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Research Article

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Giovanni Di Minno, Anna M. Cerbone, Pier L. Mattioli, Salvatore Turco, Ciro Iovine, and Mario Mancini
Istituto Di Medicina Interna e Malattie Dismetaboliche, Clinica Medica, Nuovo Policlinico, Napoli, Italy

Abstract

To elucidate the bleeding tendency that follows the administration of ticlopidine, we investigated the skin bleeding time and some ex vivo functions of platelets obtained from eight healthy volunteers before and 1 wk after daily administration of 500 mg of ticlopidine. We found the following: ticlopidine significantly ($P < 0.001$) prolonged the skin bleeding time and impaired the binding of radiolabeled fibrinogen and von Willebrand Factor, the clot retraction and the aggregation of platelets in response to ADP, epinephrine, thrombin, ionophore A23187, collagen, or arachidonic acid. In contrast, the administration of this drug did not affect intraplatelet levels of cAMP, agglutination and binding of von Willebrand Factor in response to ristocetin, shape change in response to ADP, collagen, thrombin, or arachidonic acid, or binding of prostaglandin E_1 to resting platelets. Secretion of ATP in response to ADP or epinephrine was completely inhibited, whereas secretion as well as thromboxane synthesis in response to high concentrations of collagen, arachidonic acid, calcium ionophore A23187, or thrombin was unaffected. Studies with monoclonal antibodies showed that the glycoprotein IIb-IIIa complex (the putative receptor for fibrinogen and von Willebrand Factor on the surface of platelets exposed to naturally occurring aggregating agents) was quantitatively unaffected by ticlopidine. This observation was further confirmed by densitometric scannings of Periodic Acid-Schiff-stained gels of platelet suspensions. The onset, as well as the cessation of the inhibitory effect of ticlopidine on platelets was very slow, and reached a maximum after a 3–5-d administration. In addition, ticlopidine appeared to be a much more potent inhibitor when administered to subjects than when added in vitro to platelets. Finally, abnormalities comparable to those found in volunteers taking ticlopidine were observed when platelets from untreated subjects were incubated in the plasma of ticlopidine-treated subjects.

We conclude that ticlopidine induces a thrombasthenic state in normal platelets without affecting the glycoprotein IIb-IIIa complex quantitatively. Furthermore, our data suggest that one or more active metabolites rather than the native drug mediate the abnormalities of platelet function observed in ticlopidine-treated subjects.

Introduction

The key role of blood platelets in thrombogenesis and atherogenesis has fostered the search for new drugs interfering with platelet function more selectively and effectively than aspirin.

Ticlopidine (5-[2chlorobenzyl]-4,5,6,7,tetrahydrothieno[3,2-C]pyridine hydrochloride) has been shown to inhibit ex vivo the aggregation of platelets in response to a variety of agents, to prevent the primary aggregation in response to ADP, to decrease the adhesion of platelets to glass surfaces, and to prolong the skin bleeding time (see reference 1 for a review). The interaction of fibrinogen with platelets plays a central role in platelet physiology (2–11), and recent data (12–16) have emphasized the role of ADP, thromboxane (Tx),[1] and cAMP in the exposure of specific receptors for fibrinogen on the platelet surface. In this report we present evidence that ticlopidine almost suppresses the binding of fibrinogen to platelets without affecting the intraplatelet levels of cAMP, the formation of TxB_2 and the secretion of nucleotides in response to high concentrations of collagen, arachidonic acid, ionophore A23187, or thrombin.

Abnormally prolonged bleeding time, defective adhesion of platelets to glass surfaces, defective ADP-induced first wave of aggregation and normal TxB_2 synthesis, and secretion of nucleotides in response to high concentrations of collagen, ionophore A23187, or thrombin are known to occur in Glanzmann's thrombasthenia (2, 3, 17–21). Platelets from these patients lack the glycoprotein IIb-IIIa (GPIIb-IIIa) complex (22, 23) and exhibit negligible binding of fibrinogen after stimulation with ADP (24–28) and deficient binding of von Willebrand Factor (vWF) when exposed to thrombin (29). Since GPIIb-IIIa may form complexes with fibrinogen (30), and monoclonal antibodies to GPIIb and/or IIIa may impair the binding of fibrinogen as well as of vWF to platelets (15, 31–34), such glycoproteins are currently thought to be the receptor for these adhesive proteins on the surface of platelets exposed to naturally occurring aggregating agents. To elucidate whether the impaired platelet aggregation, fibrinogen, and vWF binding observed after the administration of ticlopidine might be due to abnormalities of this receptor, we used two monoclonal antibodies specific to this complex. The data

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1. *Abbreviations used in this paper:* AA, arachidonic acid; DTT, dithiothreitol; GPIIb-IIIa, glycoprotein IIb-IIIa; PFP, platelet-free plasma; PRP, platelet-rich plasma; PG, prostaglandin; PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$, prostaglandins E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$; Tx, thromboxane; vWF, von Willebrand Factor.

obtained indicate that ticlopidine may induce the functional abnormalities of thrombasthenia in platelets in the absence of GPIIb-IIIa deficiency.

Methods

Disodium EDTA, basic fuchsin, and dithiothreitol (DTT) were obtained from Fisher Scientific Co., King of Prussia, PA. EDTA was used as a 100-mM stock solution in distilled water (pH adjusted to 7.4 with NaOH solution). SDS was from Pierce Chemical Co., Rockford, IL. Ammonium persulphate, acrylamide, N-N-methylene-bis-acrylamide, and molecular weight standards were from Bio-Rad Laboratories, Richmond, CA. Bovine serum albumin (Pentex, fraction V) was from Miles Laboratories Inc., Elkhart, IN. Prostaglandin E_1 (PGE_1), hirudin, Hepes, ATP, and ADP (ADP and triphosphate sodium salts) were from Sigma Chemical Co., St. Louis, MO. PGE_1 was dissolved and stored as previously described (35); ADP, ATP, and hirudin were dissolved in distilled water and stored at $-20°C$ in small aliquots; Hepes was prepared as a stock solution of 1 M and stored at 4°C. Epinephrine (epinephrine hydrochloride, isotonic solution) was from Elkins-Sinn Inc., Cherry Hill, NJ. Arachidonic acid (AA) and calcium ionophore A23187 were kindly provided by Dr. Renato Saggiorato (Menarini Diagnostici, Florence, Italy) and prepared and stored as previously described (36). Thrombin was obtained from Parke Davis & Co., Detroit, MI, dissolved in distilled water, and stored in small aliquots at $-70°C$. Equine tendon collagen was from Hormon Chemie, München, Federal Republic of Germany. Protosol, ^{14}C-carboxylinulin, sodium iodide (^{125}I) and radiolabeled PGE_1 (PGE_1-5,6-^3H, specific activity > 60 Ci/mmol) were from New England Nuclear, Florence, Italy. Istagel scintillation fluid and Tri-Carb 2660 beta-counter were from Packard Instruments Co. Inc. (Downers Grove, IL). Highly specific antibodies to TxB_2 (Seragen Inc., Boston, MA) exhibited <0.03% cross-reactivity with prostaglandins E_2, $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and D_2. Silicone oils (methyl silicone 1.0 DC 200 and Hi phenyl silicone 125 DC 550) were purchased from W. F. Nye, Inc., Specialty Lubricants, New Bedford, MA. Mixtures of DC 200 and DC 550 were prepared as described previously (15). Reptilase was from Pentapharm, Basel, Switzerland. Ticlopidine (powder and 250-mg tablets) and its parachlorobenzyl isomer PCR 5325 were kindly provided by Drs. Eric Vallee, Sanofi Recherche, Toulouse, France, and Luca Piovella, Clin Midy Milan, Italy. vWF, purified, characterized, and labeled as described by Ruggieri et al. (34) was a gift of Dr. A. Federici, Milan, Italy. Monoclonal antibodies B79.7 (IgG1) and B59.2 (IgG2a) were kindly provided by Dr. P. Thiagarajan, Thomas Jefferson University, Philadelphia, PA. B79.7 is similar to antibody B59.2, which has been previously shown to be specific for the GPIIb-IIIA complex (15). Indeed, like B59.2, antibody B79.7 (or its Fab fragments) inhibited aggregation and fibrinogen binding to platelet suspensions in a dose-dependent fashion and bound only negligibly (<5%) to platelets from three patients with Glanzmann's thrombasthenia. Moreover, like unlabeled B59.2, antibody B79.7 cross-competes >98.7% with labeled antibody B59.2 for the binding to stimulated or unstimulated platelets. For binding studies, antibody B79.7 and B59.2 were labeled with ^{125}I by the iodine monochloride method (37). For both aggregation and binding studies, mouse immunoglobulins were used as controls throughout.

