Biochemical Pharmacology, Vol. 40, No. 2, pp. 229–238, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

THE THIENOPYRIDINE PCR 4099 SELECTIVELY INHIBITS ADP-INDUCED PLATELET AGGREGATION AND FIBRINOGEN BINDING WITHOUT MODIFYING THE MEMBRANE GLYCOPROTEIN IIb–IIIa COMPLEX IN RAT AND IN MAN*

CHRISTIAN GACHET,† ANITA STIERLÉ,† JEAN-PIERRE CAZENAVE,†‡ PHILIPPE OHLMANN,† FRANÇOIS LANZA,† CYRILLE BOULOUX§ and JEAN-PIERRE MAFFRAND§

†INSERM U.311, Biologie et Pharmacologie des Interactions du Sang avec les Vaisseaux et les Biomatériaux, Centre Régional de Transfusion Sanguine, 10 rue Spielmann, 67085 Strasbourg Cédex; and §Sanofi Recherche, Laboratoire d'Hémobiologie, 195 route d'Espagne, 31036 Toulouse Cédex, France

(*Received* 2 *October* 1989; *accepted* 4 *February* 1990)

Abstract—The thienopyridines, ticlopidine and PCR 4099, inhibit *ex vivo* aggregation in response to ADP and other agonists. It has been shown that ticlopidine induces a functional defect in the binding of fibrinogen to its platelet membrane receptor. We have studied the effects on platelet functions of PCR 4099 in rat and in man. The aim of the study was to check the possibility of a direct modification of the fibrinogen binding site on the GP IIb–IIIa complex. Washed platelet suspensions were used for aggregation and fibrinogen binding studies. Platelet lysates were submitted to SDS–polyacrylamide gel electrophoresis, crossed immunoelectrophoresis and immunoprecipitation. We found that administration of PCR 4099 inhibited selectively and irreversibly ADP-induced aggregation. Although the effect of ADP on aggregation was blocked, PCR 4099 did not modify ADP-induced shape change. Only the effects of low concentrations of thrombin on platelet aggregation were inhibited. Fibrinogen binding was dramatically inhibited in rat and in man when platelets were stimulated with ADP and low concentrations of thrombin. At high concentration of thrombin there still remained a part of fibrinogen binding inhibition although aggregation was not impaired. Electrophoretic and immunoelectrophoretic studies showed no difference before and after treatment by PCR 4099. In particular, the GP IIb–IIIa-complex was not dissociated, its electrophoretic mobility was not changed and three monoclonal anticomplex antibodies recognized it in the same manner before and after treatment. We conclude that PCR 4099 selectively inhibits the ADP aggregation pathway and that the inhibition of fibrinogen binding is probably not due to a direct modification of the GP IIb–IIIa complex.

Ticlopidine and PCR 4099 inhibit *ex vivo* platelet aggregation induced by several agonists [1–3]. In particular, ticlopidine inhibits ADP-induced aggregation and this effect on ADP may explain the broad spectrum antiaggregating effect of the thienopyridines, since dense granule secreted ADP plays a major role in platelet activation [4]. Interaction of ADP with platelets leads to shape change, exposure of the fibrinogen receptor on the GP IIb–IIIa complex, binding of external fibrinogen to its binding site and aggregation [5–8]. Ticlopidine is known to inhibit fibrinogen binding to its receptor *in vitro* [9] and *ex vivo* [10]. It was also suggested that ticlopidine induces a "functional thrombasthenic state" of platelets [11]. We have studied the effects on platelet functions of PCR 4099, a structural analogue of ticlopidine. In the rat, at a dose of 100 mg/kg that completely blocks ADP-induced aggregation and in man at the dose of 200 mg/day for 8 days, where 10 healthy human volunteers were studied. These studies were performed in order to check the possibility that the "functional thrombasthenic state" may be due to a direct modification of the fibrinogen binding site on the GP IIb–IIIa complex.

MATERIALS AND METHODS

Chemicals. PCR 4099 (*d,l* methyl (2-chlorophenyl)-5-(4,5,6,7-tetrahydrothieno (3,2-C) pyridyl) acetate, hydrochloride, hydrate) was synthesized by Sanofi Recherche (Toulouse, France). Adenosine-5′-diphosphate, sodium salt (ADP), *N*-2-hydroxyethyl-piperazine-*N*′-2-ethane sulfonic acid (HEPES), prostacyclin (PGI_2), tris (hydroxymethyl) aminomethane (Tris), glycine, ε-aminocaproic acid, diisopropylfluorophosphate (DFP), leupeptin and lactoperoxidase were from the Sigma Chemical Co. (St Louis, MO); aprotinin was from Choay (Paris, France), bovine thrombin from Hoffmann-La Roche, (Basel, Switzerland), agarose from Litex (Glastrup, Denmark), Coomassie blue-R-250 (CB-R), sodium dodecylsulfate (SDS), acrylamide, bisacrylamide and 2-mercaptoethanol from Bio-Rad (Richmond, CA), Triton X-100 from Merck (Darm-

* Part of this work was presented at the XIth International Congress on Thrombosis and Haemostasis, Brussels, Belgium, 6–10 July, 1987 [*Thromb. Haemost.* **58**, 211, (1987)].

‡ Address reprint requests to: Dr J.-P. Cazenave, Service d'Hémostase et de Thrombose, Centre Régional de Transfusion Sanguine, 10, Rue Spielmann, 67085 Strasbourg Cédex, France.

stadt, F.R.G.), Na[^{125}I] and [^{131}I]human albumin from the Commissariat à l'Energie Atomique (Saclay, France), 1,3,4,6-tetrachloro-3α, 6α,-diphenylglycoluril (Iodo-Gen®) from the Pierce Chemical Co. (Rockford, IL), protein A-Sepharose from Pharmacia (Uppsala, Sweden), monoclonal anti-Kappa chain of human IgM antibody from Immunotech (Marseille, France), IgG anti-mouse antibody from Tebu (Paris, France) and anti-mouse anti serum and polyclonal antibodies against rat and human platelets from the Centre Régional de Transfusion Sanguine (Strasbourg, France).

Highly purified human α-thrombin (3,000 NIH Units/mg), a generous gift of J.-M. Freyssinet, was purified from prothrombin as previously described [12]. The synthetic peptide D-phenyl-L-alanyl-L-propyl-L-arginine aldehyde sulfate (D-Phe-Pro-Arg-CHO) was provided by Pierre Fabre (Castres, France). Mouse monoclonal antibody AP-2 directed against the human platelet membrane glycoprotein (GP) IIb–IIIa complex was kindly provided by Dr T. J. Kunicki (Blood Center of Southern Wisconsin, Milwaukee, WI). Mouse monoclonal antibodies C-17 and 6C9 directed against epitopes located respectively on the GP IIIa and on the GP IIb–IIIa complex were kindly provided by Dr J. A. van Mourik (Central Laboratory of the Netherland Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Human volunteers. Ten healthy male volunteers who had not taken any medication for 2 weeks before participating in the study gave their informed consent. The study protocol was approved by the local Committee of Ethics. PCR 4099 (200 mg/day) was taken *per os* (two tablets in the morning, two tablets in the evening) for 8 days. All studies were performed 1 day before PCR 4099 administration and at day 8, 2 hr after the last intake.

