The Effect of an Inhibitor of the Thrombin–Fibrinogen Reaction on Thrombin-Induced Blood-Platelet Aggregation

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We have reported the purification and some characteristics of a macromolecular inhibitor of the thrombin–fibrinogen reaction (Flengsrud *et al.*, 1972). The inhibitor had no effect on the esterase activity of thrombin when toluene-*p*-sulphonyl-arginine methyl ester was the substrate, and probably acted on the thrombin–fibrinogen complex (Flengsrud *et al.*, 1972).

Thrombin has a pronounced effect on blood platelets (Lüscher, 1956, 1967; Brayton & Zucker, 1957). Platelets contain fibrinogen as well as other proteins that are substrates for or affected by thrombin (Zieve & Greenough, 1969; Salzman & Levine, 1971; Ganguly, 1972; Baenziger *et al.*, 1971; Nachman, 1968; Cohen *et al.*, 1969; Brodie *et al.*, 1972). Brodie *et al.* (1972) found that incubation of intact platelets with thrombin resulted in the release of thrombin-sensitive protein from the platelet membrane. Cohen *et al.* (1969) purified thrombosthenin M (a contractile protein) from the membrane of horse blood platelets and showed that incubation of thrombosthenin M with thrombin resulted in the appearance of a new C-terminal amino acid in the protein.

The principal substrate for thrombin in platelet aggregation is of great interest. The role of fibrinogen in this reaction is still unclear in spite of considerable investigation (Castaldi & Caen, 1965; Morse *et al.*, 1965; Caen, 1965; Solum, 1966; Brown *et al.*, 1972). If the thrombin–fibrinogen reaction were important in the aggregation of platelets by thrombin, an inhibitor of the thrombin–fibrinogen reaction might be expected to inhibit platelet aggregation also.

We report here that the inhibitor described by Flengsrud *et al.* (1972) has only a very small effect in a thrombin-induced platelet-aggregation system even when fibrinogen is added.

Materials and methods

The preparations of bovine thrombin (Topostasine; Hoffmann–LaRoche Ltd., Basle, Switzerland), human fibrinogen (Kabi, Stockholm, Sweden) and the buffers, including veronal-buffered saline (VBS medium), have been described (Flengsrud *et al.*, 1972).

The inhibitor was obtained from rat liver and purified to homogeneity in analytical disc-gel electrophoresis as reported by Flengsrud *et al.* (1972). The final preparations contained 10–30 µg of protein/ml

as determined by the method of Lowry *et al.* (1951). Platelet suspensions were prepared by the method of Haslam (1964) by using blood drawn from healthy donors. The suspensions contained about 5×10^5 platelets/µl (Brecher & Cronkite, 1950).

Platelet aggregation was followed by recording the galvanometer deflection in an EEL titrator (Unigalvo, type 20; Evans Electroelenium, Halstead, Essex, U.K.) based on the method of Born & Cross (1962). Two different incubation mixtures were used: (a) 1.0 ml of platelet suspension + 0.8 ml of VBS medium; (b) 1.0 ml of platelet suspension + 0.8 ml of VBS medium + 0.1 ml of fibrinogen solution (7 mg/ml). After incubation (for 2 min at 37°C) 0.2 ml of thrombin–electrolyte solution (Eika, 1971) was added to give a final thrombin concentration of 0.05 unit/ml and the aggregation followed.

The inhibitor was tested by substituting solutions of it for VBS medium in the above systems.

Fig. 1. Platelet aggregation induced by thrombin

Platelet suspension (1 ml) was incubated with 0.8 ml of VBS medium for 2 min at 37°C. Thrombin–electrolyte solution (0.2 ml) was added (↓) and the aggregation followed as described under ‘Materials and methods’. ●, Final thrombin concentration 0.025 unit/ml; ○, final thrombin concentration 0.05 unit/ml; Δ, control (electrolyte solution without thrombin).

Fig. 2. Effect of inhibitor on platelet aggregation

Platelet suspension (1 ml) was incubated for 2 min at 37°C with 0.8 ml of VBS medium or inhibitor solution (inhibitory activity 64% in the thrombin-fibrinogen system). The 0.2 ml of thrombin (final concentration 0.05 unit/ml) was added and the aggregation followed as described under 'Materials and methods'. ●, With inhibitor (mean of three experiments); ○, control (mean of two experiments).

