Exposure of Platelet Fibrinogen Receptors by Zinc Ions: Role of Protein Kinase C (43580)

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Abstract. **Previous studies demonstrated that Zn^{2+} at a concentration of 50 μM increases the number of fibrinogen receptors exposed on ADP-stimulated platelets and that higher concentrations of Zn^{2+} induce platelet aggregation that appears to be mediated by receptors associated with the glycoprotein IIb/IIIa complex. The purpose of this study was to identify the mechanism by which Zn^{2+} modulates exposure of fibrinogen receptors on the surface of human washed platelets. We determined that Zn^{2+} (300–800 μM)-induced platelet aggregation that was not accompanied by the release of [^{14}C]serotonin was not blocked by ADP scavenging enzymes and 5′-*p*-fluorosulfonylbenzoyl-adenosine, an affinity label for ADP binding sites, but it was inhibited by disintegrins, staurosporine, and EDTA. Zn^{2+} (50–200 μM) showed a synergistic effect on platelet aggregation and platelet release caused by ADP and *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine, a Zn^{2+} chelator, and inhibited ADP-induced platelet aggregation that was reversed by Zn^{2+} (50 μM). Zn^{2+} (200 μM) increased the number of fibrinogen binding sites and the affinity of albolabrin (a disintegrin isolated from *Trimeresurus albolabris* snake venom that has been shown to bind to the fibrinogen receptor) on ADP-activated platelets. On the other hand, Zn^{2+} (100–800 μM) did not increase fibrinogen binding to the purified receptor. Incubation of platelets with Zn^{2+} (200 μM) resulted in the phosphorylation of a 47-kDa protein that was blocked by staurosporine, an inhibitor of protein kinase C. In conclusion, Zn^{2+} ions activate protein kinase C and enhance fibrinogen receptor exposure on the surface of platelets stimulated by ADP.**

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Several reports in the literature indicate that zinc (Zn^{2+}) deficiency causes impairment of platelet aggregation in the rat (1), guinea pig (2), and human (3). Rats deficient in Zn^{2+} also showed prolonged bleeding time (1, 4). Heyns *et al.* (5) demonstrated that Zn^{2+} (at concentration of 100–500 μM) induces aggregation of human platelets that is not accompanied by release of platelet serotonin and enhancement of thromboxane synthesis. These authors suggested that Zn^{2+}-induced platelet aggregation is mediated by fibrinogen receptors associated with glycoprotein IIb/IIIa (GPIIb/IIIa) complex, since platelets isolated from patients with Glanzmann thrombasthenia did not aggregate upon addition of Zn^{2+} chloride. It has been demonstrated in our laboratory that Zn^{2+} (50 μM) increased 2-fold the number of fibrinogen receptors on the surface of ADP-stimulated platelets without affecting the binding affinity of fibrinogen to platelets (6).

In this study, we attempted to determine the mechanism by which Zn^{2+} at higher concentrations causes platelet aggregation and the mechanism of enhancing fibrinogen receptor exposure at lower concentrations of this metal. We used two ligands: fibrinogen and albolabrin. Albolabrin (low molecular weight peptide containing Arg-Gly-Asp (RGD) sequence) is a disintegrin isolated from the venom of *Trimeresurus albolabris* (7). Previous experiments demonstrated that trigramin (a disintegrin showing 95% homology with albolabrin) binds to the glycoprotein IIb/IIIa on the surface of resting and activated platelets and that platelet activation with ADP increases the binding affinity of trigra-

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min (8). We investigated a possible role of protein kinase C in this reaction since (i) this enzyme contains the Zn^{2+} binding site (Zn^{2+}-finger-like sequence) (9); (ii) Zn^{2+} ions can increase the activity of protein kinase C and the number of phorbol ester receptors in platelets (10), and Zn^{2+} can induce specific association of protein kinase C with plasma membranes and cytoskeleton (11, 12); and (iii) protein kinase C has been implicated in the exposure of fibrinogen receptors mediated by thrombin (13). In addition, since divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+} have been shown to regulate the interaction of adhesive ligands with various integrins (14, 15), we investigated the effect of Zn^{2+} on the interaction of purified GPIIb/IIIa complex with fibrinogen. Our study suggests that by activating protein kinase C, Zn^{2+} enhances fibrinogen receptors' exposure on the surface of platelets stimulated by ADP. At higher concentrations, Zn^{2+} aggregates platelets by an ADP-independent mechanism.

Methods

Reagents. Albolabrin was purified from crude venom of *Tr. albolabris* by single-step, reverse-phase chromatography on a wide-pore C18 silica matrix column (10 × 250 mm; Vydac) according to the method described elsewhere (7). Echistatin was kindly provided by Dr. Robert J. Gould (Merck, Sharp & Dohme Research Laboratories, West Point, PA). Staurosporine came from Kamiya Biomedical Co., Thousand Oaks, CA. Fibrinogen was supplied from Kabi, Stockholm, Sweden. Phorbol 12-myristate 13-acetate (PMA), phosphocreatine, creatine phosphokinase, apyrase, prostaglandin E_1, 5′-*p*-fluorosulfonylbenzoyl adenosine, bovine serum albumin (BSA), *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), ADP, citrate dextrose, and concanavalin A-Sepharose came from Sigma Chemical Co., St. Louis, MO. Heparin was from Elkins-Sinn, Inc., Cherry Hill, NJ. EDTA was from Fisher Scientific, Fair Lawn, NJ, while $Na^{125}I$, [^{14}C]5-hydroxy tryptamine creatinine sulfate were from Amersham Inc., North Prospect, IL. Iodobeads came from BioRad Inc., Rockville Center, NY. Phosphorus-32 was obtained from New England Nuclear/DuPont, Boston, MA, and silver stain was from Pharmacia LKB, Piscataway, NJ. The antibody recognizing the 66-kDa component of GPIIIa was raised in rabbits as described previously (16). Rapid Coomassie stain was purchased from Research Products International (Mt. Prospect, IL).