Animals. Wistar female rats weighing 250–300 g were used in groups of three to five animals. PCR 4099 solubilized in ethanol and gum arabic was administered by gavage at the dose of 100 mg/kg, 1 hr before blood collection. Control rats received in a similar way ethanol and gum arabic.

Preparation of washed rat platelets. Rat blood (6 vol.) was collected from the aorta under ether anesthesia and anticoagulated with acid-citrate-dextrose (ACD, 1 vol.). Rat platelets were prepared as described for rabbit platelets [13]. Platelets were resuspended and stored at 37° in Tyrode's buffer (pH 7.3, 295 mOsm) containing 5 mmol/L HEPES, 2 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}, 0.35% (w/v) human albumin (Centre Régional de Transfusion Sanguine, Strasbourg, France) and apyrase (2 μg/mL, a concentration that converted 0.25 μM ATP to AMP within 2 min at 37°). Platelet count was measured with a Baker 810 platelet counter (Baker Instruments, Allentown, PA), and adjusted to 5×10^8 platelets/mL.

Preparation of washed human platelets. Blood was collected from a forearm vein. Six volumes of blood were collected into one volume of ACD anticoagulant. Twice washed platelet suspensions were prepared exactly as described [14]. The final resuspending medium (pH 7.35) was Tyrode's solution containing 0.35% human albumin, 5 mmol/L HEPES buffer and apyrase (2 μg/mL). Human platelet count was adjusted to 3×10^8/mL in the final suspension. The platelets were stored at 37° throughout the experiments.

Platelet aggregation studies. Platelet aggregation was measured at 37° using a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). A 0.45-mL aliquot of control or PCR 4099 treated rat platelet suspension was stirred at 1100 rpm and activated by the addition of ADP or bovine thrombin without addition of fibrinogen [15]. The conditions were identical for human platelets except that DFP-treated fibrinogen (0.8 mg/mL) [14] was added before addition of ADP.

Fibrinogen purification. Rat fibrinogen was purified by successive ammonium sulfate precipitations according to Bouma and Fuller [16] from fresh rat blood collected into ACD containing ε-aminocaproic acid (2.5 g/L) and aprotinin (1.8×10^3 Units/mL). The final product was dialysed against modified Tyrode's buffer containing no Ca^{2+} and no Mg^{2+}, pH 7.3, and stored at −80° until use. Human fibrinogen was purified by successive ether precipitations according to Kekwick *et al.* [17] from fresh human blood collected into ACD containing ε-aminocaproic acid (2.5 g/L) and aprotinin (1.8×10^3 Units/mL). The final product was dialysed against modified Tyrode's buffer. Both rat and human fibrinogen preparations were at least 96% pure and contained intact Aα, Bβ and γ chains, as demonstrated after SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing and reducing conditions. Rat fibrinogen was 100% precipitable in 10% (w/v) trichloroacetic acid and 95% clottable with thrombin. Human fibrinogen was 100% precipitable and 97% clottable. Human and rat fibrinogen were labeled with ^{125}I by the Iodo-Gen method [18]. The specific radioactivity ranged from 75,000 to 200,000 cpm/μg. The mobilities of rat and human [^{125}I]fibrinogen were identical to those of unlabeled fibrinogen as determined by SDS–PAGE and autoradiography. Human and rat [^{125}I]fibrinogen was, respectively, 92 and 96% clottable by thrombin.

Fibrinogen binding assay. The binding of purified [^{125}I]fibrinogen to ADP and thrombin stimulated platelets was measured according to a modification of the procedure described by Harfenist *et al.* [19]. ADP (5 μM) or thrombin (0.2 Units/mL bovine thrombin for rat platelets or 0.5 Units/mL human α-thrombin for human platelets) was added to 0.5 mL control or treated platelets in suspending buffer (37°) contained in a 1.2 mL polypropylene Eppendorf tube. Binding was initiated at the required time (from 0 to 3 min) by the addition of a mixture of purified fibrinogen (0.1 mg/mL), where labeled fibrinogen represented 2 to 5% of the total fibrinogen and about 200,000 cpm per tube, the synthetic peptide D-Phe-Pro-Arg-CHO (100 μmol/L) and traces amounts of ^{131}I-labeled human albumin. D-Phe-Pro-Arg-CHO was used to inhibit completely and specifically the clotting activity of thrombin [20]. [^{131}I]Albumin was used as a marker of extracellular fluid. The binding reaction was stopped by centrifugation for 1 min at 8000 *g* in an Eppendorf 5414 microcentrifuge (Roucaire, Paris, France). The

Table 1. Effect of PCR 4099 (100 mg/kg) on washed rat platelet aggregation

Agonist		Aggregation (% light transmission) Control rats	PCR 4099 treated rats
ADP (μM)	0.5	1 ± 1	0*
	1	21 ± 4	0*
	5	48 ± 2	0*
	10	56 ± 2	0*
	100	62 ± 2	0*
Thrombin (Units/mL)	0.05	55 ± 3	8 ± 6*
	0.5	70 ± 3	56 ± 4 NS

Platelets from control and PCR 4099 (100 mg/kg) treated rats, suspended in Tyrode's albumin buffer (5×10^8/mL), were activated by addition of ADP or thrombin at various concentrations. Results are expressed as mean values ± SE from three experiments where each measure was a duplicate. Experiments were performed with groups of three to five rats.

* $P < 0.05$ using a two-tailed Student's *t*-test.

NS, not significant at the 5% level.

supernatant was immediately and carefully aspirated, the tip of the tube was cut off and the radioactivity in the platelet pellet was counted in a 1282 Compugamma counter (LKB, Turku, Finland). Non specific binding of [^{125}I]fibrinogen was defined as that measured without stimulation with ADP or thrombin and was comparable to that measured in the presence of a 50-fold excess of unlabeled fibrinogen.

Platelet surface labeling. Platelets surface proteins were labeled with Na[^{125}I] using the lactoperoxidase method [21]. The labeled platelets were sedimented and washed twice in washing buffer containing albumin, apyrase and PGI_2. Finally, platelets were centrifuged and solubilized with 1% Triton X-100.

