

γ -Thrombin-induced phospholipase A₂ activation in rabbit platelets: comparison with α -thrombin

Lhousseine Touqui, Martine Jandrot-Perrus* and B. Boris Vargaftig

Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur/INSERM N° 285, 25 rue Dr. Roux, 75015 Paris and

**Laboratoire d'Hémostase et de Thrombose, Faculté Xavier Bichat, 16 rue Henri Huchard, 75018 Paris, France*

Received 2 July 1989

γ -Thrombin stimulated release of [³H]arachidonic acid ([³H]AA) accompanied by a significant production of PAF and lyso-PAF by rabbit platelets. These responses, which reflect PLA₂ activation, were observed after a prolonged lag and to a lower extent when compared to those induced by α -thrombin which evoked a much higher elevation in intracellular calcium. This elevation together with [³H]AA release were markedly reduced by EDTA. However, addition of ionophore A23187 enhanced the release of [³H]AA by γ -thrombin to the levels similar to those of α -thrombin. We conclude that γ -thrombin is able to activate PLA₂ and suggest that calcium influx may be a limiting factor for this activation.

Thrombin, γ -; Phospholipase A₂; Platelet

1. INTRODUCTION

Platelets respond to agonists, such as thrombin, by initiating a cascade of events which leads to activation of PLA₂ [1,2]. This enzyme hydrolyses the membrane phospholipids at the sn-2 position leading to a preferential release of arachidonic acid (AA) [3-5] which is then converted into active metabolites (prostaglandins, thromboxane, leukotrienes...) by cyclo-oxygenase and lipoxygenase [6]. Parallely, the hydrolysis of phospholipids by PLA₂ leads to the generation of lysophospholipids, especially lyso-PAF which is converted into PAF, a potent proinflammatory mediator [7]. It is, therefore, clear that PLA₂ plays a key role in the synthesis of a variety of proinflammatory mediators. It has been shown that PKC is involved in the

regulation of PLA₂ activity in various cell types [8-12], although the exact mechanism of this regulation remains unclear. We have proposed that lipocortin-like protein(s), whose anti-PLA₂ activity is modulated by PKC-induced phosphorylation, may be involved in the regulation of PLA₂ activity in rabbit platelets [13]. However, it has been recently shown that γ -thrombin (a product of limited proteolysis of α -thrombin), even though inducing PKC activation, has no effect on PLA₂ activity in human platelets [14]. This finding was used to suggest that PKC and/or lipocortin-like protein(s) are not involved in the regulation of PLA₂ activity in human platelets.

The results of the present study show that, in fact, γ -thrombin, at concentrations known to activate PKC, induces a significant release of AA from rabbit platelets. A concomitant synthesis of PAF and lyso-PAF was observed in the same conditions. Although γ -thrombin was less active than α -thrombin in inducing AA release, its effect was markedly potentiated by the simultaneous addition of calcium ionophore A23187. Our results indicate that, contrarily to previous findings on human platelets, γ -thrombin is capable to induce PLA₂ ac-

Correspondence address: L. Touqui, Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur/INSERM N° 285, 25 rue Dr. Roux, 75015 Paris, France

Abbreviations: AA, arachidonic acid; PAF, platelet-activating factor; PKC, protein kinase C; PLA₂, phospholipase A₂; EC₅₀, effective concentration giving 50% of maximal response; NPGb, *p*'-nitrophenyl-*p*-guanidino benzoate

tivation in rabbit platelets. They suggest that the influx of extracellular calcium is a limiting factor for this activation.

2. MATERIALS AND METHODS

2.1. Materials

Unlabelled AA, calcium ionophore A23187 and essentially fatty acid-free bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA). α - and γ -thrombin were prepared in the Laboratoire d'Hémostase et de Thrombose (Faculté Xavier Bichat, Paris) as indicated below. Tritiated AA ([5,6,8,9,11,12,14,15 ^3H]AA) (200 Ci/mmol) was from Amer-sham.

2.2. Preparation of α - and γ -thrombin

Human α - and γ -thrombin were prepared according to published methods [15,16] as previously detailed [17]. Both forms of thrombin were $98 \pm 2\%$ active as tested by active site titration with NPGb (*p*'-nitrophenyl *p*-guanidinobenzoate) [18]. Specific clotting activity was 3100 ± 200 U/mg for α -thrombin and less than 10 U/mg for γ -thrombin.

