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Identification of Glycyrrhizin as a Thrombin Inhibitor

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Glycyrrhizin (GL), an anti-inflammatory compound isolated from *Glycyrrhiza glabra*, was identified as a new thrombin inhibitor: (a) It prolonged plasma recalcification and thrombin and fibrinogen clotting times, and (b) it inhibited thrombin-induced, but not collagen-, PAF- or convulxin-induced platelet aggregation. On the other hand, GL did not block thrombin's amidolytic activity upon S-2238. Furthermore, the fluorescence emission intensity of dansyl-thrombin was increased upon GL binding. Moreover, GL displaced hirudin as an inhibitor of thrombin-catalyzed hydrolysis of S-2238. Our data provide evidence that GL is a selective inhibitor of thrombin (the first one isolated from plants) that is able to exert its anti-thrombin action by interacting with the enzyme's anion binding exosite 1. A pharmacophoric search identified GL as a sialyl Lewis X (SLe^X) mimetic compound able to inhibit selectin binding to SLe^X. However, SLe^X did not affect thrombin clotting activities, which indicates a lack of its interaction with thrombin and distinguishes both molecules. It is suggested that the anti-inflammatory effect of GL may be due to its effective anti-thrombin action.

Glycyrrhizin (GL) is a natural compound obtained from the aqueous extract of the root of the Leguminosae *Glycyrrhiza glabra*. This compound is a triterpenoid saponin composed of one molecule of glycerritinic acid (GA) and two glucuronic acid molecules (GU). GL is known for its anti-inflammatory (1;2), anti-allergenic (3), anti-viral (2;4) and anti-carcinogenic (5) as well as presenting anti-kinase-binding activities (6). GL also exerts inhibitory effect on PAF production by neutrophils (7) and inhibits the migration of leukocytes to the sites of inflammation (8). Inflammatory reactions involve a wide array of interactions between leukocytes and endothelial cells (EC) where the participation of selectins, a family of carbohydrate-binding proteins (lectins), have been established (9,10). Recently, the tetrasacaride Sialyl Lewis X (SLe^X: Neu5Ac2-3Gal1-4[Fuc1-3]GlcNAc) was found in the surface of EC and leukocytes and identified as the ligand necessary for selectin recognition and leukocyte trafficking (11). Interestingly, a pharmacophoric search using over 72,000 compounds identified GL as a SLe^X mimicker (8). Accordingly, GL presented *in vivo* and *in vitro* anti-inflammatory activities and its effect, like for SLe^X, seems to be mediated via its ability to block selectin binding to EC (8). These findings indicated that GL could be used to replace SLe^X in experimental models and for this tetrasacaride in particular, this in an important information considering that chemical or enzymatic synthesis of SLe^X are laborious or involve difficult glycosilation reactions or enzymes not generally available (12;13), making its acquisition both difficult and expensive. More recently, it has been shown that platelets also contain SLe^X in their surface (14), in addition to other negatively charged sugar residues, including sialic acid-containing oligosacharides (15). We thus raised the question whether GL could allow to identify possible contributions of SLe^X to platelet physiology, specially concerning to the mechanism of platelet activation by many inducers including thrombin, collagen, PAF and convulxin, a highly active platelet aggregating protein purified from *Crotalus durissus terrificus* venom (16;17). It was thought that GL could bind to the "thrombin anion binding exosite 1", which is a cluster of positively charged residues located at two poles of the molecule, remote from the active site. It mediates the interaction of thrombin with various physiological ligands (18) such as fibrinogen (19), GPIb (20), thrombomodulin (21;22) and heparin cofactor II (23). In this study, we used GL to investigate its effects on thrombin actions on clotting, platelet aggregation and other assays.

MATERIALS AND METHODS

Thrombin was from F-Hoffman La Roche (Basel, Switzerland). GL, GA, GU, hirudin, PAF, collagen, dansyl fluoride were from Sigma

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Abbreviations: GA, glycerritinic acid; GL, glycyrrhizic acid; GU, glucuronic acid; GP, glycoprotein; EC, endothelial cell; ELISA, enzyme-linked immunoenzymatic assay; PAF, platelet activating factor; Sle^X, Sialyl Lewis X (Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc); S-2238, H-D-Phe-Pip-Arg-*p*-nitroaniline.

