von Willebrand Factor Binding to Platelet GpIb Initiates Signals for Platelet Activation

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Abstract

The hypothesis that von Willebrand factor (vWF) binding to platelet membrane glycoprotein Ib (GpIb) initiates intracellular pathways of platelet activation was studied. We measured the biochemical responses of intact human platelets treated with ristocetin plus vWF multimers purified from human cryoprecipitate. vWF plus ristocetin causes the breakdown of phosphatidylinositol 4,5-bisphosphate, the production of phosphatidic acid (PA), the activation of protein kinase C (PKC), increase of ionized cytoplasmic calcium ($[Ca^{2+}]_i$), and the synthesis of thromboxane A_2. PA production, PKC activation, and the rise of $[Ca^{2+}]_i$ stimulated by the ristocetin-induced binding of vWF multimers to platelets are inhibited by an anti-GpIb monoclonal antibody, but are unaffected by anti-GpIIb-IIIa monoclonal antibodies. Indomethacin also inhibits these responses without impairing platelet aggregation induced by vWF plus ristocetin. These results indicate that vWF binding to platelets initiates specific intraplatelet signaling pathways. The mechanism by which this occurs involves an arachidonic acid metabolite-dependent activation of phospholipase C after vWF binding to platelet membrane GpIb. This signal then causes PKC activation and increases of $[Ca^{2+}]_i$, which promote platelet secretion and potentiate aggregation. (*J. Clin. Invest.* 1991. 88:1568–1573.) Key words: thrombosis • adhesion • phospholipases • protein kinase C • calcium

Introduction

Platelet plug formation is initiated by the adherence of platelets to sites of vascular injury. The components of this response include the subendothelial extracellular matrix, von Willebrand factor (vWF),[1] and platelet membrane glycoprotein (Gp) Ib. The multivalent vWF protein bridges constituents of the subendothelium to GpIb on the surface of circulating platelets. The fundamental importance of this adhesion event in normal hemostasis is demonstrated by the severe hemorrhagic diathesis suffered by individuals with quantitatively deficient or qualitatively aberrant vWF (1).

Under physiological conditions, platelet aggregation and secretion follow vWF-mediated adhesion. These functional responses are essential for establishing a hemostatically effective platelet plug, and are the culmination of a series of regulated intracellular biochemical reactions (2). Although much is known about the molecular mechanisms of platelet adhesion and activation, little data are available regarding the mechanism by which the adhesion and activation phenomena are coupled. Adhesion-activation coupling may be indirect, i.e., the adherent platelet may be subjected to exogenous stimuli arising from damaged endothelium, other blood cells, soluble clotting factors, or vasoreactive molecules (2–4). Alternatively, platelet adhesion may trigger activation through biochemical pathways directly coupled to the adhesive ligand-receptor interaction. Weiss et al. showed that normal platelets in platelet-rich plasma release ADP and serotonin in response to ristocetin, a macrolide antibiotic known to cause GpIb-dependent binding of vWF to unstimulated human platelets (5). More recently, it has been shown that asialo vWF, which is capable of ristocetin-independent binding to GpIb of resting platelets, causes the release of platelet adenine nucleotides, the synthesis of thromboxane A_2, and the binding of fibrinogen to GpIIb-IIIa (6–9). Moake et al. have shown that high shear forces induce large vWF multimers to bind to platelet GpIb in the absence of ristocetin or chemical modification of vWF, and this ligand-receptor interaction leads to the release of ADP and platelet aggregation (10).

This report presents data from experiments examining the hypothesis that vWF mediates adhesion-activation coupling in human platelets. Our results indicate that vWF binding to platelet GpIb initiates specific platelet signal transduction pathways that may couple adhesion to subsequent aggregate formation.

Methods

Materials. Human alpha thrombin was from United States Biochemical Corp. (Cleveland, OH). Ristocetin, creatinine phosphate (CP), creatinine phosphokinase (CPK), Sepharose 2B-300, fatty acid-free BSA, and indomethacin were from Sigma Chemical Co. (St. Louis, MO). Lipid standards were from Sigma Chemical Co. and Calbiochem-Behring Corp. (San Diego, CA). [^{32}P]Orthophosphoric acid and [^{14}C]-arachidonic acid were from New England Nuclear (Boston, MA). Fura2 acetooxymethyl ester was from Molecular Probes, Inc. (Eugene, OR). Whatman silica gel K5 and LK5D plates were from Fisher Scientific Co. (Fairlawn, NJ). HPLC grade organic solvents were from American Burdick and Johnson (Muskegon, MI). Monoclonal antibodies 7E3, 10E5, and 6D1 were provided generously by Dr. Barry Coller (SUNY Health Sciences Center, Stony Brook, NY).

