0026-895X/90/020271-07$02.00/0

MOLECULAR PHARMACOLOGY, 37: 271-277

Effect of Platelet Activation on the Agglutination of Platelets by von Willebrand Factor

DAVID C. B. MILLS, KATHRYN HUNCHAK, DANIEL W. KARL, and EDWARD P. KIRBY

Thrombosis Research Center and the Departments of Pharmacology and Biochemistry, Temple University Health Sciences Center, Philadelphia, PA 19140

Received December 16, 1988; Accepted October 15, 1989

SUMMARY

Agglutination of human platelets by bovine von Willebrand factor (vWF) or by human vWF in the presence of ristocetin is inhibited by ADP and by several other platelet agonists but not by epinephrine. Vincristine, which causes a shape change by disrupting microtubules, neither inhibited agglutination nor blocked the effect of ADP. The action of ADP was blocked by ATP, by *p*-fluorosulfonylbenzoyladenosine, and by the thiol-reactive reagents cytochalasin A and *p*-chloromercuribenzenesulfonate. In contrast to its effects on vWF, ADP enhanced agglutination induced by wheat germ lectin. ADP caused a small decrease in the number and affinity of binding sites for vWF on platelets, too small to explain the inhibition of agglutination. The ability of ADP and other agonists to inhibit agglutination appears to be related neither to inhibition of adenylate cyclase nor to the loss of their discoid shape but rather to the membrane changes that accompany the shape change.

vWF is a plasma glycoprotein required for the normal adhesion of platelets to subendothelial tissue (1). Its absence in von Willebrand's disease is associated with a bleeding tendency and prolonged bleeding time. In the presence of the antibiotic ristocetin, vWF causes platelets to agglutinate (2). This agglutination, in contrast to aggregation, does not require Ca^{2+} and occurs even when the platelets have been fixed (3). Bovine and porcine vWF can bind directly to human platelets and agglutinate them without ristocetin (3, 4). Platelets from patients with the Bernard-Soulier syndrome, which lack GPIb, are not agglutinated by vWF and do not adhere normally to the subendothelial matrix (5).

Platelet activation by ADP is associated with a change in the shape of the cell from a disk to an irregular spiny sphere with pseudopods (6), accompanied by a transient increase in the phosphorylation of platelet myosin light chain (7). ADP also inhibits adenylate cyclase in platelet membranes (8) and impedes the accumulation of cyclic AMP in platelets exposed to stimulators of adenylate cyclase (9). Many other aggregating agents inhibit adenylate cyclase; however, dideoxyadenosine, which inhibits adenylate cyclase in both membranes and intact cells by a direct effect on the catalytic subunit, neither induces nor potentiates platelet activation (10).

This work was supported by National Institutes of Health Grants HL27993 and HL37932.

Cohen *et al.* (11) showed that ADP inhibits the agglutination of thrombasthenic platelets by vWF and ristocetin, and Grant *et al.* (12) observed the same effects with normal human platelets and bovine or porcine vWF. Our goal was to determine whether the effect of ADP is associated with the shape change and platelet activation or with effects on cyclic AMP metabolism.

Materials and Methods

Buffers. Tris-saline was 10 mM Trizma base, 150 mM NaCl, adjusted to pH 7.4 with HCl. Wash Buffer A was Tyrode's buffer (13) containing 22 mM Na_3 citrate, 5.5 mM glucose, 5.5×10^{-5} M (3.5 g/liter) bovine serum albumin, and 0.05 units/ml apyrase, adjusted to pH 6.5 by addition of 1 M citric acid.

Reagents. Bovine vWF was purified from bovine plasma (14) and labeled with ^{125}I by the Iodogen procedure (15) to approximately 1 atom of iodine/230-kDa subunit. This extent of iodination had no effect on platelet agglutinating activity. 2-Methylthio-ADP was prepared from adenosine-1-*N*-oxide (16). Prostaglandins (prostaglandin E_1, prostaglandin D_2 and prostaglandin I_2) and the endoperoxide analogue U46619 were gifts from Dr. J. E. Pike, Upjohn Co. (Kalamazoo, MI). PAF was from Avanti Polar Lipids (Montgomery, AL) and was stored frozen as a solution in Tris-saline containing 1% bovine albumin. Cytochalasins A and B were from Aldrich; vincristine sulfate (Oncovin) was from Lilly; Iodogen was from Pierce. Other biochemicals were from Sigma. Inorganic chemicals were of analytical grade. ^{125}I was from New England Nuclear.

ABBREVIATIONS: vWF, von Willebrand factor; FSBA, *p*-fluorosulfonylbenzoyladenosine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 2-methylthio-ADP, 2-methylthioadenosine-5'-diphosphate; PAF (platelet-activating factor), 1-*O*-alkyl-2-acetyl-sn-glycerolphosphorylcholine; *p*CMBS, *p*-chloromercuribenzene sulfonate; PRP, platelet-rich plasma; U46619, (15-*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid; GPIb, glycoprotein Ib.

Platelet suspensions. Venous blood was collected from healthy volunteers directly into ⅛ volume of 0.122 M Na_3 citrate and was centrifuged (150 × *g* for 15 min) at room temperature to prepare citrated PRP. Platelets were washed by a modification of the method of Mustard *et al.* (17), centrifuging at room temperature and avoiding the use of heparin. Fresh PRP (1 volume) was acidified to pH 6.5 by addition of 1 M citric acid and was centrifuged at 540 × *g* for 20 min in round-bottomed 50-ml polycarbonate tubes. The pellets were resuspended in 2–3 ml of Buffer A, diluted to 1 volume with Buffer A, and incubated for 30 min at 37°. The suspension was then centrifuged at 440 × *g* for 15 min and the pellet was resuspended as before in Buffer A, diluted to ½ volume, and incubated at 37° for 20 min. At this stage any contaminating red cells were removed by centrifugation at 440 × *g* for 2 min. Finally the suspension was centrifuged at 440 × *g* for 15 min and resuspended in Tyrode's saline buffered at pH 7.4 with HEPES, and diluted to ⅒ volume. If necessary, this suspension could be kept at room temperature for 2–3 hr until needed. It was warmed to 37° for 15 min before use.