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Chemical Co (St. Louis, USA). Fibrinogen and H-D-Phe-Pip-Arg-*p*-nitroaniline (S-2238) were purchased from Kabi Diagnostica (Sweden). Convulxin was purified as described (17). SLe^X (Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc) was purchased from Oxford Glycosystems (UK). GL was diluted in 0.1 M NaOH and pH adjusted to 7.4 with 1 M Tris-HCl pH 7.4. All other reagents were of analytical grade.

Platelet aggregation and ATP secretion. Platelets were obtained from rabbit blood, washed and resuspended in Tyrode-gelatin buffer; platelet aggregation and ATP secretion were monitored in a Chrono-Log Lumi Aggregometer (Havertown, PA), as previously described (17).

Coagulation assays. These experiments were performed using a Thermomax microplate ELISA reader (Molecular Devices, USA) equipped with an apparatus mixer and heating system in addition to a kinetic module software and processed as described (24). An eight-channel multipipette was used for delivering $CaCl_2$, plasma or thrombin and the optical density was continuously recorded at 6-sec intervals during 20 min. We chose either the time taken for reaching a defined (0.05 to 0.1) absorbance value (onset O.D.) as a measure of clotting time and/or the rate of clotting formation (Vmax), expressed as mO.D./min. For recalcification time, serial dilutions (50 μl) of GL, GA and GU in Hepes-saline buffer (0.15M NaCl, 10 mM Hepes, pH 7.4) were incubated for 10 min with pre-warmed (37°C) human plasma (50 μl) followed by addition of pre-warmed $CaCl_2$ (50 μl, 7.5 mM, final concentration). For thrombin time, thrombin (50 μl) diluted in hepes-saline buffer was incubated with GL solution(50 μl) or not for 10 min at 37°C followed by addition of pre-warmed human plasma (50 μl). To determine fibrinogen clotting activity, fibrinogen (50 μl, 0.25 - 2 mg/ml, final concentration) diluted in 10 mM Hepes pH 7.4 was incubated with GL (0-750 μM) for 10 min at 37°C followed by addition of thrombin (50 μl, 0.4 U/ml, final concentration).

Thrombin hydrolysis of chromogenic substrate S-2238. Thrombin (50 μl) was diluted in 20 mM Tris-HCl, 175 mM NaCl, pH 8.4 in the presence of GL (50 μl) or not followed by addition of S-2238 (50 μl; 0.1 mM, final concentration). The absorbance at 405 nm was recorded for 20 min in a Microplate reader and the initial rate of *p*-nitroaniline liberation determined. In some experiments, hirudin in the presence of GL or not was incubated with thrombin for 10 min at 37°C followed by addition of S-2238.

Fluorescent labeling of human thrombin. Bovine thrombin was labeled at the active site in the serine residue by reacting with dansyl fluoride to form dansyl-thrombin (25). Dansyl thrombin was extensively dialyzed against 50 mM sodium phosphate, 0.75 M NaCl, pH 6.5.

Fluorescence spectroscopy. Spectral measurements were performed in a Hitachi F-4500 fluorescence spectrophotometer (Japan). The excitation and emission wavelengths were 345 and 510 nm, respectively. Titration was carried out with thrombin (4 U/ml) in 50 mM sodium phosphate, 0.75 M NaCl, pH 6.5. F/Fo data were corrected for volume changes and background readings were obtained with glycyrrhizin in the absence of dansyl-thrombin.

RESULTS

The effects of GL on platelets were first tested on platelet aggregation. Figure 1 shows that glycyrrhizin (GL) did not affect platelet aggregation induced by collagen, PAF or convulxin, a highly purified protein and potent inducer of platelet aggregation, isolated from the venom of the snake *C. d. terrificus* (16;17). With thrombin, however, GL produced a dose-dependent inhibition of platelet aggregation with an IC_{50} around 150 μM. Fig. 1 also depicts (insert) the inhibitory pattern of GL on thrombin-induced platelet aggregation and its effects on ATP secretion. For shorter (5 sec) or longer incubation times (up to 1 min) no changes in the inhibitory pattern were observed thus suggesting that GL interaction with platelets seems to be not necessary (not shown). In fact, platelets that were pre-incubated with GL, centrifuged, re-suspended in the buffer and tested again with thrombin, were fully responsive to the agonist, thus confirming that GL was completely washed out from the platelets (not shown). Taken together, these observations indicated that GL behaves as a direct and specific inhibitor of thrombin.