Preparation of von Willebrand factor. vWF was purified from normal human cryoprecipitate as previously described (11), and quantified by solid-phase immunoradiometric assay (12). The multimeric

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1. *Abbreviations used in this paper:* Gp, glycoprotein; PA, phosphatidic acid; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; TXA_2, thromboxane A_2; vWF, von Willebrand factor.

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composition of the vWF preparations was determined by SDS-agarose gel electrophoresis using 1% agarose and a continuous buffer system, followed by gel overlay with rabbit ^{125}I-antihuman vWF IgG and autoradiography (11).

Platelet preparation. Venous blood from healthy volunteer donors who had not taken medications affecting platelets for at least 10 d before blood drawing was collected in 15% (vol/vol) acid-citrate-dextrose (ACD, NIH formula A). Blood was centrifuged at 180 *g* for 12 min and the platelet-rich plasma was acidified to pH 6.5 with ACD and treated with creatine phosphate and creatine phosphokinase (5 mM and 25 U/ml, respectively). This preparation was layered over a gradient of fatty-acid free BSA and centrifuged at 1,500 *g* for 15 min, as described by Walsh et al. (13). Interface platelets were collected and subjected to repeat albumin density gradient separation. Platelets isolated in this manner were then suspended in buffer A containing 6 mM glucose, 130 mM NaCl, 9 mM $NaHCO_3$, 10 mM Na citrate, 10 mM Tris base, 3 mM KCl, 2 mM Hepes, and 0.9 mM $MgCl_2$, pH 7.35.

The collected platelets were suspended in a small volume of buffer A (except where noted) and either radiolabeled or loaded with fura2 by incubating the platelets with the appropriate reagent for 1 h at 37°C in a gently shaking water bath. Platelets were radiolabeled with 0.5 mCi [^{32}P]orthophosphate or 20 μCi [^{14}C]arachidonic acid; or were loaded with 2 μM fura2 acetooxymethyl ester. After this, the platelet suspension was gel-filtered through Sepharose 2B-300 equilibrated with buffer A, and resuspended in buffer A containing 1 mM $CaCl_2$ at a concentration of 2.5×10^8 platelets/ml (except where noted). All reactions were carried out in a dual channel aggregometer (Payton Scientific, Buffalo, NY) in stirring platelet aliquots at 37°C.

Analyses of phospholipids and protein phosphorylation. ^{32}P-labeled platelets were mixed for 30 s with 1 mg/ml ristocetin, followed by the addition of purified vWF (antigen level = 100% of normal plasma [100 U/dl], unless otherwise stated). For platelet lipid measurements, the reactions were terminated at the designated time points by adding 3.8 × reaction volume of ice-cold MeOH/$CHCl_3$ (2:1, vol/vol) and extracting the lipids as previously described (14). [^{32}P]Orthophosphate-labeled phospholipids were separated on Whatman K5 plates previously dipped in 1% K oxalate/2 mM EDTA, using the solvent system $CHCl_3$/acetone/MeOH/glacial acetic acid/H_2O (40:15:13:13:12:7, vol/vol) according to the method of van Dongen et al. (15). For platelet protein phosphorylation experiments, ^{32}P-labeled platelets were stimulated as described and the reactions stopped by the addition of a solution of 50% glycerol, 10% dithiothreitol, 8% SDS, and trace bromphenol blue, followed by immediate boiling for 5 min. SDS-PAGE was performed on 7–17% gradient slab gels as described by Laemmli (16). The gels were stained with Coomassie brilliant blue R, destained, fixed, dried, and the radiophosphorylated proteins located by autoradiography. Protein phosphorylation was quantified by transmittance densitometry using a laser densitometer (LKB Instruments, Bromma, Sweden). Lipid bands were detected by autoradiography, identified by cochromatography with unlabeled standards, scraped, and counted for radioactivity.

