Interaction of Factor VIII–von Willebrand Factor with phospholipid vesicles

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The interaction of Factor VIII–von Willebrand Factor with phospholipid vesicles has been studied by using sucrose-density-gradient ultracentrifugation. When purified Factor VIII–von Willebrand Factor was run alone, Factor VIII activity and Factor VIII_{R-AG} sedimented together to the lower half of the tube. Addition of phosphatidylserine/phosphatidylethanolamine vesicles at concentrations above 250 µg/ml resulted in complete separation of Factor VIII activity and Factor VIII_{R-AG}, the former appearing with the phospholipid on the top of the tube and the latter sedimenting as before. This separation was obtained even in the presence of proteinase inhibitors. Activation of Factor VIII–von Willebrand Factor by thrombin resulted in formation of a slow sedimenting component containing essentially all the Factor VIII activity, whereas the Factor VIII_{R-AG} sedimented towards the bottom of the tube as before. The thrombin-induced Factor VIII activity was strongly bound to phospholipid vesicles as determined by density-gradient centrifugations at various Factor VIII concentrations and low concentrations of phospholipid. Based on certain assumptions a dissociation constant of 2.5 nM was calculated, a mechanism for the formation *in vivo* of the Factor X-activator complex is suggested.

The Factor VIII coagulant activity of normal plasma is associated with a series of high-molecular-weight complexes also containing von Willebrand Factor-related activities. The major antigen of the Factor VIII–von Willebrand Factor complexes, usually called Factor VIII-related antigen (Factor VIII_{R-AG}), seems to be related only to the von Willebrand Factor part of the complexes. In agreement with that, available data indicate that the main part of the protein material in the complexes is von Willebrand Factor-related. By using several different techniques (Weiss *et al.*, 1972; Weiss & Hoyer, 1973; Cooper *et al.*, 1973; Baugh *et al.*, 1974) it has been possible to demonstrate separation between Factor VIII activity and Factor VIII_{R-AG}. In most of those cases the separated Factor VIII activity has been associated with only very small amounts of protein material.

When the Factor VIII/von Willebrand Factor is incubated with low concentrations of thrombin there is initially a strong increase in the procoagulant activity, which later is followed by a more slowly occurring inactivation. Both the activation and the subsequent inactivation reactions are probably processes of significance *in vivo*. The K_m for the

activation reaction is very low (Brodén *et al.*, 1980), suggesting that thrombin has a very high affinity for Factor VIII. The activation by thrombin has also been shown to result in separation of Factor VIII activity and Factor VIII_{R-AG} (Switzer & McKee, 1977).

In the coagulation cascade Factor VIII participates in activation of Factor X together with activated Factor IX and phospholipid. The main function of Factor VIII seems to be to increase the speed of the reaction. The interaction of Factor VIII with phospholipid has been studied previously, although with rather poorly defined phospholipid mixtures. It has been shown (Hemker *et al.*, 1970) that Factor VIII activity is bound to phospholipid in the absence and at a low level of Ca^{2+} and that high concentrations of Ca^{2+} induced dissociation.

In the present study the interaction of native as well as thrombin-activated Factor VIII–von Willebrand Factor with phospholipid vesicles of defined composition has been followed by using sucrose-density-gradient centrifugation.

Materials and methods

Materials

The phosphatidylserine and phosphatidylethanolamine preparations were obtained from Sigma

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Chemical Co., St. Louis, MO, U.S.A. Phosphatidylserine was prepared from brain and was more than 98% pure. Phosphatidylethanolamine was from brain and was more than 95% pure. It was delivered as a solution in chloroform. Phosphatidylcholine was from Supelco Inc., Bellefonte, PA, U.S.A., prepared from egg, and was more than 95% pure. It was delivered in benzene solution. Di[1- ^{14}C]-palmitoylphosphatidylcholine was obtained from New England Nuclear, Boston, MA, U.S.A., in toluene/benzene solution. The specific radioactivity was 100 Ci/mol. The di-isopropyl fluorophosphate was from Aldrich. Trasylol was obtained from FBA Pharmaceuticals, New York, NY, U.S.A.

The Factor VIII-von Willebrand Factor preparation was made by gel filtration on Bio-Gel A 15M (Bio-Rad Laboratories, Richmond, CA, U.S.A.) of Kabi high-purity Factor VIII concentrate. The void-volume fractions were collected and directly used for sucrose-density-gradient experiments or thrombin activation. The specific activity was 10–20 units/mg of protein and the ratio of Factor VIII_{R-AG}/Factor VIII activities was approx. 3. Human thrombin was prepared by activation of prothrombin with tissue thromboplastin followed by separation on Amberlite IRC-50 and SP-Sephadex ion-exchange chromatography. The specific activity was 2650 NIH units/mg of protein. Russell's Viper Venom was purchased from Sigma Chemical Co.