Preparation of Human Platelet Suspension. Blood was obtained from healthy volunteers who denied taking any medications within the previous 2 weeks. Blood was collected in acid citrate dextrose (1/7, v/v) and heparin (15 units/ml), and centrifuged (400*g*) for 17 min to obtain platelet-rich plasma. A washed platelet suspension was prepared according to the method of Mustard *et al.* (17). Platelets were resuspended in Tyrode's albumin solution (pH 7.35) containing 3.5 mg/ml of bovine serum albumin. Platelets were counted electronically (Coulter Channelyzer; Coulter Electronics, Hialeah, FL) and the concentration was adjusted to 3×10^8/ml for aggregation, release of platelet granule contents, and the phosphorylation study, and to 5×10^8 platelets/ml for the binding study.

Platelet Aggregation. Platelet aggregation was monitored with a Payton aggregometer (Scarborough, Ontario, Canada), and the extent of aggregation was expressed either as the percentage of change in optical density during aggregation or as light transmission units. Four hundred microliters of platelets (3×10^8/ml) suspended in Tyrode buffer containing Ca^{2+} (2 m*M*) and Mg^{2+} (1 m*M*) were placed in a Payton aggregometer cuvette for 5 min at 37°C. Either 15 μl of buffer or 3–15 μl of $ZnCl_2$ (for a final concentration of 50–300 μ*M*) were added to the cuvette and incubated for 1 min. Fibrinogen (500 μg/ml) was then added and incubated for 1 min, followed by the addition of 10 μl of ADP (3–60 μ*M*, final concentration). For Zn^{2+}-induced aggregation, 10 μl of fibrinogen were added to the platelets and followed by 1 min of incubation and 10 μl of $ZnCl_2$ (200–800 μ*M*, final concentration).

Determination of Zn^{2+} Level in Platelets and in Plasma. This was performed by atomic absorption spectrophotometry (18) at the SmithKline and Beecham laboratories (King of Prussia, PA). The detection level of Zn^{2+} by this method was 0.1 μg/ml. Preliminary measurements demonstrated that Zn^{2+} was not detectable by this method in all plasticware and buffers used in the experiments. Subsequently, we determined the level of Zn^{2+} in platelet suspensions prepared by the standard method, and the level of Zn^{2+} in platelet poor plasma was centrifuged at 7500*g* for 3 min.

Release of Platelet Constituents. Samples of the platelets loaded with [^{14}C]serotonin after aggregation were centrifuged for 2 min in an Eppendorf centrifuge (7500*g*). Supernatant was analyzed for β-thromboglobulin, lactate dehydrogenase, and [^{14}C]serotonin. β-Thromboglobulin released from platelets was measured by radioimmunoassay as reported previously (19). Lactate dehydrogenase activity was measured with a modification of the method of Wacker *et al.* (20). [^{14}C] Serotonin release was determined according to a method of Holmsen and Setkowsky-Dangelmaier (21).

Binding of ^{125}I-Fibrinogen and ^{125}I-Albolabrin to Platelets. Fibrinogen was radiolabeled with $Na^{125}I$ using Iodobeads (Pierce, Rockford, IL). Free iodine and labeled protein were separated by passage over a G-25 chromatography column. Specific radioactivity of ^{125}I-fibrinogen and ^{125}I-albolabrin were 1.5×10^3 cpm/μg and 17.5×10^3 cpm/μg, respectively. ^{125}I-Fibrinogen binding to washed platelets was performed as described

previously (22). Briefly, washed, ADP-stimulated (60 μM) platelets (5×10^8/ml) in Tyrode buffer containing Ca^{2+} (2 mM) and Mg^{2+} (1 mM), pH 7.35, were incubated with increasing concentrations of ^{125}I-fibrinogen (3–400 μg/ml). The total binding was measured in the presence or absence of $ZnCl_2$. Nonspecific binding of fibrinogen to platelets was measured in the presence of 6 mM EDTA. After 5 min, 400 μl of the platelet suspension were placed over silicone oil and centrifuged for 5 min at 7500g in an Eppendorf centrifuge. Both the platelet pellet and aliquots of the supernatant were counted in a gamma counter to determine the percentage that ^{125}I-fibrinogen bound. The values obtained for specific binding of fibrinogen to platelets were analyzed according to the method of Scatchard (23). Radiolabeling and binding of albolabrin (0.4–16 μg/ml) to platelets were performed in a manner similar to that of fibrinogen.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using both the Laemmli (24) system and Phast gel apparatus (Pharmacia, Uppsala, Sweden). Gels were stained with silver reagents according to the procedure supplied by the manufacturer.

Purification of GPIIb/IIIa Complex. Purification of platelet membrane GPIIb/IIIa complex was performed according to a procedure of Fitzgerald *et al.* (25) with a few modifications (26). Initially, outdated platelets were washed three times and the platelets in the final pellet were lysed by resuspending them in 7 vol of lysis buffer containing 1% Triton X-100. The lysate was centrifuged at 30,000g for 15 min and passed over a concanavalin A-Sepharose, a heparin-Sepharose and anti-fibrinogen-Sepharose column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis detected no trace of fibrinogen after this final purification step.