Arachidonic acid release experiments. [^{14}C]Arachidonate-labeled platelets were assayed for free [^{14}C]arachidonic acid on Whatman LK5D plates using the solvents diethyl ether/hexane/glacial acetic acid (60:40:1, vol/vol) as previously described (14). Lipid bands were detected and reported by autoradiography.

Thromboxane A_2 measurements. Production of thromboxane A_2 was quantified by radioimmunoassay of its stable breakdown product, TXB_2, using a polyclonal rabbit antiserum from Seragen (Boston, MA), as previously described (14).

Measurement of platelet cytosolic calcium. Measurements of platelet ionized cytosolic calcium ($[Ca^{2+}]_i$) were made in a Deltascan spectrofluorometer (Photon Technologies International, Princeton, NJ) having dual wavelength excitation capacity. Albumin-washed platelets were loaded with 2 μM fura 2 acetooxymethyl ester, gel-filtered, and resuspended in buffer B (10 mM Na Hepes, 135 mM NaCl, 5 mM KCl, 5.5 mM glucose, and 1 mM $MgCl_2$, pH 7.3) with 1 mM $CaCl_2$. $[Ca^{2+}]_i$ was measured in a 1.5-ml cuvette of stirring fura 2–loaded platelets at 37°C by measuring absorbance at 510 nm after excitation at 340 and 380 nm. The ratio of absorbance 340/380 nm was used to calculate $[Ca^{2+}]_i$, using a k_d for fura 2 of 224 nM, as described by Grynkiewicz et al. (17).

Results

The responses of ^{32}P-labeled platelets to ristocetin-induced binding of purified vWF multimers to GpIb molecules on intact human platelets were studied. Fig. 1 shows SDS-polyacrylamide and agarose gel electrophoretograms of the purified vWF used in these studies. The largest vWF multimers found in normal plasma were present in the purified vWF preparations.

Washed stirred platelets treated with 1 mg/ml ristocetin alone, or washed unstirred platelets treated with ristocetin plus vWF, do not demonstrate any biochemical responses (data not shown). Fig. 2 shows that ristocetin-induced vWF binding to platelets, under stirred conditions, stimulates platelet production of phosphatidic acid (PA), a product of phosphoinositide turnover that may be an intracellular stimulatory molecule (14). Because PA can be generated as a consequence of the phospholipase C–mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), we measured changes in PIP_2 after ristocetin-induced vWF binding. The breakdown of PIP_2 precedes PA production: PIP_2 decreases to 69.3±5.2% of basal levels (mean±SEM; n = 6) 45 s after the addition of purified vWF multimers.

Phospholipase C–mediated signal transduction in platelets results in the activation of protein kinase C (PKC) and elevation of $[Ca^{2+}]_i$. To determine if the binding of purified vWF

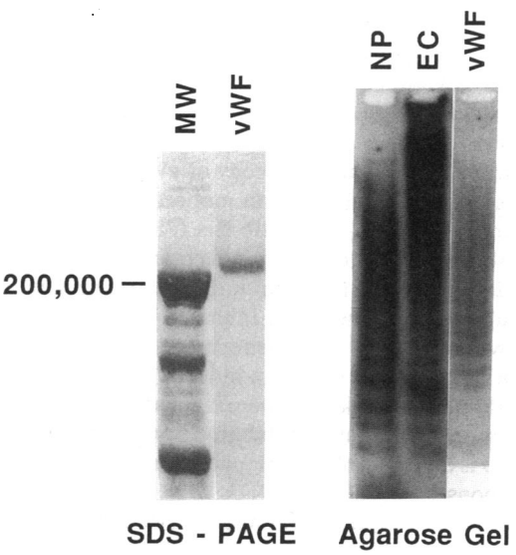

Figure 1. The homogeneity and multimeric structure of purified vWF used in these studies. vWF was purified from human cryoprecipitate as described in Methods, quantified by IRMA, and analysed by SDS-PAGE through 4% acrylamide under reducing conditions, and by electrophoresis through SDS-1% agarose. The left side of the figure shows a homogeneous Coomassie-stained band of $M_r \approx 225{,}000$, representing the reduced vWF monomer; the right side is an autoradiogram that shows the multimeric pattern compared to vWF in normal pooled platelet-poor plasma (*NP*) and vWF secreted from human umbilical vein endothelial cells (*EC*). EC vWF includes unusually large vWF forms.

