50 μg/ml). Each sample contained the amount of Triton- insoluble protein that could be obtained from the same number of platelets ($0.4x10^8$ cells) and ranged between 25 and 35 μg depending on the type of treatment that platelets had undergone. The numbers over the peaks indicate the migration rate of each band. The identification was performed by comparison with the mobility of standard proteins of known molecular weight.

| | | |
|---|---|---|
| 0.49min | 250Kd | Acting Binding Protein |
| 0.66min | 200Kd | Myosin |
| 1.12min | 1O2Kd | α-Actinin |
| 1.28-1.41min | | unidentified |
| 1.60-1.67min | 62-58Kd | Fibrinogen |
| 1.89min | 45Kd | Actin |
| 2.15min | 30Kd | Tropomiosin |

The degree of inhibition ranged from 60% to 90% when evaluated at 30 sec from the start of the experiment as well as at 20 min. Addition of 400 μM peptide to Fura 2-loaded platelets and ASA-pretreated platelets resulted in a significant decrease of $Ca^{2+}$ movement when platelets were stimulated with low thrombin(0.1 U/ml) or 10 μM ADP(Table I). When high thrombin (1 U/ml) was used, the inhibition of $Ca^{2+}$ movement, though always present, was not statistically significant. GRGDS, like MA, significantly decreased the amount of proteins recovered in the Triton-insoluble residue after thrombin stimulation(Table II)and SDS-PAGE showed CS modifications similar to that obtained in the presence of MA.

Effect of Fibrinogen on cytoplasmic free-$Ca^{2+}$increase in thrombin and ADP activated platelets.In some experiments,Fg(O.4 and 1.2 mg/ml) was used as a ligand for IIb-IIIa complex and added to Fura 2-loaded platelets just before cells were stimulated with thrombin (O.1 U/ml)or 10 μM ADP in the presence of 1 mM $CaCl_2$ . Both Fg concentrations, as well as MA and GRGDS appeared to modulate the effect of both these agonists with respect to the increase of free $Ca^{2+}$ in platelet cytoplasm (Table I).

## DISCUSSION

The GP IIb-IIIa complex plays very important roles in platelet function: it represents the receptor for fibrinogen, fibronectin and von Willebrand factor and is a high affinity binding site for $Ca^{2+}$ on the platelet surface(25). Moreover its involvement in maintaining $Ca^{2+}$ homeostasis in unstimulated platelets has been previously demonstrated (26). In the present paper, the effect of the presence of some ligands specific for GP IIb-IIIa complex on intracellular $Ca^{2+}$ modification following thrombin and ADP stimulation, was investigated. In addition, cytoskeletal organization was also studied under the same experimental conditions. The monoclonal antibody PBM 6.4 binds specifically to the GP IIb-IIIa complex of activated platelets (16) and, in the presence of $Ca^{2+}$, impairs platelet aggregation and ATP release (9) and inhibits fibrinogen binding. When platelets were stimulated by low thrombin concentrations or ADP in the presence of PBM 6.4, there was a significant inhibition in the increase of intracellular free calcium. This inhibition was much less evident, and statistically unsignificant, when higher concentrations of thrombin were used. The concomitant decrease in the degree of cytoskeleton organization in the same experimental conditions, is in agreement with the results concerning the modifications of intracellular $Ca^{2+}$ concentrations, since it is well known that cytoskeleton assembly is calcium dependent. Our results regarding intracellular free $Ca^{2+}$ determination in activated platelets in the presence of an anti IIb-IIIa monoclonal antibody confirm observations made by Powling & Hardisty (27), who hypothesized that the effect of the antibody might be due to its steric hindrance, damaging a $Ca^{2+}$ channel near to GP IIb-IIIa complex.

However similar results were obtained by Yamaguchi et al.(28),using the exapeptide GRGDSP and by us, using the pentapeptide GRGDS; both these ligands contain the same RGDS sequence present at residues 572-576 of the fibrinogen A $\alpha$-chain (29) and have a small steric hindrance, so that it can be postulated that GP IIb-IIIa complex itself must be involved in the regulation of intacellular $Ca^{2+}$ concentration. When the sinthetic peptide GRGDS was used, it displayed a higher inhibitory effect than MA PBM 6.4, reducing at a greater extent the effects of low thrombin and of ADP stimuli on cytomplasmic $Ca^{2+}$ movement; at the same time pentapeptide significantly decreased the agonist induced cytoskeleton reorganization . These results suggest that the occupancy of GP IIb-IIIa binding sites can, in some way, modulate platelet responsiveness and that this modulation is particularly effective for low stimulations. In order to verify if this mechanism could have a physiological role in platelet function, the main plasma GP IIb-IIIa ligand, fibrinogen, was used to determine whether or not the binding to platelet surfaces, besides promoting aggregation, has a regulatory role in platelet function. Our results indicate that platelet stimulation by ADP or

low thrombin concentrations, in the presence of $Ca^{2+}$ and physiological concentrations of fibrinogen, is significantly less effective with respect to the ability to increase intracellular $Ca^{2+}$ levels. The mechanisms responsible for the reported data are not fully understood and their interpretation may only be speculated. However, it appears very probable that the GP IIb-IIIa complex plays two apparently opposite roles. On one hand it is responsible for the final stage of platelet activation and for the formation of the hemostatic plug; on the other hand, for low intensity stimulation, it inhibits the intracellular transduction of surface events.