Preparation of platelet suspensions. Blood was collected from eight healthy subjects (five males, three females, 26–32-yr-old) before and 1 wk after daily administration of 250 mg of Ticlopidine b.i.d. From each subject, 9 vol of blood (90 ml) were collected 12–14 h after the last administration of Ticlopidine from the antecubital vein via a 19-gauge scalp vein needle into a plastic tube containing 1 vol (10 ml) 3.8% trisodium citrate. Platelet-rich plasma (PRP) obtained by centrifugation (200 g for 15 min) at room temperature was used as such within 2 h. Platelet-free plasma (PFP) was obtained by centrifuging

fresh PRP in an Eppendorf centrifuge (Beckman Analytical, Milan, Italy) at 12,000 g for 5 min. For preparation of platelet suspensions, aliquots of PRP were cooled on ice for 30 min and EDTA was added to a final concentration of 5.6 mM. After centrifugation at 2,000 g for 6 min at 4°C in a Beckmann J-6B centrifuge, the platelets were resuspended, as previously described (15), in a volume of buffer (0.14 M NaCl, 20 mM Tris, 5 mM glucose, and 1 mM EDTA, pH 7.4) equal to that of the discarded plasma, centrifuged at 900 g for 6 min at room temperature and resuspended at counts in the range of 1–5 × 10^8/ml in a Tyrode's buffer (0.14 M NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, and 0.4 mM NaH_2PO_4) containing 5 mM glucose, 3.5 mg/ml bovine serum albumin, and 10 mM Hepes, pH 7.4. Platelet counts were determined by phase-contrast microscopy. When trace amount of ^{125}I-fibrinogen or ^{125}I-vWF were added to the PRP, >99.9% of these proteins were removed by the washing procedure. Platelets washed and resuspended in this manner showed "swirling" as a gross indication of maintenance of discoid shape. Platelet suspensions contained <0.5 μg/ml fibrinogen as measured by the staphylococcal clumping test (Boehringer Mannheim, Milan, Italy, sensitivity < 0.5 μg/ml) (38) and did not aggregate in response to 100 μM ADP or 1 μg/ml collagen unless fibrinogen (200 nM final) was added. Studies on aggregation of washed platelets were carried out in the presence of 200 nM fibrinogen. The aggregating response of washed platelets in the presence of fibrinogen was 80–90% of that seen in autologous PRP and was unchanged for at least 2 h. All aggregation and binding studies were conducted within 2 h. Rat blood, obtained by heart puncture from 15 female CD-COBS anesthetized with ether vapor, was collected into plastic tubes containing 1 vol of 3.8% trisodium citrate. PRP was obtained by centrifugation of the blood for 15 min at 220 g and employed as such for aggregation studies or processed as described above for human PRP for the preparation of platelet suspensions. Platelet aggregation and fibrinogen binding were determined as described below for platelets from humans.

Fibrinogen purification and labeling. Fibrinogen was purified from normal donor citrated plasma by the method of Martinez et al. (39). The purified fibrinogen contained <0.8% immunoassayable fibronectin and vWF. Fibrinogen, labeled with ^{125}I by the iodine monochloride method of McFarlane (37) had a specific activity of 2.6–3.8 × 10^4 cpm/μg protein. Unlabeled and labeled fibrinogen showed the characteristic A_α, B_β, and γ peaks, without any degradation products, when electrophoresed on reduced SDS-polyacrylamide gels (40). Further details of the properties of fibrinogen prepared and labeled in this manner have been previously reported (39).

Platelet aggregation and thromboxane formation. Platelet aggregation tests were performed in plastic cuvettes in the Elvi 840 aggregometer (Elvi-Logos, Milan, Italy). The apparatus was adjusted so that PRP (or suspensions of washed platelets) and PFP (or buffer) produced 10 and 90% light transmittance, respectively. When thrombin was the stimulus used, hirudin (0.2–10 U/ml) was employed to prevent clotting. TxB_2 levels were measured by a radioimmunoassay (41) in aliquots of the supernatant solution of tests for platelet aggregation. The sensitivity was such that as little as 0.5 pmol $TxB_2/3$ × 10^8 platelets could be detected.

Platelet secretion of ATP. Secretion of ATP was determined as described elsewhere (41) in a Lumi-aggregometer (Mascia Brunelli, Milan, Italy). This apparatus records the luminescence resulting from the interaction of released ATP (secreted simultaneously with ADP) with firefly luciferase and luciferin (Chronolume 395, Chrono-log Corp., Havertown, PA). The sensitivity was such that concentrations of released ATP as low as 0.1 μM could be detected.

cAMP determination. cAMP was measured on samples of PRP and of platelet suspensions essentially as described by Remuzzi et al. (42) employing a commercially available kit for radioimmunoassay (Beckton-Dickinson, Novate Milanese, Italy). Briefly, 1 ml of 5% TCA was added to 1 ml of PFP or PRP that had been previously incubated for 1 min with microliter amounts of buffer or with concentrations of

PGE₁ (1 μM) that completely inhibited the aggregation of PRP from normals. The sample was immediately frozen in liquid nitrogen and thawed at room temperature, then shaken at 4°C for 30 min. After centrifugation at 3,000 g for 30 min at 4°C, the supernatant was extracted with water saturated ether and the residual ether evaporated under nitrogen. When trace amounts of ^{3}H-cAMP were added to the PRP, >90% of this nucleotide could be recovered at the end of the procedure. For each PRP the amount of cAMP present in the PFP was subtracted. cAMP of the PFP was always <10 pmol/ml. cAMP of rat platelets was determined in a similar fashion.

Platelet clot retraction. The platelet-fibrin clot retraction was measured on PRP as described elsewhere (43). Briefly, a series of samples, each containing 0.5 ml of unstirred PRP, were incubated at 37°C with 1–10 μl of each retracting agent or vehicle. After 3 min, 0.1 ml Reptilase was added and the mixture was stirred with a small glass rod then left inside the tube. At time intervals between 10 and 60 min after the addition of Reptilase, the rod, to which the clot was attached, was gently removed from duplicate samples and the serum was measured in a microliter syringe. The amount of clot retraction was calculated by relating the volume of serum to the total amount of initial mixture and expressed as a percentage. When thrombin was the retracting agent used, Reptilase was omitted.