Platelet function. Aggregation, agglutination, and shape change were studied in a Payton aggregometer at 37° with stirring at 1400 rpm. Agglutination was studied with PRP or suspensions containing approximately 3 × 10^8 platelets/ml, after addition of 5–10 mM $MgNa_2$ EDTA to prevent platelet secretion, aggregation, and the exposure of GPIIb/IIIa receptors. Shape change was measured in platelet suspensions diluted with Tris-saline/EDTA to 0.5–1 × 10^8/ml. EDTA was added no more than 2 min before each test to avoid the shape change that EDTA causes on prolonged exposure (18). Aggregation and agglutination rates are expressed as maximum rates of pen deflection, and the extent of shape change as the maximum pen deflection, occurring approximately 1 min after addition of agonist.

Cyclic AMP measurements. Stimulation of adenylate cyclase was measured as the accumulation of [^{14}C]cyclic AMP in platelets prelabeled by incubation with [^{14}C]adenine (19). Cyclic AMP was isolated from other nucleotides by sequential chromatography on Dowex 50 and alumina (20).

Results

Fig. 1 shows that platelets in normal human citrated PRP agglutinate at about the same rate on addition of 1.9 mg/ml ristocetin or of 7 μg/ml purified bovine vWF. Prior addition of 30 μM ADP caused a similar degree of inhibition of each effect.

Many aggregating agents induce platelets to change shape. Several of these appear to act independently of ADP, inasmuch as their effects are not inhibited by ATP, a specific competitive antagonist of ADP (19). Fig. 2 shows that, at maximally effective concentrations, ADP, PAF, and the thromboxane A_2 analogue U46619 all caused a shape change and inhibited agglutination by vWF. Both vasopressin and 5-hydroxytryptamine caused a shape change that was less rapid and less extensive than that seen with the other agents, and epinephrine caused no detectable shape change. Epinephrine did not inhibit vWF-induced agglutination, and the effects of 5-hydroxytryptamine and vasopressin were very slight.

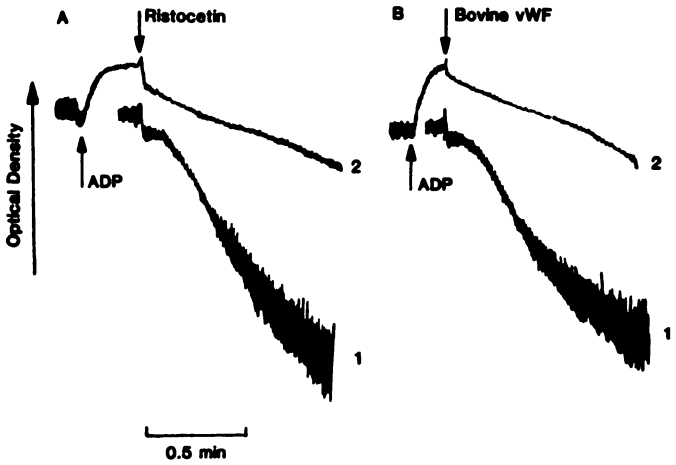

Fig. 1. Inhibition of platelet agglutination by ADP. PRP was stirred in the aggregometer for 30 sec with 6.3 mM EDTA and then either buffer (*1*) or 29 μM ADP (*2*) was added. Agglutination was then induced by the addition of 1.9 mg/ml ristocetin (A) or 7 μg/ml purified bovine vWF (B).

A common property of the agents that induce platelet aggregation is their ability to inhibit the activation of adenylate cyclase, both in intact platelets and in platelet membranes, although these properties do not appear to be well correlated. To determine whether platelet activation or inhibition of adenylate cyclase was more closely related to the inhibition of vWF-induced agglutination, we attempted to measure agglutination and adenylate cyclase activity under as closely similar conditions as possible. Adenylate cyclase activity was measured indirectly by the accumulation of [^{14}C]cyclic AMP in cells prelabeled with [^{14}C]adenine and stimulated by prostaglandin E_1 in the presence of a phosphodiesterase inhibitor. The effects of several agonists on platelet adenylate cyclase activities are shown in Fig. 3A. The same PRP sample was used to measure agglutination, and in both cases the PRP was diluted with Tris-saline containing EDTA immediately before testing. Fig. 3B shows that, with five different platelet agonists, there was no general correlation between the ability to prevent the increase in cyclic AMP caused by prostaglandin E_1 and inhibition of agglutination by vWF. Under these conditions, epinephrine strongly inhibited adenylate cyclase and had no effect on agglutination; U46619 and PAF were comparatively weak inhibitors of adenylate cyclase yet strongly inhibited agglutination. 3′,5′-Dideoxyadenosine had no effect on vWF-induced agglutination at concentrations up to 100 μM.

The ADP analogue 2-methylthio-ADP has effects similar to those of ADP on platelets, although the potency difference between the two compounds is different for induction of shape change and inhibition of adenylate cyclase (16). We compared ADP and 2-methylthio-ADP as inhibitors of vWF-induced agglutination, as inducers of shape change and aggregation, and as inhibitors of adenylate cyclase in the same experiment. The different measurements were made under as closely similar conditions as possible. Table 1 shows that the ratio of potency of ADP and 2-methylthio-ADP for inhibition of vWF-induced agglutination fell between the ratios found for inhibition of adenylate cyclase on the one hand and for induction of shape change and aggregation on the other.

ATP is a competitive antagonist of the effects of ADP on platelets. At high concentrations, ATP blocked both the shape change and the inhibition of vWF-induced agglutination due to ADP but had no effect on the actions of U46619 (Fig. 4) or PAF (not shown).

