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TYPE IIB VON WILLEBRAND FACTOR INDUCES PHOSPHOLIPASE A2 ACTIVATION AND CYTOSOLIC Ca^{2+} INCREASE IN PLATELETS[+]

Mariangela Francesconi, *Alessandra Casonato, *Elena Pontara, Lisa Dalla Via,*Antonio Girolami, and Renzo Deana**

Department of Biological Chemistry, Unit for the Study of Mitochondrial Physiology of C.N.R., *Institute of Medical Semeiotics and Fourth Chair of Internal Medicine, University of Padova, **Padova**, Italy

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SUMMARY: Von Willebrand factor (vWF) is a large glycoprotein which plays a central role in thrombus formation and blood clotting. The type IIB variant of vWF is characterized by an abnormally high affinity for the platelet receptor GPIb. Type IIB vWF purified from plasma and added to a platelet suspension induced a rapid, dose-dependent (1.2-9 μg/ml) increase in the cytosolic Ca^{2+} concentration. ATP secretion and platelet aggregation also occurred with type IIB vWF concentrations higher than about 5 μg/ml, which corresponds to the original plasmatic level. The IIB vWF-evoked (3 μg/ml) cytosolic Ca^{2+} increase was negligibly affected by ADP scavengers or protein kinase C inhibitors; it was drastically reduced by EGTA, La^{3+}, Ni^{2+} or acetylsalicylate and abolished by the phospholipase A2 inhibitors ONO-RS-082 or oleolyloxyethyl-phosphocholine. Platelet exposure to IIB vWF caused arachidonic acid release, thromboxane B2 and inositoltrisphosphate formation. LJIB1, a monoclonal antibody against GPIb, completely suppressed all platelet responses, whereas LJCP8, an antibody against the receptor GPIIb-IIIa ($\alpha_{IIb}\beta_3$ integrin), or the tetrapeptide RGDS, caused a complete inhibition of the aggregation but a partial inhibition of the activation-linked parameters. It is concluded that type IIB vWF-binding to GPIb induces phospholipase A2 activation, arachidonic acid release and GPIIb-IIIa dependent cellular Ca^{2+} influx. These events may lead to platelet secretion and aggregation.

Von Willebrand factor (vWF) is a large multimeric plasma glycoprotein which plays an essential role in the formation of platelet plugs at sites of vascular injury by mediating platelet adhesion to the exposed subendothelium [see reviews 1,2]. Two membrane glycoprotein receptors for vWF have been identified in platelets [3]. Unstimulated platelets bind vWF through the GPIb complex, whereas activated cells bind vWF, as well as other adhesive proteins, through the GPIIb-IIIa ($\alpha_{IIb}\beta_3$ integrin) [1,4,5].

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**Correspondence to R. Deana, Department of Biological Chemistry, Via Trieste 75, University of Padova, 35121 Padova, Italy. FAX #: +39-49-8073310.

Abbreviations used: vWF, von Willebrand factor; GP, glycoprotein; PL, phospholipase; PKC, protein kinase C; TBX, thromboxane; IP3, inositoltrisphosphate; ASA, acetylsalicylic acid; ONO-RS-082, 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid.

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The type IIB variant of vWF is characterized by an abnormally high affinity of vWF for the glycoprotein GPIb-IX, with the consequence that the largest multimers are cleared from the bloodstream [4,6]. In addition to a bleeding tendency, enhanced responsiveness of platelet rich plasma to ristocetin, spontaneous platelet aggregation and occasionally thrombocytopenia are the characteristics of the type IIB vW disease [7].

It has been demonstrated that molecular mutations, located in a single disulfide loop of the A1 domain, between the 509 and 695 aminoacid residues, are responsible for the increased affinity of IIB vWF for the receptor GPIb [1,2].

It is well known that platelet activation is generally associated with an increase in the cytosolic Ca^{2+} concentration evoked by inositol phosphates generated by a receptorial agonist-induced phospholipase C-mediated breaking of membrane phosphatidylinositols [8].

We have recently found that platelet aggregation induced by plasma from type IIB vW patients occurred in parallel with an increase in cytosolic Ca^{2+} concentration [9].