Binding of Purified GPIIb/IIIa to Immobilized Fibrinogen. This was studied by means of enzyme-linked immunoassay (26, 27). Briefly, 200-μl wells were coated with 1 μg/well of fibrinogen and bleached with 1% BSA. Subsequently, increasing concentrations of purified GPIIb/IIIa complex (2–200 nM) in buffer containing 0.5 mM calcium were added in the presence or absence of 50–600 μM $ZnCl_2$. The amount of GPIIb/IIIa complex bound to fibrinogen was measured using a polyclonal antibody against a 66-kDa component of human GPIIIa raised in rabbit (16). Binding of the complex to BSA was accepted as nonspecific binding (NSB). The quantities of GPIIb/IIIa complex bound to BSA and to fibrinogen in the absence of calcium were similar (16).

Phosphorylation of Platelet Proteins. Phosphorylation of platelet proteins with ^{32}P was studied according to Parise *et al.* (28) with a few modifications. In brief, platelets were washed as described in the Methods and resuspended in buffer A (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, and 10 mM HEPES). One-milliliter samples of platelet suspension (3×10^8 platelets/ml) were aliquoted into microcentrifuge tubes. One microcurie of [^{32}P]disodium phosphate was added to each tube and the mixture was incubated for 30 min at room temperature. Afterward, platelets were centrifuged (1000g for 5 min) and resuspended in the same volume of Tyrode buffer containing calcium, magnesium, and albumin. Platelet aliquots were incubated for 5 min with inhibitor (staurosporine or TPEN) and for 3 min with agonist ($ZnCl_2$, PMA, or ADP), and centrifuged pellets were solubilized in 200 μl of buffer B (2% Triton X-100, 20 mM Tris, 2 mM sodium metavanadate, 40 mM molybdic acid, 80 mM sodium pyrophosphate, 4 mM EGTA, 0.2 mM trifluoroperazine, 0.2 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 40 mM KH_2PO_4). Samples were electrophoresed in 15% Laemmli (24) gel in reduced system. Gels were stained with rapid Coomassie stain and dried. Autoradiography was performed using Kodak X-Omat AR films.

Results

Effect of Zn^{2+} on Platelet Aggregation. Zn^{2+} chloride ($ZnCl_2$) at concentrations of 300–800 μM caused aggregation of human washed platelets in the presence of fibrinogen and in the absence of ADP (Fig. 1). Zn^{2+}-induced platelet aggregation was accompanied by shape change and it showed a pattern similar to PMA-induced platelet aggregation. Zn^{2+}-induced platelet aggregation was delayed as compared with ADP-induced platelet aggregation (data not shown). Aggregation induced by Zn^{2+} was completely blocked by prostaglandin E_1 (20 μM), the protein kinase C inhibitor staurosporine (1 μM), echistatin (0.3 μM), albolabrin (0.6 μM), and

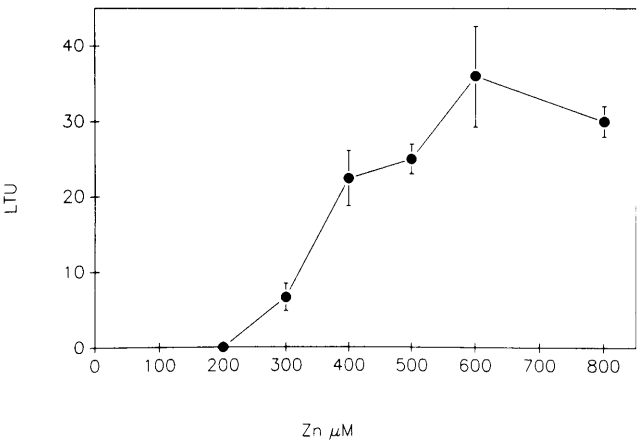


Figure 1. Effect of various concentrations of Zn^{2+} on the aggregation of washed human platelets. Fibrinogen (10 μl; 500 μg/ml; incubated for 1 min) and Zn^{2+} (200–800 μM) were added to the 0.4-ml platelet suspension. Values refer to the final concentrations. Data show Zn^{2+}-induced aggregation expressed as light transmission units (LTU). Values represent mean ± SE of seven experiments.

EDTA (3 mM). The inhibitory effect of staurosporine on Zn^{2+}-induced platelet aggregation suggested that this reaction can be mediated through the activation of protein kinase C. 5′-*p*-Fluorosulfonylbenzoyl adenosine- and ADP-removing enzymes had only a partial inhibitory effect on Zn^{2+}-induced platelet aggregation. PMA- and Zn^{2+}-induced aggregation was inhibited in a very similar manner by certain antagonists, except prostaglandin E_1 (Table I). Zn^{2+} (200 μM) and ADP (3 μM) had a synergistic effect on washed platelet aggregation in the presence of fibrinogen (500 μg) (Fig. 2). This suggests that Zn^{2+}-induced platelet aggregation and ADP-induced platelet aggregation are triggered by different mechanisms. The minimum concentration of Zn^{2+} required to potentiate ADP-induced platelet aggregation was 50 μM. By contrast, Zn^{2+} and PMA did not show a synergistic effect on platelet aggregation; however, Zn^{2+} (200 μM) enhanced platelet aggregation induced by 200 nM PMA (data not shown).