The adenosine derivative FSBA reacts covalently with nucleotide binding sites on a variety of enzymes and blocks platelet activation by ADP, although at concentrations up to 60 μM it does not block the inhibitory effect of ADP on adenylate cyclase (21). Fig. 5 shows that FSBA had no effect of its own on agglutination of washed platelets by vWF but blocked the inhibitory effect of ADP. Similar results were obtained when adenosine deaminase was included to eliminate

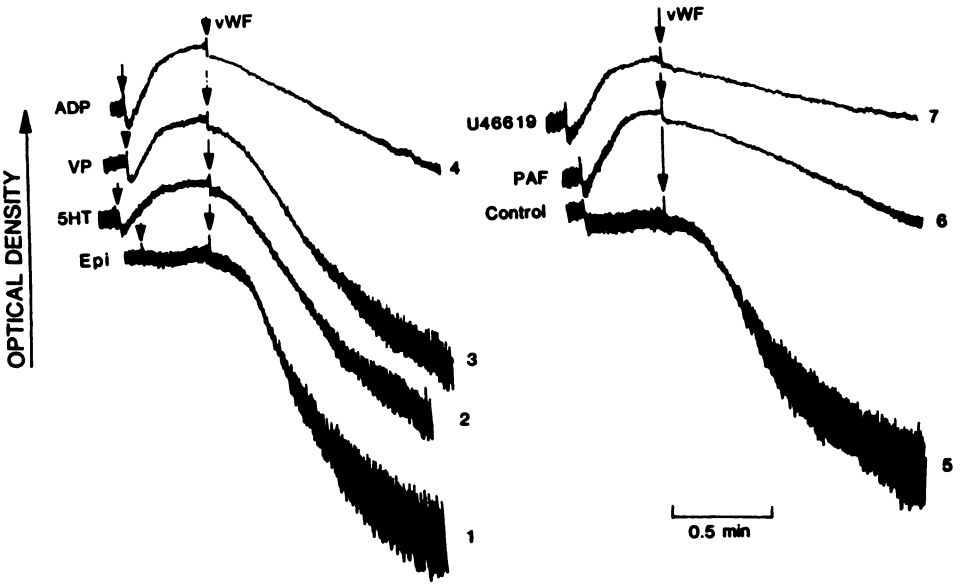

Fig. 2. Effects of different agonists on agglutination. Agonists were added to stirred PRP in the presence of 5.6 mM EDTA. Bovine vWF (1.2 μg/ml) was added shortly after the point of maximum shape change. The agonists were: *1*, epinephrine (*Epi*), 500 μM; *2*, 5-hydroxytryptamine (*5HT*), 2.8 μM; *3*, arginine vasopressin (*VP*), 0.5 units/ml; *4*, ADP, 5.5 μM; *5*, control; *6*, PAF, 2.5 μM; and *7*, U46619, 0.3 μM.

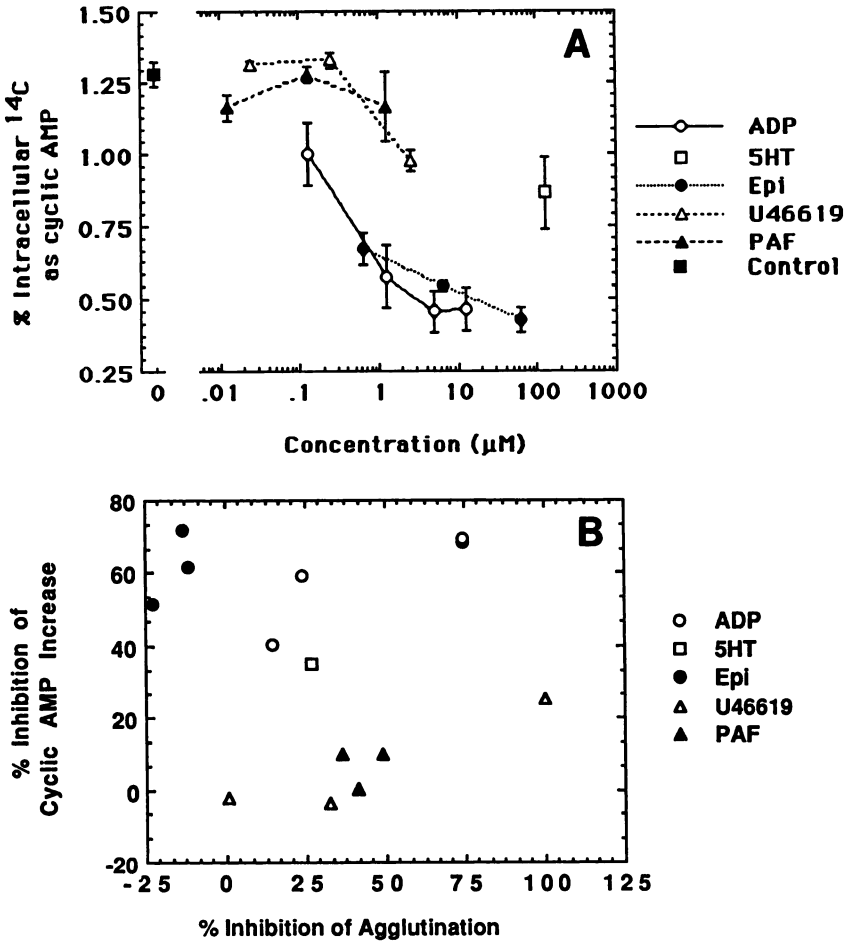

Fig. 3. Effects of different agonists on adenylate cyclase. A, PRP was incubated with [^{14}C]adenine to label the platelet adenine nucleotides. Samples of the labeled platelets were diluted with an equal volume of Tris-saline containing 10 mM EDTA and were incubated for 30 sec with the agonists. Then a mixture of prostaglandin E_1 (2 μM) and the phosphodiesterase inhibitor RA233 (0.2 mM) was added. After 2 min the reaction was stopped with ice-cold trichloroacetic acid. After a sample of [^{3}H]cyclic AMP was added as a recovery standard, [^{14}C]cyclic AMP was isolated by Dowex 50 and alumina chromatography. Results are plotted as the percentage of the intracellular radioactivity present as cyclic AMP. B, The same concentrations of the agonists used in A were added to the samples of the same PRP diluted with Tris-saline/EDTA in an aggregometer cuvette 30 sec before agglutination was induced with 4.6 μg/ml vWF. The percentage of inhibition of vWF-induced agglutination is plotted against the percentage of inhibition of cyclic AMP formation from A. *Epi*, epinephrine; *5HT*, 5-hydroxytryptamine.

any possible effects of adenosine present as either a breakdown product or contaminant of FSBA (21).