Crossed immunoelectrophoresis. Platelets were sedimented and washed in Tyrode's buffer to remove excess albumin. They were centrifuged and the pellet resuspended in 0.1 mmol/L glycine, 0.038 mol/L Tris, pH 8.7 (Tris–glycine), prechilled to 4°. Platelet solubilization was performed by incubation for 30 min at 4° with 1% (v/v) Triton X-100. Triton insoluble material was removed at 100,000 *g* for 10 min by ultracentrifugation in an Air-Fuge ultracentrifuge (Beckman, Palo Alto, CA) and the supernatant stored at −80° until use. Crossed immunoelectrophoresis of platelet extracts was carried out as described by Kunicki *et al.* [22]. Immunoprecipitates were located by CB-R250 staining and by autoradiography (Kodak X-Omat film) when [^{125}I] platelets were used.

SDS–PAGE. Human platelets and/or rat platelets were solubilized in a 2% SDS containing buffer. Polyacrylamide gel electrophoresis in the presence of SDS was carried out by the discontinuous buffer method of Laemmli [23] on a 7–12% linear gradient polyacrylamide slab gel. The gel was stained with CB-R250. Glycoproteins were also located by periodic acid Schiff (PAS) staining of 7.5% acrylamide rod gels [24].

Immunoprecipitation. The monoclonal antibodies AP-2, 6C9 and C17 were used for immunoprecipitation of the GP IIb–IIIa complex of human platelets. Surface labeled platelets were solubilized (5×10^8/mL) in PBS containing 1% Triton X-100, DFP (0.5 mM), leupeptin (1 mM) and aprotinin (20 Units/mL). The solubilized platelets were stirred at 4° for 30 min and then centrifuged at 100,000 *g* in an Air-Fuge ultracentrifuge and the supernatant was precleared by successive incubations 30 min at 4° with mouse serum, an irrelevant monoclonal antibody directed against the Kappa chain of IgM and protein A-Sepharose coupled to an antimouse antibody. After incubation, this mixture was centrifuged 5 min at 12,000 *g* in an Eppendorf 5415 microcentrifuge. The supernatant was then incubated 2 hr at 4° with the monoclonal antibody AP-2, 6C9 or C17. Then, protein A-Sepharose coupled to an antimouse antibody was added for 90 min. After centrifugation, the pellet was washed three times in PBS containing 1% Triton X-100 and then suspended in the SDS–PAGE lysis buffer. Samples were heated to 100° for 10 min and immediately electrophoresed or stored at −80° until use. Immunoprecipitates were revealed by autoradiography.

RESULTS

Effect of PCR 4099 on the bleeding time in man

The bleeding time was measured in 10 volunteers, using a Simplate device with a constant arm pressure of 40 mmHg. It was between 4 and 8 min in each case before oral administration of PCR 4099. At day 8, it was prolonged in all cases, above 30 min in four cases, above 15 min in five cases and in one case poorly prolonged from 6 to 9 min.

Effect of PCR 4099 on platelet aggregation in rat and in man

PCR 4099 treatment (100 mg/kg, 1 hr before blood collection) resulted in complete inhibition of washed rat platelet aggregation induced by ADP, whatever the concentration of ADP used (0.5 to 100 μM, Table 1). In contrast, when washed rat platelets were stimulated with thrombin, significant inhibition occurred at low concentrations of thrombin (0.05 Units/mL) but not at high concentrations (0.5 Units/mL).

ADP-induced aggregation of washed human platelets after oral administration of PCR 4099 (200 mg/

Table 2. Effect of PCR 4099 (200 mg/day for 8 days) on washed human platelet aggregation

Agonist		Aggregation (% light transmission) Before treatment	After treatment
ADP (μM)	0.5	3 ± 2	0 ± 0*
	1	4 ± 2	0 ± 0.2*
	5	30 ± 2	5 ± 1*
	10	36 ± 2	7 ± 2*
Thrombin (Units/mL)	0.05	58 ± 2	43 ± 7 NS
	0.1	67 ± 2	61 ± 1 NS
	0.5	58 ± 2	58 ± 2 NS

Washed human platelets from 10 healthy human volunteers before and after treatment with PCR 4099 (200 mg/day for 8 days), suspended in Tyrode's albumin buffer (3×10^8/mL), were incubated with fibrinogen (0.08%) and various concentrations of ADP, or with thrombin without fibrinogen. Results are expressed as mean values ± SE from the 10 experiments.

* $P < 0.05$ using a two-tailed Student's t-test.

NS, not significant at the 5% level.

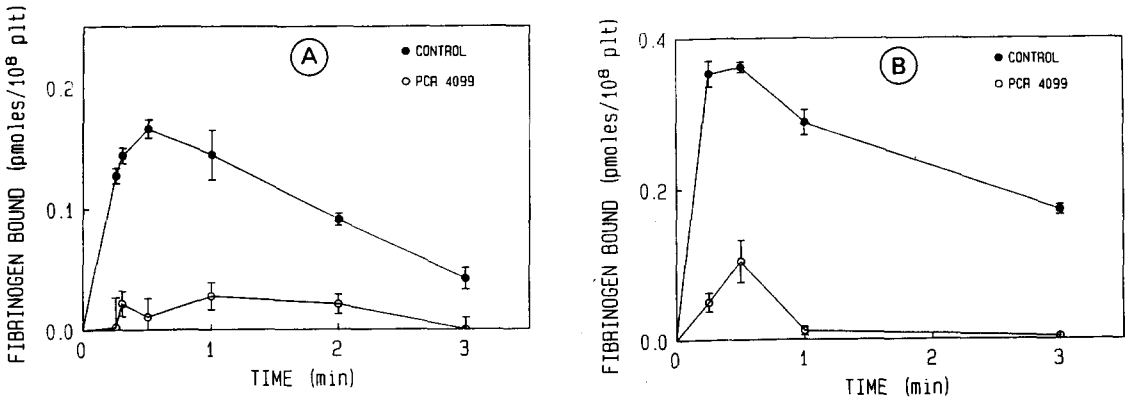

Fig. 1. Binding of rat [^{125}I]fibrinogen to intact washed rat platelets. In (A) platelets are activated by 5 μM ADP. (●) Control rats, (○) PCR 4099 treated rats (100 mg/kg, 1 hr before blood collection). This represents the specific binding i.e. the total binding to ADP stimulated platelets minus the binding to non-stimulated platelets. (B) Specific rat [^{125}I]fibrinogen binding to intact washed rat platelets stimulated with 0.2 Units/mL thrombin. (●) Control rats, (○) PCR 4099 treated rats (100 mg/kg, 1 hr before blood collection). Results are expressed as mean values ± SE from three separate experiments performed in triplicate.

day, 8 days) was also strongly inhibited, about 80%, at the highest concentration of ADP tested (10 μM). Thrombin induced aggregation was not significantly modified after PCR 4099 treatment (Table 2) even at lower thrombin concentrations (0.05 Units/mL). It is to be noted that in man and even in rat, where ADP-induced aggregation was fully abolished, shape change was never inhibited even at low concentrations of ADP (0.5 μM).