2.3. Preparation and labelling of platelets

Whole blood was obtained from the central ear artery of adult New Zealand rabbits and anticoagulated with EDTA (5 mM). Platelets were then separated from blood and washed as previously described [19]. The platelet suspension (5×10^8 cells/ml) was in a calcium-free Tyrode's buffer consisting of 2.6 mM KCl, 3.6 mM MgCl_2 , 140 mM NaCl, 12 mM NaHCO_3 , 10 mM Tris, 0.2 mM EGTA, 5.5 mM glucose, 2.5 mg/ml gelatin at pH 6.5. Cyclo-oxygenase was inhibited by 10 min incubation with 500 μM aspirin before incubating platelets with 0.2 $\mu\text{Ci/ml}$ [^3H]AA. After 90 min incubation at 37°C, the platelets were washed twice to remove unincorporated radioactivity. The uptake of [^3H]AA by platelets was estimated at 70–80% of the total added [^3H]AA. The final suspension (5×10^8 cells per ml) was in a calcium-Tyrode's buffer (pH 7.4) which consisted essentially of the same reagents as above except that EGTA was replaced by 1.72 mM CaCl_2 and gelatin by 2.5 mg/ml BSA.

2.4. Measurement of AA release

Aliquots (0.5 ml) of labelled platelets were incubated with α - or γ -thrombin at concentrations indicated in the legends of figures and stimulations carried out at 37°C in the absence or in the presence of 5 mM EDTA. In some experiments platelets were incubated with 200 nM of calcium ionophore A23187 immediately before the addition of thrombin. After the intervals indicated in the figures, the reactions were terminated by the addition of 0.5 ml saline containing 5 mM EDTA and 10 mg/ml BSA (pH 7.4) and maintained at 4°C. Platelets were then removed by 2 min centrifugation at 12000 rpm. The radioactivity in the 100 μl of supernatant was then determined by liquid scintillation spectrophotometry. Lipids in the remaining supernatants (900 μl) were rapidly extracted according to the method of Bligh and Dyer [20]. The organic phases were carefully removed and brought to dryness under a stream of nitrogen and finally redissolved in 50 μl chloroform/methanol (2:1). Then,

extracts were subjected to thin-layer chromatography (TLC) on plastic silicagel plates using chloroform/methanol/acetic acid/water (65:43:1:3) as the mobile phase to separate lipids. Authentic unlabelled AA was also spotted on the same plates as standard to confirm the identity of released [^3H]AA. Lipids were visualized by iodine vapour and plates scraped off in order to measure their radioactivity by liquid scintillation spectrophotometry.

2.5. Measurement of PAF and lyso-PAF synthesis

Aliquots (1 ml) from unlabelled platelets were incubated with 18 nM of α - or γ -thrombin at 37°C. Then, the reactions were terminated at the times indicated, by the addition of 1 ml methanol and lipid extraction carried out as indicated above. Thereafter, lipids were separated on TLC using chloroform/methanol/water (70:35:7) as mobile phase. Areas containing PAF or lyso-PAF (located by referring to corresponding standards) were scraped off, resuspended in 1 ml methanol/water (2:1) and extracted as indicated above. The organic phases were then removed and brought to dryness under nitrogen. The assay of PAF and lyso-PAF was carried out as previously described [21].

2.6. Measurement of cytosolic free calcium concentrations

Washed platelets were incubated in calcium-free buffer with 3 μM of Fura-2 AM at 37°C. Fura-2 was liberated from its methyl ester Fura-2 AM by cytosolic esterase inside the cell [22]. After 30 min, loaded platelets were centrifuged to remove extraneous Fura-2 AM and resuspended in calcium- and EDTA-free buffer. Calcium (1 mM) or EDTA (5 mM) were then added to platelets before their stimulation with agonists. Fluorescence changes were monitored in 2 ml platelet samples with a Jobin-Yvon (JY 3D) spectrophotometer. The intracellular calcium levels were calculated as previously described [22].

3. RESULTS AND DISCUSSION

Stimulation of [^3H]AA labelled platelets with α - or γ -thrombin led to a time-dependent release of [^3H]AA in the medium (fig.1). While [^3H]AA release was rapidly observed with α -thrombin, a prolonged lag phase was observed when using γ -thrombin. Half-maximal responses were observed 1 min and 3 min after platelet stimulation with α - and γ -thrombin, respectively. [^3H]AA release increased rapidly to reach a maximum (8–10% of total incorporated radioactivity) after 5 min incubation with α -thrombin. In the presence of γ -thrombin, this release increased with a lower velocity and reached its maximum (5–6%) after 10 min stimulation.