Studies on fibrinogen, antibody and vWF binding to platelets. Binding of ^{125}I-fibrinogen (or of ^{125}I-B79.7) to platelet suspensions was performed as reported previously (15) with some modifications. Briefly, the time dependence of binding was studied by incubating at room temperature (22°C) 6 ml of unstirred platelet suspension (3–5 × 10⁸/ml) with 10 μM AA, 0.5 μg/ml collagen, 10 μM epinephrine, 10 μM ADP, and 15 μM A23187, or with microliter amounts of buffer. After a 3-min incubation, labelled fibrinogen (or B79.7) was added at the final concentration of 100 μg/ml. At intervals (30 s–120 min), 0.4 ml of the platelet suspension was removed and layered onto 50 μl of silicone oil in a 0.5 ml micro-Eppendorf tube, and free and platelet-bound ligand were separated by centrifugation for 2 min at 12,000 g in an Eppendorf centrifuge and counted in a Beckman DP 5,500 gammacounter. The effect of increasing concentrations of ADP, collagen, AA, A23187, or epinephrine on the extent of fibrinogen binding to platelets was assessed by incubating unstirred platelet suspensions with ADP (5–30 μM), collagen (0.5–3 μg/ml), AA (5–30 μM), thrombin (5–30 mU/ml), ionophore A23187 (5–30 μM), or epinephrine (5–30 μM). After 3 min, labeled fibrinogen (200 nM, final concentration) was added and the extent of binding was determined 5 min later. For dose-response studies, 500-μl aliquots of unstirred platelets were incubated with each aggregating agent (or with equal amounts of buffer) for 3 min, after which labeled fibrinogen (or B79.7) was added in final concentrations of 1.5–2,400 nM. Fibrinogen binding was measured 5 min later while B79.7 binding was measured after 1 min. Specific fibrinogen binding was calculated by subtracting from the total bound the binding measured in the presence of a 20-fold excess of unlabeled protein. This latter "nonspecific" binding represented <20% of the total bound. Specific fibrinogen binding was not (51) corrected for the amount of this protein bound in the absence of stimulation, which was <4–5% of stimulated binding. Studies on the inhibition of aggregation and fibrinogen binding to platelets by antibody B79.7 were carried out as previously described for antibody B59.2 (15). Binding of ^{125}I-vWF to thrombin-, collagen-, AA-, ionophore A23187-, and ADP-stimulated platelets was performed as previously reported (44), while binding of ^{125}I-vWF to ristocetin-stimulated platelets was carried out as described by Kao et al. (45).

Whole platelet glycoprotein analysis. Reduced samples for whole platelet glycoprotein analysis were prepared by mixing 500 μl platelet suspension (containing ~1–2 × 10⁹ platelets) with an equal volume of a 6% SDS solution and 250 μl of a 0.2 M solution of DTT. The samples were then heated at 100°C for 3 min and centrifuged at 12,000 g for 10 min in an Eppendorf centrifuge. Polyacrylamide gels, 1.5 mm thick, contained a *bis*-acrylamide ratio of 0.67% with a 7.5%

(wt/vol) acrylamide. Aliquots (~300 μg protein) of the supernatant were loaded on the gels and run in a discontinuous SDS-Tris-glycine system employing a vertical slab gel electrophoresis cell (Bio-Rad Laboratories). Gels were run at 40 mA constant current until the front (visible as refractive index difference) reached the bottom of the gel. Running time was ~4–5 h. Gels, fixed and stained with Schiff's reagents according to Clementson et al. (46), were soaked twice in 7.5% acetic acid and scanned at 550 nm in a spectrodensitometer model SD 3,000 connected with a SDC 300 density computer (Schoeffel Instruments Corp., Westwood, NJ).

Binding of PGE₁ to resting platelets. Binding of radiolabeled PGE₁ to resting platelets was carried out essentially as described by McDonald and Stuart (47) with some modifications. Briefly, appropriate amounts of ^{3}H-PGE₁ were added to a solution of unlabeled ligand to yield a 5-μM solution of specific activity 0.5 Ci/mmol. Binding procedures were performed at 37°C. The time dependence was determined by incubating 3 ml of unstirred PRP (obtained from four normal subjects before and 1 wk after daily administration of 500 mg of ticlopidine) with 32 nM (final) PGE₁. At time intervals between 1 and 60 min, 0.4 ml of PRP were removed and free and platelet-bound PGE₁ separated in silicone oil. After centrifugation, the supernatant plasma and silicone oil were carefully aspirated with a Pasteur pipette, and a cotton tip applicator was used to remove residual plasma from the walls of the conical tubes and the top of the platelet pellet. The tips of the tubes and known amounts of the supernatants were placed in scintillation vials containing 1 ml of Protosol and allowed to stand overnight to dissolve the pellet. A 10-ml vol of scintillation fluid was then added and the vials counted for radioactivity. For dose-response studies, 500-μl aliquots of PRP were incubated with increasing concentrations (from 16 to 512 nM, final) of PGE₁ and binding determined 5 min later. In some experiments, the quantity of radioactivity trapped with the pelleted platelets was measured by adding trace amounts of ^{14}C-inulin to PRP 5 min before centrifugation and determining the amount of ^{14}C-inulin present in the platelet pellet. Trapping never exceeded 0.5% of the total bound, and this value was subtracted from the total binding. Specific binding was also corrected for the amount of this prostaglandin bound in the presence of a 20-fold excess of unlabeled PGE₁ that was 23–26% of the total bound.

Bleeding time. The skin bleeding time was determined with a Simplete II apparatus (General Diagnostic, Warner Lambert Co., Morris Plains, NJ) according to the manufacturer's recommendations.

Protein concentration. Protein concentration was calculated from the absorbance at 280 nm using an extinction coefficient of 15.1 for fibrinogen and 14.3 for antibodies.

Statistical analysis. t Test for paired comparison was used for statistical analysis.

Informed consent. Informed consent was obtained from all the volunteers in accordance with accepted institutional practices. The studies were carried out according to the principles of the declaration of Helsinki.

Results

Prolongation of the bleeding time after the administration of ticlopidine. After 1 wk of ticlopidine administration there was a significant prolongation of the skin bleeding time in all the subjects studied (4.96±1.1 min before vs. 9.16±1.12 after, P < 0.001). One volunteer had nose bleeding, and five out of eight exhibited easy bruising. No further prolongation of the bleeding time was observed in four volunteers to whom ticlopidine (500 mg daily) was administered for 1 mo.