Effect of PCR 4099 on fibrinogen binding to activated rat and human platelets

Binding of purified rat [^{125}I]fibrinogen to intact washed rat platelets stimulated by 5 μM ADP was maximum (about 0.17 picomol/10^8 platelets) in less than 1 min, and then decreased. Fibrinogen binding to non-stimulated platelets was considered as non specific. Figure 1A represents the specific binding, i.e., the total binding to ADP stimulated platelets minus the binding to non-stimulated platelets. The binding of rat fibrinogen to intact washed platelets from rats treated by PCR 4099 was dramatically reduced (maximum was 0.025 picomol/10^8 platelets). Similarly, when rat platelets were stimulated with 0.2 Units/mL thrombin, a concentration which induced aggregation (60% increase in light transmission), a marked decrease in the amount of fibrinogen bound was observed (Fig. 1B).

Similar experiments were performed in man before and after PCR 4099 treatment. Binding of human [^{125}I]fibrinogen to ADP stimulated platelets was inhibited by 60% at maximum binding. This paralleled inhibition of human platelet aggregation induced by ADP (Fig. 2A). At low thrombin concentrations (0.05 Units/mL), where fibrinogen binding was low in control platelets, 50% inhibition was

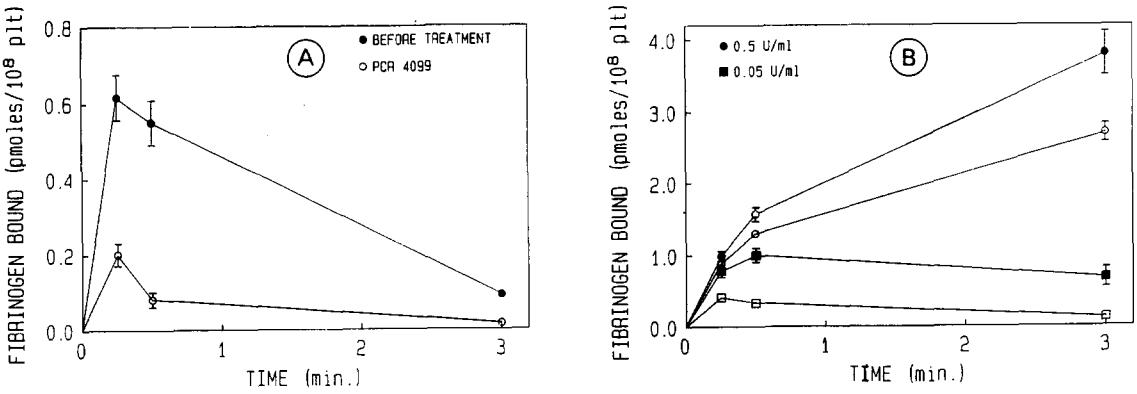

Fig. 2. Specific human [^{125}I]fibrinogen binding to intact washed human platelets. (A) Platelets are stimulated with 5 μM ADP, (●) before treatment with PCR 4099 (200 mg/day), (○) after 8 days treatment. (B) Platelets are stimulated with 0.05 Units/mL thrombin (■) or with 0.5 Units/mL thrombin (●), before (filled symbols) and after (open symbols) PCR 4099 treatment (200 mg/day for 8 days). Results are expressed as mean values ± SE from 10 experiments performed in triplicate.

observed. However, at high concentrations of thrombin (0.5 Units/mL), despite a slight decrease of binding at 3 min, no significant inhibition was measured. Again this is in agreement with the aggregation data (Fig. 2B).

SDS–PAGE. Rat platelet lysates were submitted to SDS–PAGE on 7.5% acrylamide rod gels under reducing and non-reducing conditions. The electrophoretic mobilities of the major platelet glycoproteins stained by PAS were unchanged after PCR 4099 treatment. Human platelets were submitted to SDS–PAGE on a 7–12% linear gradient polyacrylamide slab gel. After electrophoresis the gels were stained with CB-R250. Again no modification of the protein profile was seen in any of the 10 cases studied (data not shown).

Crossed immunoelectrophoresis. Rat or human washed platelets prepared for aggregation and fibrinogen binding studies were labeled with ^{125}I using the lactoperoxydase technique, solubilized in Triton X-100 and analysed by crossed immunoelectrophoresis against rabbit antiserum to whole platelets proteins (Fig. 3). PCR 4099 did not modify the pattern of immunoprecipitates. In particular, the GP IIb–IIIa complex was not dissociated and its electrophoretic mobility was not changed. The size of the immunoprecipitates either on the Coomassie blue stained gels (Fig. 3, upper panel) or after autoradiography (Fig. 3, bottom panel) was the same before and after PCR 4099 treatment. This was the case in experiments with rat platelets and with the 10 cases of human platelets. In two cases non-labeled human platelets were solubilized in Triton X-100 and analysed by crossed immunoelectrophoresis against the rabbit antiserum depleted in anti-fibrinogen antibodies. [^{125}I]Fibrinogen was incorporated in the intermediate gel of the C.I.E. (Fig. 4). After autoradiography, [^{125}I]fibrinogen revealed the GP IIb–IIIa immunoprecipitate. There was no difference before and after PCR 4099 treatment.

A similar experiment was performed with rat platelets except [^{125}I]fibrinogen was mixed with the platelet lysate and the rabbit antiserum was not depleted in antifibrinogen antibodies (Fig. 5). The autoradiography revealed that [^{125}I]fibrinogen migrated as the native fibrinogen and that an immunoprecipitate not corresponding to the rat GP IIb–IIIa was labeled. We think that this arc may correspond to fibrinogen associated with the GP IIb–IIIa complex. Again, no difference was detectable in control and PCR 4099 treated rats.

Immunoprecipitations. In order to further examine the hypothesis of a structural modification of the GP IIb–IIIa complex, immunoprecipitations were performed with several different monoclonal antibodies raised against GP IIb–IIIa. ^{125}I-Labeled human platelets solubilized with Triton X-100 were precleared by incubation with murine serum, irrelevant monoclonal antibody and antimouse IgG linked to protein A-Sepharose. After centrifugation the supernatant was incubated with a GP IIb–IIIa complex specific monoclonal antibody (AP-2, C17 or 6C9) and with anti-mouse IgG linked to protein A-Sepharose. The immunoprecipitates were applied to an SDS–polyacrylamide gel under reducing conditions. Figure 6 is the autoradiogram of such immunoprecipitations with AP-2, 6C9 and C17. No difference was detectable before and after treatment with PCR 4099.