The concentration-response curve of [^3H]AA release was studied by increasing the concentration of thrombin from 5 to 50 nM. Fig.1 shows that [^3H]AA release increased with the thrombin concentration, γ -thrombin being approximately two

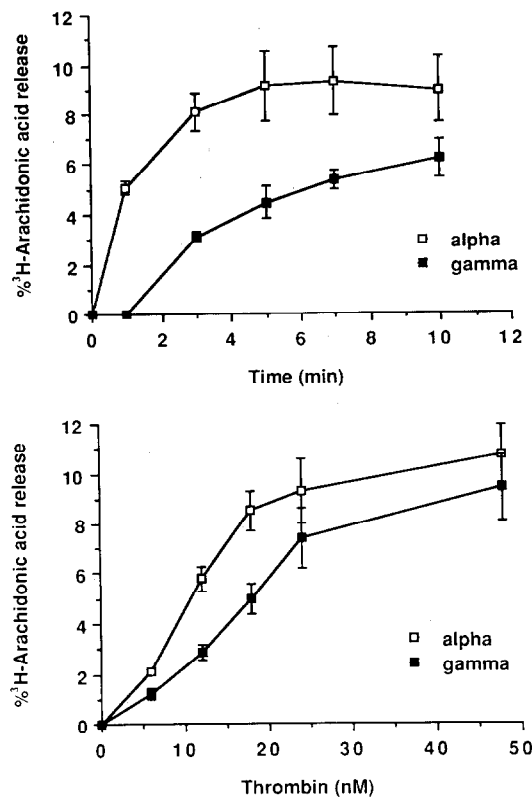


Fig.1. α - and γ -thrombin-induced release of AA from rabbit platelets. Time course (upper panel) and concentration-response curve (lower panel). Rabbit platelets were pretreated with aspirin and labelled with 0.2 $\mu\text{Ci}/\text{ml}$ [^3H]AA as indicated in section 2. Aliquots (0.5 ml) of platelet suspensions were stimulated with 18 nM thrombin for the indicated times or with increasing concentrations of thrombin for 3 min at 37°C. The reactions were terminated by the addition of 0.5 ml of saline solution containing 10 mM EGTA and 10 mg/ml BSA. The release of [^3H]AA was then measured as detailed in section 2. The figures represent the release of [^3H]AA expressed as percent of total radioactivity incorporated in the cells and are the mean $\pm$ SD of 3 separate experiments.

times less potent than α -thrombin. Indeed, the EC_{50} values were estimated at 9–10 and 18–20 nM, for α - and γ -thrombin, respectively.

Using washed human platelets, Jandrot-Perrus et al. (manuscript in preparation) have observed that γ -thrombin induced an increase of thromboxane B_2 synthesis. The latter was detected after a prolonged delay and at a lower amount when compared to that induced by α -thrombin. Thus, it is clear that, although γ -thrombin is a less potent activator than α -thrombin, it is still effective in inducing PLA_2 activation.