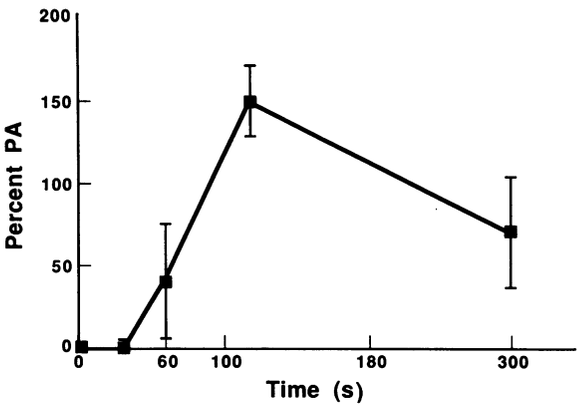

Figure 2. Ristocetin-induced vWF multimer binding stimulates platelet phosphatidic acid (PA) generation. Stirring ^{32}P-loaded aliquots of 2.5×10^8 platelets/ml in buffer A with 1 mM $CaCl_2$ were pretreated for 30 s with ristocetin (1 mg/ml) to which was added purified vWF multimers (antigen level = 100%). Radiolabeled PA was extracted from platelet phospholipids, separated by thin-layer chromatography, and quantified by liquid scintillation counting. The ordinate is the percent increase in PA production above basal levels at time 0. Each point represents the mean±SEM of four separate experiments.

multimers to platelets activates PKC, the phosphorylation of its M_r 47,000 substrate (p47) was measured in ^{32}P-labeled platelets exposed to ristocetin. Fig. 3 shows that p47 phosphorylation of platelets binding vWF increases over a period of 4 min. To determine if platelet $[Ca^{2+}]_i$ changes after ristocetin-induced binding of purified vWF multimers, fura 2–loaded platelets were analyzed spectrofluorometrically. Fig. 4 shows that platelet $[Ca^{2+}]_i$ responses to ristocetin plus vWF occur, and that $[Ca^{2+}]_i$ increases as the vWF antigen concentration is raised from 10 to 100%. A similar dose-response relationship was observed for platelet PA production and p47 phosphorylation (data not shown).

To determine the receptor specificity of vWF-induced signals, platelets were preincubated with monoclonal antibodies to platelet GpIb (6D1, [18]) or GpIIb-IIIa (7E3 [19] or 10E5 [20]). Fig. 5 shows that platelet PA production and p47 phosphorylation 2 min after the ristocetin-induced binding of purified vWF multimers are completely inhibited by 6D1, but are unaffected by 7E3. Fig. 6 shows similar results for $[Ca^{2+}]_i$ changes associated with the ristocetin-induced binding of purified vWF multimers after platelets were incubated with 6D1 or 10E5, and demonstrates that the inhibition by 6D1 observed at 2 min in Fig. 5 is due to an ablated response, rather than due to a delayed response.

An important consequence of some platelet activation signals is the production of thromboxane A_2 (TXA_2). This eicosanoid has proaggregatory and vasoconstrictor properties that amplify the initiating stimulus and contribute to platelet plug formation. It is produced as a consequence of release of free arachidonic acid from membrane phospholipids, followed by the metabolism of this substrate to prostagandin endoperoxides (catalyzed by cyclooxygenase) which are converted to TXA_2 (catalyzed by TXA_2 synthase). To directly demonstrate this, we measured the release of [^{14}C]arachidonic acid and the production of TXB_2, the stable breakdown product of TXA_2, in platelets after the ristocetin-induced binding of purified vWF multimers. Fig. 7 demonstrates the time-course of platelet arachidonic acid release and TXB_2 production after ristocetin-induced binding of purified vWF multimers to platelets. This figure also shows that TXB_2 generation is slower than with thrombin (1 U/ml). The release and metabolism of arachidonic acid observed in these experiments appear to occur after the elevation of $[Ca^{2+}]_i$ (Fig. 4). This suggests that this eicosanoid-generating signal pathway is activated as a consequence of the rise of $[Ca^{2+}]_i$, probably resulting in the stimulation of platelet phospholipase A_2 (2).