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## REFERENCES

1- Lam S.C.T., Plow E.F., Smith M.A.,Andrieux A., Ryckwaert J. S.,Marguerie G.&Ginsberg M.H. (1987) J.Biol.Chem. 262, 947-950
2- Kloczewiak M., Timmons S.,Lukas T.J.&Hawiger J.(1984) Biochemistry, 23, 1767-1774
3- Plow E.F., Sronji A.H.,Meyer D.,Marguerie G.&Ginsberg M.H.(1984) J.Biol. Chem. 259, 5388-5391
4- Gartner T.K.&Bennett J.S.(1985) J.Biol.Chem. 260,11891-11894
5- Ginsberg M.H.,Pierschbacher M.D.,Ruoslahti E.,Marguerie G.A.&Plow E.F.(1985) J.Biol.Chem. 260, 3931-3936
6- Plow E.F.,Pierschbacher M.D.,Ruoslahti E., Marguerie G.A.&Ginsberg M.H. (1985) Proc. Natl. Acad. Sci. USA 82, 8057-8061
7- Haverstick D.M.,Cowan J.F.,Yamada K.M.& Santoro S.A.(1985) Blood 66, 946-952
8 -Coller B.S. (1984) Thromb. Haemost. 51, 169-173
9- Balduini C.L.,Sinigaglia F.,Damiani G.,Bertolino G.,Bisio A.&Zocchi E. (1986) Exp. Hematol., 14, 338-342
10-Balduini C.L.,Grignani G.,Sinigaglia F.,Bisio A.,Pacchiarini L.,Rota Scalabrini D.,Balduini C.,Mauri C.&Ascari E.(1987) Haemostasis 7, 98-104
11-Di Minno G., Coraggio F., Cerbone A.M., Capitanio A.M., Manzo C., Scarpato P., Dattoli G.M.R., Mattioli P.L., Mancini M.,(1986) J.Clin.Invest. 77,157-64
12-Parise L.V.,Helgerson S.L.,Steiner B,Naurizzi L.&Phillips D.R.(1987) J. Biol. Chem. 262, 12597-12602
13-Isenberg W.M.,McEver R.P.,Phillips D.R.,Schuman M.A.&Bainton D.F. (1987) J Cell Biol. 104, 1655-1663
14-Banga H.S.,Simons E.R.,Brass L.F.&Rittenhouse S.H. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9197-9201
15-Phillips D.R., Jennings L.K.&Edwards H.H. (1980) J Cell. Biol. 86 77-86
16-Damiani G., Zocchi E., Fabbi M., Bargellesi A.&Patrone F., (1983) Exp. Hematol. 11, 169-177
17-Pollock W.K., Rink T.J.,&Irvine R.F.,(1986) Biochem.J., 235, 869-877
18-Vila V., Reganòn E., Llopis F.&Aznar J., (1985) Thromb. Res. 39, 651-656
19-Smith P.K. ,Krohn R.I., Hermanson G.T., Mallia A.K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J. & Klenk D.C. (1985) Anal. Biochem. 150, 76-85
20-Laemmli U.K.(1970) Nature (London) 227, 680-85
21-Sinigaglia F., Balduini C.L., Bisio A.&Balduini C.(1985)Br.J.Haemat. 59, 587-592
22-Baldassare J.J., Bakshian S., Knipp M.A.&Fisher G.J.,(1985) J. Biol. Chem., 260, 10531-35
23-Di Minno G., Thiagarajan P., Perussia B., Martinez J., Shapiro S.,Trinchieri G.&Murphy S. (1983) Blood, 61, 140-148
24-Sinigaglia F., Balduini C.L., Bisio A.&Balduini C.(1985) Biochem. J. 232, 305-308
25-Brass L.F.&Shattil S.J.(1984)J.Clin.Invest.73,626-632
26-Brass L.F. (1985) J.Biol.Chem. 260,2231-2236
27-Powling M.J. & Hardisty R.M. (1985) Blood 66, 731-734
28-Yamaguchi A., Yamamoto N., Kitagawa H., Tanoue K.& Yamazaki H. (1987) FEBS Lett. 255,228-232
29-Plow E.F., Pierschbacher M.D., Ruoslahti E., Marguerie G. &Ginsberg M.H.(1987) Blood 70,110-115