Preparation of phospholipid vesicles

To 4.5 mg of phosphatidylserine in a polypropylene tube was added 4.5 mg of phosphatidylethanolamine in chloroform solution and 15 μg of ^{14}C -labelled phosphatidylcholine in toluene/benzene solution. After mixing, the solvents were evaporated by immersing the tube in a water bath and blowing N_2 through. When the tube was completely dry, 6 ml of 0.02 M-imidazole/0.15 M-NaCl buffer, pH 7.3, was added and N_2 was bubbled through the solution for about 5 min. Then the tube was put in an ice bath and the solution was sonicated for 3×5 min in a sonicator (Heatsystems-Ultrasonics Inc., Plainview, NY, U.S.A.). Interruptions were made every fifth minute to cool the solution for 1 or 2 min. The solution was then centrifuged for 1 h at 100 000 g and after that the concentration of phospholipid remaining in solution was determined and buffer added to give a phospholipid concentration of 1.0 mg/ml. Phosphatidylserine/phosphatidylcholine vesicles were prepared in the same way.

Sucrose-density-gradient centrifugation

Sucrose gradients were made with an ISCO model 570 gradient former. The gradients were from 30% to 5% sucrose and the buffer used was 0.02 M-imidazole/0.15 M-NaCl, pH 7.3. The centrifuge tubes contained 13.0 ml of gradient solution

and 0.4 ml or 0.5 ml of sample solution was applied on the top. The tubes were then put in the ultracentrifuge (Beckman) and run for 16 h at 5°C and 36 000 rev./min in an SW 40 rotor corresponding to 160 000 g. After the run, holes were made in the bottom of the tubes and 1.0 ml fractions were collected by gravity and analysed.

Factor VIII determinations

Factor VIII coagulant activity was determined by the one-stage assay of Langdell *et al.* (1953) using haemophilic plasma with cephalin and kaolin as activator. The assay was not affected by the amounts of phospholipid present in the density-gradient-centrifugation experiments.

Factor VIII_{R-AG} was assayed by rocket immunoelectrophoresis (Laurell, 1966).

Phospholipid coagulant activity

Stypven clotting times were measured as described by Sandberg & Andersson (1976) using Russell's Viper Venom. Platelet-poor plasma was prepared by centrifugation of platelet-rich plasma for 20 min at 3000 g.

Radioactivity measurements

Concentration of ^{14}C -labelled material was measured in a Packard Tri-carb liquid-scintillation counter with PCS as scintillant (Amersham, Arlington Heights, IL, U.S.A.).

Results

Preparation and properties of phospholipid vesicles

The sizes and properties of phospholipid vesicles differ considerably depending on the way they are prepared (Zwaal, 1978). Phosphatidylserine/phosphatidylcholine and phosphatidylserine/phosphatidylethanolamine vesicles containing ^{14}C -labelled phosphatidylcholine were prepared as described above and sonicated between 3 and 15 min in 3 min periods with cooling periods in between.

The density-gradient centrifugation of the various preparations showed that in all cases the main part of the phospholipid material, as measured by ^{14}C counting, appeared in the upper half of the centrifuge tube. However, the positions of the peaks of material varied considerably as well as the distribution pattern. Fig. 1 shows the pattern obtained with phosphatidylserine/phosphatidylethanolamine vesicles prepared by sonication for 15 min. In this case there is essentially one peak of material banding close to the top of the tube. When shorter sonication times were used more heterogeneous patterns were obtained, with peaks of material appearing both on the top of the tube and at positions corresponding to fractions 7–10. As it is advantageous to have the phospholipid vesicles behave differently from the

proteins, we chose to use vesicles that appeared on the top of the tube.

To remove traces of larger vesicles those vesicle preparations were also subjected to centrifugation for 1 h at 100000 *g*. Gel filtration on Bio-gel A 150 M of the phosphatidylserine/phosphatidylethanolamine vesicle preparation showed one main peak containing more than 90% of the material with a K_{av} of 0.78.