Effect of ticlopidine on platelet aggregation, ATP secretion, TxB₂ synthesis, and intraplatelet cAMP. In vitro addition of ticlopidine (100 μM, final) to PRP or to washed platelet

suspensions did not change the sensitivity of these cells to aggregating agents. In contrast, after 1 wk of administration, the aggregation of PRP in response to ADP (5–15 μM), epinephrine (5–15 μM), collagen (0.125–0.75 μg/ml), thrombin (0.1–0.5 U/ml), or AA (0.2–0.6 mM), was almost suppressed (Fig. 1). Similar impairment of platelet aggregation was observed when the calcium ionophore A23187 (5–15 μM) was the stimulus employed (not shown). The administration of ticlopidine did not affect agglutination of PRP and of formaldehyde-fixed platelets in response to ristocetin and shape change in response to ionophore A23187 (not shown) or to ADP, collagen, thrombin, and AA (Fig. 1). Likewise, the intraplatelet levels of cAMP, determined before and after exposure to PGE_1 (1 μM), were unaffected by ticlopidine (Table I). When the secretion of ATP was measured simultaneously with the aggregation, it appeared that ticlopidine suppressed secretion in response to ADP or epinephrine, whereas it only partially affected secretion in response to AA, ionophore A23187, collagen, or thrombin. This partial inhibition of secretion was further evaluated. Dose-response studies revealed (Fig. 2) that the inhibition of secretion by ticlopidine could only be detected when low concentrations of AA, collagen, or thrombin were used. Furthermore, normal secretion of ATP (i.e., 3.7±0.72 μM) was also observed when unstirred platelet suspensions from ticlopidine-treated subjects were preincubated in vitro with 5 mM EDTA (in order to avoid aggregation) and 25–50 μM of ionophore A23187 were added 5 min later. In parallel
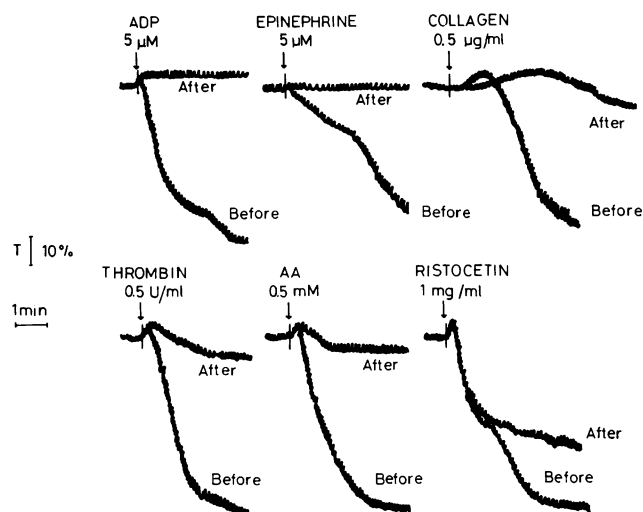
Figure 1. Effect of the administration of ticlopidine on platelet aggregation. Microliter amounts of each aggregating agent were incubated with 0.25 ml of PRP which had been stirred at 37°C for 1 min at 1,000 rpm. The apparatus was calibrated so that PRP and PFP produced 10 and 90% light transmittance, respectively. The extent of platelet aggregation was determined 3 min after the addition of each aggregating agent. The inhibitory effect of ticlopidine on the aggregation induced by all the agents (but ristocetin) and the lack of inhibition of the shape change in response to ADP, collagen, AA, or thrombin are evident in this figure. The seven other PRPs studied gave similar results. Similar inhibitory effects of ticlopidine were also observed on suspensions of washed platelets (5 × 10^8/ml) stirred in the presence of fibrinogen (200 nM final) for 1 min before adding each aggregating agent. No shape change was seen before or after ticlopidine when epinephrine was the stimulus employed.

Table I. Changes of cAMP Content in Platelets Obtained Before and After the Administration of Ticlopidine*

Stimulus	cAMP (pmol/10^9 platelets)	
	Before	After
Buffer	67.8±14.1	72.2±16.3
PGE$_1$ (1 μM)	188.9±21.2‡	201.3±29.1‡

A 1-ml solution of 5% TCA was added to 1 ml of PRP or PFP previously incubated for 1 min with buffer or with a concentration of PGE$_1$ (1 μM) that completely suppressed the aggregation of platelets. The sample was immediately frozen in liquid nitrogen and thawed at room temperature, then shaken at 4°C for 30 min. After centrifugation at 3,000 g for 30 min at 4°C, the supernatant was extracted with water-saturated ether and cAMP measured by a radioimmunoassay. For each PRP the amount of cAMP present in the PFP was subtracted.
* Mean ± SEM of composite data from all eight subjects.
‡ $P < 0.001$ buffer vs. PGE$_1$-treated platelets ($P > 0.05$ for all the other comparisons).

with secretion, TxB$_2$ synthesis was only affected in response to low concentrations of these agents (Fig. 3).

Results similar to those seen employing PRP were found when aggregation, ATP secretion, TxB$_2$ synthesis, and intra-platelet cAMP were determined in washed platelet suspensions from normal subjects and in PRP or washed platelet suspensions from rats treated with ticlopidine (data not shown).

Inhibition of clot retraction by ticlopidine. In parallel with the inhibition of platelet aggregation, ticlopidine almost suppressed the retraction of PRP clotted with Reptilase and challenged with ADP, epinephrine, collagen, or AA as inducers of platelet contractile activity. Inhibition by ticlopidine of ADP-induced clot retraction is shown in Fig. 4. Similar results were found employing 5–15 μM epinephrine, 0.2–1 μg/ml collagen, 0.2–0.6 mM AA, or 1–3 U/ml thrombin (data not shown). When thrombin was the stimulus, Reptilase was omitted. It is also important to note that in control tubes, full retraction was achieved within 45–60 min, whereas no complete retraction was found in samples obtained following the administration of ticlopidine even after 6 h of incubation at 37°C.

Inhibition of ^{125}I-fibrinogen binding to platelets by ticlopidine. Exposure of platelet suspensions to ADP, epinephrine, collagen, thrombin, or AA induced an immediate time-dependent binding of ^{125}I-fibrinogen which was complete within 3–5 min (the time period within which platelet aggregation is complete). Between 5 and 15 min after addition of labeled fibrinogen, >85% of this could be displaced by a 20-fold excess of unlabeled protein. Similar results were obtained when the labeled and unlabeled fibrinogen were added together at the initiation of the binding reaction. EDTA (10 mM) inhibited 75–83% of the fibrinogen binding to stimulated platelets. Binding of fibrinogen was not affected at all when ticlopidine was added to platelets in vitro at concentrations as high as 100 μM. However, when administered in vivo, ticlopidine almost suppressed the interaction of fibrinogen with platelets exposed to ADP, epinephrine, collagen, AA, or thrombin (Fig. 5). The affinity of fibrinogen for platelets obtained before and after ticlopidine was studied quantitatively by incubating platelet
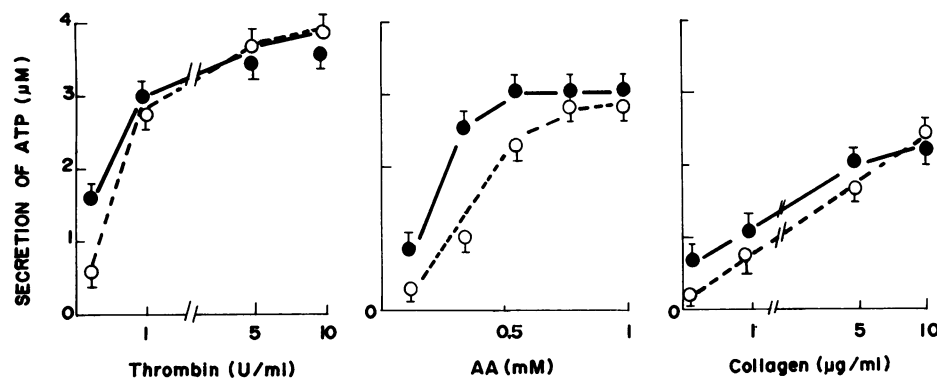
Figure 2. Effect of ticlopidine on the secretion of ATP from platelets. 50 μl of firefly luciferase and luciferin (or of the buffer) were incubated with 0.5 ml PRP that had been stirred at 37°C for 1 min at 1,000 rpm in a lumi-aggregometer. After 30 s, increasing concentrations of thrombin, AA, or collagen were added. ATP secretion was measured by comparing the luminescence produced by platelets 3 min after addition of each aggregating agent with that produced by known amounts of ATP added to unstimulated platelet suspensions. The sensitivity was such that<0.1 μM secreted ATP could be detected. Each circle is the mean±SEM of composite data from all eight subjects tested. Closed circles refer to pre-ticlopidine and open circles to post-ticlopidine data. A statistically significant effect of ticlopidine ($P < 0.01$) on ATP secretion was only evident in response to thrombin (0.2 U/ml), AA (0.2–0.4 mM), or collagen (0.25 μg/ml).

