Journal of Thrombosis and Thrombolysis 2000;9:23–28

Role of Extracellular Ionized Calcium in the In Vitro Assessment of GPIIb/IIIa Receptor Antagonists

Sam S. Rebello, Jinbao Huang, Jessica D. Faul, and Benedict R. Lucchesi

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan, USA

Abstract. **Several preclinical studies have found a poor correlation between the ex vivo platelet inhibitory potency and the in vivo antithrombotic efficacy of GPIIb/IIIa receptor antagonists. The present study was designed to examine the differential in vitro potencies of c7E3, MK-383, DMP-728, and SM-20302 in inhibiting ex vivo platelet aggregation under normocalcemic and hypocalcemic conditions. Human blood was collected in either trisodium citrate (0.37%) or PPACK (20 μg/mL). Platelet aggregation assays were performed in platelet-rich plasma from citrate-anticoagulated blood (cPRP) and PPACK-anticoagulated blood (pPRP) using ADP (20 μM) and TRAP (10 μM) as agonists in the presence of c7E3, MK-383, DMP-728, or SM-20302. The concentrations of ionized calcium in cPRP was 16–19 times lower than that in pPRP. The IC_{50} of c7E3 for inhibiting ADP-induced platelet aggregation in cPRP (2.76 ± 0.11 μg/mL) was 1.6 times lower than that in pPRP (4.46 ± 0.48 μ/mL; $P < 0.05$). Similarly, the IC_{50} for c7E3 for inhibiting TRAP-induced platelet aggregation in cPRP (4.52 ± 0.34 μg/mL) was 1.7 times lower than that in pPRP (7.69 ± 0.43 μg/mL; $P < 0.05$). MK-383, DMP-728, and SM-20302 also demonstrated 1.96-, 1.15-, and 1.43-fold lower IC_{50} values, respectively, in cPRP as compared with pPRP. Chelation of ionized calcium in pPRP led to a progressive increase in platelet inhibition by all the antagonists. These results suggest that the observed in vitro inhibitory potency of a GPIIb/IIIa receptor antagonist is markedly enhanced when trisodium citrate is used as an anticoagulant to collect blood for ex vivo assay. These findings indicate that dosing regiments for GPIIb/IIIa receptor antagonists based on the platelet inhibition profile in citrate may provide misleading information with respect to their true in vivo antithrombotic efficacy.**

Key Words. **GPIIb/IIIa, trisodium citrate, PPACK (Phe-Pro-Arg chloromethyl ketone), Platelet aggregation**

Preclinical and clinical evaluations of GPIIb/IIIa receptor antagonists rely heavily on the relationship between *ex vivo* platelet inhibition assays, and *in vivo* antithrombotic efficacy. The *ex vivo* platelet aggregation assay utilizes trisodium citrate as the conventional anticoagulant for the collection of whole blood to be used for the preparation of platelet-rich plasma (PRP). Unfortunately, the reduction or removal of the ionized calcium concentration from the PRP by trisodium citrate induces a morphological change in the platelets [1] and influences the stability of the GPIIb/IIIa complex [2–4] and the divalent cation-dependent binding of RGD ligands to the GPIIb/IIIa receptor [5,6]. Under such circumstances, the *ex vivo* platelet aggreagation studies may falsely reveal a greater antiplatelet potency of a particular antagonist of the glycoprotein receptor.

It is important to note that both intracellular and extracellular calcium play an important role in platelet aggregation. Intracellular calcium influences the platelet shape change, exposure of GPIIb/IIIa receptors, and fibrinogen binding [7–9]. On the other hand, control of the extracellular ionized calcium concentration, within the physiologic range (1 m/M), is essential for maintaining the GPIIb/IIIa receptor complex in a conformation capable of interacting with soluble fibrinogen on exposure of the platelet to an agonist. Complexing extracellular ionized calcium ions to the four repetitive domains (resembling calmodulin or troponin C) on the GPIIb subunit of the integrin receptor plays a role in the stability of the receptor complex [10,11]. In addition to the four calcium-binding sites, there is a proposed fifth site, known as the metal ion-dependent adhesion site (MIDAS) domain, on the GPIIIa [12–14]. Divalent cations, such as Ca^{2+} and Mn^{2+}, can compete with RGD-containing ligands or synthetic antagonists to bind to the activated GPIIb/IIIa receptor [15,16]. Although trisodium citrate may not alter the intracellular calcium ion concentration, it can certainly do so extracellularly in the platelet suspension, and consequentially alter receptor function. In addition, removal of calcium from the receptor sites may enhance the binding of GPIIb/IIIa antagonists and may, therefore, introduce an artifact in evaluating the *in vivo* efficacy of the antagonists.