The purpose of this study was to further understand the mechanisms through which the IIB vWF promotes cytosolic Ca^{2+} increase and platelet activation. Our findings indicate that type IIB vWF binding to platelet GPIb induces activation of phospholipase A2, production of arachidonic acid and a GPIIb-IIIa dependent Ca^{2+} uptake; these events may lead to platelet secretion and aggregation.

MATERIALS AND METHODS

Materials. Apyrase, prostacyclin, acetylsalicylic acid, and peptide Arg-Gly-Asp-Ser (RGDS), were purchased from Sigma. D-myo-inositol-[^{3}H]1,4,5-trisphosphate assay system was from Amersham; fura 2/AM, oleoyloxyethyl-phosphocholine and ionomycin from Calbiochem. ATP monitoring reagent was from LKB, 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082) and thromboxane B2 ELISA kit from Cascade Biochemicals. Bisindolylmaleimide was from Boehringer Mannheim, [^{3}H]arachidonic acid from Du Pont, NEN, and the anti-phosphotyrosine PV20 (Mouse Hybridomas) from ICN Biochemical. Ro 31-8220 was a kind gift from Roche, Herts, U.K. The anti-GPIb (LJIB1) and anti-GPIIb-IIIa (LJCP8) monoclonal antibodies were generously provided by Dr. Z. M. Ruggeri.

Methods: Preparation of platelet suspensions. Blood samples were obtained from patients and healthy volunteers with their informed consent and in accordance with the declaration of Helsinki. Platelet suspensions ($2\text{-}5\cdot10^8$ cells in a medium consisting of 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES, pH 7.4), were prepared from citrated fresh blood as previously reported [9].

Determination of cytosolic free Ca^{2+} concentration and cellular Mn^{2+} influx. Cytosolic Ca^{2+} concentration was determined by the fluorescent probe fura 2 as described in Ref. [10]. Fluorescence was measured at 37° C in a thermostated, magnetically stirred cuvette. The Mn^{2+} uptake was followed using excitation and emission wavelengths of 360 nm and and 505 nm respectively, and a cellular suspending medium containing 1 mM Mn^{2+} instead of Ca^{2+}.

Platelet aggregation and secretion. Platelet aggregation and ATP secretion were monitored as described in [11], whereas serotonin secretion was measured according to Lapetina et al. [12].

Arachidonic acid release. The release of arachidonic acid was followed as described in [13]. Platelets loaded with [^{3}H]arachidonic acid (13.5 nM, 3μCi/ml) were incubated according to the experimental protocols and the reactions were terminated by addition of 3% glutardialdehyde containing 2 mM EDTA.

Measurements of thromboxane B2 formation. The production of TXB2 was assayed with the Cascade's "Thromboxane B_2 ELISA kit". The incubations of platelet suspensions ($3\cdot10^8$ cells/ml) with IIB vWF were terminated by the addition of glutardialdehyde (3% w/v) plus 2 mM EDTA. The samples were then immediately centrifuged and the supernatants used for the determination of TXB_2.

Assay for the inositoltrisphosphate formation. IP3 formation was measured with the Amersham's D-myo-inositol-[^{3}H]1,4,5-trisphosphate (IP3) assay system in samples ($5\cdot10^8$ cells/ml) incubated with IIB vWF for fixed times and then deproteinized with 10% (v/v)

perchloric acid. After neutralization of the supernatant with $KHCO_3$ and removal of excess K-perchlorate by centrifugation, aliquots of the latter supernatant were used for the IP3 determination.

Purification of vWF. The purification of vWF was performed from human plasma cryoprecipitates as described in [3]. Two different preparations of type IIB vWF were obtained from two patients belonging to the same family (C. Ar. and F. I.), who fulfilled the criteria for IIB vW disease as previously described in detail [6,9,14]. Normal vWF was purified from 1.5 liters of plasma obtained from three different healthy donors. The multimeric composition of purified vWF preparations was analyzed by SDS-containing agarose gel electrophoresis and autoradiography after reaction with radiolabeled specific anti-vWF antibody. Analysis of the purified vWF on reduced polyacrylamide gels, stained with Coomassie Blue, showed a major band with apparent M.W. of 220 kDa. Protein concentration was evaluated using the Bradford's method [15]. Purified vWFs were stored at -80°C.