We determined that aggregation induced by Zn^{2+} was inhibited by TPEN, a heavy metal chelator. Subsequent studies showed that aggregation of washed human platelets induced by ADP in the presence of fibrinogen was also inhibited by this compound. This effect could be totally reversed by the concomitant addition of $ZnCl_2$, whereas Ca^{2+} and Mg^{2+} had no effect (Fig. 3). TPEN (50 μM) also inhibited aggregation induced by either PMA (200 nM) or low doses of thrombin (0.02–0.04 units/ml). Aggregation induced by higher doses of thrombin (0.1–1.0 U/ml) was not inhibited by TPEN (data not shown). Platelets washed in the presence of TPEN (100 μM) had reduced response to ADP that was partially corrected after 2 min of incubation with $ZnCl_2$ (100 μM) (data not shown).

Zn^{2+} in Plasma and in Platelets. We determined the Zn^{2+} level in plasma and in platelets of eight blood donors. The level of Zn^{2+} in plasma was 0.642 ± 0.06 μg/ml (mean ± SE). The level of Zn^{2+} in control platelets was 0.32 ± 0.05 $\mu g/10^9$ platelets (mean ± SE), and in platelets washed in the presence of TPEN (100

Table I. Comparison of Aggregation of the Suspension of Washed Platelets Induced by Zn (500 μM) and PMA (200 nM)[a]

Inhibitor	Percentage of inhibition	
	PMA	Zn^{2+}
Staurosporine	100 ± 10	100 ± 10
Echistatin	100 ± 0	100 ± 0
Albolabrin	100 ± 0	100 ± 0
EDTA	100 ± 0	100 ± 0
Prostaglandin E_1	57 ± 7	100 ± 0
FSBA	53 ± 9	62 ± 9
Apyrase	51 ± 6	50 ± 10
CP-CPK	64 ± 9	50 ± 8

[a] Data show inhibition of aggregation as compared with control samples and represent mean values ± SD of three experiments using blood of three different donors: staurosporine, 1 μM; echistatin, 0.3 μM; albolabrin, 0.6 μM; EDTA, 3 mM; prostaglandin E_1, 20 μM; FSBA (5′-*p*-fluorosulfonylbenzoyl-adenosine), 30 μM; apyrase, 2 units/0.4 ml; CP (phosphocreatine), 1.5 mM; CPK (creatine phosphokinase), 14 units/ml.

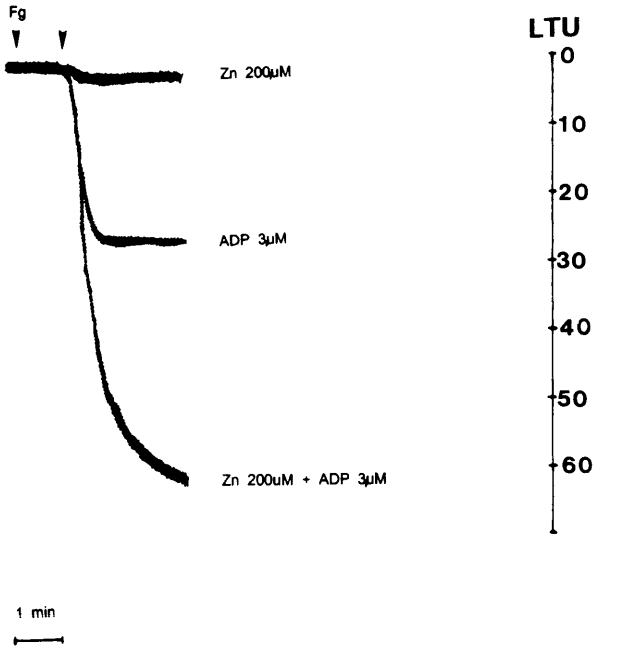


Figure 2. Synergistic effect of Zn^{2+} and ADP on platelet aggregation. $ZnCl_2$ (200 μM) in the presence of fibrinogen (1.5 μM) did not cause platelet aggregation. ADP (3 μM) had a slight effect on platelet aggregation, as revealed by measuring light transmission. However, both agents in combination caused an extensive platelet aggregation, suggesting a synergistic effect.

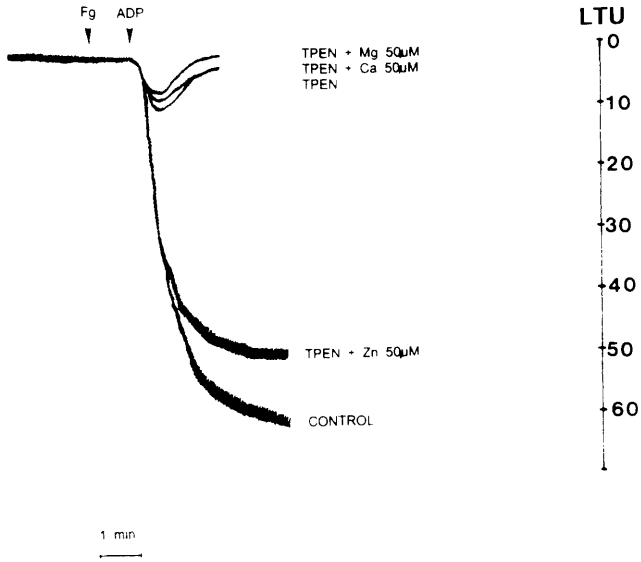


Figure 3. Effect of TPEN on platelet aggregation. Aggregation of washed human platelets induced by ADP (60 μM) in the presence of fibrinogen (400 μg/ml) was inhibited by intracellular heavy metal chelator TPEN (50 μM). This effect could be totally reversed by concomitant addition of 50 μM of Zn^{2+}, whereas Ca^{2+} (50 μM) and Mg^{2+} (50 μM) had no effect. This is a representative tracing from three similar experiments.