Fig. 6 shows that the inhibitory effect of ADP was also blocked by cytochalasin A, a lipophilic thiol-reactive agent with a high affinity for membranes. Similar results were obtained when PAF was used in place of ADP. The action of cytochalasin A was essentially reversed by the inclusion of cysteine to block the thiol reactivity. The inhibitory effect of ADP on agglutination was also blocked (Fig. 7) by the polar mercurial thiol reagent *p*CMBS. *p*CMBS was only effective with PRP at concentrations high enough to saturate the free thiol content of plasma proteins (0.4–0.5 mM); in washed platelet suspensions it was active at 10–20 μM. In contrast to ATP and FSBA, neither *p*CMBS nor cytochalasin A, at the concentrations used here, had any detectable effect on the shape change induced by ADP or by PAF.

TABLE 1

Concentrations of ADP and 2-methylthio-ADP giving half-maximal effects on different platelet functions

Inhibition of adenylate cyclase was determined by the ability of the compound to block the accumulation of [^{14}C]cyclic AMP in platelets incubated for 30 sec with 0.4 μM prostaglandin I_2. All measurements were made with the same sample of PRP, which was prelabeled for the cyclic AMP measurements by incubation with [^{14}C]adenine. All samples contained 5 mM EDTA, except those used for measuring aggregation.

Function	Concentration for half-maximal effect		Ratio	95% Confidence limits	
	ADP	2-MethylthioADP			
	nM				
Shape change (Extent)	92.2	19.1	4.8	3.0	7.3
Aggregation	1304	247	5.3	4.1	6.9
Inhibition of adenylate cyclase	1350	23.1	58.4	43.3	78.8
Inhibition of agglutination	550	27.5	20.0	12.6	38.8

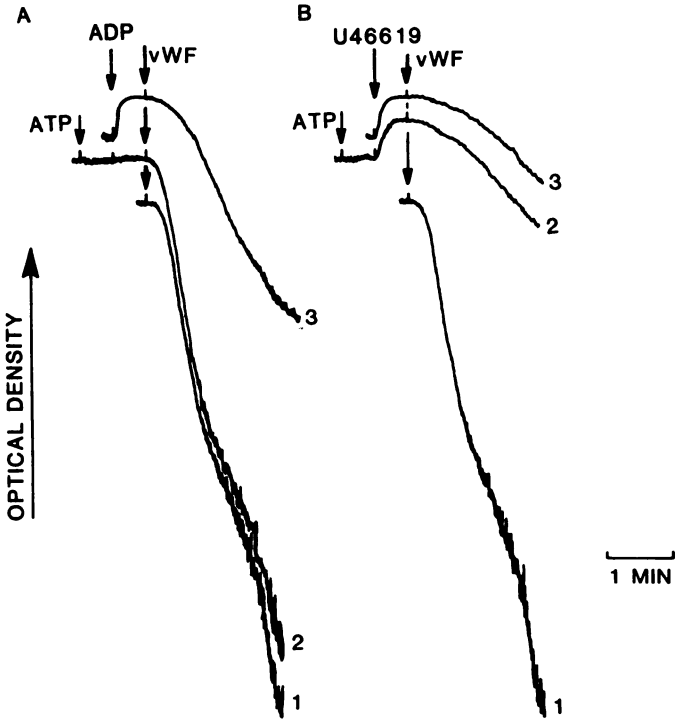

Fig. 4. Effect of ATP on inhibition of vWF-induced platelet agglutination. Agglutination was induced by addition of bovine vWF to washed platelets diluted before use to 0.5 × 10^8 ml in Tris-saline buffer containing 8.8 mM EDTA. A, Samples were treated with 0.6 μg/ml bovine vWF (*1*), 500 μM ATP followed by ADP and vWF (*2*), or 5 μM ADP followed by vWF (*3*). B, Samples were treated as in A but with 1 μM U46619 instead of ADP.

The normal disk shape of platelets is maintained by a marginal array of microtubules. Vincristine interferes with the polymerization of tubulin and consequently causes platelets slowly to lose their disk shape (22). Incubation of PRP for 15 min with vincristine had no effect on agglutination induced by vWF (Fig. 8), although it caused a loss of the normal disk shape of the platelets and the disappearance of the baseline oscillations of the aggregometer tracings. This was accompanied by an apparent inhibition of ADP-induced shape change. The inhibitory effect of ADP on agglutination induced by vWF was not affected by vincristine, indicating that platelets can lose their disk shape without losing either their responsiveness to vWF or the inhibition of this agglutination by ADP.

It has been suggested (12) that the shape change induced by ADP may be associated with a reduction in the availability of

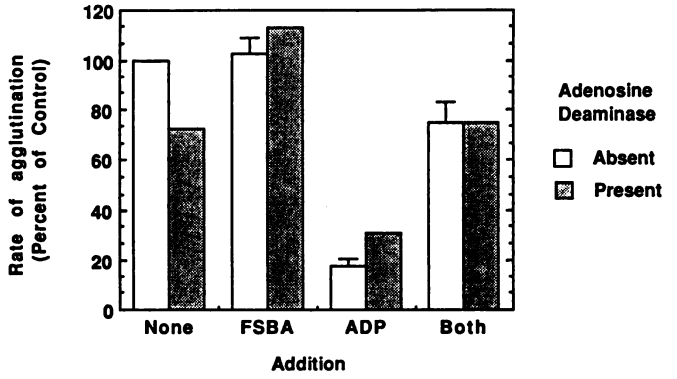

Fig. 5. Effects of FSBA on agglutination of washed platelets. Washed platelets (1–1.5 × 10^9/ml) in HEPES-buffered saline were diluted 8-fold with 5 mM EDTA in resuspension buffer immediately before testing. FSBA was added as 1–3 μl of a freshly prepared solution in *N,N*-dimethylformamide to a final concentration of 55–165 μM. An equal volume of dimethylformamide was used in controls. After 30 sec, saline or ADP (63 μM) was added, followed after 30 sec by 5 μg/ml bovine vWF. Results are expressed as percentages of the rates of agglutination in the controls with dimethylformamide and no ADP. *Error bars* indicate the mean plus standard error for 5 to 12 comparisons. In one experiment (□), 1 unit/ml adenosine deaminase was added before the other reagents to exclude the effects of adenosine.