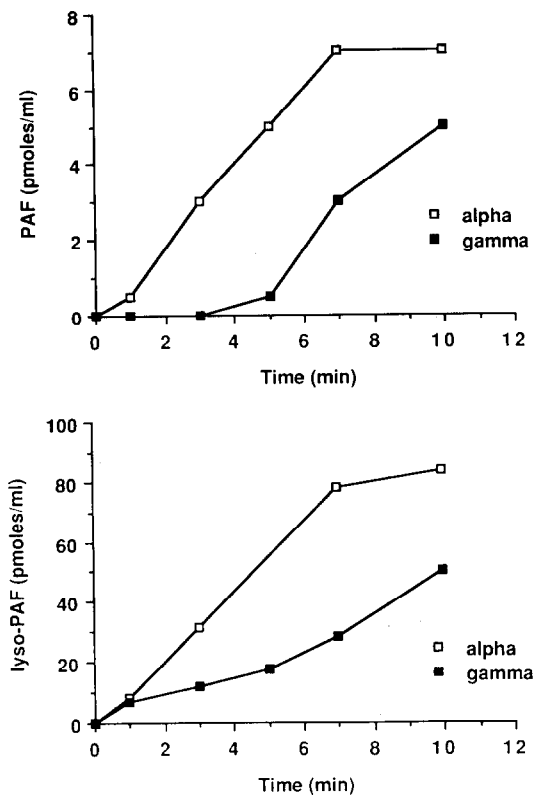


Fig.2. Effects of α - and γ -thrombin on the production of PAF and lyso-PAF by rabbit platelets. 0.5 ml aliquots of unlabelled platelets were stimulated with 18 nM of α - or γ -thrombin and the reactions terminated at the indicated times by the addition of 1 ml methanol. PAF and lyso-PAF were extracted and their amounts determined as indicated in section 2. The results show the production of PAF (upper panel) and lyso-PAF (lower panel) expressed in pmol/ml platelets and are representative of 2 separate experiments.

We have also investigated the effect of γ -thrombin on the production of PAF and its immediate precursor lyso-PAF. Fig.2 shows that stimulation of platelets by γ -thrombin led to a significant production of PAF and lyso-PAF. This was observed after a delay of 2–3 min, whereas in the presence of α -thrombin the production of PAF and lyso-PAF was detected after a short time of stimulation. The enhancement of lyso-PAF production is of particular interest since this compound directly reflects the activation of PLA_2 [7].

On the other hand, we have investigated the effect of extracellular calcium on the release of [^3H]AA induced by the two forms of thrombin. Fig.3 shows that this release was completely blocked by

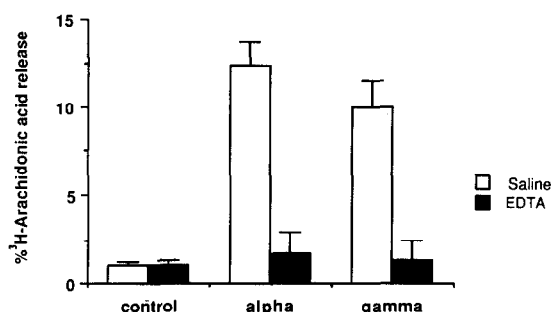


Fig.3. Effect of EDTA on thrombin-induced release of AA from rabbit platelets. 0.5 ml aliquots of [³H]AA prelabelled platelets were incubated with 5 mM EDTA or 50 μ l saline, 10 min before the addition of 18 nM thrombin. Stimulations were carried out for 3 min at 37°C and the release of [³H]AA measured as indicated above.

the chelation of extracellular calcium by EDTA suggesting that the activation of PLA₂ by thrombin is dependent on the influx of extracellular calcium rather than on the mobilization of intracellular calcium. Therefore, we have compared the effect of γ - and α -thrombin on the increase of cytosolic calcium concentration using Fura-2 loaded platelets. Fig.4 shows that both α - and γ -thrombin

produced a dose-dependent increase of intracellular calcium concentration which was markedly reduced in the presence of EDTA. The responses induced by γ -thrombin occurred at a lesser extent and with a lower velocity than those induced by α -thrombin. For example, incubation of platelets with 12 nM of α -thrombin led to an increase in intracellular calcium concentration reaching a value 6 times higher than that induced by the same concentration of γ -thrombin (fig.4, left panel). Thus, we have postulated that the low potency of γ -thrombin in inducing PLA₂ activation may be due to its limited capacity to induce calcium influx. To examine this hypothesis we have investigated the effect of calcium ionophore A23187 on the release of AA by thrombin-stimulated platelets in the presence of calcium. Our results show that A23187 (200 nM) potentiates the release of [³H]AA induced by both forms of thrombin (fig.5). When used alone at the same concentration, A23187 had no significant effect on the release of [³H]AA (less than 1%). The synergistic effect of A23187 was more pronounced in the presence of γ -thrombin. Indeed, A23187 reduced by 5 times the EC₅₀ of γ -thrombin-induced [³H]AA release (EC₅₀ = 20 and 4 nM, in the absence and in the presence of