Another molecule that is released from aggregating platelets is ADP. ADP is secreted from platelet dense granules, and can further activate and thereby recruit circulating platelets into the developing thrombus (2). To determine if ADP released as a consequence of platelet aggregation affects platelet signal generation, we pretreated platelets with CP/CPK (5 mM/25 U/ml, respectively), and measured PA production and p47 phosphorylation in response to risocetin and vWF. CP/CPK, which scavenges ADP and eliminates the effect of released ADP on platelets, has no significant inhibitory effect on platelet PA production and p47 phosphorylation in response to risocetin and vWF (data not shown).

The weak agonists ADP and epinephrine activate platelets through a mechanism that depends on the release and metabolism of small (and generally unmeasurable) amounts of arachidonic acid (2, 21). To determine if the vWF-GpIb interaction initiates a cyclooxygenase-dependent pathway of intracellular signal generation that subsequently activates platelet phospholipase C, platelets were pretreated with indomethacin, stimulated with purified vWF multimers and ristocetin, and assayed

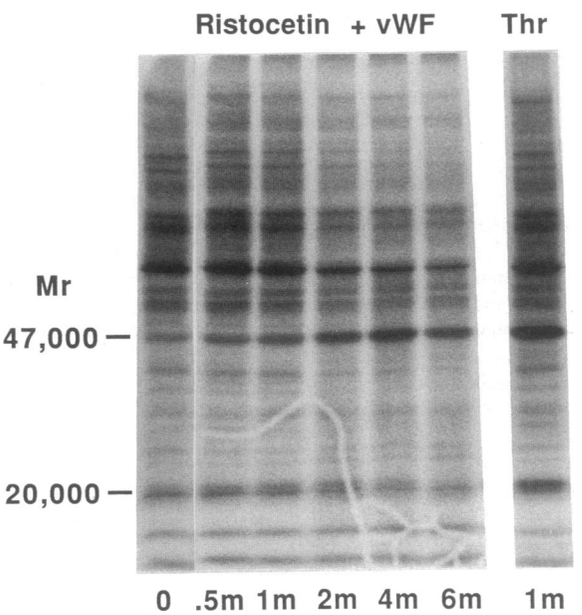

Figure 3. The ristocetin-induced binding of vWF multimers to platelets is associated with an increase in the phosphorylation of p47, a M_r 47,000 substrate of platelet protein kinase C. Stirring ^{32}P-loaded aliquots of 2.5×10^8 platelets/ml in buffer A with 1 mM $CaCl_2$ were treated for 30 s with ristocetin (1 mg/ml) and then mixed with purified vWF multimers (antigen level = 100%). Platelet phosphoproteins were separated by 7–17% gradient SDS-PAGE, fixed, dried, and reported by autoradiography. The phosphorylation response of platelets treated with 1 U/ml thrombin (*Thr*) is given for comparison. This autoradiogram is representative of eight separate experiments.

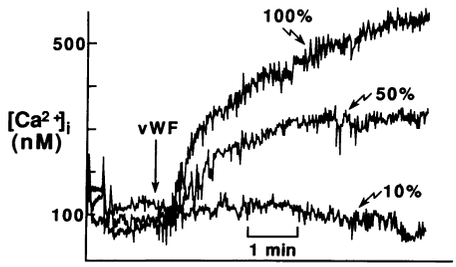

Figure 4. Ristocetin-induced binding of vWF multimers is associated with elevations of platelet-ionized cytosolic calcium ($[Ca^{2+}]_i$). Washed platelets loaded with fura 2, in buffer B with 1 mM $CaCl_2$, were treated for 30 s with ristocetin (1 mg/ml) followed by purified vWF multimers (antigen levels of 100, 50, or 10%). $[Ca^{2+}]_i$ was determined by measuring fluorescence emmission at 510 nm after the dual excitation of the platelets at 340 and 380 nm. The ratio of emission at 510 nm after excitation at 340 nm and 380 nm (denominator) was used to calculate $[Ca^{2+}]_i$ as described in Methods. This figure is representative of four separate experiments.