The effect of the inhibitor on the thrombin-fibrinogen reaction was determined as described by Flengsrud *et al.* (1972). The inhibitor activity was calculated from a standard curve based on thrombin dilutions.

Results and discussion

The time-course of platelet aggregation (i.e. galvanometer deflexion) obtained with 0.05 unit of thrombin/ml differs substantially from that obtained with 0.025 unit/ml (Fig. 1). This difference corresponds to that obtained if an inhibitor producing a 50% inhibition of thrombin action had been added. With concentrations of the inhibitor described here, however, which inhibited the thrombin-fibrinogen reaction by more than 60%, only minimal (10–12%) inhibition of platelet aggregation was observed (Fig. 2). The inhibition was also unchanged when fibrinogen (incubation mixture *b*, final concentration 0.33 mg/ml) was added to the platelet incubation mixture. When the inhibitor was incubated for the same length of time as required for the platelet-aggregation experiments, and then tested in a thrombin-fibrinogen system, it was fully active. Its lack of effect on platelet aggregation was therefore not due to thermal or other instability.

Our results suggest that the reaction of thrombin with plasma fibrinogen is unimportant in platelet aggregation. Platelet fibrinogen, however, is apparently somewhat different from plasma fibrinogen (Solum & Lopaciuk, 1969), probably in its carbohydrate component (Ganguly, 1972). This might influence the binding of the inhibitor to the thrombin-fibrinogen complex and render it less active.

A more likely explanation may be that the principal substrate of thrombin in triggering platelet aggregation is another protein and that the reaction with fibrinogen is unimportant in this connexion. Brown *et al.* (1972) compared the action of Arvin and thrombin on blood platelets and arrived at the same conclusion. Gross *et al.* (1963) reported that platelets from patients with congenital afibrinogenemia, in which fibrinogen could not be detected with immunological techniques, were aggregated by thrombin. This suggests that another substrate is essential for the effect of thrombin on platelets.

- Baenziger, N. L., Brodie, G. N. & Majerus, P. W. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68**, 240–243
- Born, G. V. R. & Cross, M. J. (1962) *J. Physiol. (London)* **168**, 178–195
- Brayton, R. G. & Zucker, M. B. (1957) *Proc. Soc. Exp. Biol. Med.* **96**, 418–421
- Brecher, G. & Cronkite, E. P. (1950) *J. Appl. Physiol.* **3**, 365–377
- Brodie, G. N., Baenziger, N. L., Chase, L. R. & Majerus, P. W. (1972) *J. Clin. Invest.* **51**, 81–87
- Brown, C. H., III, Bell, W. R., Schreiner, D. P. & Jackson, D. P. (1972) *J. Lab. Clin. Med.* **79**, 758–769
- Caen, J. P. (1965) *Nature (London)* **205**, 1120–1121
- Castaldi, P. A. & Caen, J. (1965) *J. Clin. Pathol.* **18**, 579–585
- Cohen, J., Bohak, Z., Vries, D. A. & Katchalski, E. (1969) *Eur. J. Biochem.* **10**, 388–394
- Eika, C. (1971) *Scand. J. Haematol.* **8**, 216–222
- Flengsrud, R., Østerud, B. & Prydz, H. (1972) *Biochem. J.* **129**, 83–89
- Ganguly, P. (1972) *J. Biol. Chem.* **247**, 1809–1816
- Gross, R., Schwick, G., Lang, N., Nies, D., Rahn, B., Becker, M. & Hengstmann, H. (1963) *Klin. Wochenschr.* **41**, 695–706
- Haslam, R. J. (1964) *Nature (London)* **202**, 765–768
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lüscher, E. F. (1956) *Vox Sang.* **1**, 133–154
- Lüscher, E. F. (1967) *Brit. J. Haematol.* **13**, 1–9
- Morse, E. E., Jackson, D. P. & Conley, C. L. (1965) *J. Clin. Invest.* **44**, 809–816
- Nachman, R. (1968) *Semin. Hematol.* **5**, 18–31
- Salzman, E. W. & Levine, L. (1971) *J. Clin. Invest.* **50**, 131–141
- Solum, N. O. (1966) *Scand. J. Clin. Lab. Invest.* **18**, 577–587
- Solum, N. O. & Lopaciuk, S. (1969) *Thromb. Diath. Haemorrh.* **21**, 428–440
- Zieve, P. D. & Greenough, W. B. (1969) *Biochem. Biophys. Res. Commun.* **35**, 462–466