The effects of GL on blood coagulation tests were then studied. Consistent with the finding on thrombin-induced platelet aggregation, 150 μM GL slightly prolonged the onset time for clotting plasma on the recalcification time test. Concentrations higher than 525 μ M were increasingly inhibitory and at 1.8 mM plasma was incoagulable (data not shown). The aglycon of GL, GA, and GU (up to 1.8 mM) did not prolong recalcification time; on the contrary, a small decrease in the clotting onset time was recorded (data not shown). These findings suggest that the sugar moiety may be important for the expression of the inhibitory effect of GL. Additional experiments were then performed in order to explore the mechanism of thrombin inhibition by GL. Table 1A shows that GL prolongs thrombin time in a dose-dependent manner, as determined by the decrease in the rate of fibrin formation, expressed as Vmax in mO.D./min. In addition, it also inhibited thrombin-induced fibrinogen clotting activity in a competitive manner with Ki of 240 ± 20 μM (Table 1B; Fig. 2), thus suggesting that GL is a competitive inhibitor of throm-

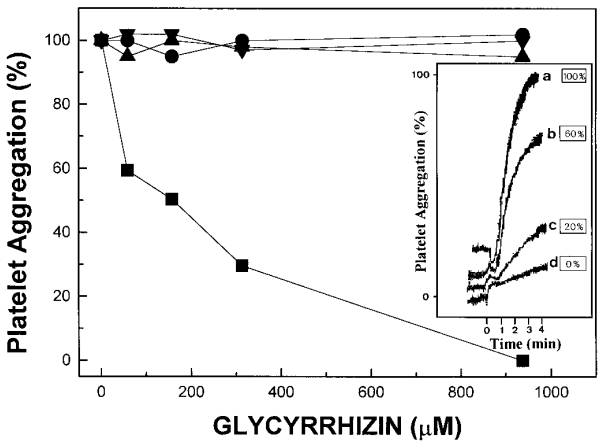

FIG. 1. Effects of GL on platelet aggregation. Platelets, suspended in Tyrode-gelatin buffer were incubated for 30-s with GL followed by addition of thrombin (0.112U/ml) (■); collagen (10 μg/ml) (▲); convulxin (0.8 nM) (▼) or PAF (100 pM) (●). Inset: Representative tracings show the effect of GL on thrombin: platelets were stimulated by thrombin (0.112 U/ml) in the absence (a) or in the presence of GL: 58 μM (b); 156 μM (c) or 312 μM (d). The number inside the boxes indicates the % of inhibition of thrombin-induced ATP secretion, in the presence of GL.

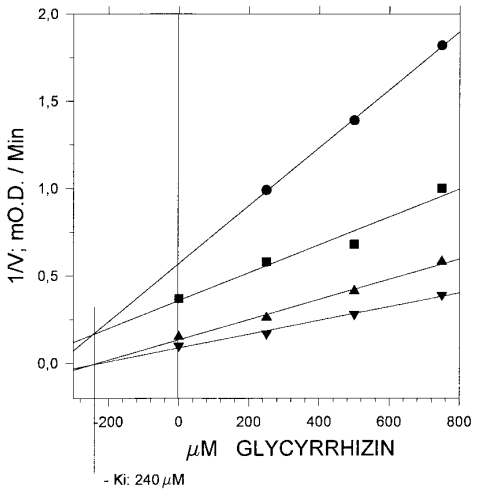

FIG. 2. Dixon's plot for the inhibitory pattern of GL on thrombin upon fibrinogen-clot formation. GL (0-750 μM) diluted in 10 mM Hepes pH 7.4 was incubated for 10 min at 37°C with fibrinogen (0.25 mg/ml) (●); (0.50 mg/ml) (■); (0.75 mg/ml) (▲) or (1.00 mg/ml) (▼), followed by addition of thrombin (0.4 U/ml).

bin anion binding exosite 1. More recently, it has been shown that GL is a SLe^X mimetic compound and it inhibits selectin binding to SLe^X as shown by pharmacological assays (8). However, SLe^X (up to 460 μM) did not prolong thrombin time or fibrinogen clotting activity induced by low concentrations of thrombin (data not shown) suggesting that SLe^X does not bind to thrombin.