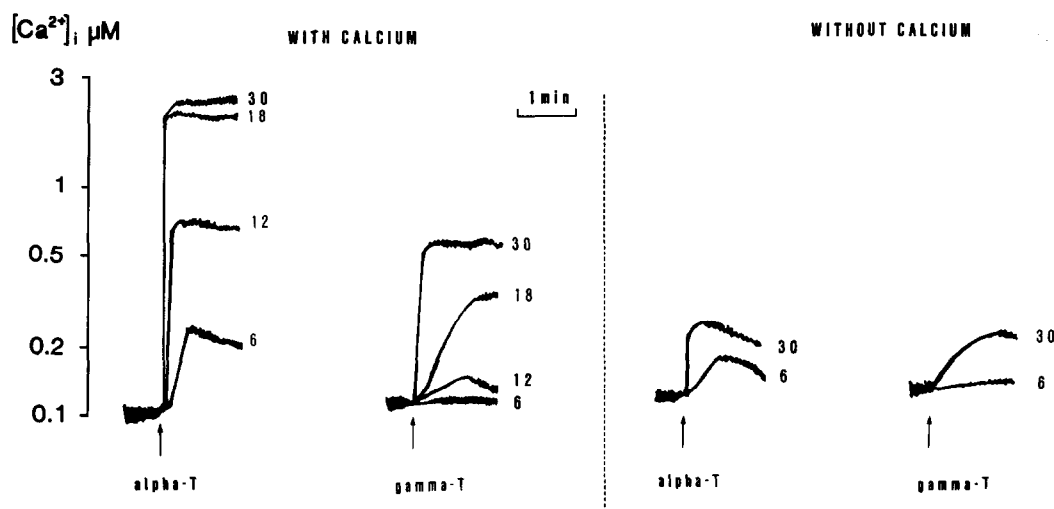


Fig.4. Changes in cytosolic calcium concentration in response to α - and γ -thrombin. 2 ml aliquots from Fura-2 loaded platelets (see section 2) were stimulated with α - (α -T) or γ -thrombin (γ -T) at the concentrations indicated next to each trace and expressed in nM. Stimulations were carried out in the presence of 1 mM calcium (left panel) or 5 mM EDTA (right panel). The fluorescence calibration for intracellular calcium levels was calculated as previously described [22]. The intracellular calcium concentrations vary from a basal value of 100 nM (resting platelets) to 2550 nM in the presence of 30 nM α -thrombin. The figure shows traces representative of 3 experiments.

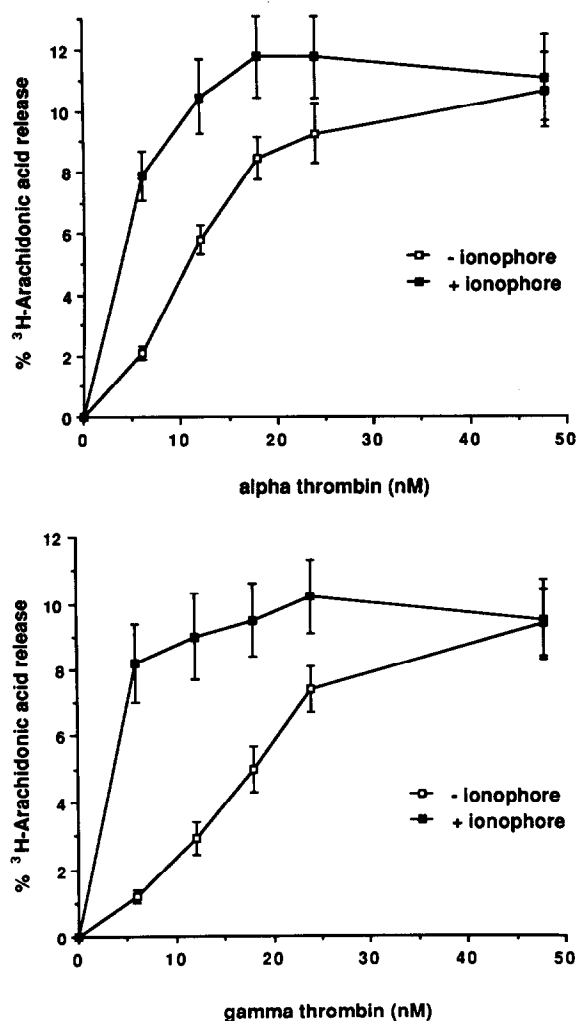


Fig.5. Synergistic effect of calcium ionophore A23187 on thrombin-induced release of AA by platelets. 0.5 ml aliquots of [^3H]AA prelabelled platelets were incubated with 200 nM A23187 or with its solvent dimethylsulfoxide (2 μl) as a control. α - or γ -thrombin were immediately added to platelets at the indicated concentrations. The reactions were stopped after 3 min stimulation at 37°C and the release of [^3H]AA measured as indicated in section 2. The figures show the release of [^3H]AA by α -thrombin (upper panel) and γ -thrombin (lower panel) in the presence (+ ionophore) or in the absence (- ionophore) of calcium ionophore A23187.