μM), it was 0.27 ± 0.05 $\mu g/10^9$ platelets. Therefore, washing of platelets in the presence of TPEN did not significantly change the level of Zn^{2+} in platelets. The correlation coefficient between Zn^{2+} level in plasma and in platelets was $r = 0.6$.

Release of Platelet Constituents During Zn^{2+}-Induced Platelet Aggregation. Platelet aggregation induced by Zn^{2+} (500 μM) was associated with a low level of release of β-thromboglobulin from α-granules (Table II). There was no significant release of dense granule content as measured by [^{14}C]serotonin release. However, Zn^{2+} significantly potentiated release induced by ADP (Table II). Zn^{2+} did not cause release of lactate dehydrogenase, indicating that platelets remained intact (data not shown).

Effect of Zn^{2+} on Fibrinogen and Albolabrin Binding to Platelet Fibrinogen Receptors. In these experiments, we compared the effect of Zn^{2+} on the binding of ^{125}I-fibrinogen and on the binding of ^{125}I-albolabrin, a disintegrin isolated from *Tr. albolabris*, to ADP-stimulated platelets. Suspension of washed, ADP-stimulated platelets (5×10^8 platelets/ml) in Tyrode's buffer containing Ca^{2+} (2 mM) and Mg^{2+} (1 mM), pH 7.35, was incubated with increasing concentrations of ^{125}I-fibrinogen. The total binding was measured in the presence or absence of $ZnCl_2$ (200 μM). Nonspecific binding (NSB) of fibrinogen to platelets was measured in the presence of 6 mM EDTA. It was established in our laboratory that the values of NSB determined in this way correspond to the values of NSB determined by the addition of a 20-fold molar excess of unlabeled fibrinogen to the incubation mixture. Figure 4A shows that the addition of Zn^{2+} to the final concentration of 200 μM caused about a 4-fold increase of the number of fibrinogen binding sites per platelet, whereas the binding affinity was not altered. Binding of ^{125}I-albolabrin to platelets was performed in a similar manner. Analysis of a binding isotherm by Scatchard equation revealed one class of binding sites on ADP-activated platelets in the presence and in the absence of Zn^{2+} (200 μM) (Fig. 4B). The presence of Zn^{2+} in the incubation mixture caused only a slight increase in the number of binding sites, but it resulted in a 2-fold increase of binding affinity of albolabrin to platelets.

Effect of Zn^{2+} on the Interaction of Fibrinogen with Purified GPIIb/IIIa Complex. Figure 5 shows that Zn^{2+} at a concentration of 100 μM had no effect on the amount of GPIIb/IIIa bound to immobilized fibrinogen in the presence of 0.5 mM calcium, as measured by enzyme-linked immunosorbent assay using an antibody to a 66-kDa component of GPIIIa. At the range of concentration from 100 to 800 μM, Zn^{2+} did not alter the interaction between purified GPIIb/IIIa and immobilized fibrinogen. At the higher concentration (800–1000 μM), Zn^{2+} significantly increased the amount of GPIIb/IIIa bound to fibrinogen (data not shown).

Effect of Zn^{2+} on the Phosphorylation of Platelet Proteins. Incubation of platelets with Zn^{2+} did not cause any significant changes in the pattern of platelet proteins separated by the method of Laemmli (24) in the reduced system (data not shown). Figure 6A shows that incubation of platelets with Zn^{2+} (200–500 μM) enhanced phosphorylation of a platelet protein migrating with an apparent molecular mass of 47 kDa. This component likely corresponds to pleckstrin. Phosphorylation of pleckstrin occurring at 500 μM Zn^{2+} was completely prevented by staurosporine (Fig. 6A) and partially by TPEN (not shown). Similarly, phosphorylation of pleckstrin was induced by a well-known activator of protein kinase C, phorbol ester (PMA, 200 nM) (Fig. 6B), and by thrombin (2 units/ml) (Fig. 6C). This phosphorylation was completely blocked by staurosporine (1 μM) and partially by TPEN (50 μM). In this experimental system, ADP had no effect on the phosphorylation of the 47 kDa protein. However, it potentiated the effect of Zn^{2+} on the phosphorylation of this component. Minimal concentrations of Zn^{2+} required to cause phosphorylation of the 47-kDa component in the absence and in the presence of ADP were 200 μM and 50 μM, respectively (data not shown).

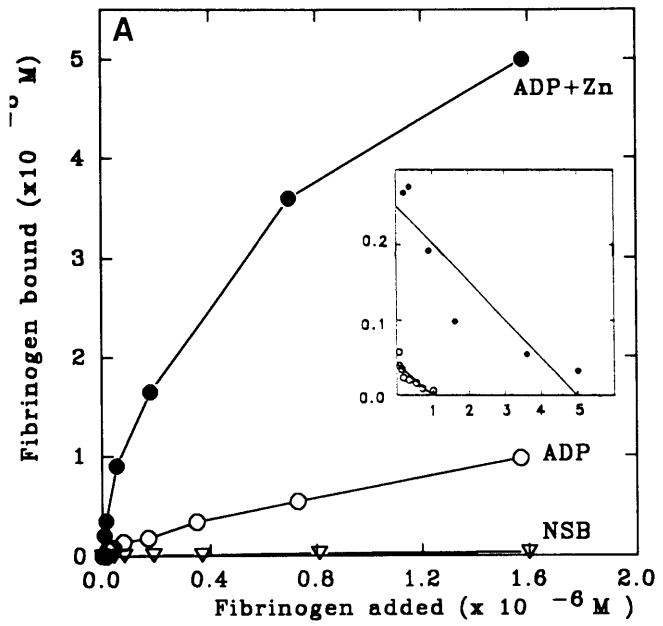
Discussion

In this study, we evaluated two effects of Zn^{2+} on platelets. At a lower concentration (50–200 μM), Zn^{2+}