Many thrombin inhibitors described to date such as DFP, PMSF and serpins block the catalytic site of thrombin (26). Experiments were then performed to evaluate the effect of GL on thrombin-catalyzed hydrolysis of the small chromogenic substrate S-2238. It was found that GL was not an inhibitor of thrombin's catalytic site, on the contrary, GL at the concentrations of 225 μM and 525 μM increased 41% and 100 % thrombin's catalytic activity upon this substrate (not shown). These results suggest that a possible conformational change in thrombin's active site may took place after GL's binding to the enzyme. To test this possibility, we chemically modified the enzyme's active site by covalently linking a dansyl (5-dimethylaminonaphthalene-1-sulphonyl) group to its serine residue. We then examined the influence of GL on the emission intensity of the modified protein. Titration of dansyl-thrombin with GL resulted in a 20% decrease in fluorescence emission intensity (Fig. 3), thus confirming that GL induces the expected conformational change into the enzyme's active site.

Several anti-thrombins which do not block the catalytic site of the enzyme, exert their inhibitory effect through interactions with "thombin anion binding exosite 1" (27;28). In addition, most of these inhibitors are able to overcome the anion binding exosite 1-dependent inhibi-

TABLE 1

Effects of Glycyrrhizin on Thrombin Coagulant Activity

A: Rate of clot formation thrombin + plasma

	Thrombin concentration (Units/ml)			
Glycyrrhizin (μM)	0.046 m O.D./Min.	0.093 m O.D./Min.	0.187 m O.D./Min.	0.375 m O.D./Min.
0	0.02 ± 0.02	0.38 ± 0.13	1.42 ± 0.65	3.52 ± 0.33
225	0.04 ± 0	0.49 ± 0.06	1.45 ± 0.04	3.00 ± 0.08
525	0.0	0.0	0.72 ± 0.05	2.40 ± 0.05
900	0.0	0.0	0.20 ± 0.03	0.68 ± 0.08

B: Rate of clot formation: Thrombin + fibrinogen

	Fibrinogen concentration (mg/ml)			
Glycyrrhizin (μM)	0.20 m O.D./Min.	0.40 m O.D./Min.	0.75 m O.D./Min.	1.00 m O.D./Min.
0	0.88 ± 0.02	2.67 ± 0.16	6.78 ± 0.17	10.23 ± 0.28
250	1.01 ± 0.02	1.72 ± 0.36	3.78 ± 0.06	5.96 ± 0.28
500	0.72 ± 0.01	1.48 ± 0.05	2.42 ± 0.03	3.64 ± 0.10
750	0.55 ± 0.01	1.00 ± 0.05	1.73 ± 0.04	2.39 ± 0.09

A. Thrombin (0.046–0.375 U/ml) diluted in Hepes-Saline buffer was incubated with GL solution (0–900 μM) for 10 min at 37°C followed by addition of pre-warmed human plasma.

B. Fibrinogen-clot formation. Fibrinogen: (0.25–2.00 mg/ml) diluted in 10 mM Hepes pH 7.4 was incubated with GL (0–750 μM) for 10 min at 37°C followed by addition of thrombin (0.4 U/ml).