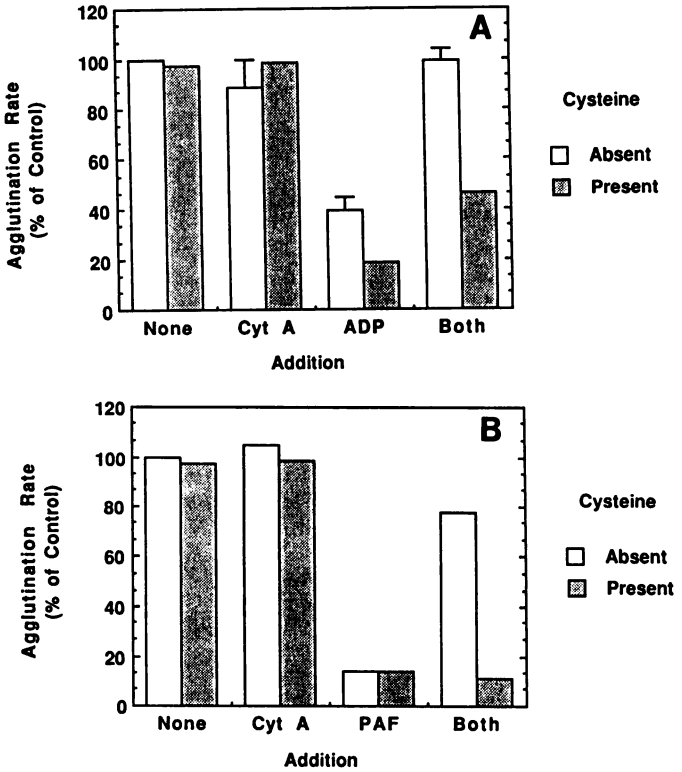

Fig. 6. Effects of cytochalasin A on agglutination of washed platelets. Cytochalasin A (1 μM) or its solvent, dimethyl sulfoxide (1 μl/ml), were added to washed platelets 30 sec before ADP. Agglutination was induced 30 sec later by adding vWF. The conditions were the same as for Fig. 5. *Error bars* indicate the mean plus standard error of three measurements. In one experiment (□), 200 μM cysteine was added before the other reagents to neutralize the thiol reactivity of cytochalasin A.

the membrane receptors with which vWF interacts to induce agglutination. We measured the binding of labeled vWF to platelets in the presence and absence of ADP. Fig. 9 shows that at saturating concentrations the amount of vWF bound to

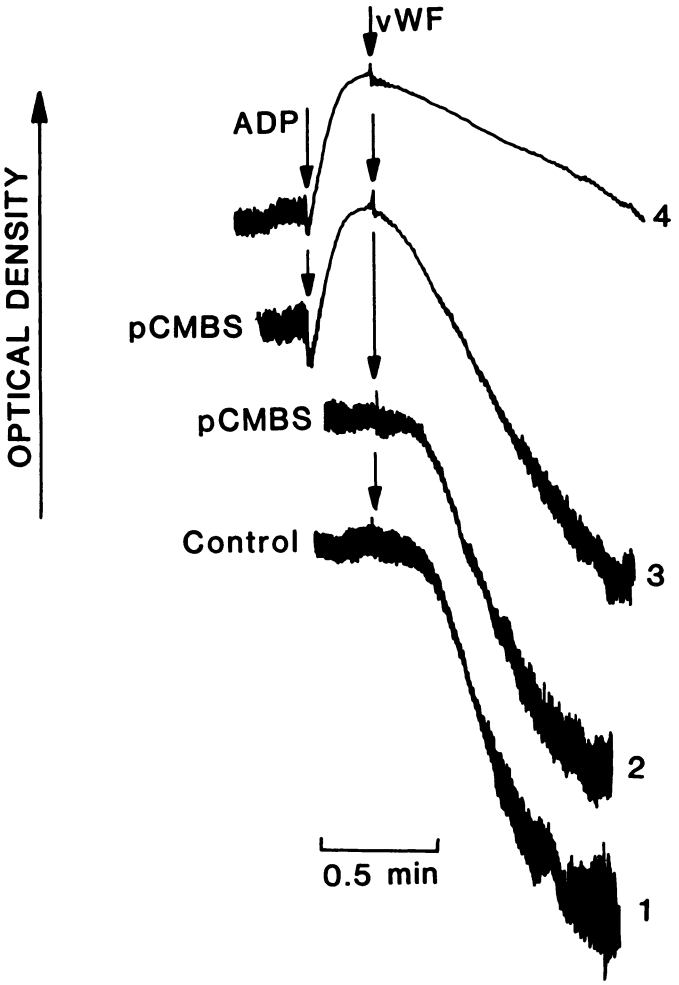

Fig. 7. Effect of *p*CMBS on the inhibitory effect of ADP. PRP was incubated with 1.5 mM *p*CMBS for 30 sec before addition of 14 μM ADP or saline. Agglutination was induced 15 sec later by the addition of 1.5 μg/ml bovine vWF. *1*, Control agglutination with vWF; *2*; *p*CMBS added before vWF; *3*, *p*CMBS followed by ADP and vWF; *4*, ADP followed by vWF.