A23187, respectively). However, in the case of α -thrombin, this value decreased only by 2.5 times (from 10 to 4 nM).

Our results clearly show that γ -thrombin, although less active than α -thrombin, is capable of inducing PLA₂ activation in rabbit platelets. How-

ever, Crouch and Lapetina [14] have shown that γ -thrombin, at a concentration which fully activated PKC, failed to induce [^3H]AA release by human platelets. This observation was used to suggest that PKC may not play a role in the regulation of PLA₂ activity. In fact, their results are probably accounted for by the lower sensitivity of human platelets to γ -thrombin or to the relatively short time of incubation they used in their study. Indeed, in our conditions, the release of [^3H]AA induced by γ -thrombin was observed 1–2 min later than that induced by α -thrombin. We suggest that the lower potency of γ -thrombin to induce PLA₂ activation may result from its limited capacity to induce extracellular calcium influx. This is supported by the finding that, in the presence of calcium ionophore A23187, γ -thrombin became as active as α -thrombin in inducing [^3H]AA release.

Acknowledgements: We are grateful to Miss Patricia Renesto for her technical help in the studies with Fura-2.

REFERENCES

- [1] Billah, M.M., Lapetina, E.G. and Cuatrecasas, P. (1980) *J. Biol. Chem.* 255, 10227–10231.
- [2] Billah, M.M., Lapetina, E.G. and Cuatrecasas, P. (1981) *J. Biol. Chem.* 256, 5399–5403.
- [3] Irvine, R.F. (1982) *Biochem. J.* 204, 3–16.
- [4] Bills, T.K., Smith, J.B. and Silver, M.J. (1977) *J. Clin. Invest.* 60, 1–6.
- [5] Kannagi, R., Koisumi, K. and Masuda, T. (1981) *J. Biol. Chem.* 256, 1177–1184.
- [6] Siess, W., Siegel, F.L. and Lapetina, E.G. (1983) *J. Biol. Chem.* 258, 11236–11242.
- [7] Braquet, P., Touqui, L., Shen, T.Y. and Vargaftig, B.B. (1987) *Pharmacol. Rev.* 39, 97–145.
- [8] Volpi, M., Molski, T.F.P., Naccache, P.H., Feinstein, M.B. and Sha'afi, R.L. (1985) *Biochem. Biophys. Res. Commun.* 128, 594–600.
- [9] Mobley, A. and Tai, H.H. (1985) *Biochem. Biophys. Res. Commun.* 130, 717–723.
- [10] Parker, J., Daniel, L.W. and Waite, M. (1987) *J. Biol. Chem.* 262, 5385–5393.
- [11] Halenda, S.P. and Rhem, A.G. (1987) *Biochem. J.* 248, 471–475.
- [12] Portilla, D., Mordhorst, M., Bertrand, W. and Morisson, A.R. (1988) *Biochem. Biophys. Res. Commun.* 153, 454–462.
- [13] Touqui, L., Rothhut, B., Shaw, A.M., Fradin, A., Vargaftig, B.B. and Russo-Marie, F. (1986) *Nature* 321, 177–180.
- [14] Crouch, M.F. and Lapetina, E.G. (1986) *Biochem. Biophys. Res. Commun.* 141, 459–465.

- [15] Bezeaud, A., Denninger M.H. and Guillin, M.C. (1985) *Eur. J. Biochem.* 153, 491-496.
- [16] Fenton, J.W., Landis, B.H., Walz, D.A. et al. (1979) in: *Chemistry of the Human Plasma Proteins* (Bing, D.H., ed.) pp. 151-183, Pergamon.
- [17] Jandrot-Perrus, M., Didry, D., Guillin, M.C. and Nurden, A.T. (1988) *Eur. J. Biochem.* 174, 359-367.
- [18] Chase, T. and Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* 29, 508-514.
- [19] Ardlie, N.G., Packham, M.A. and Mustard, J.F. (1970) *Br. J. Haematol.* 19, 7-17.
- [20] Bligh, E.G. and Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* 37, 911-918.
- [21] Benveniste, J., Chignard, M., Le Couedic, J.P. and Vargaftig, B.B. (1982) *Thromb. Res.* 25, 275-285.
- [22] Pollock, W.K., Rink, T.J. and Irvine, R.F. (1986) *Biochem. J.* 235, 869-877.