Monoclonal antibodies against GPIb (LJIB1) and GPIIb-IIIa complex (LJCP8) were prepared and characterized by the Ruggeri's group as described elsewhere [16,17].

RESULTS

vWF was purified from plasma of both healthy people and patients fulfilling the criteria for type IIB vWdisease including lifelong bleeding tendency, heightened response of platelet rich plasma to ristocetin and reduced factor VIII activity [6,9]. Plasmatic as well as purified type IIB vWF lacked the larger multimeric forms present in the normal vWF (not shown).

The structural defect in the vWF of these patients was found to be a molecular modification consisting in the substitution of a cysteine for an arginine at position 545; (personal communication kindly provided by Dr. Judith Dent and Jerry Ware of the Dr. Z. M. Ruggeri's laboratory, Scripps Clinic and Research Foundation, La Jolla, California).

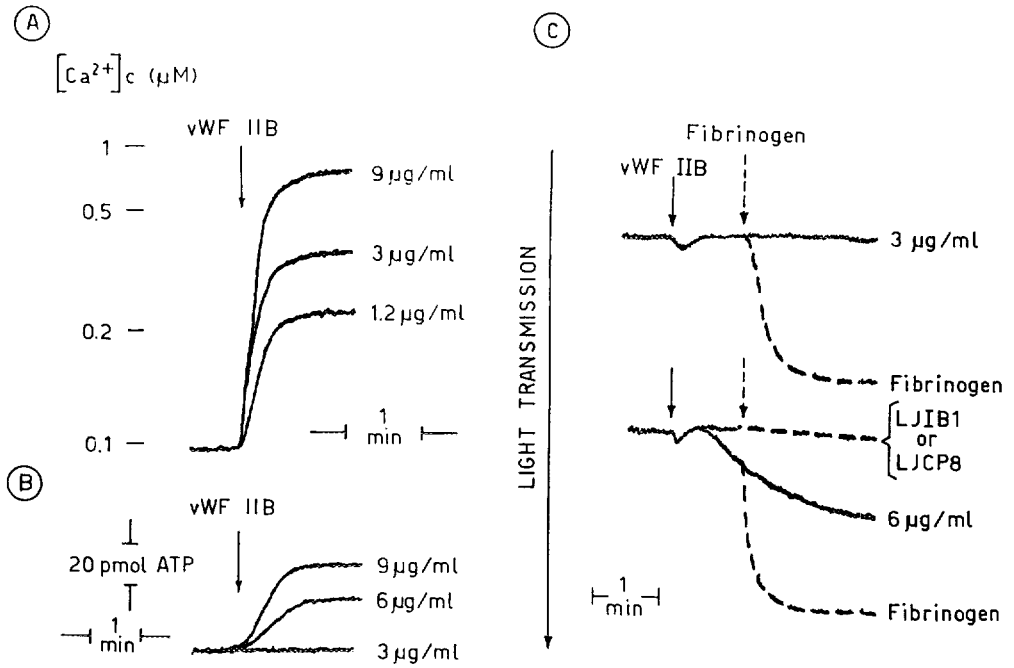

Fig. 1. Increase in cytosolic Ca^{2+} concentration (A), secretion (B) and aggregation (C) of platelets challenged with type IIB vWF. The indicated concentration of vWF IIB and 400 μg/ml of fibrinogen were added at the arrow. Where indicated, 50 μg/ml of monoclonal anti-GPIb-IX (LJIB1) and 120 μg/ml of anti-GPIIb-IIIa (LJCP8) were preincubated for 10 min at 37°C prior to IIB vWF addition. Tracings are representative of at least four different experiments.