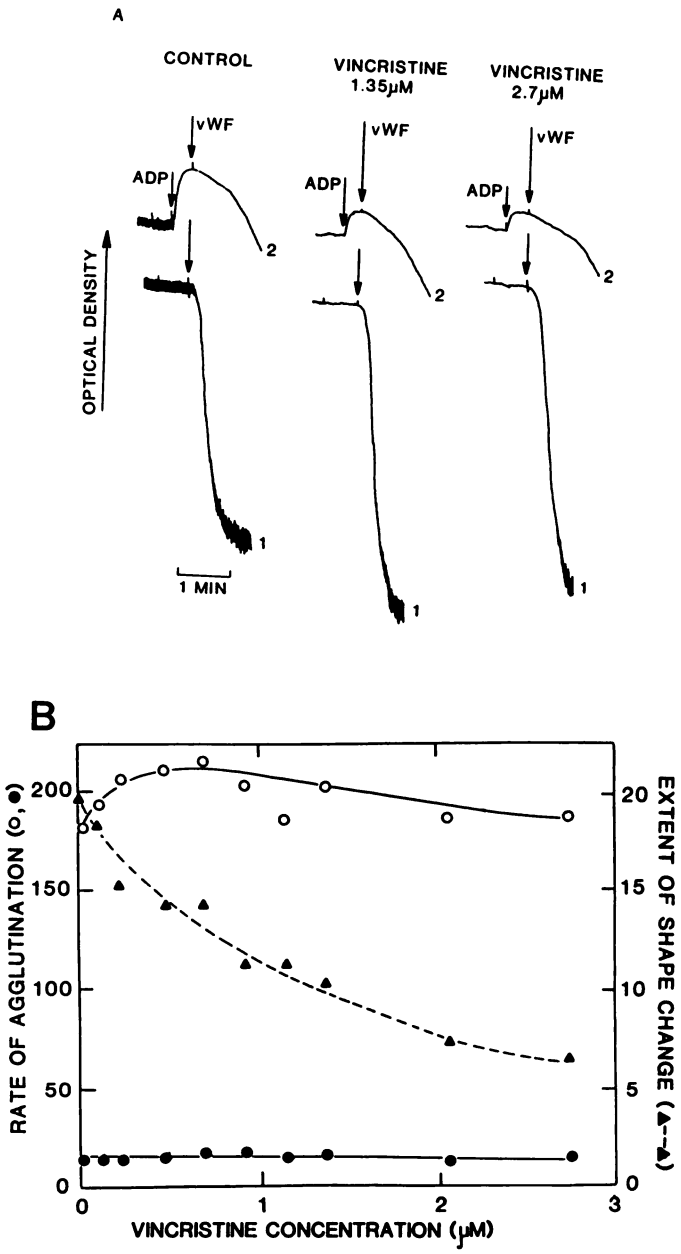

Fig. 8. Vincristine alters platelet shape but not the response to vWF. A, PRP, diluted 4-fold with Tris-saline buffer, was incubated with the indicated concentrations of vincristine sulfate for 15 min and then 3 mM EDTA was added with either buffer (*1*) or 5 μM ADP (*2*). After 10–15 sec, when the shape change was complete, 1.25 μg/ml bovine vWF was added. Aggregometer *tracings* show loss of baseline oscillations at high vincristine concentrations. B, Extent of shape change caused by ADP (▲) and rate of agglutination induced by vWF in the absence of ADP (○) and in the presence of 5 μM ADP (●) are plotted against the concentration of vincristine.

platelets was about 20% less in the presence of ADP than in its absence and the affinity of binding was about 10% lower. This small difference in binding is insufficient to explain the 2- to 10-fold shift in the dose-response curve for vWF observed when ADP is added to PRP.

Platelets can also be agglutinated by wheat germ lectin, but this agglutination is potentiated by ADP (Fig. 10). The extent of binding of labeled wheat germ lectin or vWF, measured under the same conditions as the agglutination responses but without stirring, was not substantially affected in either case.

Discussion

Agglutination of human platelets by bovine vWF appears to be similar in many respects to agglutination caused by human vWF in the presence of ristocetin (12, 23). This model system is useful for investigating the adhesion of platelets to subendothelial tissue and the early stages of the formation of hemostatic platelet plugs and possibly also of mural thrombi (24). That vWF-induced agglutination can be inhibited by agents that promote platelet aggregation is unexpected; in the case of other pairs of activating agents, mutual potentiation is usually observed. In contrast to the effect of ADP on agglutination induced by vWF, we found that agglutination by wheat germ lectin was enhanced by ADP. The receptors for wheat germ lectin and vWF are reported to be distinct (25), although GPIb binds to columns of immobilized wheat germ lectin (26).

ADP has at least two major distinguishable effects on platelets. It blocks the accumulation of cyclic AMP in platelets stimulated by prostaglandins and other activators of adenylate cyclase, and it also induces platelet activation, causing shape

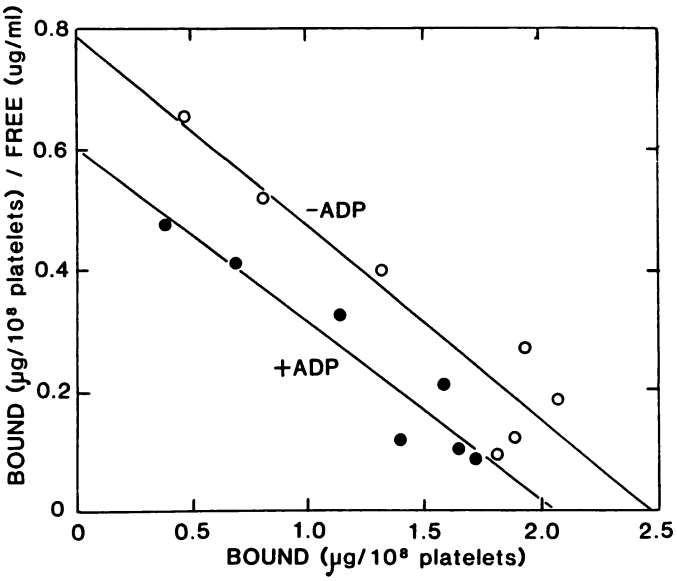

Fig. 9. Binding of labeled vWF to platelets. ^{125}I-labeled vWF was diluted with unlabeled vWF and added to EDTA-treated PRP diluted to 1.11 × 10^8 platelets/ml, to give concentrations between 1.1 and 21.8 μg/ml. One set of platelet samples was pretreated with 7.6 μM ADP. After incubation for 5 min at 37° without stirring, the platelets were sedimented and the supernatant was drawn off. The tips of the tubes containing the pellets were cut off with a razor blade and counted to determine the amount of vWF bound. Nonspecific binding (estimated by extrapolation of the binding isotherm to saturating vWF concentrations) was 6.9%, and this was subtracted from the total binding at each vWF concentration.

