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Comparison of the properties of phospholipid surfaces formed on HPA and L1 biosensor chips for the binding of the coagulation factor VIII

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Abstract

Binding of a coagulation factor VIII to phosphatidylserine-containing membranes is critical for exerting its cofactor activity. The use of surface plasmon resonance allows studying factor VIII interaction with immobilized phospholipids. In the present study we compared factor VIII-binding properties of phospholipid surfaces immobilized on L1 and HPA Biacore chips in the form of a flexible bilayer and rigid monolayer, respectively. We demonstrated that immobilized phospholipid surfaces with physiological contents of PS and PE formed on L1 but not on HPA chip closely mimic intact phospholipid vesicles in their factor VIII and thrombin-activated factor VIII (factor VIIIa) binding properties.

Keywords: Biosensors; Chip technology; Surface plasmon resonance detection; Factor VIII; Phospholipids; Phosphatidylserine; Phosphatidylethanolamine

1. Introduction

Factor VIII is an essential component of the intrinsic pathway of blood coagulation. In this pathway, thrombin-activated VIII (factor VIIIa) functions as a cofactor for the serine protease factor IXa. The formed membrane-bound complex (factor Xase) activates factor X to factor Xa. In its turn, factor Xa participates in activation of prothrombin into thrombin, the key enzyme of the coagulation cascade. The role of activated factor VIII is to increase the catalytic rate constant (k_{cat}) of factor X conversion into factor Xa by several orders of magnitude. Deficiency or defects in factor VIII results in a severe bleeding disorder hemophilia A, a genetic disease that occurs in one per 5000 males. The factor VIII protein consists of the homologous A and C domains and the unique B domain which are arranged in the order A1–A2–B–A3–C1–C2. Thrombin activates factor VIII by proteolysis at three specific sites [1]. Activated factor VIII is a heterotrimer A1/A2/A3–C1–C2 [2] in which domains A1 and A3 retain the metal ion linkage and the stable dimer A1/A3–C1–C2 is weakly associated with A2 subunit through electrostatic forces [2].

The major role of the phospholipid surface is to

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reduce interactions between the components of factor Xase complex from three- to two-dimensional space, which dramatically decreases K_m for factor X. The major physiological phospholipid surface is provided by membranes of activated platelets. Formation of factor VIII binding sites on platelets occurs only upon their activation by thrombin or other agonists [3,4]. This leads to the reorientation of phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the inner to the outer layer of the plasma membrane [5] to provide sufficient concentrations of PE and PS for the formation of factor VIII binding sites. Upon translocation of PS and PE to the outer membrane, these phospholipids may constitute 4–10 and 10–30% of the lipid surface, respectively [6,7]. Thus, in physiological membranes, the simultaneous presence of PS and PE is required for the formation of high affinity binding sites for factor VIII.

Consistent with observations made for membranes of activated platelets, synthetic membranes require inclusion of at least 4% PS to form factor VIII binding sites [8,9]. Addition of PE to synthetic vesicles containing the physiological concentration of PS (4%) dramatically increases the number of factor VIII binding sites [10]. The affinity of factor VIII for synthetic vesicles containing PS and PE is similar to that for activated platelets [9–11]. In addition, factor VIII-binding sites on PS-containing synthetic membranes, like those on activated platelets, were shown to be highly specific for factor VIII [12]. These findings justify a wide use of phospholipid vesicles as a model of physiological phospholipid surfaces for factor VIII binding and assembly of the Xase complex.

The use of plasmon resonance allows studying factor VIII interaction with immobilized phospholipid surfaces. It was shown that the hydrophobic alkanethiol-coated surface of a HPA chip provides formation of a flat rigid lipid monolayer upon immobilization of phospholipid vesicles [13]. In the resulting monolayer, the hydrophilic groups of phospholipids are directed towards the solution phase. In contrast, immobilization of vesicles on the surface of an L1 chip is mediated by interaction of the hydrophobic portions of phospholipids with the lipophilic groups covalently attached to the dextran-coated surface of the L1 chip. Characterization of phospholipid surfaces formed on L1 chips by atomic force microscopy and fluorescent microscopy revealed that liposomes fuse and form a flexible lipid bilayer on the chip surface [14]. In the present study we compared the factor VIII binding properties of PS-containing phospholipid surfaces immobilized on HPA and L1 Biacore chips in order to determine which surface most closely resembles the membrane of intact phospholipid vesicles.