suspensions with 10 μM ADP, 20 μM epinephrine, 15 mU/ ml thrombin, 10 μM AA, or 1 μg/ml collagen. After 3 min, increasing concentrations (from 1.5 to 2,400 nM, final) of fibrinogen were added, and 5 min later free and platelet-bound fibrinogen were separated in silicone oil and counted. In suspensions of platelets obtained before ticlopidine administration, fibrinogen binding was concentration-related and reached a maximum at a final concentration of ∼200 nM. Ticlopidine did not affect this pattern, but reduced by 94–97% the binding in response to all agents tested (Table II). Scatchard analysis of the data (15) showed that ticlopidine did not affect the apparent affinity for fibrinogen of the "high affinity sites," whereas it significantly decreased the affinity of the platelet "low affinity sites." Therefore, the dissociation constant of the low affinity sites was 8–12-fold higher than normal ($P < 0.01$) in platelets obtained after the administration of the drug.

Results similar to those reported for the aggregation, the clot retraction, the secretion of ADP, the synthesis of TxB₂, the binding of fibrinogen, and the measurement of cAMP of

platelets from subjects who had ingested ticlopidine were found in platelets from three patients who fulfilled all the criteria for the diagnosis of thrombasthenia (17–29).

Effect of ticlopidine on PGE₁ and vWF binding to platelets. Drugs like carbenecillin and penicillin G inhibit platelet function by impairing the interaction of several agonists with their specific receptors on the platelet surface membrane (48). We wondered, therefore, whether the inhibition of fibrinogen was a selective event or that ticlopidine might inhibit platelet function by interfering with the binding of other agonists to the platelet surface. We studied the binding to platelets of an inhibitor of platelet aggregation, PGE₁, as well as that of the plasma protein vWF. PGE₁ was studied because recent reports (49, 50) showed that, at variance with aspirin (35), ticlopidine induces hypersensitivity of platelets to this prostaglandin. The binding of vWF was determined because this plasma protein is involved in the adhesion of platelets to the vascular subendothelium, a phenomenon thought to play a role in the arrest of bleeding (29, 36, 44, 45).

Following exposure to PGE₁, resting platelets in PRP from

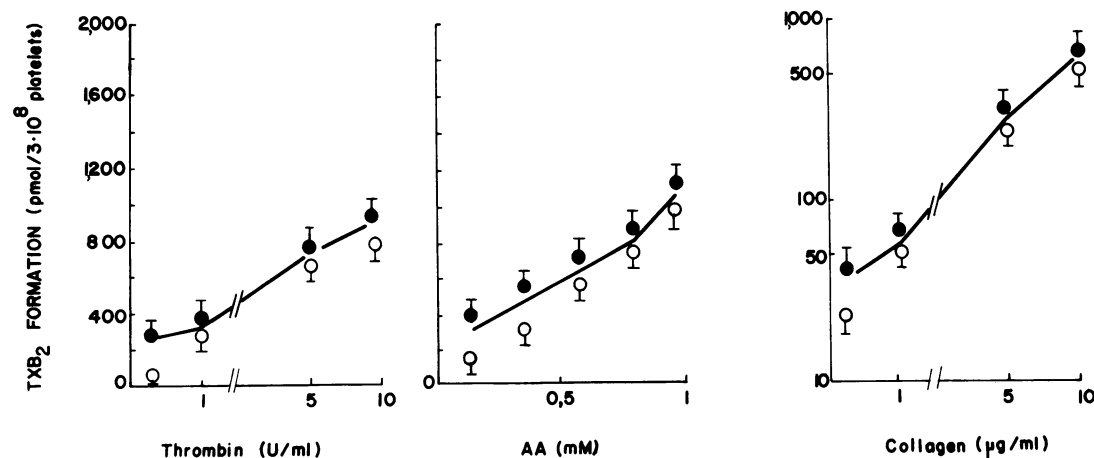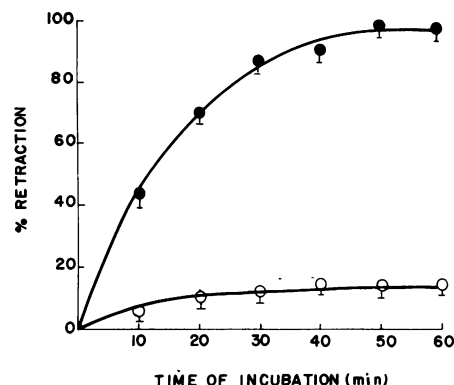*Figure 3.* Effect of ticlopidine on platelet TxB₂ synthesis. TxB₂ was measured in aliquots of the supernatant solution after completion of tests for platelet aggregation. Each circle is the mean±SEM of composite data from all eight volunteers studied. Closed circles refer to pre-ticlopidine and open circles to post-ticlopidine data. Significant

differences ($P < 0.05$) between the amounts of TxB₂ produced before and after ticlopidine administration could only be found in response to thrombin (0.2 U/ml), AA (0.2 and 0.4 mM), and collagen (0.25 μg/ml).
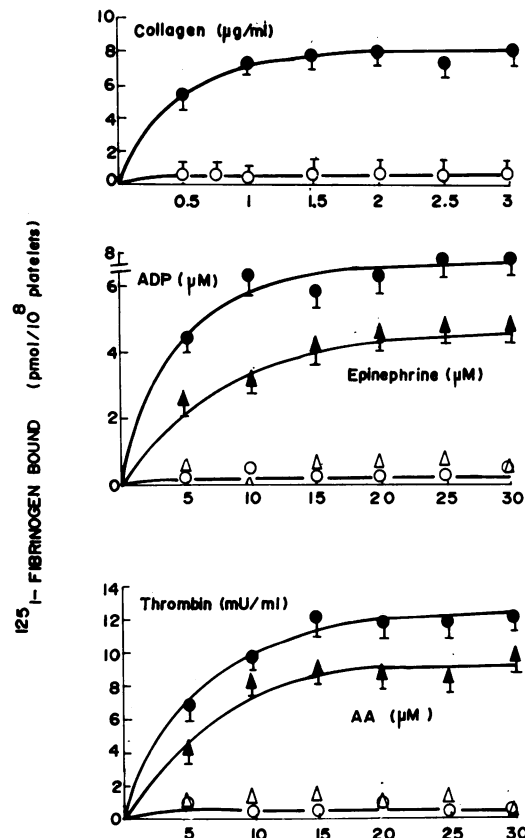
Figure 4. Effect of the administration of ticlopidine on the platelet-fibrin clot retraction in response to 5 μM ADP. A series of samples containing 0.5 ml of unstirred PRP were incubated at 37°C with 5 μM (final) ADP. After 3 min, 0.1 ml Reptilase was added and the mixture stirred with a small glass rod, then left inside the tube. At time intervals between 10 and 60 min after the addition of Reptilase the rod, to which the clot was attached, was gently removed by duplicate samples and the serum was measured in a microliter syringe. The amount of clot retraction was calculated by relating the volume of serum to the total amount of initial mixture and expressed as a percentage. Each circle is the mean±SEM of composite data from all eight subjects studied. Closed circles refer to pre-ticlopidine and open circles to post-ticlopidine data. Statistical analysis showed significant differences (*P* always <0.01) between the data obtained before and after the administration of this drug.