DISCUSSION

This study was performed in order to check the possibility that the so called "functional thrombasthenic state" of platelets induced by ticlopidine [10] is due to a modification of the GP IIb–IIIa complex that can explain the inhibition of aggregation. As expected from general knowledge [1–3], oral administration of PCR 4099, the thienopyridine analog of ticlopidine, to rats led to complete inhibition of aggregation induced by ADP whatever the concentration used up to 100 μM. In man, where the dose was 200 mg/day for 8 days, inhibition of aggregation induced by ADP was important if not complete. This *ex vivo* effect of PCR 4099 was irreversible as it is for ticlopidine since washed platelets

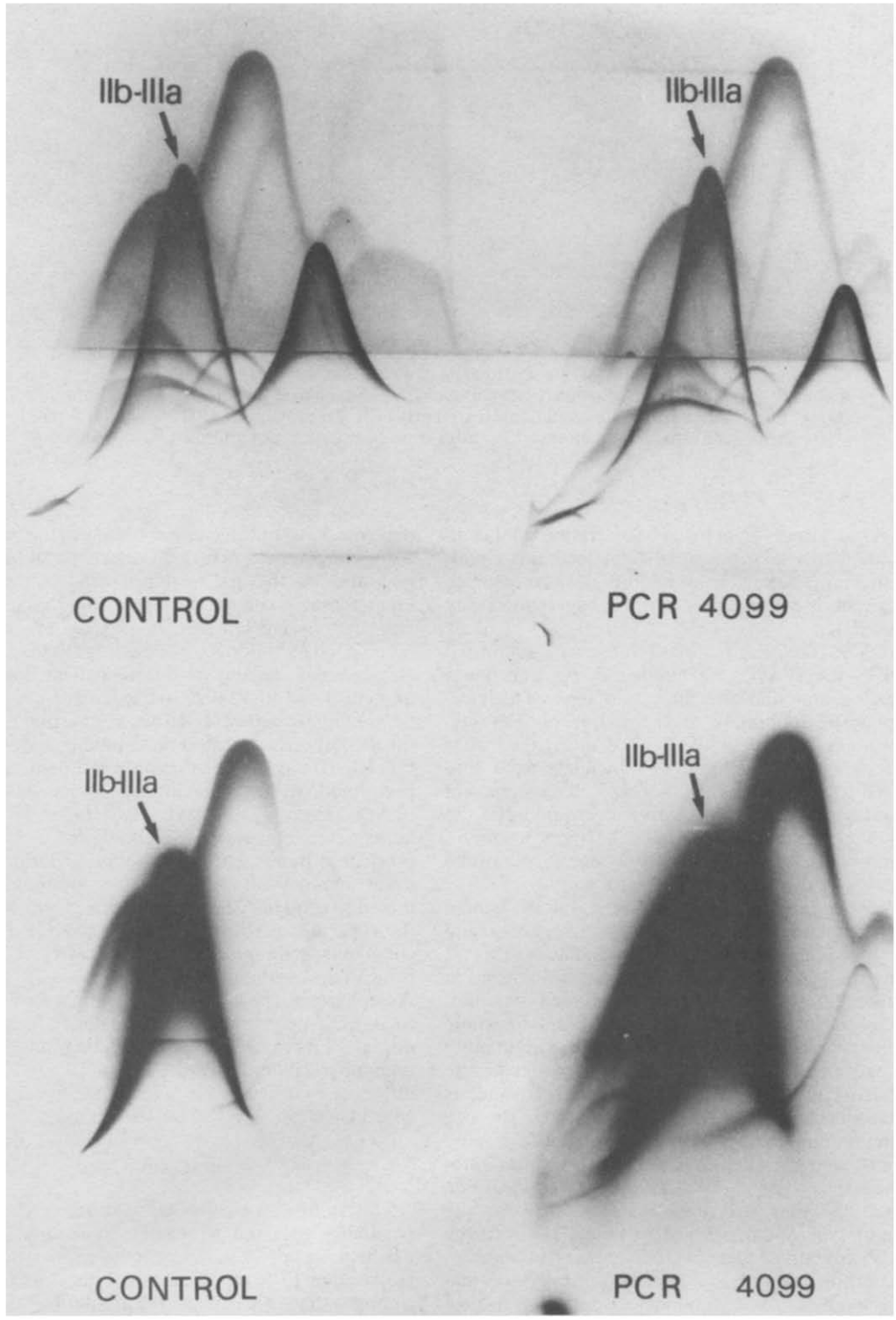

Fig. 3. C.I.E. of human platelet lysates. [^{125}I]membrane labeled platelets were solubilized with 1% Triton X-100 and submitted to crossed immunoelectrophoresis against rabbit antiserum to whole human platelet proteins. The upper panel is the Coomassie blue stained dry gel. The bottom panel is the autoradiography of the gel. The GP IIb–IIIa immunoprecipitate is not modified after treatment with PCR 4099. This figure is representative of the 10 cases studied.