Increase in the cytosolic Ca^{2+} concentration, secretion and aggregation of platelets challenged with type IIB vWF. Addition of type IIB vWF to a suspension of normal platelets induced a rapid cytosolic Ca^{2+} increase roughly proportional to the amount (1.2-9 μg/ml) of vWF added (Fig. 1A). By contrast, appreciable IIB vWF-induced secretion of ATP (Fig. 1B) and serotonin (data not shown) was evident only at IIB vWF concentrations higher than about 5 μg/ml, which is the concentration originally present in plasma. An equivalent concentration was found to be necessary for the occurrence of platelet aggregation, which was preceded by a small shape change (Fig. 1C). The rate and extent of this process was markedly increased by the addition of fibrinogen, which *per se* did not induce any detectable platelet aggregation. As expected no aggregation was found upon preincubation with monoclonal antibodies against platelet receptors GPIb and GPIIb-IIIa. None of the above reported platelet responses was found upon addition of normal vWF (9 μg/ml) (not shown).

All the following experiments were performed with 3 μg/ml of IIB vWF, i.e. in the absence of the meddling interference of both secretion and aggregation. The IIB vWF-evoked cytosolic Ca^{2+} increase was negligibly affected by addition of ADP scavengers (apyrase or the creatinephosphate/creatine kinase system, CK/CP) whereas it was drastically reduced in the presence of either the calcium-chelator EGTA or La^{3+} and Ni^{2+} inhibitors of the Ca^{2+} transport pathway of the plasma membrane [18,19] (Fig. 2A). Type IIB vWF induced a platelet uptake of Mn^{2+} similar to that of Ca^{2+} (not shown).

The intracellular calcium increase was also markedly inhibited by platelet preincubation with the cyclooxygenase-inhibitor acetylsalicylic acid (ASA) and abolished in the presence of the phospholipase A2 (PLA2) inhibitors ONO-RS-082 or oleoyloxyethyl-phosphocholine (Fig. 2B). The latter however induced *per se* a slight calcium concentration rise (not shown). Addition of the protein kinase C (PKC) inhibitor bisindolylmaleimide (Fig. 2B) or of fibrinogen (Fig. 2C) did not significantly affect the cytosolic Ca^{2+} concentration rise. Platelet preincubation with the monoclonal antibody against the receptor GPIb (LJIB1) caused a total block of calcium increase, whereas pretreatment with LJCP8, a monoclonal antibody against GPIIb-IIIa ($\alpha_{IIb}\beta_3$ integrin) or with the tetrapeptide RGDS, inhibitor of adhesive protein binding to this receptor (20), caused a marked but only partial inhibition of Ca^{2+} increase (Fig. 2C).

Arachidonic acid release and thromboxane formation. Platelets challenged with IIB vWF released arachidonic acid indicating that IIB vWF activates the phospholipase A2. The maximum release of arachidonic acid occurred after an approximate incubation period of 10 s (Fig. 3A). EGTA, ASA, RGDS and the monoclonal antibody LJCP8 partially inhibited, albeit to varying degrees, the release of arachidonic acid, whereas the anti-GP1b antibody LJIB1 and the PLA2-inhibitor ONO-RS-082 completely suppressed the acid formation.

Since arachidonic acid is converted to a variety of compounds including thromboxanes which act as potent platelet activators [8,21], we measured the thromboxane formation in IIB vWF-treated platelets and found that 1.25 ± 0.19 ng of TXB2 (mean ± S.D., n = 4) were released by 10^8 cells incubated for 2 min with 3 μg/ml IIB vWF. As expected, the TXB2 formation was suppressed in platelets preincubated with ASA.

Formation of inositoltrisphosphate (IP3). In order to verify whether phospholipase C (PLC) was involved in the IIB vWF-promoted platelet activation, the formation of the PLC-product inositoltrisphosphate was measured. Platelet incubation with IIB vWF brought about a transient increase in the IP3 concentration. The inhibition of IP3 formation was only partial with EGTA and LJCP8, whilst it was complete with ASA or LJIB1.