Figure 5. Effect of ticlopidine on the binding of fibrinogen to platelets. Aliquots of 0.5 ml of unstirred suspensions of platelets (5 × 10⁸/ ml) from each volunteer were incubated at room temperature with 5–30 μM ADP (circles), epinephrine (triangles), or AA (triangles), 0.5–3 μg/ml collagen (circles) or 5–30 mU/ml thrombin (circles). After 3 min, 200 nM (final concentration) of ¹²⁵I-fibrinogen was added and 5 min later aliquots (0.4 ml) of the mixture were layered onto 50 μl of silicone oil in a 0.5 ml micro-Eppendorf tube. Free and platelet-bound ¹²⁵I-fibrinogen were separated by centrifugation for 2 min at 12,000 *g* at room temperature and counted separately. Specific fibrinogen binding was calculated by subtracting from the total bound the amount of binding in the presence of a 20-fold excess of unlabeled protein. Specific fibrinogen binding was not corrected for the amount of this protein bound in the absence of stimulation. Closed symbols refer to pre-ticlopidine, open symbols to post-ticlopidine data. Note the almost complete suppression of the binding to platelets observed after the administration of ticlopidine (*P* always <0.001). The values reported are mean±SEM of composite data from all eight subjects studied.

four untreated subjects bound this prostaglandin completely within 3–5 min. At 5 min and up to 30 min, >75% of the labeled ligand could be displaced by a 100-fold excess of unlabeled PGE₁ but not by a similar molar excess of PGF₂α or TxB₂. Ticlopidine either added in vitro at concentrations as high as 100 μM or administered in vivo for 1 wk at the dose of 250 mg b.i.d. did not affect the patterns and the extent of this binding. This lack of effect was further confirmed in studies in which PRP obtained before and after the administration of ticlopidine was incubated with increasing concentration (from 16 to 512 nM, final) of PGE₁ and free and platelet-bound ligand determined 5 min later (Fig. 6).

When the binding of vWF to platelets was studied, it appeared that washed platelet suspensions exposed to ristocetin bound as much ¹²⁵I-vWF after the administration of ticlopidine as before. However, further studies (Table II) showed that when exposed to thrombin, collagen, AA, or ADP, platelets obtained after the administration of ticlopidine bound 40–60% less vWF than those from untreated subjects. Evidence is now accumulating (15, 31–34) to indicate that GPIIb-IIIa complex is the receptor for fibrinogen as well as for vWF on platelets exposed to naturally occurring aggregating agents. Therefore, we studied the effects of the administration of ticlopidine on this complex.

Effect of ticlopidine on the GPIIb-IIIa complex. To evaluate the potential effect of ticlopidine on GPIIb-IIIa complex we utilized monoclonal antibodies specific to this complex. Regardless of ticlopidine, binding of radiolabeled antibody B79.7 (or B59.2) to unstimulated platelets was complete in 1 min. The amount of specific B79.7 binding at saturation was similar in platelets obtained before and after the administration of

ticlopidine (3.62±1.16 pmol/10⁸ platelets before vs. 3.47±2.16 after ticlopidine, *P* > 0.1). Pretreatment of platelets with ADP (30 μM), AA (30 μM), or thrombin (30 mU/ml) did not affect the amount of binding observed in suspensions of platelets obtained before and after ticlopidine. Likewise, binding of antibody B59.2 to platelets was unaffected after 1 wk of administration of ticlopidine (not shown). In order to confirm that ticlopidine did not affect the GPIIb-IIIa complex quantitatively, we employed densitometric scannings of Periodic Acid-Schiff-stained gels of suspensions of platelets obtained before and after the administration of this drug. Fig. 7 shows a densitometric scan of one of these gels. It is representative

Stimulus	pmols ^{125}I-fibrinogen-bound/10^8 platelets		μg ^{125}I-vWF-bound/10^9 platelets	
	Before	After	Before	After
ADP (10 μM)	6.73±0.46*	0.26±0.04‡	3.76±0.54*	1.07±0.03‡
Epinephrine (20 μM)	4.83±0.36*	0.18±0.06‡	2.03±0.12*	0.16±0.06‡
Collagen (1 μg/ml)	7.68±0.15*	0.29±0.01‡	6.42±0.39*	2.40±0.01‡
AA (10 μM)	8.57±0.51*	0.31±0.06‡	7.52±0.61*	3.05±0.13‡
Thrombin (15 mU/ml)	12.63±1.13	0.50±0.03‡	15.07±1.83*	6.04±1.14‡
Ionophore A23187 (15 μM)	9.36±0.73*	0.41±0.09‡	10.04±0.97*	4.03±1.16‡
Ristocetin (1.5 mg/ml)	ND	ND	19.36±2.13	20.16±1.96

Mean±SEM of composite data from eight subjects for fibrinogen and four for vWF. Platelet suspensions (5 × 10^8/ml) were incubated with 10 μM ADP, 20 μM epinephrine, 1 μg/ml collagen, 10 μM AA, 15 mU/ml thrombin, 15 μM ionophore A23187, or 1.5 mg/ml ristocetin. After 3 min, increasing concentrations of fibrinogen (from 1.5 to 2,400 nM, final) or of vWF (from 1.25 to 40 μg/ml) were added and, at appropriate time intervals (i.e., 5 min for fibrinogen and 45 min for vWF binding), free and platelet-bound ligands were separated in silicone oil and counted. When ristocetin was used, formaldehyde-fixed platelets were employed. Both before and after ticlopidine administration, fibrinogen binding reached saturation at a final concentration of $\sim$200 nM, while vWF at a concentration of $\sim$20 μg/ml. No difference ($P > 0.05$) was found between thrombin- and ristocetin-induced vWF binding to platelets. * $P < 0.01$ thrombin- vs. ADP-, epinephrine-, collagen-, ionophore A23187-, or AA-induced binding (pre-ticlopidine). ‡ $P < 0.001$ before vs. after ticlopidine. ND, not detectable.

of the seven other platelet preparations tested. At variance with what was observed in gels of platelets from thrombasthenics (bottom of the figure), no apparent change in the region of glycoprotein IIb and IIIa was found in platelets obtained from ticlopidine-treated subjects.