We have provided evidence for this phenomenon with TP-9201, a small, peptidomimetic, cyclic GPIIb/IIIa receptor antagonist [17]. In this study two dosing regimens (120 μ/kg + 3 μ/kg/min and 185 μ/kg + 5 μ/kg/min) were used, with the expectation of a dose-

Address for correspondence: Benedict R. Lucchesi, Ph.D., M.D., Professor, Department of Pharmacology, University of Michigan Medical School, 1301C Medical Science Research Building III, Ann Arbor, 48109-0632, USA E-mail: benluc@umich.edu

related effect on platelet inhibition and antithrombotic efficacy. However, both the dosing regimens completely inhibited (>90%) ADP-induced platelet aggregation in citrated PRP (cPRP). In heparinized PRP (hPRP), the low and high doses of TP-9201 produced 21% and 45% inhibition of platelet aggregation, respectively. Dose-dependent prolongation of bleeding time and prevention of thrombosis were also observed. A similar observation has been reported by Collen et al. using TP-9201 [18]. In these studies the GPIIb/IIIa receptor antagonist exhibited enhanced potency based on the ex vivo assay in citrated blood. We aimed to investigate whether chelation of calcium by trisodium citrate was responsible for this phenomenon. Accordingly, the objective of the present investigation was to assess the differential in vitro potencies of structurally different GPIIb/IIIa antagonists under hypocalcemic and normocalcemic conditions.

Methods

Reagents

c7E3 (ReoPro) was provided by Dr. Robert E. Jordan (Centocor, Inc., Malvern, PA). DMP-728 was a gift from Dr. Shaker A. Mousa (DuPont Merck Pharmaceutical Co., Wilmington, DE). MK-383 was obtained from Dr. Claus J. Schmitges (E. Merck, Darmstadt, Germany). SM-20302 was provided by Sumitomo Pharmaceuticals (Osaka, Japan). All drugs were dissolved in sterile saline. Trisodium citrate, adenosine diphosphate (ADP), PPACK (D-phenylalanyl-L-propyl-L-arginyl-chloromethyl ketone), and standard reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Heparin sodium injection, USP (1000 U/mL) was purchased from Elkins-Sinn. (Cherry Hill, NJ). TRAP (SFLLRN-NH, 10 μM) was synthesized in the Protein and Carbohydrate Structure Core Laboratory of the University of Michigan Multipurpose Arthritis Center.

Blood collection

After informed consent, blood was collected from healthy, drug-free human volunteers in either trisodium citrate (1:10 citrate to blood; final concentration, 0.37%), PPACK (final concentration, 20 μg/mL of blood), or heparin (final concentration, 10 U/mL).

Platelet aggregation studies

The whole blood cell count was determined with an H-10 cell counter (Texas International Laboratories, Houston, TX). Platelet-rich plasma (PRP) was obtained by centrifuging anticoagulated whole blood at 1000 rpm for 5 minutes (140 g). PPP was prepared after the PRP was removed by centrifuging the remaining blood at 2000 g for 10 minutes and discarding the bottom cellular layer. All centrifugations were done at 21°C. The PRP obtained from citrated blood (cPRP) and PPACK-anticoagulated blood (pPRP) was diluted with the respective PPP to achieve a platelet count of 200,000/μL. *Ex vivo* platelet aggregation was assessed by established spectrophotometric methods with a four-channel aggregometer (BioData-PAP-4, Bio Data Corp., Hatboro, PA) by recording the increase in light transmission through a stirred suspension of PRP (410 μL). Aggregation studies were performed after samples were rewarmed to 37°C. Aggregation was induced with ADP (20 μM) or TRAP (SFLLRN-NH2; 10 μM). All antagonists were incubated in PRP for 1 minute, after which the platelet agonists were added. Agonists and antagonists (c7E3, DMP-728, MK-383, and SM-20302) were added in a volume of 50 μL, and the extent of inhibition was examined by light transmittance aggregometry. Values for platelet aggregation were expressed as the percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively. The IC_{50} values were determined from a sigmoidal dose-response relationship using GraphPad Prism (Version 2.0a, GraphPad Software, Inc.). A minimum of 8–10 concentrations were used for all antagonists.