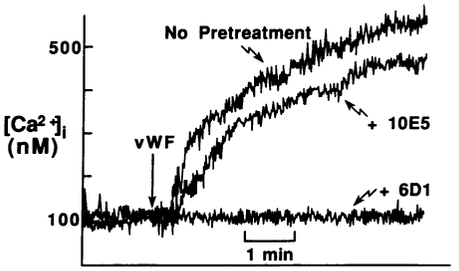

Figure 6. vWF-induced elevations of platelet ionized cytosolic calcium ($[Ca^{2+}]_i$) are inhibited by a monoclonal antibody to GpIb (6D1) but less affected by a monoclonal antibody to GpIIb-IIIa (10E5). Washed platelets loaded with fura 2, in buffer B with 1 mM $CaCl_2$, were treated for 30 s with ristocetin (1 mg/ml) and then mixed with purified vWF multimers (antigen level = 100%). $[Ca^{2+}]_i$ was determined as described in Methods. This figure is representative of four separate experiments.

for changes in lipid and protein phosphorylation. Fig. 8 shows that platelet PA production and p47 phosphorylation 2 min after the initiation of vWF-mediated aggregation are inhibited by indomethacin, and Fig. 9 shows that indomethacin inhibits the platelet $[Ca^{2+}]_i$ response to ristocetin plus vWF.

The aggregation of platelets induced by vWF multimers and ristocetin does not require a living cell. To corroborate that platelet signals generated during ristocetin-induced vWF binding are not due to passive platelet agglutination, we measured simultaneously, in separate aliquots, aggregation and $[Ca^{2+}]_i$ of indomethacin-pretreated, fura 2–loaded platelets. Fig. 9 shows that indomethacin inhibits changes of $[Ca^{2+}]_i$ without inhibiting vWF-induced platelet aggregation. This divergence of the platelet aggregation response from the $[Ca^{2+}]_i$ response is also observed with a lower concentration of purified vWF multimers in the absence of indomethacin: a 10% antigen level causes full aggregation of intact platelets (not shown) but no change in their $[Ca^{2+}]_i$ (Fig. 4).

Discussion

Data presented here demonstrate that ristocetin-induced vWF binding to platelets initiates specific intracellular signals for platelet activation. We have found that vWF and ristocetin cause platelets to hydrolyze PIP_2, generate PA, phosphorylate p47, and increase $[Ca^{2+}]_i$. Because the platelet has two binding sites for vWF, GpIb, and GpIIb-IIIa, the receptor specificity of platelet biochemical responses to vWF and ristocetin was examined. We found that platelet signals in response to vWF and ristocetin are inhibited by a monoclonal antibody to platelet GpIb, but not by antibodies to GpIIb-IIIa. This suggests that

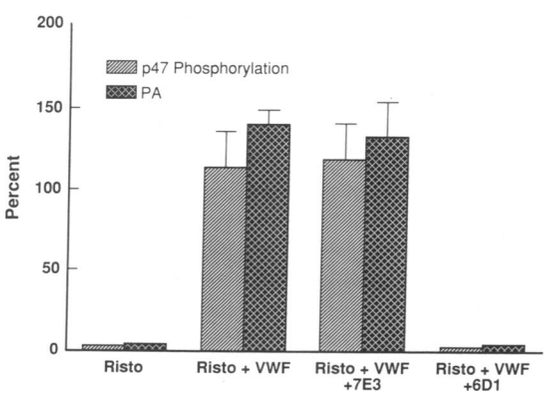

Figure 5. Platelet phosphatidic acid (PA) production and protein kinase C–dependent protein phosphorylation (p47) are inhibited by a monoclonal antibody to GpIb (6D1) but unaffected by a monoclonal antibody to GpIIb-IIIa (7E3). Stirring ^{32}P-loaded aliquots of 2.5×10^8 platelets/ml in buffer A with 1 mM $CaCl_2$ were treated for 30 s with ristocetin (1 mg/ml) followed by purified vWF multimers (antigen level = 100%) for 2 min. p47 phosphorylation and PA production were quantified as described in Methods. Each bar represents the mean±SEM of the increase from basal levels for three to six separate experiments.