Onset and cessation of the effect of ticlopidine on platelets. Following the cessation of ticlopidine, an immediate time-dependent recovery of platelet aggregation and fibrinogen binding in response to 5 μM ADP occurred which approached completion within 3–5 d (Fig. 8 *A*). To further evaluate this observation, 250 mg b.i.d. of ticlopidine was administered daily for 1 wk to four volunteers and the aggregation of PRP and the binding of fibrinogen in response to 5 μM ADP was determined every 24 h. As shown in Fig. 8 *B*, the inhibitory effect of ticlopidine was very slow, and reached a maximum after a 3–5-d administration. These observations, together with
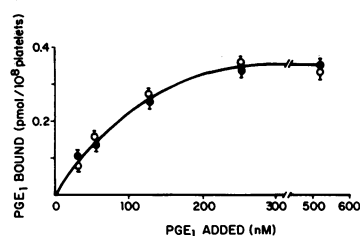
the fact that ticlopidine is a potent inhibitor of platelet function only when administered in vivo, suggested that the platelet effect of ticlopidine could be mediated by a metabolite rather than the native drug. To further evaluate this possibility, washed platelets from untreated subjects were incubated either in control PFP or in the PFP of four subjects to whom 500 mg of ticlopidine had been administered for 1 wk. PFP of
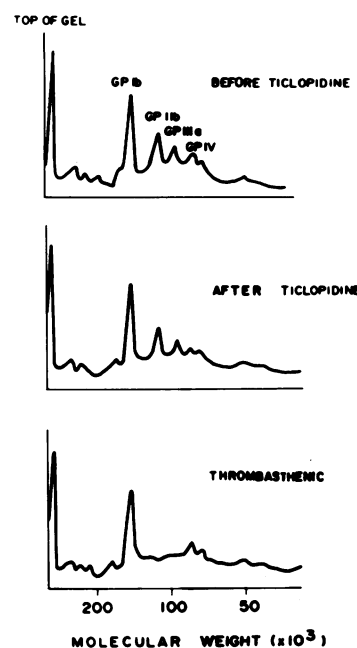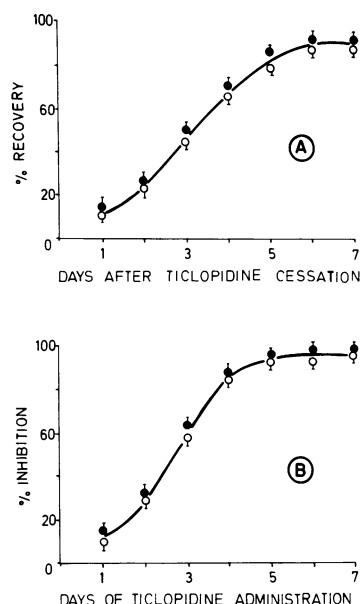

Figure 6. Effect of ticlopidine on the binding of prostaglandin E_1 (PGE$_1$) to platelets. Each point is the mean±SEM of composite data from four subjects. Aliquots of 0.5 ml of unstirred PRP were incubated at 37°C with increasing amounts (from 16 to 512nM, final) of PGE$_1$ and, 5 min later, free and platelet-bound PGE$_1$ were separated by centrifugation at 12,000 g for 2 min. After centrifugation, the supernatant plasma and silicone oil were carefully aspirated with a Pasteur pipette and a cotton tip applicator was used to remove residual plasma from the walls of the tubes and the top of the platelet pellet. The tips of the tubes and appropriate amounts of the supernatant were placed in scintillation vials containing 1 ml of Protosol and allowed to stand overnight to dissolve the pellet. A 10-ml vol of scintillation fluid was then added and the vials counted for radioactivity. Closed symbols refer to pre-ticlopidine, open symbols to post-ticlopidine data.

Figure 7. Typical scan records of Periodic Acid-Schiff-stained SDS-polyacrylamide gels representing whole platelet glycoproteins from platelets obtained before and 1 wk after daily administration of ticlopidine. Reduced samples for whole platelet glycoprotein analysis were prepared by mixing 500 μl of platelet suspension (containing $\sim$1–2 × 10^9 platelets) with an equal volume of 6% SDS solution and 250 μl of a 0.2-M solution of DTT. The samples were then heated at 100°C for 3 min, centrifuged at 12,000 g for 10 min, and loaded on polyacrylamide gels that were 1.5 mm thick and contained a *bis*-acrylamide ratio of 0.67% with a 7.5% (wt/vol) acrylamide. Gels were run at 40 mA constant current for 4–5 h in a vertical slab gel, fixed and stained with Schiff's reagent, and scanned in a spectrodensitometer connected with a density computer. The bottom part of the figure refers to the scan of a gel of whole platelet glycoproteins from one of the thrombasthenic patients studied. The lack of GPIIb-IIIa is evident in this record.

Figure 8. Onset and cessation of the effect of ticlopidine on platelets. (A) After 1 wk of administration, ticlopidine was withdrawn and platelet aggregation (closed circles) in response to 5 μM was determined for 7 d in parallel with the binding of ^{125}I-fibrinogen (open circles). At least 5 d were required to achieve 90% of the aggregation and the binding found before starting the administration. The values reported are mean±SEM of composite data from all eight subjects studied. (B) Four normal subjects (two males and two females aged 28–32 yr) who denied taking any medication donated blood. 9 vol of blood (45 ml) were drawn into 1 vol (5 ml) 3.8% trisodium citrate. The subjects then ingested 500 mg ticlopidine daily for 1 wk. Blood samples (45 ml) were taken at daily intervals for 7 d and the aggregation of PRP (closed circles) in response to 5 μM ADP and the binding of fibrinogen to washed platelets (open circles) determined. At least 4–5 d of ticlopidine administration were required to achieve 90% inhibition of the aggregation and of the binding of fibrinogen.

ticlopidine-treated subjects was obtained from blood collected 12–14 h after the last administration of this drug. When platelet aggregation, secretion, and TxB$_2$ formation were determined after 90 min incubation in PFP, it appeared that plasma from subjects taking ticlopidine induced abnormalities of platelet function comparable to those described above for platelets from ticlopidine-treated subjects.

Effect of ticlopidine on the aggregation and binding of fibrinogen to rat platelets. Ticlopidine, as well as its isomer PCR 5325 (Fig. 9), is a weak lipophilic substance and can affect the fluidity of membrane lipids in rat platelets (Vallee, E., unpublished observations). Since platelet activation involves changes in membrane fluidity (51, 52), we wondered whether ticlopidine could induce abnormalities of platelet function by altering this membrane-associated process. To evaluate this, ticlopidine or its isomer were given by mouth to rats and, after 3 d of administration, platelet aggregation and fibrinogen binding were determined. Table III reports data from these studies. In spite of the fact that ticlopidine and PCR 5325 are able to induce comparable changes in the fluidity of platelet membrane lipids, only ticlopidine inhibited the aggregation and the binding of fibrinogen to platelets.
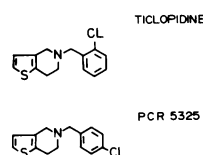
Figure 9. Structure of ticlopidine and its isomer PCR 5325. Pka of ticlopidine was 7.64.