To study the effect of calcium chelation on the inhibitory potency of GPIIb/IIIa receptor antagonists, varying concentrations of trisodium citrate were added to pPRP and the aggregatory response of ADP (20 μM) was examined in the presence of a subthreshold concentration of each antagonist.

Concentration of ionized calcium in plasma

Whole blood samples were collected in individual tubes containing either trisodium citrate, PPACK, or heparin as the anticoagulant. The blood samples were processed for the preparation of PPP and PRP as described previously and were analyzed for ionized calcium concentration using an ion analyzer (Nova-6, Nova Biomedical Instruments, Waltham, MA).

Statistical analysis

The data are expressed as the mean ± SEM. An unpaired *t*-test was used to assess differences in values obtained in cPRP and pPRP. Values were determined to be statistically different at a level of $P < 0.05$.

Results

Concentration of ionized calcium in plasma using different anticoagulants

The concentration of ionized calcium was measured in PRP and PPP samples that were anticoagulated initially with either trisodium citrate, PPACK, or heparin (Table 1). The mean ionized calcium concentrations in the PRP prepared from citrate, PPACK, and heparin were 0.056, 1.08, and 0.98 mM, respectively. Similarly, the mean ionized calcium concentrations in the PPP samples prepared from citrate, PPACK, or heparin were 0.059, 1.13, and 0.971 mM, respectively. The concentration of ionized calcium in citrated plasma was

Table 1. *Effect of different anticoagulants on the concentration of ionized calcium in human plasma samples*

Anticoagulant	PPP $[Ca^{2+}]$ mM	PRP $[Ca^{2+}]$ mM
Sodium citrate (0.37%, n = 7)	0.056 ± 0.003	0.059 ± 0.003
Heparin (10 U/mL, n = 5)	1.08 ± 0.05[a]	1.13 ± 0.02[a]
PPACK (20 μg/mL, n = 7)	0.980 ± 0.017[a]	0.971 ± 0.015[a]

[a]$P < 0.05$ as compared with trisodium citrate.
PRP = platelet-rich plasma.
Values are presented as the mean ± SEM.
PPP = platelet-poor plasma;

19 times lower than that in heparinized plasma ($P < 0.05$) and 16–17 times lower than in PPACK anticoagulated plasma ($P < 0.05$).

In vitro potency of GPIIb/IIIa receptor antagonists

The platelet inhibition profile in cPRP differed significantly from that in pPRP (Table 2). For example, the estimated IC_{50} for c7E3 for inhibiting ADP-induced platelet aggregation in cPRP was 1.6 times lower than that in pPRP. Figure 1 is a representative platelet inhibition profile of c7E3 (5 μg/mL) in cPRP and pPRP treated with 20 μM ADP. It is evident that 5 μg/mL of c7E3, which is the IC_{50} for ADP-induced platelet aggregation in pPRP, produced complete inhibition of the aggregation response in cPRP. The IC_{50} values for inhibiting TRAP-induced platelet aggregation were higher, but the difference in cPRP and pPRP was 1.7-fold ($P < 0.05$). MK-383, DMP-728, and SM-20302 also demonstrated 1.96-, 1.15-, and 1.43-fold lower IC_{50} values, respectively, in cPRP compared with that in pPRP.

Effect of calcium chelation on the platelet inhibitory action of GPIIb/IIIa receptor antagonists

To examine the inhibitory effects of the antagonists in the presence of varying calcium concentrations, trisodium citrate was added to pPRP samples (Figure 2). Irrespective of the structure, all the antagonists produced negligeable inhibition when the ionized calcium was 0.9 mM to 1 mM. Further depletion of calcium led to accentuation of the inhibitory response of the subthreshold concentrations of all the antagonists.