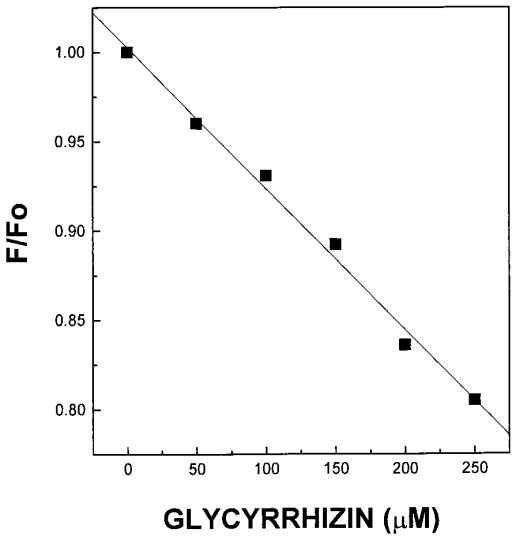

FIG. 3. Sensitivity of dansyl-thrombin emission intensity to GL. Dansyl thrombin (4 U/ml) was titrated with GL (pH 7.4) as described in Materials and Methods.

tory action of hirudin on thrombin-catalyzed hydrolysis of S-2238. Here, we confirm that the inhibitory effect of hirudin on thrombin was gradually displaced by GL (Fig. 4). This effect was due to competition between GL and hirudin for thrombin's anion binding exosite with apparent Kis ranging from 200 to 600 μM GL.

DISCUSSION

Many thrombin inhibitors isolated from distinct sources have been described so far (26-29). Some of them affect thrombin's catalytic site while others bind to "thrombin anion binding exosites". These regions are clusters of positively charged residues located at two different poles of the protein molecule remote from the active site (18). One pole, known as "anion binding exosite 1" forms a recognition site for the carboxy-terminal acidic chain of hirudin and various physiological ligands, including fibrinogen (19), GPIb (20), thrombomodulin (21;22) and heparin cofactor II (23). The second positively charged patch is located at the top of the molecule, is referred to as "anion binding exosite 2" and is involved in heparin binding (30). Thus, different anti-thrombin compounds may affect thrombin's functions by exerting specific interactions with the enzyme.

Accordingly, glycyrrhizin was identified here as a compound, the first one isolated from plants, displaying most remarkable and striking effects on thrombin. Furthermore, substantial evidence was obtained to indicate that GL exerts its anti-thrombin activity through interactions with anion binding exosite 1. In fact: a) GL selectively inhibited thrombin-induced platelet aggregation and fibrinogen clotting, two events dependent on thrombin anion binding exosite 1; b) it did not block thrombin's catalytic activity upon small substrate S-2238; c) Like other molecules such as TR 52-69 (31), which interacts with thrombin via anion binding exosite 1, GL also increased thrombin activity toward its chromogenic substrate. In addition, GL also produced a change in the fluorescence emission of dansylated-thrombin which is consistent with conformation changes at the enzyme's active site; d) GL displaced hirudin binding and inhibitory effect to thrombin as seen with thrombin-catalyzed hydrolysis of S-2238.

Although inhibition of thrombin by GL was achieved in the μM range, suggesting that GL-thrombin interaction is of low affinity, the findings are not fortuitous and the data seem to be relevant. In fact, considering that glycerritinic acid (GA), the aglycon moiety of GL as well as glucoronic acid (GU), its dissaccharidic unit, were both devoid of anti-thrombin activities, GL-thrombin interaction proved to be dependent of the intact GL molecule. In this context, it has been shown that the interactions of many proteins with its non-proteic ligands (32-36), or the inhibition of thrombin by peptides derived from physiological molecules such as thrombin receptor-peptide (37) or glycoprotein GPIb (38), occurs in the μM to low mM range. Actually, SLe^X, a tetrasaccharide recently found in the surface of various cells, including platelets (14), has been identified as the physiological ligand for selectins with a Kd around 0.2 - 0.5 mM (9;39). The selectins, on the other hand, are molecules expressed in the surface of activated leukocytes, endothelial cells (9) and platelets (14), and shown to be involved in the initial phase of leukocyte adhesion as a necessary event for inflammatory response (9;10). Interestingly, Rao *et al.* (8) re-