Determination of procoagulant activity in the Russell-Viper-Venom assay showed that, at a phospholipid concentration of 50 $\mu\text{g}/\text{ml}$, the clotting times were 18.0 s for the phosphatidylserine/phosphatidylethanolamine vesicles and 32.5 s for the phosphatidylserine/phosphatidylcholine vesicles corresponding to about one-quarter of the activity of the phosphatidylserine/phosphatidylethanolamine vesicles. The time of sonication only had minor influence on the procoagulant activity of the vesicles.

Interaction between Factor VIII–von Willebrand Factor and phospholipid vesicles

When Factor VIII–von Willebrand Factor complex, purified by gel filtration of Factor VIII concentrate, was studied by sucrose-density-gradient centrifugation, the pattern shown in Fig. 2(a) was obtained. In agreement with previous studies (Weiss & Kochawa, 1970) it is found that the Factor VIII activity and Factor VIII_{R-Ag} behave identically and sediment to the lower half of the centrifuge tube. Recoveries of Factor VIII activity were between 29 and 38% and recoveries of Factor VIII_{R-Ag} between 81 and 96% (four experiments).

Fig. 2. Sucrose-density-gradient (30–5%) centrifugation of Factor VIII–von Willebrand Factor with and without phospholipid

Sample volume was 0.4 ml and 1.0 ml fractions were collected. Buffer was 0.02 M-imidazole/0.15 M-NaCl, pH 7.3. (a) Sample was purified Factor VIII–von Willebrand Factor (5.6 units of Factor VIII activity/ml). O, Factor VIII activity; ▲, Factor VIII_{R-Ag}. (b) Sample was purified Factor VIII–von Willebrand Factor (5.6 units of Factor VIII activity/ml) and phosphatidylethanolamine/phosphatidylserine vesicles (0.5 mg/ml). O, Factor VIII activity; ●, ^{14}C radioactivity; ▲, Factor VIII_{R-Ag}. (c) O, purified Factor VIII–von Willebrand Factor (5.6 units of Factor VIII activity/ml) and phosphatidylethanolamine/phosphatidylserine vesicles (250 $\mu\text{g}/\text{ml}$); ▲, purified Factor VIII–von Willebrand Factor (5.6 units of Factor VIII activity/ml) and phosphatidylethanolamine/phosphatidylserine vesicles (125 $\mu\text{g}/\text{ml}$); ■, purified Factor VIII–von Willebrand Factor (5.6 units of Factor VIII activity/ml) and phosphatidylethanolamine/phosphatidylserine vesicles (62 $\mu\text{g}/\text{ml}$).

Fig. 1. Sucrose-density-gradient (30–5%) centrifugation of phosphatidylserine/phosphatidylethanolamine vesicles (0.5 mg/ml)

Sample volume was 0.4 ml and 1.0 ml fractions were collected. Buffer was 0.02 M-imidazole/0.15 M-NaCl, pH 7.3. O, ^{14}C radioactivity, ●, procoagulant activity.

Fig. 3. Sucrose-density-gradient centrifugation (30–5%) of thrombin-activated Factor VIII–von Willebrand Factor with and without phospholipid.

Sample volume was 0.4 ml and 1.0 ml fractions were collected. Buffer was 0.02 M-imidazole/0.15 M-NaCl, pH 7.3. (a) Sample was thrombin-activated Factor VIII–von Willebrand Factor (26 units of Factor VIII activity/ml). O, Factor VIII activity; ▲, Factor VIII_{R-AG}. (b) ▲, Thrombin-activated Factor VIII–von Willebrand Factor (26 units of Factor VIII activity/ml) and phosphatidylethanolamine/phosphatidylserine vesicles (10 µg/ml); O, thrombin-activated Factor VIII–von Willebrand Factor (26 units of Factor VIII activity/ml) and phosphatidylethanolamine/phosphatidylserine vesicles (2.5 µg/ml) (containing 9 ng/ml PC). (c) Sample was thrombin-activated Factor VIII–von Willebrand Factor (26 units of Factor VIII activity/ml) and phosphatidylethanolamine/phosphatidylserine

When the Factor VIII–von Willebrand Factor complex is mixed with phosphatidylserine/phosphatidylethanolamine vesicles at a concentration of 0.5 mg/ml and subjected to density-gradient centrifugation the pattern shown in Fig. 2(b) was obtained. Now the Factor VIII activity is found at the top of the tube together with the phospholipid vesicles, whereas the Factor VIII_{R-AG} sediments to the lower half of the tube as before. Factor VIII, but not von Willebrand Factor, is bound to the phospholipid vesicles. No Factor VIII_{R-AG} can be detected at the top of the tube, indicating that the complex Factor VIII–von Willebrand Factor is not bound to the phospholipid vesicles. Recoveries of Factor VIII activity were between 51 and 72% and recoveries of Factor VIII_{R-AG} between 77 and 92% (five experiments). This experiment was also repeated with 1 mM-di-isopropyl fluorophosphate and 5 units of Trasylol/ml added to the sample solution and 0.1 mM-di-isopropyl fluorophosphate in gradient solution to inhibit potential proteinase activity. The pattern obtained was identical with that obtained in the absence of di-isopropyl fluorophosphate and Trasylol.