Discussion

The discordance between the IC_{50} values obtained using trisodium citrate and PPACK can be explained by the varying concentration of ionized calcium in the two assay media. We noted that the ionized calcium concentration in cPRP was 16–19 times less than that in hPRP and pPRP. A physiologic concentration of extracellular ionized calcium is critical to maintain the integrity of the GPIIb/IIIa heterodimer complex on the platelet surface [2,19] and fibrinogen binding to the activated integrin receptor [20–22]. The concentration of ionized calcium can also influence the formation of GPIIb/IIIa heterodimers [23], and the GPIIb/IIIa and fibrinogen interaction [5]. Human platelets incubated with the extracellular calcium chelators, EDTA and EGTA, undergo morphological changes [1] and fail to aggregate [2,3,24], or bind to fibrinogen in response to ADP [9,20,22]. Winters et al. [25] demonstrated that the binding of MoAb 10E5, a complex specific anti-GPIIb/IIIa antibody, to the platelets was reduced by 80% under the conditions of complete absence of calcium and a temperature of 37°C. The extent to which the platelet response is altered depends on the degree of calcium chelation. Trisodium citrate is not as effective as EDTA in chelating extracellular calcium, but it does induce hypocalcemic conditions as indicated by our data. In the present study, the presence of a GPIIb/IIIa receptor antagonist in cPRP may have acted in concert with low Ca^{+2} concentration to reduce the platelet aggregation response, thereby yielding a false indication of the drug's in vivo potency in terms of preventing platelet aggregation.

Investigation of the current literature on the preclinical evaluation of GPIIb/IIIa receptor antagonists indicates that trisodium citrate was the anticoagulant used to collect blood for ex vivo platelet inhibition studies, and as a result there was discordance between the ex vivo platelet inhibition data and the in vivo efficacy

Table 2. *Effect of different anticoagulants on the potency (IC_{50}) of GPIIb/IIIa antagonists in inhibiting human platelet aggregation*

	ADP (20 μM)		TRAP (10 μM)	
Drugs	Citrate	PPACK	Citrate	PPACK
c7E3 (n=5, μg/mL)	2.76 ± 0.11	4.46 ± 0.48[a]	4.52 ± 0.34	7.69 ± 0.43[a]
MK-383 (n=5, ng/mL)	25.00 ± 1.76	49.04 ± 1.10[a]	41.31 ± 1.64	70.97 ± 6.01[a]
DMP728 (n=6, ng/mL)	29.55 ± 1.89	34.21 ± 2.31	38.64 ± 1.41	55.21 ± 3.33[a]
SM-20302 (n=4, ng/mL)	11.59 ± 0.87	16.64 ± 0.94[a]	14.36 ± 1.44	18.49 ± 1.16

[a]$P < 0.05$ as compared with trisodum citrate.
Values are presented as the mean ± SEM.