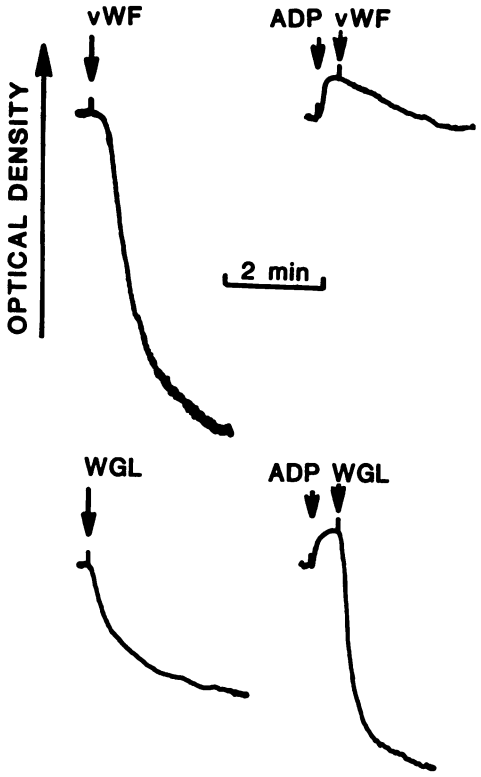

Fig. 10. Effects of ADP on agglutination and binding of vWF and wheat germ lectin. vWF or wheat germ lectin (*WGL*), labeled with ^{125}I, were incubated with PRP in the absence and presence of 5 μM ADP in aggregometer cuvettes. After 2.5 min, the platelets were centrifuged and the amount of vWF or wheat germ lectin bound was determined. The amounts of vWF and wheat germ lectin bound in the controls were 27.6 and 25.1%, respectively. In the presence of ADP, the amounts bound were 25.8% for vWF and 26.4% for wheat germ lectin.

change and aggregation. ADP-induced aggregation is believed to be mediated by the binding of fibrinogen to GPIIb/IIIa (26). In the presence of extracellular Ca^{2+}, vWF can also bind to the GPIIb/IIIa receptor and can cause platelet aggregation (27). To avoid confusion arising from the binding of vWF to GPIIb/IIIa, all of our experiments were done in the presence of EDTA.

Three lines of evidence suggest that inhibition of vWF-induced agglutination by ADP is associated with platelet activation and shape change and is unrelated to the effects of ADP on adenylate cyclase. (a) Epinephrine and 2′,5′-dideoxyadenosine both inhibit adenylate cyclase without causing shape change. Neither compound inhibited vWF-induced agglutination. (b) PAF and the prostaglandin H_2 analogue U46619 are less active than ADP as inhibitors of the cyclase, as indicated by their effects on cyclic AMP accumulation in cells exposed to prostaglandin stimulators of adenylate cyclase, but they are both more active than ADP in inducing a shape change. Both PAF and U46619 strongly inhibited vWF-induced agglutination. (c) FSBA inhibits platelet shape change without affecting the inhibition of adenylate cyclase by ADP. FSBA blocked the inhibitory effect of ADP on vWF-induced agglutination.

Further experiments suggest that it is not the change in platelet shape itself that interferes with agglutination but some closely associated event. (a) Serotonin and vasopressin both cause a shape change, although the optical change is somewhat slower and less extensive than with ADP. Neither serotonin nor vasopressin affected vWF-induced agglutination. (b) Vincristine, which inhibits the reversible polymerization of microtubules and slowly causes platelets to lose their disc shape, has no effect on vWF-induced agglutination nor does it affect the ability of ADP to inhibit agglutination, even though the optical effect of the shape change is reduced. (c) The ratio of the potencies of ADP and 2-methylthio-ADP with respect to the inhibition of agglutination is different from the ratio for induction of shape change or aggregation or for inhibition of adenylate cyclase. This suggests that inhibition of vWF-induced agglutination is not exclusively associated with induction of shape change but may represent a third effect associated with activation of the ADP receptor. (d) The thiol reagent *p*CMBS, at concentrations that have no effect on ADP-induced shape change, blocks the ability of ADP to inhibit vWF-induced agglutination. Another thiol reagent, cytochalasin A, which carries a —CO—CH═CH—CO— group analogous to the reactive group in maleimides, also blocks the effect of ADP on vWF-induced agglutination, and its action was inhibited by cysteine. Under the same conditions, cytochalasin B, which lacks the reactive double bond responsible for thiol reactivity, was inactive.

Inhibition of vWF-induced agglutination cannot be simply due to a reduction in the availability or affinity of the platelet membrane receptors for vWF, because the binding of vWF was not greatly affected by exposure of the cells to ADP. Although a small reduction in binding was seen, it was hardly enough to account for the inhibition of agglutination. A similarly small decrease in the availability of GPIb to a monoclonal antibody after treatment of platelets with ADP has been reported by Shapiro (28). It is possible that the orientation or association of receptors on the cell surface is involved in agglutination

caused by the multimeric vWF, and the processes are influenced by ADP.

The events that accompany the platelet shape change are clearly complex and may involve changes in the spatial distribution of receptors on the platelet surface. GPIb is linked to an actin-binding protein on the inner surface of the membrane (29) and the phosphorylation of the α-subunit of GPIb is increased when the intracellular concentration of cyclic AMP is raised (30). Clustering of GPIb molecules has been observed in activated platelets (29). The effects of *p*CMBS and cytochalasin A, two thiol reagents of different types, suggest that a thiol-containing membrane protein is involved in these events. Because *p*CMBS penetrates the platelet membrane very slowly, the postulated mediator protein can be presumed to be near the external surface of the membrane.