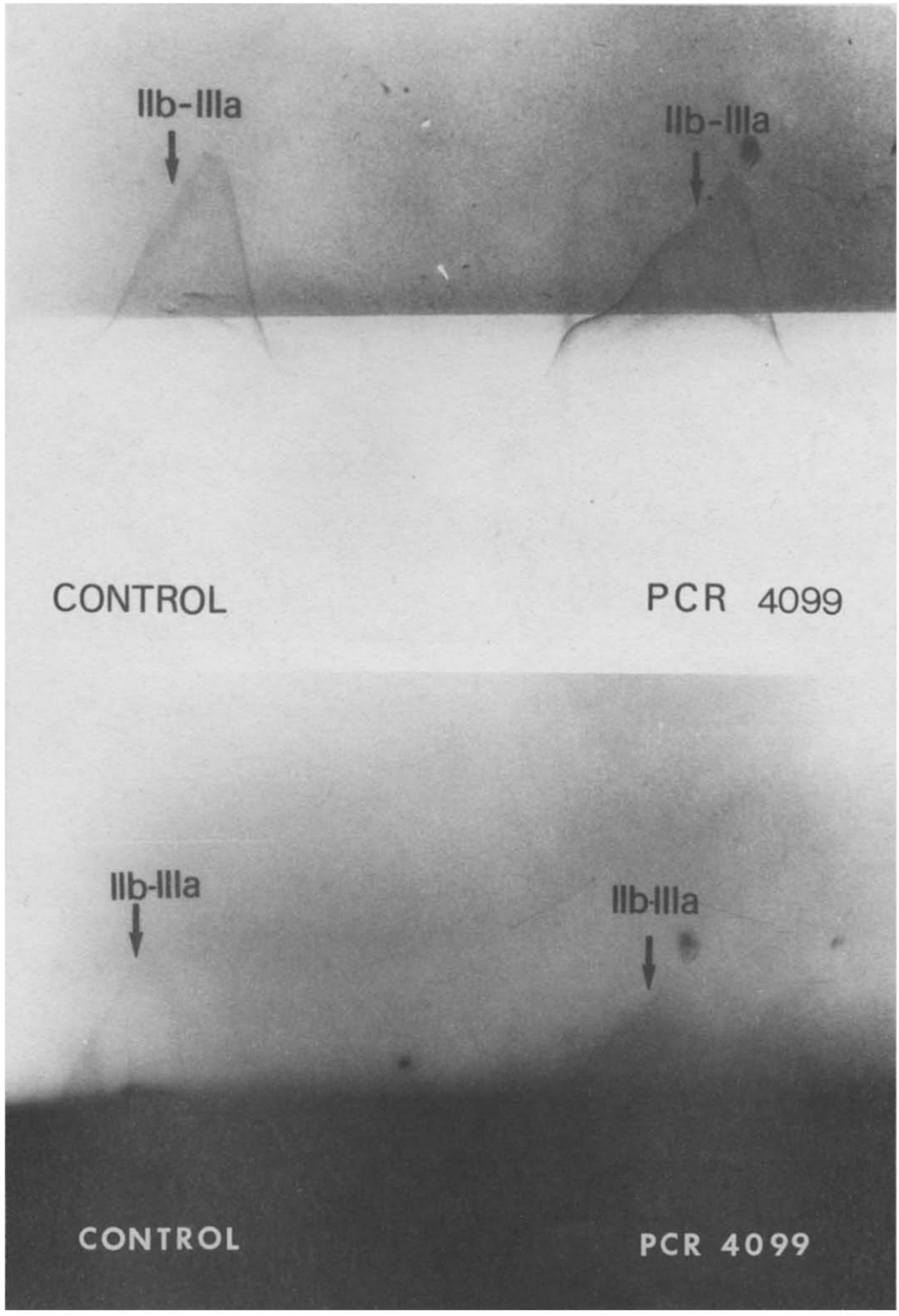

Fig. 4. C.I.E. of human platelet lysates. In this experiment, non labeled human platelet lysates were analysed by C.I.E. Human [^{125}I]fibrinogen was added to the intermediate gel of the C.I.E. The rabbit antiserum was depleted in antibodies directed against fibrinogen to avoid the immunoprecipitation of fibrinogen which recognized and bound to the GP IIb–IIIa. Again, no difference is detectable after PCR 4099 treatment. This figure is typical of two cases studied. The upper panel is a Coomassie blue stained gel and the bottom panel the corresponding autoradiogram.

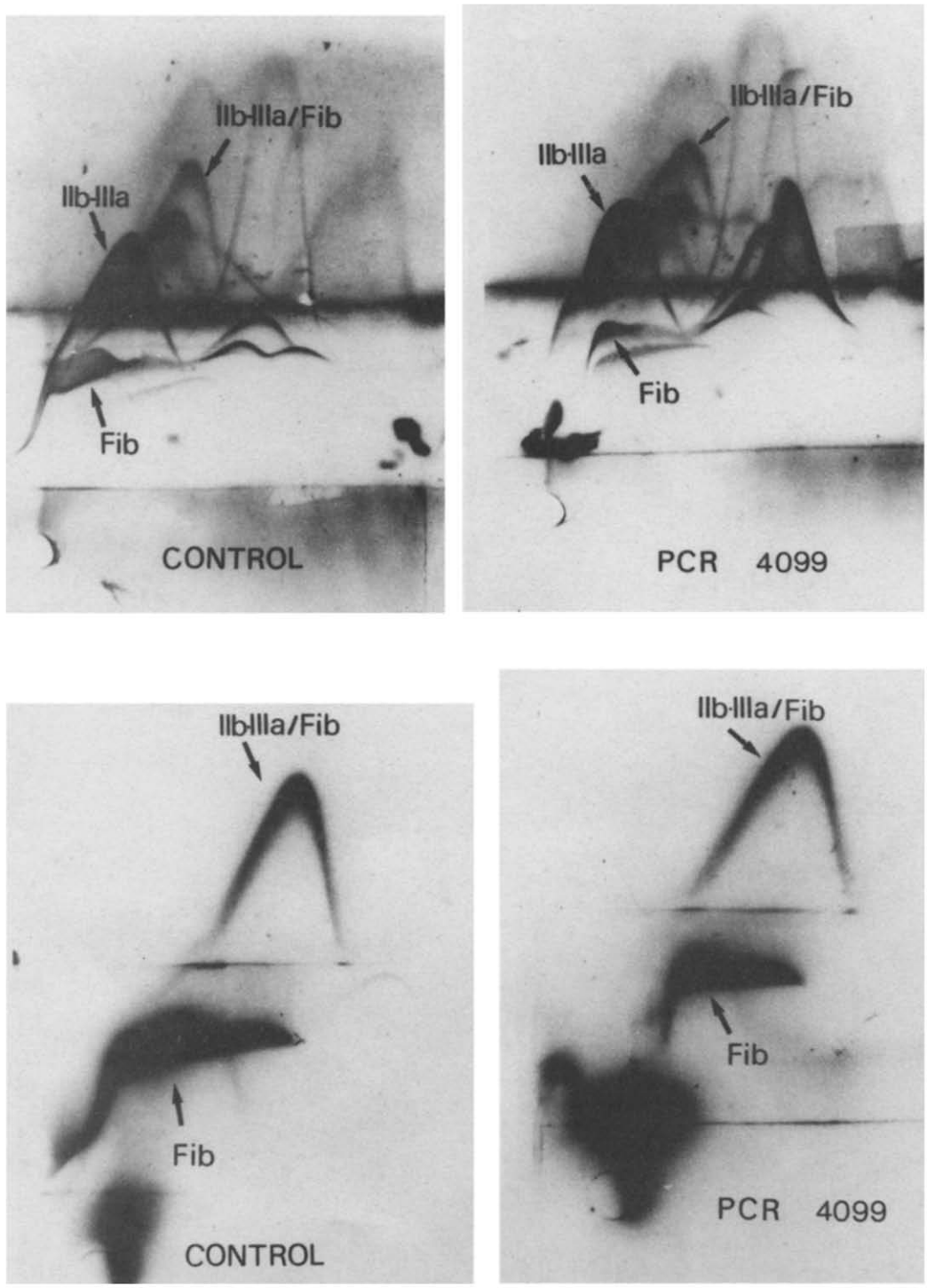

Fig. 5. C.I.E. of rat platelet lysates. Rat platelet lysates from control and PCR 4099 treated rats were analysed by crossed immunoelectrophoresis against rabbit antiserum to whole rat platelet proteins. Rat [^{125}I]fibrinogen was added to the platelet lysate. The upper panel is the Coomassie blue stained dry gel. As can be seen, the rat GP IIb–IIIa complex is not modified after treatment. The bottom panel is the autoradiogram of the upper gel. The rat [^{125}I]fibrinogen has migrated to the fibrinogen place and has also labeled an immunoprecipitate which probably corresponds to the bound rat GP IIb–IIIa and fibrinogen, migrating differently from free GP IIb–IIIa. There is no difference after PCR 4099 treatment.