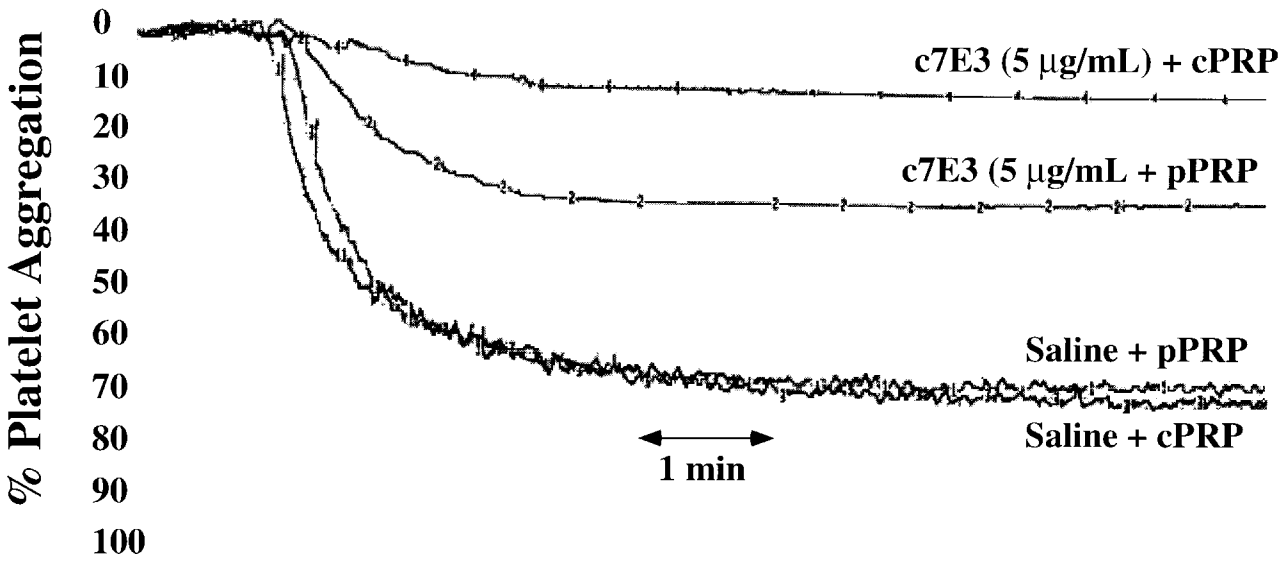

Fig. 1. *A representative trace illustrating the effect of c7E3 (5 μg/mL) on platelet aggregation induced by ADP (20 μM). Platelet aggregation was performed using PRP isolated from blood anticoagulated with trisodium citrate (cPRP) and PPACK (pPRP). Saline or c7E3 was added to a stirred suspension of PRP 1 minute prior to the addition of ADP, and the run time for the assay was 10 minutes.*

data. For example, using c7E3 in a primate model of carotid artery thrombosis, it was shown that the 0.15 mg/kg and 0.25 mg/kg doses had a similar ex vivo platelet inhibition profile (80–90%), but the incidence of occlusion was 66% and 0% for the low and high doses, respectively [26]. Lynch et al. [27] observed that a 3 μg/kg/min and 10 μg/kg/min infusion of MK-383 was associated with a 90–100% and 100% inhibition of collagen-induced platelet aggregation. However, in a canine model of primary coronary artery thrombosis, only the 10 μg/kg/min regimen was effective. Similarly, the 0.1 and 1.0 mg/kg doses of DMP-728 produced 75–100% and 90–100% inhibition of ADP-induced platelet aggregation in cPRP, and the incidences of carotid artery occlusion occlusion were 17% and 0% respectively. The lack of correlation between platelet inhibition in cPRP and the in vivo antithrombotic efficacy was also evident in previous studies employing L-738, 167 [28], SC-49992 [29], TP-9201 [17], and SM-20302 [30]. These studies reinforce the concept that platelet aggregation performed in cPRP is a poor indicator of the in vivo activity of GPIIb/IIIa receptor antagonists.

Our in vitro data with GPIIb/IIIa receptor antagonists may help to provide a better understanding of the outcome of the integrilin trials. Using four different dosing strategies of integrilin (Eptifibatide) in patients scheduled for elective percutaneous interventions, it was observed that administration of a bolus (90–180 μg/kg) followed by an infusion (0.5–1 μg/kg/min for 20 hours) did not result in a dose-related inhibition of ADP-induced platelet aggregation in cPRP. The maximum inhibition produced by all four dosing regimens was 90% [31]. In the IMPACT-II study [32] it was noted that despite 70–78% inhibition of ADP-induced platelet aggregation in cPRP, integrilin treatment did not influence 6-month clinical outcomes after PTCA. Recently Phillips et al. [33] provided evidence for the integrilin discrepancy by demonstrating that the IC_{50} values for ADP and TRAP-induced platelet aggregation in cPRP were 4- and 7.5-fold lower than those in pPRP. Furthermore, these investigators made use of PAC1, an antibody having binding properties that mimic the binding of fibrinogen to GPIIb/IIIa. PAC1, like fibrinogen, has similar divalent cation requirements for binding, requires platelet activation before binding can occur, and its binding can be affected by inhibitors of GPIIb/IIIa. The concentration of integrilin required to inhibit PAC1 binding in cPRP was lower than that required in pPRP, indicating that enhanced inhibition by integrilin in cPRP was the result of enhanced inhibition of fibrinogen binding. The pharmacodynamic determinations of the platelet aggregation inhibitory activity of integrilin, therefore, is enhanced by collection of blood samples with citrate as the anticoagulant [33]. The integrilin trials indicate that an effective dosing regimen for in vivo inhibition of platelet reactivity may correlate with less than 80% inhibition of ex vivo platelet aggregation responses to ADP and/or TRAP. Later the PURSUIT trials revealed that a modified and clinically effective dosing regimen, guided by ex vivo aggregation studies done in PPACK-PRP, and which did not inhibit completely the platelet aggregation response, could be used with relative safety. In contrast to integrilin, the differences