By decreasing the concentration of phospholipid it was possible to find a region where part of Factor VIII activity was associated with phospholipid and part with Factor VIII_{R-AG}. Fig. 2(c) shows the Factor VIII activity patterns obtained at the phospholipid concentrations 250, 125 and 62 µg/ml. In all these runs Factor VIII_{R-AG} is found in tubes 1–5.

Interaction of thrombin-activated Factor VIII with phospholipid vesicles

Activation of purified Factor VIII–von Willebrand Factor with thrombin at a concentration of 10⁻³ units/ml resulted in an increase in the apparent one-stage coagulant activity reaching the maximum, corresponding to a 6-fold increase, after about 1.5 h. Analysis of this thrombin-activated Factor VIII–von Willebrand Factor by density-gradient centrifugation yielded the pattern shown in Fig. 3(a), where it is seen that the Factor VIII coagulant activity is now separated from the Factor VIII_{R-AG} and appears in the upper half of the tube. This is in agreement with what has been observed previously (Weiss & Kochawa, 1970). The recovery of Factor VIII activity was between 12 and 18% (three experiments). This low recovery is not surprising as thrombin-activated Factor VIII is known to be very labile. When the thrombin-activated Factor VIII–von Willebrand sample is mixed with phosphatidylserine/phosphatidylethanolamine vesicles at a

vesicles (0.5 mg/ml). Both sample and gradient solution concentrations 5 mM with respect to CaCl₂. O, Factor VIII activity; ●, ¹⁴C radioactivity.

concentration of 0.5 mg/ml and run in the density-gradient centrifugation all the Factor VIII activity is found associated with the phospholipid at fractions 11–13 and Factor VIII_{R-AG} is found at the same position as before at fractions 2–5. Recoveries of Factor VIII activity were between 41 and 61% (five experiments). By using lower concentrations of phospholipid it was possible to reach a region where part of Factor VIII activity was associated with phospholipid and part was not (Fig. 3b). This concentration of phospholipid was found to be about 10-fold lower than when native Factor VIII–von Willebrand Factor complex was studied (Fig. 2b). Phospholipid seems to have a stabilizing effect on the activity of the thrombin-activated Factor VIII as recoveries of activity are considerably higher when phospholipid is present.

To estimate a binding constant between the thrombin-activated Factor VIII and the phospholipid a series of density-gradient runs were performed at the phospholipid concentration 5 µg/ml and at various concentrations of thrombin-activated Factor VIII. The relative amount of free and phospholipid-bound Factor VIII at various Factor VIII concentrations was determined and is shown in a Scatchard plot in Fig. 4. For the calculations of the molar concentrations of thrombin-activated Factor VIII, the values for bovine Factor VIII (Vehar & Davie, 1980) were used, that is mol. wt. 90000 and a concentration in normal plasma of 0.2 µg/ml for the native protein. The corresponding values are not yet available for the human protein. Further, the molar concentration of thrombin-activated Factor VIII has been set equal to the starting molar concentration of native Factor VIII. This is an approximation as the molar concentration of thrombin-activated Factor VIII probably is somewhat lower because of incomplete activation and degradation reactions and

thus the dissociation constant obtained is likely to be somewhat high. The Scatchard plot seems to be linear indicating a single class of sites. The value obtained for the dissociation constant was 2.5 nM for the phospholipid concentration 5 µg/ml, showing a very strong association between the thrombin-activated Factor VIII and phospholipid.

The effect of Ca²⁺ on the binding of thrombin-activated Factor VIII to phospholipid was studied by making the centrifugation medium and the sample solution 5 mM with respect to CaCl₂. The pattern obtained is shown in Fig. 3(c). Both phospholipid and Factor VIII activity now peak at fractions 7–8. When phospholipid was run alone but under otherwise the same conditions it also appeared at the same fractions. In the presence of Ca²⁺ the vesicles probably aggregate, resulting in formation of larger vesicles that sediment faster. This is also supported by the observation that the phospholipid vesicle solution became opalescent when CaCl₂ solution was added. The thrombin-activated Factor VIII is evidently still bound to phospholipid even if the vesicles are larger and Ca²⁺ is present. Whether the binding affinity of Factor VIII for the phospholipid was altered by the presence of Ca²⁺ was not determined.