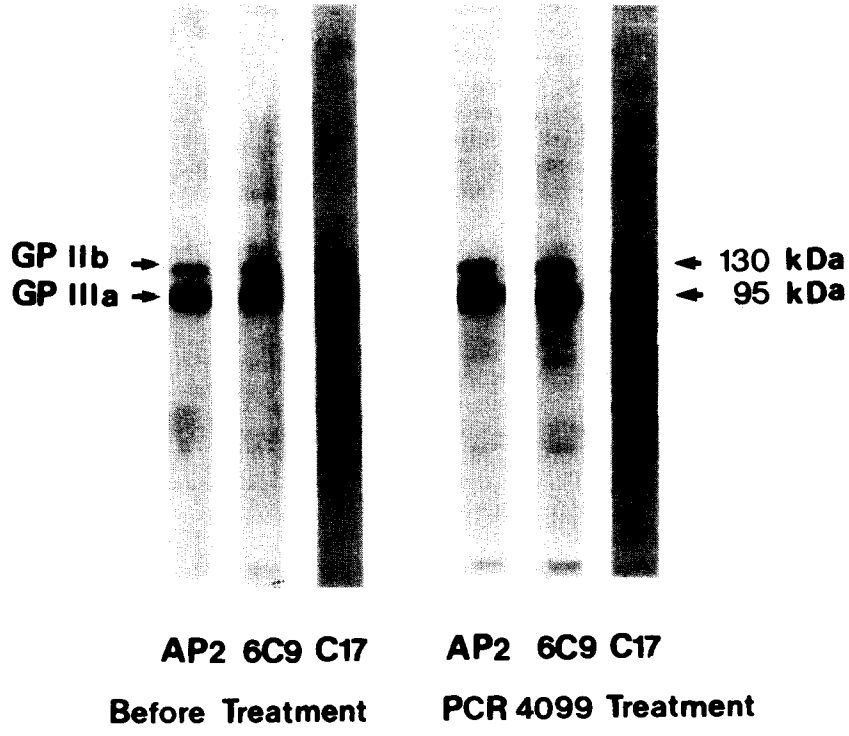

Fig. 6. Immunoprecipitation of the GP IIb–IIIa from human platelets using three different monoclonal anti-GP IIb–IIIa complex antibodies, before and after PCR 4099 treatment. This view is representative of the 10 human volunteers studied.

resuspended in a physiological medium are unable to recover their ability to respond to ADP. The effect on ADP stimulation was selective: thrombin stimulation was inhibited at low concentrations of thrombin, where secreted ADP is essential for aggregation [25]. At higher concentrations of thrombin, despite a slight decrease in light transmission, inhibition was not significant. These data are consistent with other studies [4] where ADP scavengers were used that mimicked the effects of thienopyridine. In addition, arachidonate (0.25 mM) induced aggregation of washed human platelets was not inhibited after oral administration of ticlopidine 500 mg daily for a week (unpublished data). The fact that even in rat where the PCR 4099 dose was high, with very low concentrations of ADP, the shape change was never inhibited, led us to consider that PCR 4099 acts at a later stage of the activation process than the binding of ADP to its receptor. Although we have not measured the binding of ADP to its putative receptor(s), the absence of inhibition of shape change induced by a low concentration of ADP (0.5 μM) is not in favor of a modification of the high affinity ADP receptor. This is in agreement with previous work [26] showing that ticlopidine does not inhibit the binding of ADP at low concentration (1 μM) to the high affinity ADP receptor. Although ticlopidine was able to inhibit ADP binding to the low affinity receptor, its relevance to platelet aggregation is probably of little value [26] except if high ADP concentrations are locally involved in thrombogenesis. We thus measured the binding of purified fibrinogen to intact platelets stimulated with ADP or with thrombin. The same platelet suspensions were used for the aggregation and binding studies. Under our conditions, binding of fibrinogen to platelets stimulated with 5 μM ADP was markedly reduced if not fully abolished. This correlates well with the complete inhibition of ADP induced aggregation. However, when rat platelets were stimulated with 0.2 Units/mL thrombin, a concentration which induced aggregation, the binding of rat fibrinogen was decreased. In man, where the dose of PCR 4099 was lower than in rat, we observed the same correlation between the inhibition of ADP induced aggregation and that of fibrinogen binding. Again, when platelets were stimulated with thrombin, we observed a discrepancy between aggregation and fibrinogen binding. At 0.05 Units/mL thrombin, where aggregation was not significantly reduced, the binding of fibrinogen was inhibited. At higher concentrations, where aggregation was not different before and after treatment, the binding of fibrinogen was still slightly reduced. At this concentration of thrombin, the small inhibition of fibrinogen binding detected is obviously not sufficient to impair aggregation. But these fibrinogen binding data, compared to the aggregation data, are consistent with the hypothesis of a covalent modification of the fibrinogen receptor or of a certain number of the fibrinogen binding sites involved in the ADP-induced aggregation pathway. In order to verify this idea we studied the GP IIb–IIIa complex by electrophoretic and immunoelectrophoretic methods, both in rat and in

man. SDS–PAGE showed no difference before and after treatment. Crossed immunoelectrophoresis using rabbit polyclonal antibodies directed against all platelet proteins was not different before and after treatment. In particular, the GP IIb–IIIa complex was not dissociated, its electrophoretic mobility was not changed and the size of its immunoprecipitate was the same before and after PCR 4099 treatment. This was examined in the 10 men studied and in rats. Moreover, when [^{125}I]fibrinogen was incorporated in the intermediate gel of a crossed immunoelectrophoresis or mixed with the platelet lysate, it bound to the GP IIb–IIIa arc, indicating that this interaction could still occur. Since we found that anti-human GP IIb–IIIa complex monoclonal antibodies did not cross-react with the rat GP IIb–IIIa complex, we could not perform immunoprecipitations with rat platelets. However, we submitted human platelets to immunoprecipitation with three different anti-GP IIb–IIIa complex monoclonal antibodies. AP2, 6C9 and C17 recognized the glycoprotein complex in the same manner before and after PCR 4099 treatment. We conclude from this information and from other authors [27], who have reported that ticlopidine does not inhibit the binding of monoclonal antibodies to the GP IIb–IIIa complex in flow cytofluorimetry, that the mechanism of inhibition of fibrinogen binding does not reside at the GP IIb–IIIa level itself but more probably at a stage just before the conformational change of the GP IIb–IIIa, on an ADP specific pathway unknown at this time.