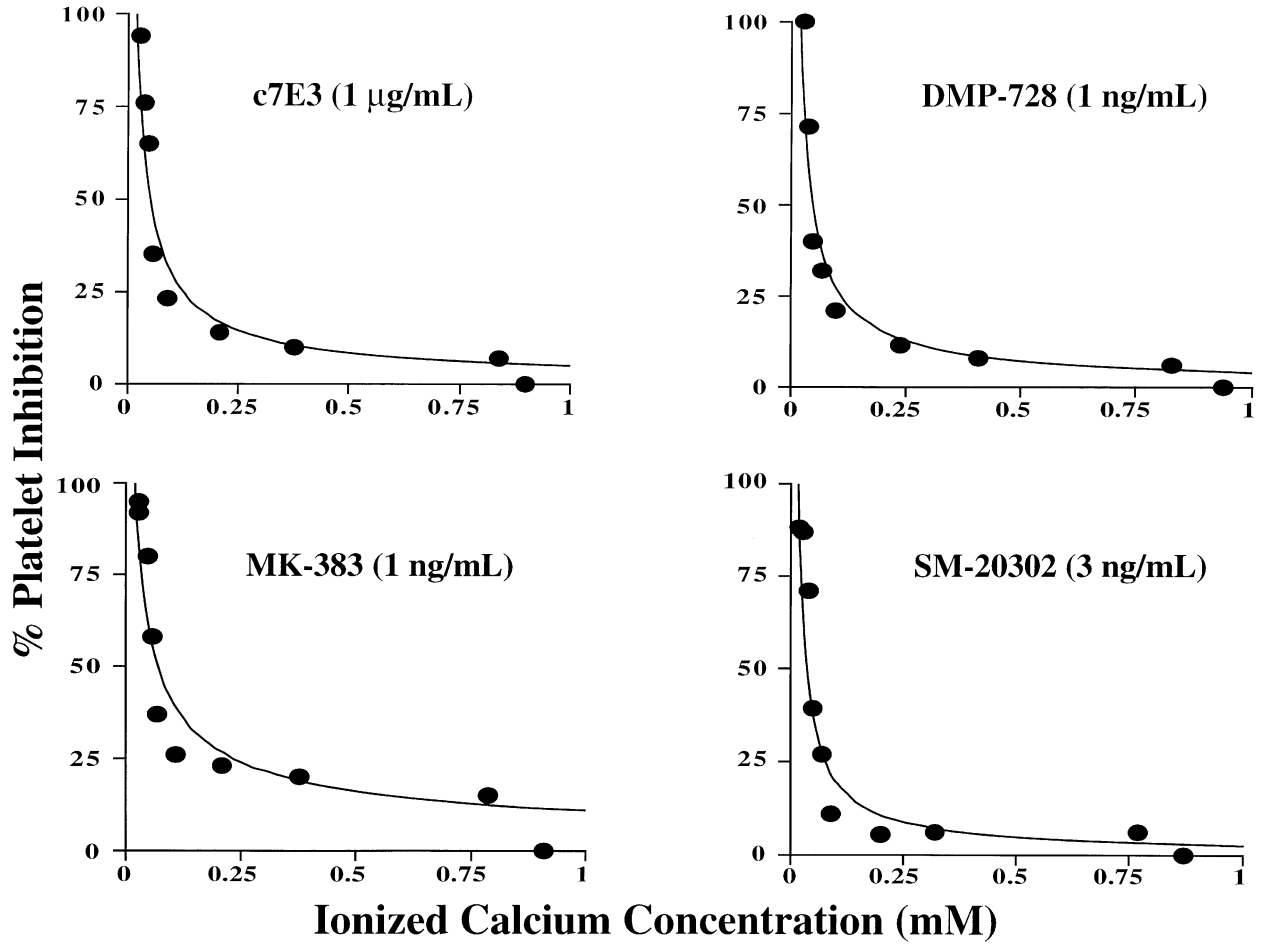

Fig. 2. *Effect of ionized calcium on the inhibitory effect of subthreshold concentrations of GPIIb/IIIa receptor antagonists. Platelet aggregation was induced by ADP (20 μM) using pPRP. Varying calcium concentrations were achieved by adding different concentrations of trisodium citrate to pPRP.*

in the IC_{50} values between cPRP and pPRP in our study were smaller (about two fold for MK-383). This may be due to the extent of calcium chelation in the two studies. We used 0.37% of trisodium citrate, whereas Phillips et al. used 0.57%.