DISCUSSION

This study explored the mechanism of the type IIB vWF-evoked platelet aggregation. Consistently with our previous reports [6,9] the present results indicate that both vWF binding

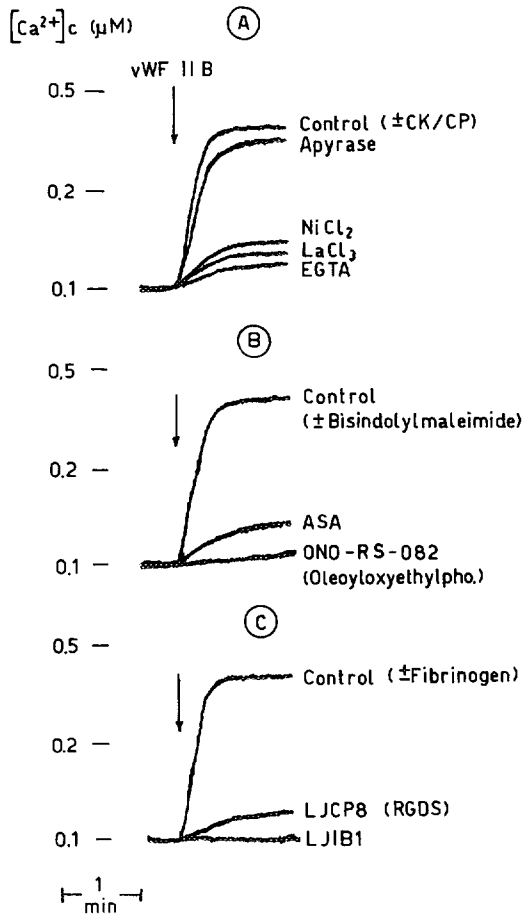

Fig. 2. Changes in the cytosolic Ca^{2+} concentration in platelets exposed to type IIB vWF under different conditions. Additions: 30 U/ml creatine kinase (CK), plus 5 mM creatine phosphate (CP), 20 µg/ml apyrase, 4 mM $NiCl_2$, 50 µM $LaCl_3$, 0.2 mM EGTA, 3 µM bisindolylmaleimide, 5 µM ONO-RS-082, 20 µM oleolyloxyethyl-phosphocholine, and 400 µg/ml fibrinogen were added 1 min prior to IIB vWF (3 µg/ml); 500 µM ASA, 50 µg/ml LJIB1, 120 µg/ml LJCP8, 100 µM RGDS were preincubated for 10 min at 37°C prior to IIB vWF addition. Representative tracings of at least three different experiments.

sites on the platelet membrane, i.e. GPIb and GPIIb-IIIa are involved in the IIB vWF-induced platelet activation. While GPIb appears to be absolutely essential for IIB vWF-promoted platelet activation to occur, GPIIb-IIIa seems to play a role in potentiating platelet responses. Indeed LJIB1, a monoclonal antibody against GPIb, completely abolishes all the activation-linked events i.e. arachidonic acid release, increase in the cytosolic Ca^{2+} concentration, secretion and aggregation, while either LJCP8, an anti-GPIIb-IIIa antibody, or the tetrapeptide RGDS, inhibit drastically but only partially, these processes, with the obvious exception of aggregation which requires both GPIb and GPIIb-IIIa to take place.

The main source of the type IIB vWF-elicited cytosolic Ca^{2+} increase appears to be the extracellular fluid. This conclusion is based, on the one hand, on the drastic inhibition in the Ca^{2+} rise observed in the presence of either the extracellular calcium chelator EGTA, or inhibitors of the cellular calcium uptake La^{3+} and Ni^{2+} and, on the other, on the similarity between IIB vWF-induced platelet uptake of Mn^{2+} with that of Ca^{2+}. The marked inhibition of