In summary, several conclusions can be drawn: (i) The thienopyridines ticlopidine and PCR 4099 are specific inhibitors of the ADP aggregation pathway. (ii) Inhibition of fibrinogen binding correlates very well with inhibition of aggregation when platelets are stimulated with ADP or low concentrations of thrombin. At higher concentrations of thrombin there still remains a slight inhibition of binding suggesting that a proportion of binding sites are not available. (iii) The inhibition of fibrinogen binding is not due to a direct modification of the GP IIb–IIIa complex. One point is not completely elucidated, i.e., whether ticlopidine and its analogues interfere with the binding of ADP to its receptor(s) and if not, at what stage of the aggregation pathway do they act. Some authors have established that intracellular Ca^{2+} mobilization induced by ADP, but not Ca^{2+} influx, is inhibited by PCR 4099 in rat [28] and by ticlopidine in rabbit [29]. The significance of these data remain to be clarified and further investigations should lead us to a better understanding of the mechanism of action of these drugs. It has been shown that the antiaggregating activity of PCR 4099 is due to its dextrogyre enantiomer (SR25990C) whereas SR25989C, the levogyre enantiomer is inactive [30]. It is reasonable to assume that the effects observed for PCR 4099 are due to SR25990C.

Acknowledgements—The authors wish to thank Mrs M.-O. Bernhard for typing the manuscript, Mrs J. Mulvihill for reviewing the English and Mrs A. Sutter-Bay, J. Eberhardt and J. Launay for their skilful technical assistance. Christian Gachet was supported by a predoctoral grant from the Ministère de la Recherche et de la Technologie. This work was also supported by a grant from Sanofi, France.

REFERENCES

1. E. Panak, J. P. Maffrand, C. Picard-Fraire, E. Vallée, J. Blanchard and R. Roncucci, *Haemostasis* **13** (Suppl. 1), (1983).
2. J. P. Maffrand, E. Vallée, A. Bernat, D. Delebassée, E. Millou, A. Tissinier and R. Roncucci, *Thromb. Haemost.* **54**, 133 (1985).
3. E. Saltiel and A. Ward, *Drugs* **34**, 222 (1987).
4. R. Feliste, D. Delebassée, M. F. Simon, H. Chap, G. Defreyn, E. Vallée, L. Douste-Blazy and J. P. Maffrand, *Thromb. Res.* **48**, 403 (1987).
5. G. V. R. Born, *J. Physiol. (Lond)* **209**, 487 (1970).
6. J. F. Mustard, M. A. Packham, R. L. Kinlough-Rathbone, D. W. Perry and E. Regoeczi, *Blood* **52**, 453 (1978).
7. J. S. Bennett and G. Vilaire, *J. clin. Invest.* **64**, 1393 (1979).
8. G. A. Marguerie, T. S. Edgington and E. F. Plow, *J. biol. Chem.* **255**, 154 (1980).
9. H. Lee, R. C. Paton and J. P. Caen, *Thromb. Haemost.* **46**, 590 (1981).
10. F. W. Dunn, J. Soria, C. Soria, A. Thomaidis, H. Lee and J. P. Caen, *Agents Actions* **15**, 97 (1984).
11. G. Di Minno, A. M. Cerbone, P. L. Mattioli, S. Turco, C. Lovine and M. Mancini, *J. clin. Invest.* **75**, 328 (1985).
12. J. M. Freyssinet, J. Gauchy and J. P. Cazenave, *Biochem. J.* **238**, 151 (1986).
13. N. G. Ardlie, M. A. Packham and J. F. Mustard, *Br. J. Haematol.* **19**, 7 (1970).
14. J. P. Cazenave, S. Hemmendinger, A. Beretz, A. Sutter-Bay and J. Launay, *Ann. Biol. Clin.* **41**, 167 (1983).
15. E. J. Harfenist, M. A. Packham and J. F. Mustard, *Thromb. Haemost.* **59**, 319 (1988).
16. H. Bouma and G. M. Fuller, *J. biol. Chem.* **250**, 4678 (1975).
17. R. A. Kekwick, M. E. MacKay, M. H. Nance and B. R. Record, *Biochem. J.* **60**, 671 (1955).
18. E. Regoeczi, *Iodine-labeled Plasma Proteins*, Vol. 1, p. 49. CRC Press, Boca Raton, FL. (1984).
19. E. J. Harfenist, M. A. Packham, R. L. Kinlough-Rathbone and J. F. Mustard, *J. Lab. clin. Med.* **97**, 680 (1981).
20. F. Markwardt, *Ann. N.Y. Acad. Sci.* **370**, 757 (1981).
21. D. R. Phillips and P. P. Agin, *J. biol. Chem.* **252**, 2121 (1977).
22. T. J. Kunicki, A. T. Nurden, D. Pidard, N. R. Russell and J. P. Caen, *Blood* **58**, 1190 (1981).
23. U. K. Laemmli, *Nature* **227**, 680 (1970).
24. R. M. Zacharius, T. E. Zell, J. H. Morrison and J. J. Woodlock, *Anal. Biochem.* **30**, 148 (1969).
25. G. Marguerie and E. F. Plow, *Ann. N.Y. Acad. Sci.* **408**, 556 (1983).
26. J. P. M. Lips, J. J. Sixma and M. E. Schiphorst, *Thromb. Res.* **17**, 19 (1980).
27. M. J. Powling, TJ. C. Nokes and R. M. Hardisty, *Xth International Congress on Thrombosis. The Mediterranean League against Thrombo-Embolic Diseases*, May 22 (1988) (Abstract).
28. R. Feliste, M. F. Simon, H. Chap, L. Douste-Blazy, G. Defreyn and J. P. Maffrand, *Biochem. Pharmac.* **37**, 2559 (1988).
29. C. K. Derian and P. A. Friedman, *Thromb. Res.* **50**, 65 (1988).
30. D. Delebassée, A. Bernat, D. Frehel, G. Defreyn and J. P. Maffrand, *Xth International Congress on Thrombosis. The Mediterranean League against Thrombo-Embolic Diseases*, May 22 (1988) (Abstract).