In conclusion, using several structurally unrelated inhibitors of the platelet GPIIb/IIIa receptor, our observations, which are in agreement with those of Phillips et al. [33], suggest that the decrease in ionized calcium ion concentration renders the platelets more susceptible to inhibition by GPIIb/IIIa antagonists when challenged with platelet agonists. The use of PPACK, heparin, or hirudin as an anticoagulant during the collection of the blood sample maintains the ionized calcium concentration similar to that in the in vivo situation. The latter approach may provide a more accurate ex vivo assessment of platelet reactivity in response to aggregating agents, and may be more suited to the mechanistic study of platelet function, where it becomes essential to correlate the ex vivo observation with an anticipated in vivo effect, that is, prevention of arterial thrombosis. The use of a proper anticoagulant should result in a better correlation between the ex vivo and in vivo findings, and assist in maintaining an optimal dosing regimen while avoiding or reducing the incidence of untoward effects related to hemostasis.

Acknowledgments

This study was supported in part by the Cardiovascular Pharmacology Research Fund. The Protein and Carbohydrate Structure Core Laboratory support was from NIH Center Grant UM-MAC P60-AR20557. During the tenure of this study, SSR was the recipient of an Advanced Postdoctoral Fellowship from the American Heart Association, Michigan Affiliate (Lathrup Village, MI), and a Merck Postdoctoral Fellowship (Merch, West Point, PA).