Discussion

Previous reports have shown that ticlopidine significantly prolongs the skin bleeding time and inhibits the aggregation of platelets in response to a variety of agents (1). We have confirmed these observations, and relate them to a dramatic inhibition of fibrinogen and vWF binding to platelets in the absence of quantitative abnormalities of platelet membrane glycoproteins. Inhibition of platelet aggregation and clot retraction cannot be attributed to a direct effect of ticlopidine on fibrinogen, since similar inhibitory effects were found in PRP and in platelets washed free of fibrinogen. In addition, fibrinogen purified from four volunteers before and after 1 mo of administration of ticlopidine (250 mg b.i.d.) behaved identically in supporting the aggregation of washed platelets.

In agreement with our data, other groups have found a very slow onset and cessation of the effect of ticlopidine and an apparent discrepancy between the in vitro and ex vivo effects of this drug (1). This suggests that either ticlopidine acts through one or more active metabolites, or that 3–5 d are required to achieve plasma concentrations of the drug sufficient to cause the inhibitory effect observed. The latter possibility seems unlikely, since ticlopidine is completely cleared from the plasma within 6 h and no signs of accumulation have been found after repeated doses (53). Furthermore, abnormalities of platelet function comparable to those of ticlopidine-treated subjects can be observed when platelets from untreated subjects are incubated in PFP from subjects to whom ticlopidine has been administered.

We have found that, following the administration of ticlopidine, platelets exhibit impaired aggregation, clot retraction, and fibrinogen and vWF binding in response to a variety of agents. These in vitro abnormalities were associated with a prolonged skin bleeding time and are comparable to those found in patients with Glanzmann's thrombasthenia. Moreover, as in thrombasthenia (1–3, 17–21, 54), platelets obtained after ticlopidine administration have normal shape change in response to ADP, thrombin, collagen, or AA, normal agglutination and binding of vWF in response to ristocetin, and decreased adhesion to glass surfaces.

It is known that prostacyclin either administered in vivo or added in vitro may inhibit the first wave of aggregation in response to ADP (35, 55), the clot retraction (43), and the exposure of fibrinogen and vWF receptors on platelets (56, 57). However, at variance with ticlopidine, prostacyclin cannot be said to induce a thrombasthenic-like state in normal platelets, since these effects are associated with increases in intraplatelet cAMP able to inhibit platelet activation (16, 58).

The binding of fibrinogen or vWF to platelets involves activation of these cells (24–29, 31–34, 44), and such activation is known to be triggered by a variety of cellular reactions. Among these, thromboxane synthesis, changes in intracellular cAMP, and translocations of calcium ions across the membranes are thought to be major events (48, 51, 52). Our data make it unlikely that ticlopidine acts by affecting one or more of these basic events of platelet activation since, as in thrombasthenia, these processes are normal in platelets from ticlopidine-treated subjects. However, we observed that the hypersensitivity to PGE$_1$ of ticlopidine-treated platelets from rats does not depend on abnormalities of the platelet receptor for this prostaglandin

Table III. Aggregation and Binding of ^{125}I-Fibrinogen to Rat Platelets*

| | Aggregation | | Fibrinogen binding | |
Inhibitor	Before	After	Before	After
Vehicle				
Collagen (1 μg/ml)	5.34±1.56	5.16±1.32	7.14±1.64	7.57±0.65
ADP (10 μM)	3.29±1.51	4.12±1.60	4.09±0.78	4.64±0.16
Ticlopidine				
Collagen (1 μg/ml)	4.91±1.19	0.76±0.10‡§	7.52±0.46	0.94±0.09‡§
ADP (10 μM)	3.21±0.13	0.20±0.03‡§	4.26±1.12	0.36±0.01‡§
PCR 5325				
Collagen (1 μg/ml)	4.86±1.31	3.15±1.24	6.97±0.86	5.78±1.01
ADP (10 μM)	3.62±1.14	2.97±0.66	4.06±0.93	3.24±0.77

15 CD-COBS rats (five times each group) weighing 200–250 g were given daily by mouth 200 mg/kg per body weight of ticlopidine or of its isomer PCR 5325 or of vehicle (distilled water). After 3 d of administration the animals were anesthetized by immersion in ether vapor and blood was collected by heart puncture. PRP was employed as such for aggregation studies or processed as described in methods for the preparation of platelet suspensions (5–8 × 10^8 platelets/ml). Fibrinogen binding was determined as described above for human platelets. Platelet aggregation, studied as maximum light transmittance, was measured 3 min after the addition of an aggregating agent and expressed in units, 1 U = 10% light transmittance. Fibrinogen binding is reported as picomoles of ligand bound/10^8 platelets. Binding of fibrinogen to rat platelets showed patterns quantitatively and qualitatively comparable to that of human platelets. * Each value is the mean of composite data from five animals. ‡ $P < 0.01$ before vs. after. § $P < 0.01$ vehicle vs. ticlopidine (both after). All other comparisons were not significant ($P > 0.05$).

or on the stimulation of abnormally high levels of intraplatelet cAMP following this binding. Therefore, it is conceivable that other poorly understood mechanisms of platelet activation are affected by ticlopidine.

The nature of fibrinogen and vWF receptors exposed on the platelet surface following stimulation with naturally occurring aggregating agents is not precisely known, although most evidence suggests that they are, or are closely associated with GPIIb and IIIa. Platelets from thrombasthenic patients lack the GPIIb-IIIa complex (22, 23) and have absent binding of fibrinogen after stimulation with ADP (24–28) and impaired binding of vWF following exposure to thrombin (29); GPIIb-IIIa may form complexes with fibrinogen (30) and monoclonal antibodies to GPIIb and/or IIIa inhibit the binding of fibrinogen and vWF to platelets (15, 31–34). Studies using ticlopidine indicate that this drug induces a dramatic inhibition of the binding of these adhesive proteins to the platelet surface without affecting the GPIIb-IIIa complex quantitatively. This suggests that either ticlopidine induces a qualitative defect in these glycoproteins or that molecular defects other than GPIIb-IIIa deficiency may lead to a thrombasthenic state. The latter possibility is also suggested by the observations that: thrombasthenic platelets have abnormalities of surface proteins other than GPIIb and IIIa (59); more than one class of binding sites for fibrinogen and vWF have been described on the platelet surface (14, 15, 25, 28, 34); and thrombasthenic platelets may bind fibrinogen and aggregate when exposed to chymotrypsin (26, 28).

Current evidence (48, 52, 60) suggests that plasma membrane receptors are glycoproteins presumably embedded within the membrane's lipid bilayer. Since ticlopidine is a lipophilic substance and can alter the fluidity of platelet membranes in the rat, it might interfere with the binding of several agonists to the platelet surface, either by interacting directly with

specific receptors or by affecting the surrounding lipid milieu. However, in spite of the fact that ticlopidine and its isomer PCR 5325 induce comparable changes in the fluidity of platelet membranes, our data show that only ticlopidine significantly inhibits the aggregation and the binding of fibrinogen to rat platelets (Table III). In addition, ticlopidine does not affect the binding of ADP to its high affinity receptor (61), or that of PGE$_1$ to resting platelets (Fig. 6), and this drug impairs the binding of vWF only when platelets are exposed to some agents (Table II). Finally, the interaction of thromboxane or ADP with platelets is known to account only for 30–50% of the binding of fibrinogen or vWF observed in response to ADP or collagen (12–15, 44).

Taken together, these data suggest that the effect of ticlopidine on platelets is a novel and unique platelet phenomenon that, in addition to its obvious pharmacologic significance, could serve as an important probe for understanding the basic events leading to platelet activation.

Acknowledgments

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