2. Experimental

2.1. Protein purification

Plasma factor VIII was purified from therapeutic concentrates (American Red Cross, Rockville, MD, USA) [15]. Factor VIII concentration was determined by absorbance at 280 nm, using the extinction coefficient of 1.2 [16]. Activated factor VIII was prepared by treatment of factor VIII (2.2 μ*M*) with thrombin (0.08 μ*M*) for 8 min at 37°C in 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethenanesulfonic acid (Hepes), pH 7.4, 0.15 *M* NaCl (Hepes-buffered saline, HBS) containing 5 m*M* $CaCl_2$. The reaction was stopped by addition of hirudin (0.15 μ*M*) and lowering pH to 6.0 with 0.2 *M* 2-(*N*-morpholino)ethanesulfonic acid (MES). Purification of the A2 subunit was performed using ion-exchange chromatography of thrombin-activated heterotrimeric factor VIII (A1/A2/A3–C1–C2) on a Resource S column (Amersham Pharmacia Biotech) [17]. The protein concentration was determined by the method of Bradford [18] and its molar concentration was calculated based on the A2 molecular mass of 40 000 [19].

2.2. Preparation of phospholipid vesicles

Phospholipids PS, phosphatidylcholine (PC) and PE were purchased from Sigma (St. Louis, MO, USA). Phospholipid vesicles with varying PS and PE content, and the balancing content of PC were prepared as described [20].

2.3. Protein–phospholipid binding measurements using surface plasmon resonance

Supported PS/PC, PS/PC/PE or PC surfaces

were formed on HPA or L1 chips[1] (Biacore, Uppsala, Sweden) by incubating unilamellar vesicles at 400 μg/ml in HBS for 20 min at 22°C, which produced signals of approximately 1000 and 1200 resonance units (RU) for HPA and L1, respectively. The phospholipid-coated chips were blocked with 0.25 mg/ml BSA for 20 min.

Binding of factor VIII or factor VIIIa to phospholipid surfaces formed on HPA and L1 chips was measured using Biacore-3000 (Uppsala, Sweden), where 1 ng of protein bound per mm^2 of the sensor chip produces a resonance signal of 1000 RU. Binding and subsequent dissociation of factor VIII were measured for 10 and 8 min, respectively. The measurements for factor VIII were performed in HBS, pH 7.4, 5 m*M* $CaCl_2$ at 22°C and for factor VIIIa in 0.02 *M* MES, pH 6.0, 0.1 *M* NaCl, 5 m*M* $CaCl_2$ in the presence of 200 n*M* A2 subunit. In all experiments the flow-rate was 10 μl/min. To regenerate the chip, complete dissociation of bound ligands was achieved by addition of 10 m*M* NaOH for 30 s.

2.4. Calculation of the kinetic parameters from surface plasmon resonance data

The rate constants (k_{off}) for dissociation of factor VIII and factor VIIIa from phospholipid surfaces were determined by fitting the dissociation kinetics data to the following equation describing a single phase dissociation process:

$$dR/dt = -k_{off}R \quad (1)$$

where the surface plasmon resonance signal observed, *R*, is proportional to the formation of a complex between immobilized component and added ligand. It was shown [21] that:

$$dR/dt = k_{on}CR_{max} - (k_{on}C + k_{off})R \quad (2)$$

where R_{max} is the maximal binding capacity of the immobilized ligand surface expressed in RU and *C* is the concentration of protein in solution.