References

1. White JG. Effects of ethylenediamine tetracetic acid (EDTA) on platelet structure. *Scand J Haematol* 1968;5: 241–254.
2. Pidard D, Didry D, Kunicki TJ, Nurden AT. Temperature-dependent effects of EDTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregability. *Blood* 1986;67: 604–611.
3. Shattil SJ, Brass LF, Bennett JS, Pandhi P. Biochemical and functional consequences of dissociation of the platelet membrane glycoprotein IIb-IIIa complex. *Blood* 1985;66:92–98.
4. Fujimura K, Philips DR. Calcium cation regulation of glycoprotein IIb-IIIa complex formation in platelet plasma membranes. *J Biol Chem* 1983;258:10247–10252.
5. Steiner B, Cousot D, Trzeciak A, Gillessen D, Hadvary P. Ca^{+2};-dependent binding of a synthetic Arg-Gly-Asp (RGD) peptide to a single site on the purified platelet glycoprotein IIb-IIIa complex. *J Biol Chem* 1989;264:13102–13108.
6. Marguerie GA, Edgington TS, Plow EF. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J Biol Chem* 1980;255:154–161.
7. Dandona P, Thusu K, Khurana U, Love J, Aljada A, Mousa S. Calcium, calmodulin and protein kinase C dependence of platlet shape change. *Thromb Res* 1996;81:163–175.
8. Lefkovitz J, Plow EF, Topol EJ. Platelet glycopotein IIb/IIIa receptors in cardiovascular medicine. *N Engl J Med* 1995;332:1553–1559.
9. Lanza F, Stierle A, Gachet C, Cazenave JP. Differential effects of extra and intracellular calcium chelation on human platelet function and glycoprotein IIb/IIIa complex stability. *Nouv Rev Fr Hematol* 1992;34:123–131.
10. Heidenreich R, Eisman R, Delgrosso K, Surrey S, Bennett JS, Schwartz E, Poncz M. Organization of the gene for platelet glycoprotein IIb. *Biochemistry* 1990;29:1232–1244.
11. Kieffer N, Phillips DR. Platelet membrane glycoproteins: Functions in cellular interactions. *Annu Rev Cell Biol* 1990;6:329–357.
12. Lee JO, Rieu P, Arnaout MA, Liddington R. Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). *Cell* 1995;80:631–638.
13. Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger AL, Ginsberg MH. A beta 3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science* 1990;249:915–918.
14. Tozer EC, Liddington RC, Sutcliffe MJ, Smeeton AH, Loftus JC. Ligand binding to integrin alphaII beta3 is dependent on a MIDAS-like domain in the beta3 subunit. *J Biol Chem* 1996;271:21978–21984.
15. D'souza SE, Haas TA, Piotrowicz RS, et al. Ligand and cation binding are dual functions of a discrete segment of the integrin β3 subunit: Cation displacement is involved in ligand binding. *Cell* 1994;79:659–667.
16. Hu DD, Barbas CF, Smith JW. An allosteric Ca2+ binding site on the beta3-integrins that regulates the dissociation rate for RGD ligands. *J Biol Chem* 1996;271:21745–21751.
17. Rebello SS, Driscoll EM, Lucchesi BR. TP-9201, a glycoprotein IIb/IIIa platelet receptor antagonist, prevents rethrombosis after successful arterial thrombolysis in the dog. *Stroke* 1997;28:1–8.
18. Collen D, Lu HR, Stassen JM, Vreys I, Yasuda T, Bunting S, Gold HK. Antithrombotic effects and bleeding time prolongation with synthetic platelet GPIIb/IIIa inhibitors in animal models of platelet-mediated thrombosis. *Thromb Haemost* 1994;71:95–102.
19. Philips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb/IIIa complex. *Blood* 1988;71: 831–843.
20. Marguerie GA, Plow EF, Edgington TS. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J Biol Chem* 1979;254:5357–5363.
21. Mustard JF, Packham MA, Kinlough-Rathbone RL, Perry DW, Regoeczi E. Fibrinogen and ADP-induced platelet aggregation. *Blood* 1978;52:453–466.
22. Peerschke EI, Zucker MB, Grant RA, Egan JJ, Johnson MM. Correlation between fibrinogen binding to human platelets and platelet aggregability. *Blood* 1980;55:841–847.
23. Lam SC. Isolation and characterization of a chymotryptic fragment of platelet glycoprotein IIb/IIIa retaining Arg-Gly-Asp binding activity. *J Biol Chem* 1992;267:5649–5655.
24. Zucker MB, Grant RA. Nonreversible loss of platelet aggregability induced by calcium deprivation. *Blood* 1978;52: 505–514.
25. Winters KJ, Eisenberg PR, Jaffe AS, Santoro SA. Dependence of plasmin-mediated degragdation of platelet adhesive receptors on temperature and Ca^{2+}. *Blood* 1990;76: 1546–1557.
26. Rote WE, Nedelman MA, Mu DX, Manley PJ, Weisman H, Cunningham MR, Lucchesi BR. Chimeric 7E3 prevents carotid artery thrombosis in cynomolgus monkeys. *Stroke* 1994;25:1223–1233.
27. Lynch JJ, Cook JJ, Sitko GR, et al. Nonpeptide glycoprotein IIb/IIIa inhibitors. 5. Antithrombotic effects of MK-0383. *J Pharmacol Exp Ther* 1995;272:20–32.
28. Cook JJ, Sitko GR, Holahan MA, et al. Nonpeptide glycoprotein IIb/IIIa inhibitors. 15. Antithrombotic efficacy of L-738,167, a long-acting GPIIb/IIIa antagonist, correlates with inhibition of adenosine diphosphate-induced platelet aggregation but not with bleeding time prolongation. *J Pharmacol Exp Ther* 1997;281:677–689.
29. Rote WE, Werns SW, Davis JH, Feigen LP, Kilgore KS, Lucchesi BR. Platelet GPIIb/IIIa receptor inhibition by SC-49992 prevents thrombosis and rethrombosis in the canine carotid artery. *Cardiovascular Res* 1993;27:500–507.
30. Rebello SS, Huang J, Saito K, Lucchesi BR. The *in vivo* efficacy of SM-20302, a GPIIb/IIIa receptor antagonist, correlates with *ex vivo* platelet inhibition in heparinized blood but not in citrated blood. *Arterioscl Thromb Vasc Biol* 1998, accepted.
31. Ohman EM, Harrington RA, Lincoff AM, Kitt MM, Kleiman NS, Tcheng JE. Early clinical experience with integrilin, an inhibitor of the platelet glycoprotein IIb/IIIa integrin receptor. *Eur Heart J* 1995;16(Suppl. L):50–55.
32. Resar JR, Brinker JA, Gerstenblith G, Blumenthal RS, Dudek A, Coombs VJ, Goldschmidt-Clermont PJ. Disparity of Integrilin inhibition of platelet aggregation and GP IIb/IIIa fibrinogen binding in angioplasty patients. *Circulation* 1996;94(Suppl. I):1–98.
33. Phillips DR, Teng W, Arfsten A, et al. Effect of Ca^{2+} on GPIIb-IIIa interactions with Integrilin. *Circulation* 1997; 96:1488–1494.