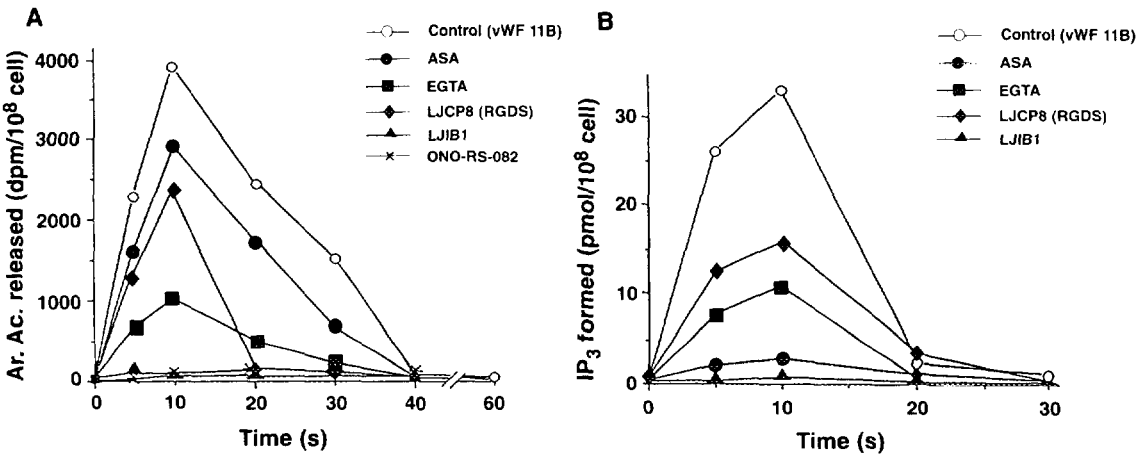

Fig. 3. Arachidonic acid release (A) and inositoltrisphosphate (IP3) formation (B) in platelets incubated with IIB vWF. Experimental conditions as in Fig. 2.

intracellular Ca^{2+} increase exerted by the antiboby LJCP8, or the tetrapeptide RGDS, indicates that the GPIIb-IIIa complex is involved in the calcium flux through the platelet plasma membrane. This conclusion is consistent with previous reports [22-24], whereas it is in disagreement with the reported findings on calcium influx induced by normal vWF in platelets exposed to high shear stress [25] or pretreated with ristocetin [26], an antibiotic which renders the GPIb highly reactive towards vWF [27]. In both cases the cytosolic Ca^{2+} increase was not affected by the anti-GPIIb-IIIa antibody LJCP8. It has been proposed that the intracellular calcium influx induced by normal vWF in platelets subjected to high shear stress is the first event promoting platelet activation and aggregation [24,25,28]. By contrast the present results suggest that the IIB vWF-induced cytosolic calcium ion rise follows the PLA2-dependent production of arachidonic acid. Indeed the cytosolic Ca^{2+} increase was suppressed by the PLA2 inhibitors in parallel with the prevention of arachidonate release.

The marked inhibition exerted by ASA on the cytosolic Ca^{2+} increase induced by IIB vWF indicates that the arachidonic acid metabolites thromboxanes and prostaglandin endoperoxides are chiefly responsible for the observed IIB vWF-promoted calcium uptake [29].

It may be worth to underline that the amount of arachidonic acid produced in the presence of anti-GPIIb-IIIa antibody is higher than that observed in the presence of EGTA. This finding suggests that the extracellular physiological level of Ca^{2+} is required for the PLA2 activation, independently of its flux through the GPIIb-IIIa pathway.

Two mammalian PLA2 have been identified: an abundant PLA2, released upon platelet activation [30], and a recently identified cytosolic PLA2, [31], whose specific functions are not yet fully defined [32]. At present we do not know the identity of the IIB vWF-activated PLA2. Interestingly, it has recently been shown that GPIb is associated with a protein having physicochemical characteristics and aminoacid sequence identical to the ζ-isoform of the human PLA2 and it has been suggested that the ligand occupancy of this receptor may directly activate this enzyme [33].

Taken together, the experimental evidences herein reported indicate that the interaction of IIB vWF with the platelet membrane GPIb brings about the activation of phospholipase A2 which produces arachidonic acid; this in turn causes an increase in the concentration of cytosolic Ca^{2+}, which derives partly from the intracellular stores but mainly from the extracellular fluid through a

GPIIb-IIIa dependent influx pathway. Even if arachidonic acid itself was sufficient to produce an appreciable increase in the cytosolic Ca^{2+} level, the latter was greatly enhanced by the cyclooxygenase metabolites [29] which bring about the phospholipase C-dependent production of the second messengers diacylglycerols and inositol-trisphosphates. The latter compounds can lead to platelet secretion and aggregation by activating the PKC.

It has been demonstrated elsewhere that the thrombin-induced protein tyrosine phosphorylation is regulated by GPIIb-IIIa and linked to the GPIIb-IIIa-mediated aggregation [34,35]. It may be worth mentioning that preliminary experiments have shown that tyrosine phosphorylation is also associated with the type IIB vWF-promoted platelet activation.

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