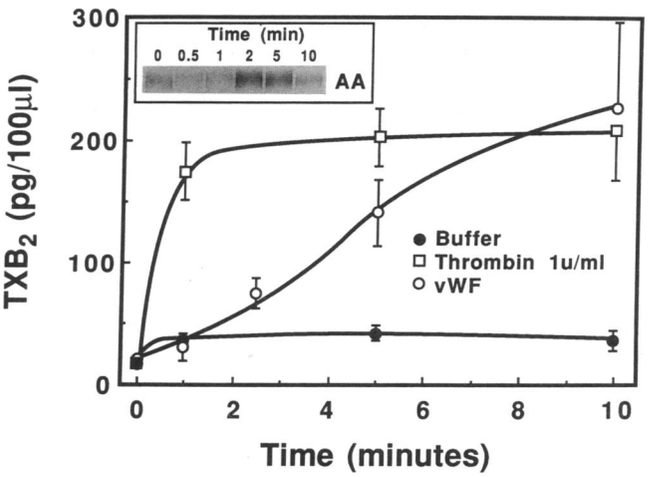

Figure 7. Platelets treated with purified vWF multimers (antigen level = 100%) in the presence of ristocetin (1 mg/ml) release free [^{14}C]-arachidonic acid and synthesize thromboxane A_2 (TXA_2). The production of [^{14}C]arachidonic acid was measured as described in Methods, and reported by autoradiography. TXA_2 was measured by radioimmunoassay of its stable breakdown product TXB_2. The time course of platelet TXA_2 production in response to vWF binding is compared to both a buffer control and thrombin (1 U/ml). Each point represents the mean±SEM of three to six determinations.

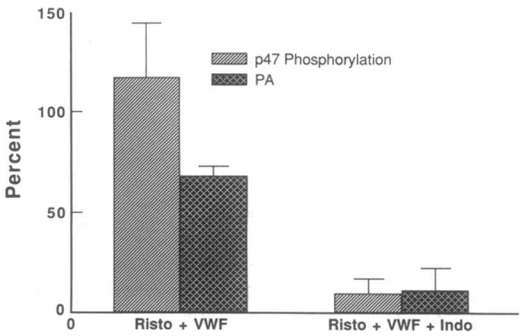

Figure 8. Indomethacin (0.27 mM) inhibits vWF-induced platelet phosphatidic acid (PA) production and protein kinase C–dependent protein (p47) phosphorylation. Stirring ^{32}P-loaded aliquots of 2.5 $\times 10^8$ platelets/ml in buffer A with 1 mM $CaCl_2$ were treated for 30 s with ristocetin (1 mg/ml) followed by vWF (antigen level = 100%) for 2 min. Platelet phosphoproteins were separated by 7–17% gradient SDS-PAGE, fixed, dried, and autoradiographed. p47 phosphorylation and PA production were quantified as described in Methods. Each bar represents the mean±SEM of the increase from basal levels for three separate experiments.

the specific interaction between vWF and platelet GpIb leads directly to intraplatelet biochemical responses.

The kinetics of vWF-mediated generation of platelet signals are different from those observed with the strong agonist thrombin, which rapidly (within 15 s) activates platelet PKC and stimulates changes in $[Ca^{2+}]_i$ (2). The lag phase of signal generation that was observed in our experiments is similar to that reported by Weiss et al. who measured the release of ADP and serotonin from intact platelets in platelet-rich plasma treated with ristocetin (5). The reason for this delay is not known. We have considered that the lag phase of platelet signal generation in response to the binding of large vWF multimers may be due to an initial requirement for the release and metabolism of arachidonic acid, as occurs with the weak platelet agonists ADP or epinephrine (21). When ADP or epinephrine binds to its specific platelet receptor, phospholipase C is activated as a consequence of an initiating cyclooxygenase metabolite-dependent signal. Our observations that indomethacin inhibits vWF/ristocetin-mediated platelet PA production, PKC activation, and changes of $[Ca^{2+}]_i$, without suppressing platelet aggregation, are consistent with this hypothesis. Because phospholipase A_2 (PLA_2)-mediated hydrolysis of membrane phospholipids is the major pathway of mobilization of endogenous arachidonic acid for prostaglandin endoperoxide and TXA_2 synthesis in stimulated platelets, our data are consistent with the hypothesis that there is an initial activation of PLA_2 after vWF binding to GpIb, and that this then leads to the release of free arachidonic acid and to the formation of cyclooxygenase products capable of activating phospholipase C.