Table II. Effect of Zn^{2+} and Thrombin on the Release of Platelet Constituents[a]

Agonist	Serotonin release[b]	β-Thromboglobulin release[b]
Zn^{2+}, 200 μM	2.0 ± 0.25	6.9 ± 0.6
Zn^{2+}, 500 μM	1.9 ± 0.1	14.5 ± 5.8
Thrombin, 2 μM	76.0	75.0 ± 14.4
ADP, 6 μM	11.0	—
ADP, 30 μM	10.0	—
ADP, 6 μM, Zn^{2+}, 200 μM	46.0	—
Control (buffer)	1.7	8.5 ± 4.3

[a] Data represent as mean values ± SE of three similar experiments.
[b] The values correspond to the percentage released as compared with the total values in platelet suspension disrupted by Triton X-100.



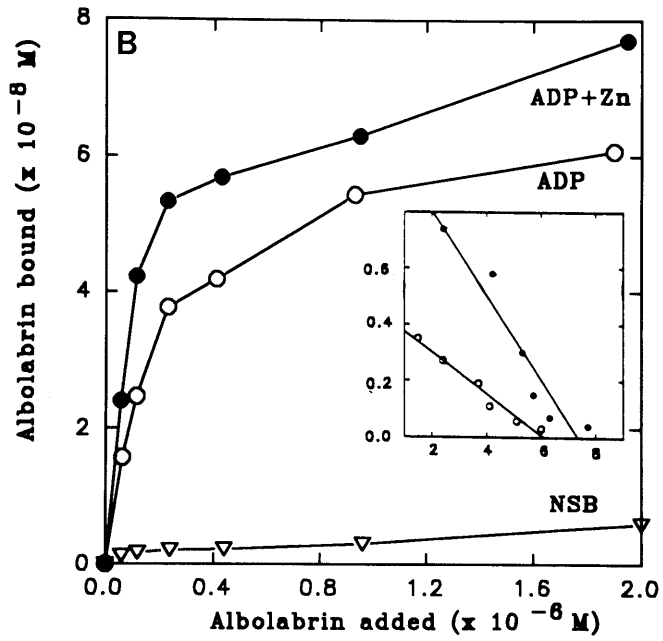


Figure 4. Effect of Zn^{2+} on ^{125}I-albolabrin binding to ADP-stimulated platelets. (A) Binding isotherms of ^{125}I-fibrinogen to washed, ADP (10 μM)-stimulated platelets (5 × 10^8/ml) in Tyrode's buffer in the absence (○) and in the presence (●) of Zn^{2+} (200 μM). The nonspecific binding (▽) was determined in the presence of 6 mM EDTA. Inset represents Scatchard analysis of the data (y axis = bound/free; x axis = bound × 10^{-8} M). There were 14,500 (K_d = 2.3 × 10^{-7} M; r = 0.86) binding sites/ADP-stimulated platelet and 74,000 (K_d = 1.9 × 10^{-7} M; r = 0.92) binding sites/ADP-stimulated platelets in the presence of Zn^{2+}. This experiment is representative of four similar experiments. (B) Binding isotherms of ^{125}I-albolabrin to washed, ADP (10 μM)-stimulated platelets in the absence (○) and in the presence (●) of Zn^{2+} (200 μM). NSB was measured in the presence of 6 mM EDTA (▽) (y axis = bound/free; x axis = bound × 10^{-8} M). Inset represents Scatchard analysis of the data. There were 70,300 binding sites/platelet (K_d = 1.1 × 10^{-7} M; r = 0.98) on ADP-stimulated platelets and 84,000 binding sites (K_d = 5.3 × 10^{-8} M; r = 0.95) on platelets stimulated by ADP in the presence of Zn^{2+}. This is a representative experiment of four similar experiments.

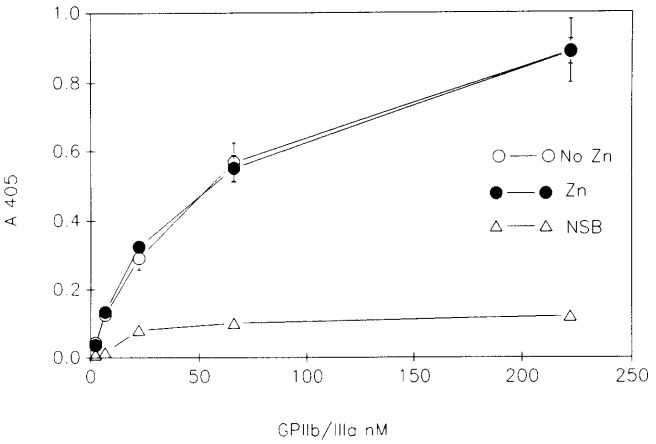


Figure 5. Effect of Zn^{2+} (100 μM) on the interaction between GPIIb/IIIa complex and fibrinogen. GPIIb/IIIa complex was incubated for 2 hr with immobilized fibrinogen in the absence or presence of $ZnCl_2$ (200 μM). Binding to wells coated with BSA was minimal and was accepted as NSB. Values represent mean ± SE of four experiments.

potentiated ADP-induced platelet aggregation, and it increased the number of fibrinogen receptors exposed by ADP. At a higher concentration (300–800 μM), Zn^{2+} induced platelet aggregation and caused exposure of fibrinogen receptors. It is not clear whether the latter effect, similar to that observed by Heyns *et al.* (5), is physiologically important. However, under physiologic conditions, Zn^{2+} may exert a modulatory effect on platelet aggregability, as suggested by experiments with TPEN, a Zn^{2+} chelator.