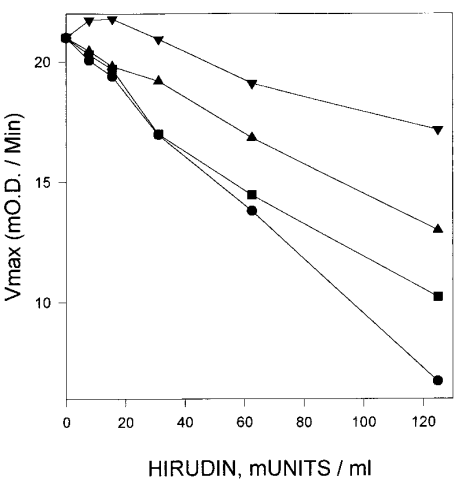

FIG. 4. Displacement of hirudin inhibition of thrombin by GL. Hirudin (0.0078-0.25 U/ml) was incubated at 37°C for 5 min with 50 mM Tris-HCl, 175 mM NaCl, pH 8.4 (●); 525 μM GL (■); 900 μM GL (▲) or 1800 μM GL (▼), followed by addition of thrombin (0.125 U/ml), as described in Materials and Methods. Data are mean of triplicate determinations.

cently performed a pharmacophoric search using 72,000 compounds, where GL was identified as the most similar to SLe^x. Like SLe^x, GL also expressed anti-inflammatory activity *in vivo* and *in vitro* and it inhibited selectin binding to SLe^x. As we found that SLe^x (up to 460 μM) did not affect thrombin clotting activities, it is concluded that SLe^x does not interact with thrombin anion binding exosite 1. Since thrombin is produced locally in response to an injury, a process that is followed by many events composing the final inflammatory acute response (40), it is attempting to speculate that the anti-inflammatory action of GL may be explained, at least in part, by its anti-thrombin effect. We finally conclude that the unexpected and unique anti-thrombin properties of glycyrrhizin shown in this work, may help to confirm the validity of performing pharmacophoric search in order to obtain additional compounds of important biological interest.