The molecular mechanisms of vWF/GpIb-induced platelet signal generation are not known. Studies of thrombin-platelet interactions have demonstrated that this platelet agonist binds to GpIb, but the consequences of this for platelet signal transduction are uncertain (22, 23). GpIb is a transmembranous heterodimer that may interact with other platelet surface glycoproteins, including the Fc receptor and the CD9 complex, both of which are putative extracellular signal-transducing proteins (24, 25). No unequivocally established signaling function has been demonstrated for platelet GpIb although there is, in addition to evidence for its possible role in mediating thrombin-induced platelet activation, further indirect evidence that GpIb is a signal transducing protein: cAMP phosphorylates the β chain of GpIb (26), decreases thrombin binding to platelets (27), and inhibits platelet activation (2). Our data suggest the possibility that platelet membrane GpIb, after the binding of vWF, undergoes a conformational change that directly, or indirectly through a coupling protein, causes the activation of platelet PLA_2 to initiate the sequence of intracellular signaling events reported in this study. vWF/GpIb-mediated signaling may also require interplatelet bridging through this specific receptor/ligand interaction. This hypothesis is based on our observations that the biochemical responses of platelets to ristocetin plus vWF do not occur in unstirred platelet suspensions where platelet–platelet interactions are minimal. Such platelet–platelet interactions, although they may be required for vWF-induced activation to occur, are insufficient for platelet signals to be generated: indomethacin inhibits signals in response to vWF binding to GpIb *without* inhibiting aggregation.

In summary, we have demonstrated that ristocetin-induced vWF binding to platelet membrane GpIb initiates specific biochemical pathways of platelet activation. These results suggest that platelet adhesion mediated by vWF multimers may lead directly to platelet secretion and aggregation through a mechanism that is intrinsic to the adherent platelet. The relevant ligand is vWF (derived from plasma, platelets, or the subendothelium), and the receptor that couples this extracellular signal to an intracellular response is platelet membrane GpIb: vWF binds to GpIb, thus mediating not only the process of adhesion, but also simultaneously activating intracellular signal pathways that cause PKC activation and the elevation of $[Ca^{2+}]_i$. These signals then lead to the release reaction and the

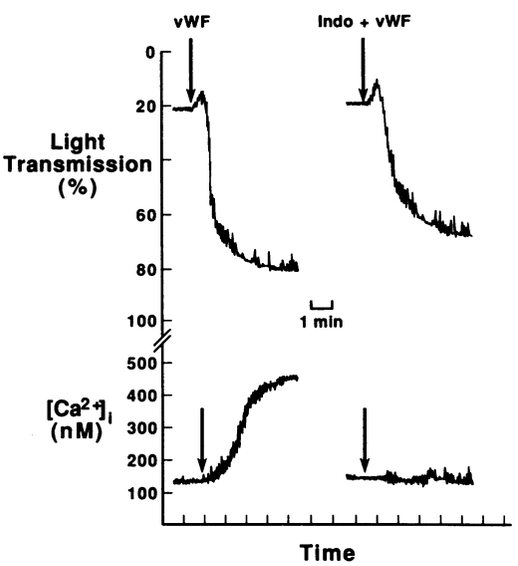

Figure 9. Indomethacin (0.27 mM) inhibits vWF-induced elevations of platelet cytosolic calcium ($[Ca^{2+}]_i$) without inhibiting platelet aggregation. Washed platelets loaded with fura 2, in buffer B with 1 mM $CaCl_2$, were treated for 30 s with ristocetin (1 mg/ml) and then mixed with purified vWF multimers (antigen level = 100%). $[Ca^{2+}]_i$ was determined as described in Methods. Aggregation of identically prepared platelets was measured simultaneously by light transmittance in an aggregometer (Chronolog, Haverton, PA).

recruitment of circulating platelets to the site of vascular injury. It is not yet known if, in vivo, vWF-mediated platelet adhesion to subendothelium is directly coupled to activation pathways that promote platelet aggregate formation. Studies of the molecular mechanisms of these responses, and of ristocetin-independent vWF binding to platelets, should help to clarify the physiological importance of vWF-mediated adhesion-activation coupling.

Acknowledgments

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