Discussion

The nature of the Factor VIII–von Willebrand Factor complex has been the subject of some controversy. Most investigators in the field believe that Factor VIII and von Willebrand Factor are separate molecules but are present in the circulation as non-covalently linked complexes of various sizes. This is supported by various experiments demonstrating separation between Factor VIII activity and von Willebrand Factor-related protein (Weiss *et al.*, 1972; Weiss & Hoyer, 1973; Cooper *et al.*, 1973; Baugh *et al.*, 1974; Holmberg & Ljung, 1978; Horowitz *et al.*, 1979). It has been questioned (Switzer & McKee, 1976, 1977), however, if those separations obtained are not the result of limited proteinase action cleaving off the Factor VIII activity part from a Factor VIII–von Willebrand Factor molecule. This opinion has been supported by observations that the separation of Factor VIII activity from von Willebrand Factor protein is influenced by the presence of proteinase inhibitors, less separation being obtained in the presence of proteinase inhibitors (Beck *et al.*, 1976).

The results obtained in the present study show that the Factor VIII–von Willebrand Factor complex can be dissociated through binding of the Factor VIII part to phospholipid vesicles. The presence or absence of proteinase inhibitors does not change the patterns obtained. These data thus support the concept of Factor VIII and von

Fig. 4. Scatchard plot of binding of thrombin-activated Factor VIII to phosphatidylethanolamine/phosphatidylserine vesicles (5 mg/ml)

Willebrand Factor being two separate molecules bound in a tight complex. It does not exclude, however, that proteinase activity may have been involved in the previously used methods to obtain separation of Factor VIII and von Willebrand Factor. The interaction between Factor VIII and phospholipid is probably stronger than any of the forces involved in most of the previous experiments resulting in separation. Thus it cannot be concluded from the experiments in the present study that the previous separations have not involved proteinase

VIII to phospholipid excludes simultaneous binding to von Willebrand Factor.

The main and so far only known biological function of Factor VIII is to participate in the activation of Factor X. This probably takes place on phospholipid-containing structures on the surface of released platelets. The data obtained in the present study, together with earlier observations, suggest that the sequence of events given below describes how Factor VIII is made part of the Factor X-activator complex.

action. The ability of thrombin, even at very low concentration, to cleave off a Factor VIII active part from the Factor VIII-von Willebrand Factor complex indicates that Factor VIII contains at least one especially sensitive bond, the cleavage of which results in separation from the von Willebrand Factor.

Binding of Factor VIII activity to phospholipid occurs both with the native Factor VIII-von Willebrand Factor complex, as well as with thrombin-activated Factor VIII. One important difference, however, is that thrombin-activated Factor VIII is bound at much lower concentrations of phospholipid. This is probably at least in part dependent on the fact that native Factor VIII binds strongly both to phospholipid and to von Willebrand Factor; more phospholipid must be added to effectively compete with von Willebrand Factor for Factor VIII binding. Thrombin-activated Factor VIII does not seem to be bound to von Willebrand Factor and thus less phospholipid is necessary to get binding.

The observation that the Factor VIII-von Willebrand Factor complex is not bound as such to phospholipid vesicles might suggest that the phospholipid-binding site in Factor VIII is identical with the von Willebrand Factor binding site. However, the presence of a strong phospholipid-binding site and no apparent von Willebrand Factor binding in thrombin-activated Factor VIII indicates that although the binding sites may be close to each other, they are not identical. Steric hindrance effects, for instance, might explain why binding of Factor

The first traces of thrombin formed splits one or several especially sensitive bonds in the Factor VIII, resulting in separation of activated Factor VIII (Factor VIII_a) from von Willebrand Factor. The activated Factor VIII is then rapidly bound to the surface of released platelets, where activated Factor IX can then be bound, resulting in formation of Factor X activator. It is likely that Factor VIII is present only transiently in non-complexed form. The observed instability of thrombin-activated Factor VIII, as well as the Factor VIII coagulant activities separated from von Willebrand Factor by various methods, may be related to the fact that the tertiary structure of Factor VIII is stable only when the protein is complexed to von Willebrand Factor or to membrane phospholipids.

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