REFERENCES

1. Finney, R. S. H., and Somers, G. F. (1959) *J. Pharmacol.* **10,** 613–620.
2. Pompei, R., Flore, O., Marccialis, M. A., Pani, A., and Loddo, B. (1979) *Nature* **281,** 689–690.
3. Takeda, H., Ohta, K., Niki, H., Matsumoto, Y., Tanaka, K., Machimura, H., Yagame, M., Inoue, W., Endoh, M., and Kaneshige, H. (1991) *Tokai J. Exp. Clin. Med.* **16,** 3–4.
4. Ito, M., Sato, A., Hirabayashi, K., Tanabe, F., Shigeta, S., Baba, M., De Clercq, E., Nakashima, H., Yamamoto, N. (1988) *Antiviral Res.* **10,** 289–298.
5. Nishino, H., Kitagawa, K., and Iwashima, A. I. (1984) *Carcinogenesis* **5,** 1529. Jacob, G. S., Kirmaier, C., Abbas, S. Z., Howard, S. C., Steininger, C. N., Welply, J. K., Scudder, P. (1995) *Biochemistry* **34,** 1210–1217.
6. Numazaki, K., Nagata, N., Sato, T., Chiba, S. (1994) *J. Leuk. Biol.* **55,** 24–28.
7. Nakamura, T., Kuriyama, M., Ishihara, K., Matsumura, Y., and Miyamoto, T. (1991) *Lipids* **26,** 1297–1300.
8. Rao, B. N. N., Anderson, M. B., Musser, J. H., Gilbert, J. H., Schaefer, M. E., Foxall, C., and Brandley, B. K. (1994) *J. Biol. Chem.* **269,** 19663–19666.
9. Mc Ever, R. P. (1991) *Thromb. Haemost.* **66,** 80–87.
10. Bevilacqua, M. P. (1993) *Annu. Rev. Immunol.* **11,** 767–804.
11. Foxall, C., Watson, S. R., Dowbenko, D., Fennie, C., Lasky, L. A., Kiso, M., Hasegawa, A., Asa, D., and Brandley, B. K. (1992) *J. Cell Biol.* **117,** 895–902.
12. Bertozzi, C. R., Fukuda, S., and Rosen, S. D. (1995) *Biochemistry* **34,** 14271–14278.
13. Yuen, C-T., Bezouska, K., O' Brien, J., Stoll, M., Lemoine, R., Lubineau, A., Kiso, M., Hasegawa, A., Bockovich, N. J., Nicolau, K. C., Feizi, T. (1994) *J. Biol. Chem.* **269,** 1595–1598.
14. Cooling, L. L., Zhang, D. S., Walker, K. E., and Koerner, T. A. (1995) *Glycobiology* **5,** 571–581.
15. Clemetson, K. J. (1995) *Thromb. Haemost.* **74,** 111–116.
16. Prado-Franceschi, J., and Vital-Brazil, O. (1981) *Toxicon* **19,** 875–890.
17. Faili, A., Randon, J., Francischetti, I. M. B., Vargaftig, B. B., and Hatmi, M. (1994) *Biochem. J.* **298,** 87–91.
18. Guillin, M. C., Bezeaud, A., Bouton, M. C., Jandrot-Perrus, M. (1995) *Thromb. Haemost.* **74,** 129–133.
19. Naski, M. C., Fenton, J. W., Maraganore, J. M., Olson, S. T., Shafer, J. A. (1990) *J. Biol. Chem.* **265,** 13484–13489.
20. Jandrot-Perrus, M., Clemetson, K. J., Huisse, M. G., Guillin, M. C. (1992) *Blood* **80,** 2781–2786.
21. Tsiang, M., Lentz, S. R., and Sadler, J. E. (1992) *J. Biol. Chem.* **267,** 6164–6170.
22. Wu, Q., Tsiang, M., Lentz, S. R., and Sadler, J. E. (1992) *J. Biol. Chem.* **267,** 7083–7088.
23. Tollefsen, D. M. (1995) *Thromb. Haemost.* **74,** 1209–1214.
24. Ribeiro, J. M. C., Schneider, M., and Guimarães, J. A. (1995) *Biochem. J.* **308,** 243–249.
25. Berliner, L. J., and Shen, Y. Y. L. (1977) *Thromb. Res.* **12,** 15–25.
26. Maraganore, J. M. (1993) *Thromb. Haemost.* **70,** 208–211.
27. Zingali, R. B., Jandrot-Perrus, M., Guillin, M. C., and Bon, C. (1993) *Biochemistry* **32,** 10794–10802.
28. Noeske-Jungblut, C., Haendler, B., Donner, P., Alagon, A., Possani, L., and Schleuning, W. (1995) *J. Biol. Chem.* **270,** 28629–28634.
29. Maffrand, J. P. (1992) *Nouv. Rev. Fr. Hematol.* **34,** 405–419.
30. Sheehan, J. P., and Sadler, J. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5518–5522.
31. Liu, L., Vu, T., Esmon, C. T., Coughlin, S. R. (1991) *J. Biol. Chem.* **266,** 16977–16980.
32. Sauter, N. K., Bednarski, M. D., Wurzburg, B. A., Hanson, J. E., Whitesides, G. M., Skehel, J. J., and Wiley, D. C. (1987) *Biochemistry* **28,** 8388–8396.
33. Lee, R. T., Ichikawa, Y., Fay, M., Drickamer, K., Shao, M.-C., and Lee, Y. C. (1991) *J. Biol. Chem.* **266,** 4810–4815.
34. Tong, P. Y., Gregory, W., Kornfeld, S. (1989) *J. Biol. Chem.* **264,** 7962–7969.
35. Lewis, S. D., Shafer, J. A., Goldstein, I. J. (1976) *Arch. Biochem. Biophys.* **172,** 689–695.
36. Jacob, G. S., Kirmaier, C., Abbas, S. Z., Howard, S. C., Steininger, C. N., Welply, J. K., and Scudder, P. (1995) *Biochemistry* **34,** 1210–1217.
37. Bouton, M. C., Jandrot-Perrus, M., Moog, S., Cazenave, J. P., Guillin, M. C., and Lanza, F. (1995) *Biochem. J.* **305,** 635–641.
38. De Marco, L., Mazzucato, M., Masotti, A., and Ruggeri, Z. M. (1994) *J. Biol. Chem.* **269,** 678–6484.
39. Nelson, R. M., Dolich, S., Aruffo, A., Cecconi, O., Bevilacqua, M. P. (1993) *J. Clin. Invest.* **91,** 1157–1166.
40. Marcus, A. J., and Safier, L. (1993) *FASEB J.* **7,** 516–522.