References

1. Weiss, H. J., H. R. Baumgartner, T. B. Tschopp, V. T. Turito, and D. Cohen. Correction by Factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand's disease. *Blood* **51**:267–279 (1978).
2. Howard, M. A., and B. G. Firkin. Ristocetin: a new tool in the investigation of platelet aggregation. *Thromb. Diath. Haemorrh.* **23**:362–369 (1971).
3. Kirby, E. P., and D. C. B. Mills. The interaction of bovine Factor VIII with human platelets. *J. Clin. Invest.* **56**:491–502 (1975).
4. Forbes, C. D. and C. R. M. Prentice. Aggregation of human platelets by purified porcine and bovine antihaemophilic factor. *Nature (Lond.)* **241**:149–150 (1973).
5. Jamieson, G. A., T. Okumura, B. Fishback, M. M. Johnson, J. J. Egan, and H. J. Weiss. Platelet membrane glycoproteins in thrombasthenia, Bernard-Soulier syndrome, and storage pool disease. *J. Lab. Clin. Med.* **93**:652–660 (1979).
6. Born, G. V. R., R. Dearnley, J. E. Foulks, and D. E. Sharp. Quantification of the morphological reaction of platelets by aggregating agents and its reversal by aggregation inhibitors. *J. Physiol. (Lond.)* **280**:193–212 (1978).
7. Daniel, J. L., I. R. Molish, M. Rigmaiden, and G. Stewart. Evidence for a role of myosin phosphorylation in the initiation of platelet shape change. *J. Biol. Chem.* **259**:9826–9831 (1984).
8. Cole, B., G. A. Robison, R. C. Hartmann. Studies of the role of cyclic AMP in platelet function. *Ann. N. Y. Acad. Sci.* **185**:477–487 (1971).
9. Mills, D. C. B., and J. B. Smith. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3′:5′ cyclic monophosphate in platelets. *Biochem. J.* **121**:185–196 (1971).
10. Haslam, R. J., M. M. L. Davidson, and J. V. Desjardins. Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets: evidence for the unidirectional control of platelet function by cyclic AMP. *Biochem. J.* **176**:83–95 (1978).
11. Cohen, I., T. Glaser, and U. Seligsohn. Effects of ATP and ADP on bovine fibrinogen- and ristocetin-induced platelet aggregation in Glanzmann's thrombasthenia. *Br. J. Haematol.* **31**:343–347 (1975).
12. Grant, R. A., M. B. Zucker, and J. McPherson. ADP-induced inhibition of von Willebrand factor-mediated platelet agglutination. *Am. J. Physiol.* **230**:1406–1410 (1976).
13. Parker, R. C. *Methods of Tissue Culture*, 3rd Ed. Hoeber Medical Division, Harper and Row, New York, 55–58 (1961).
14. Kirby, E. P. The agglutination of human platelets by bovine factor VIII: R J. Lab. Clin. Med. **100**:963–976 (1982).
15. Markwell, M. A. K., and C. F. Fox. Surface-specific iodination of membrane protein of viruses and eukaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. *Biochemistry* **17**:4807–4817 (1978).
16. Macfarlane, D. E., P. C. Srivastava, and D. C. B. Mills. 2-Methylthioadenosine-[β-^{32}P]diphosphate: an agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets. *J. Clin. Invest.* **71**:421–428 (1983).
17. Mustard, J. F., D. W. Perry, N. G. Ardlie, and M. A. Packham. Preparation of suspensions of washed platelets from humans. *Br. J. Haematol.* **22**:193–204 (1972).
18. Zucker, M. B., and R. A. Grant. Non-reversible loss of platelet aggregability induced by Ca^{2+} deprivation. *Blood* **52**:505–514 (1978).
19. Macfarlane, D. E., and D. C. B. Mills. The effects of ATP on platelets: evidence against the central role of ADP in primary aggregation. *Blood* **46**:309–320 (1975).
20. Haslam, R. J., and M. D. McGlenahan. Measurement of circulating prostacyclin. *Nature (Lond.)* **292**:364–366 (1981).
21. Mills, D. C. B., W. F. Figures, L. M. Scearce, G. J. Stewart, R. F. Colman, and R. W. Colman. Two mechanisms for inhibition of ADP-induced platelet shape change by 5′-*p*-fluorosulfonylbenzoyladenosine. *J. Biol. Chem.* **260**:8078–8083 (1985).
22. White, J. G. Effects of colchicine and *Vinca* alkaloids on human platelets. III. Influence on primary external contraction and secondary aggregation. *Am. J. Pathol.* **54**:467–473 (1969).
23. Kirby, E. P. Factor VIII-associated platelet aggregation. *Thromb. Haemostasis* **38**:1054–1072 (1977).
24. Kirby, E. P., and D. C. B. Mills. Methods for studying the von Willebrand factor-platelet interaction. *Modern Methods Pharmacol.* **4**:65–88 (1987).
25. Rock, G. A., A. Ordinas, J. Drouin, S.-C. Wong, and G. A. Jamieson. Glycoprotein I is not involved in aggregation of platelets by wheat germ agglutinin (WGA). *Thromb. Res.* **19**:725–729 (1980).
26. Clemetson, K. J. Glycoproteins of the platelet membrane, *Platelet Membrane Glycoproteins* (J. N. George, A. T. Nurden, and D. R. Phillips, Eds.). Plenum Press, New York, 51–85 (1985).
27. Fujimoto, T., and J. Hawiger. Adenosine diphosphate induces binding of von Willebrand factor to human platelets. *Nature (Lond.)* **297**:154–156 (1982).
28. Shapiro, S. S. Characterization of Factor VIII receptors, *Mechanisms of Stimulus-Response Coupling in Platelets* (J. Westwick, M. F. Scully, D. E. MacIntyre, and V. V. Kakkar, Eds.). Plenum Press, New York, 55–66 (1984).
29. Fox, J. E. B., O. C. Reynolds, and M. M. Johnson. Identification of glycoprotein Ib *beta* as one of the major proteins phosphorylated during exposure of intact platelets to agents that activate cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **262**:12627–12631 (1987).
30. Fox, J. E. B., and M. C. Berndt. Cyclic AMP-dependent phosphorylation of glycoprotein Ib inhibits collagen-induced polymerization of actin in platelets. *J. Biol. Chem.* **264**:9520–9526 (1989).

Send reprint requests to: David C. B. Mills, Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA 19140.