Foley *et al.* (29) determined that the level of Zn^{2+} in human plasma is about 1 μg/ml (range, 70–130 μg/ml) or 15.4 μM (range, 10.8–20 μM), and in platelets, 0.3 μg/10^9 (range, 0.21–0.45 μg/10^9). In our experiments, eight normal blood donors had similar values of Zn^{2+} in plasma and in platelets.

We propose that protein kinase C mediates fibrinogen receptor exposure and platelet aggregation induced by Zn^{2+}. This hypothesis is supported by the following experiments: (i) incubation of platelets with Zn^{2+} resulted in a phosphorylation of a 47-kDa component that appeared to be identical with pleckstrin, considered to be a specific substrate for protein kinase C (30, 31) (Fig. 6). This phosphorylation was blocked by staurosporine, an inhibitor of protein kinase C (32). (ii) Staurosporine inhibited Zn^{2+}-induced platelet aggregation. (iii) Phorbol ester, an activator of protein kinase C, and Zn^{2+} produced a similar pattern of platelet aggregation that was inhibited to the same degree by a number of antagonists (Table I). Although fibrinogen contains a Zn^{2+} binding site (33), it is unlikely that Zn^{2+} interaction with fibrinogen plays any role in the described phenomenon under physiologic conditions. Potentiation of GPIIb/IIIa binding to fibrinogen in the purified system did not occur at concentrations of $ZnCl_2$ between 100 and 750 μM. Previous experiments

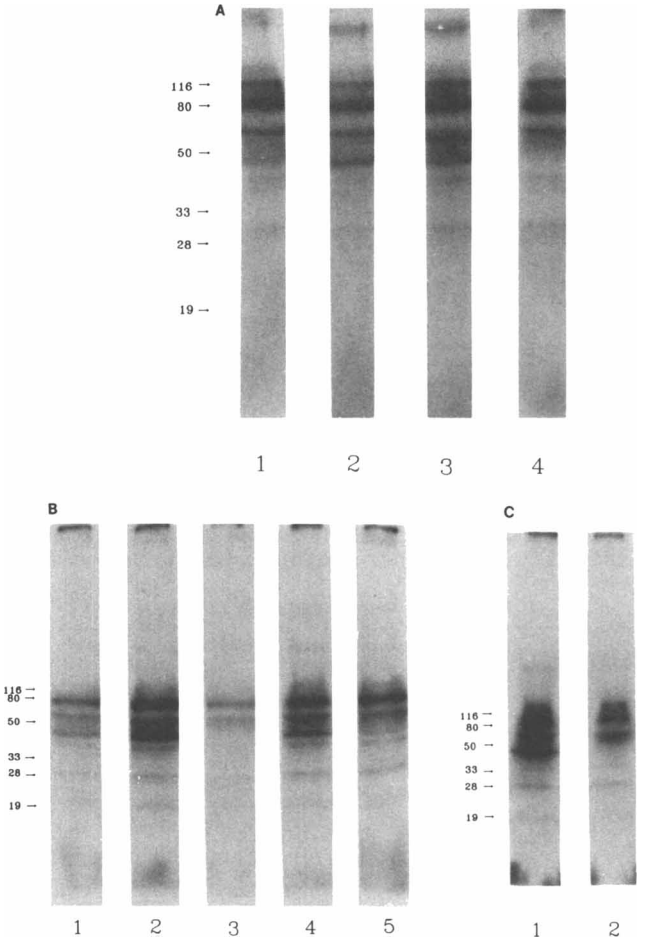


Figure 6. Effect of Zn^{2+} and PMA on phosphorylation of platelet proteins. Platelets were prepared as described in Methods and incubated as described in Methods. (A) Lane 1, buffer (control); Lane 2, Zn^{2+} (200 μM); Lane 3, Zn^{2+} (500 μM); Lane 4, staurosporine (1 μM) + Zn^{2+} (200 μM). (B) Lane 1, buffer; Lane 2, PMA (200 nM); Lane 3, staurosporine (1 μM) + PMA (1.5 mM), Lane 4, TPEN (50 μM) + PMA (200 nM); Lane 5, ADP (60 μM). (C) Lane 1, thrombin (2 units); Lane 2, staurosporine (1 μM) + thrombin (2 units). After lysis in buffer B (2% Triton X-100, 20 mM Tris, 2 mM sodium metavanadate, 40 mM molybdic acid, 80 mM sodium pyrophosphate, 4 mM EGTA, 0.2 mM trifluoroperazine, 0.2 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 40 mM KH_2PO_4), platelet proteins were separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% gel). The bands were identified by means of autoradiography. Molecular weight standards are indicated by the arrows.

demonstrated that the low concentration of Zn^{2+} used in this study does not aggregate fibrinogen (6). Therefore, it seems that the exposure of fibrinogen binding sites by Zn^{2+} is mediated through the protein kinase C-dependent pathway rather than by direct interaction with the GPIIb/IIIa complex.

The protein kinase C family of phospholipid-dependent protein kinases plays a key role in signal transduction (34, 35). Protein kinase C isoenzymes contain a catalytic domain located in the carboxy-terminal region of the molecule and a regulatory domain located in the amino-terminal region. The regulatory domain contains a tandem repeat of a cysteine-rich sequence. This region is essential for phorbol ester and for diacylglycerol binding to protein kinase C. The tandem repeats of a cysteine-rich motif resemble the "Zn^{2+} finger domain" that is found in some DNA-binding proteins. A Zn^{2+} finger comprises a repeat of approximately 30 amino acids with a Zn^{2+} atom tetrahedrally coordinated between pairs of cysteine and histidine residues. Ono *et al.* (9) studied various mutants of recombinant protein kinase C and demonstrated that phorbol ester binding to protein kinase C requires a cysteine-rich Zn^{2+} finger-like sequence. Although binding of Zn^{2+} to this region of isolated protein kinase C has not been reported as yet (35), there is evidence that Zn^{2+} activates protein kinase C in a purified system (12, 36). Forbes *et al.* (10) demonstrated that Zn^{2+} increases the number of phorbol ester receptors in intact B lymphocytes, neutrophils, and platelets. Zn^{2+}-induced translocation of protein kinase C from cytoplasm to cytoskeleton in platelets and lymphocytes has also been reported (37). It is conceivable that Zn^{2+} may cause translocation of protein kinase C to platelet membranes, and that this reaction may lead to the phosphorylation of platelet membranes or cytoskeletal proteins and subsequently expose fibrinogen receptors. Most recently, Peerschke (38) demonstrated that Zn^{2+} (concentration 300 μM) or PMA (500 nM) prevent formation of irreversible bonds between activated platelets and fibrinogen and inhibit incorporation of fibrin into cytoskeleton.

It has been suggested that under physiologic conditions, platelet fibrinogen receptors can be exposed on the platelet surface by different mechanisms. Shattil and Brass (39) proposed that thrombin and thromboxane A_2 induced exposure of fibrinogen receptors by activating phospholipase C and protein kinase C. By contrast, ADP or epinephrine expose fibrinogen receptors without detectable activation of phospholipase C and protein kinase C (40, 41). This is consistent with our observations that Zn^{2+}-induced exposure of fibrinogen receptors is not mediated by ADP and that these two agonists exert a synergistic effect on fibrinogen receptor exposure, platelet release reaction, and platelet aggregation.

The present study confirmed our previous report (6) that Zn^{2+} increases the number of fibrinogen binding sites on ADP-stimulated platelets. Moreover, we demonstrated that Zn^{2+} increased ^{125}I-albolabrin binding to platelets. Albolabrin is a disintegrin that binds to cell surface integrins by an Arg-Gly-Asp (RGD)-dependent mechanism. It contains a single-cell recognition site (42). Huang *et al.* (8) demonstrated that trigramin, an analog of albolabrin, bound to the same number of GPIIb/IIIa receptors on the surface of resting and activated platelets. However, activation of platelets by ADP increased significantly the binding affinity of trigramin to platelets. We made similar observations by studying binding of radiolabeled albolabrin to resting and to

ADP-activated platelets (43). Zn^{2+} increased further binding affinity of albolabrin to ADP-activated platelets.

We used TPEN, a heavy metal chelator, introduced by Arslan *et al.* (44), in our studies. TPEN is a lipid-soluble compound that crosses cell membranes and is nontoxic to cells. It chelates Zn^{2+} and Cu^{2+} with high affinity, and has much lower affinity to Mn^{2+} and Fe^{2+}. Its effect on calcium and magnesium is negligible. Since concentrations of copper and iron in platelets are 50 times and 20 times lower, respectively, than concentrations of Zn^{2+} (45), we consider TPEN in platelets to be a highly specific Zn^{2+} chelator. Platelets isolated by means of washing in the presence of TPEN (100 μM) showed a significant decrease of ADP-induced platelet aggregation that could be fully reversed upon the addition of Zn^{2+}, suggesting that TPEN does not damage the platelets. However, the Zn^{2+} level in TPEN-treated platelets was only slightly decreased. This is consistent with observations by Arslan *et al.* (44), who demonstrated that TPEN can enter the cells and chelate Zn^{2+} intracellularly. The inhibitory effect of TPEN on ADP-induced platelet aggregation suggests that intracellular Zn^{2+} may act as a physiologic mediator during platelet stimulation by ADP.

Several reports in the literature suggest that the variability of Zn^{2+} level in plasma *in vivo* could affect human platelet aggregability and hemostasis. Gordon *et al.* (3) demonstrated that a decrease of Zn^{2+} in plasma results in impaired platelet aggregation induced by ADP and arachidonate. Marx *et al.* (46) demonstrated that ingestion of 220 mg of Zn^{2+} sulfate, resulting in an increase of Zn^{2+} level in plasma by 0.2–0.4 μg/ml, causes significant improvement of platelet aggregation elicited with suboptimal concentrations of thrombin, ADP, epinephrine, collagen, and platelet-activating factor. It has been suggested that Zn^{2+} deficiency in humans may contribute to the bleeding in liver cirrhosis and to atonic bleeding in pregnancy (47).

Our experiments suggest the physiologic significance of Zn^{2+} in the modulation of expression of fibrinogen receptors on the surface of human platelets. Further investigations are required to identify the role of platelet Zn^{2+} in hemostatic disorders.

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