The k_{on} values were determined from individual association kinetics data using the integrated form of the rate Eq. (2):

$$R = Ck_{on}R_{max}\{1 - \exp[-(Ck_{on} + k_{off})t]\}/(Ck_{on} + k_{off}) \quad (3)$$

The values of k_{on} and R_{max} were derived from nonlinear regression analysis by fitting *R* vs. *t* to Eq. (3). The value of the k_{off} constant used in Eq. (3) was derived from the dissociation kinetics data fitted to Eq. (1). The values of equilibrium dissociation constants (K_d) were calculated as k_{off}/k_{on}. All the fitting procedures were performed using Sigmaplot 1.02 (Jandel Scientific). All the values of the kinetic parameters are the mean±standard deviation of the values derived from three independent kinetic measurements.

3. Results

3.1. Effect of PS content on factor VIII binding to phospholipid surfaces formed on HPA and L1 biosensor chips

Since intact synthetic PS/PC vesicles with the physiologically low content of PS (4%) are able to bind factor VIII [9], we tested whether phospholipid surfaces formed on the HPA and L1 chips have the same ability. Fig. 1 shows interaction of factor VIII with the phospholipid surfaces containing different PS concentrations and formed on HPA (A) or L1 (B) chips. As seen from Table 1, the kinetic parameters (k_{on}, k_{off}, and K_d) of factor VIII interaction with the lipid surfaces formed on HPA and L1 chips were similar at the PS concentrations (10 and 25%) exceeding the physiological level. In contrast, at the physiological PS concentration (4%), the signal produced by factor VIII binding to the surface formed on the HPA chip was close to the baseline, whereas the use of the L1 chip provided reliable measurements of factor VIII association and dissociation. The maximal binding capacity of this surface (R_{max}) was 0.4 fmol of factor VIII per mm^2 of the

[1]The HPA chip (Biacore) is a long-chain alkanethiol-coated chip designed to facilitate liposome adsorption to form polar lipid monolayers; the L1 chip (Biacore), chip is coated with lipophilically modified dextran and designed to capture liposomes in the form of lipid bilayers.

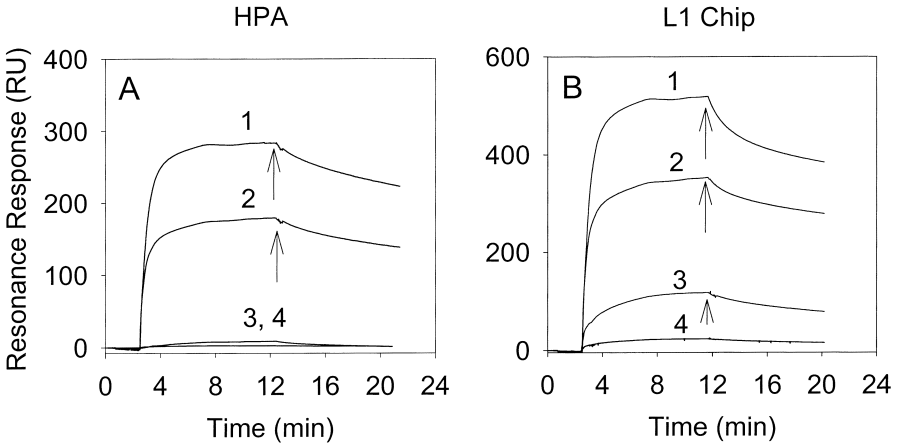

Fig. 1. Effect of the PS content in phospholipid surfaces formed on HPA and L1 chips on factor VIII binding. Interaction of factor VIII (20 n*M*) with phospholipid surfaces formed on HPA (A) and L1 (B) chips was studied as described in Section 2. Arrows indicate time points at which factor VIII solution was replaced with dissociation buffer. Curves 1, 2 and 3 represent factor VIII binding to the PS/PC surfaces containing 25, 10 and 4% of PS, respectively. Curve 4 represents control experiment and shows binding of factor VIII to 100% PC surfaces. The kinetic parameters were derived from association and dissociation kinetic curves as described in Section 2 and are presented in Table 1.

chip surface, which is 6.3 and 3.2 times lower than the corresponding R_{max} values for the surfaces containing 10 and 25% of PS. This PS-dependent decrease in R_{max} is quantitatively similar to that observed for small unilamellar vesicles in solution [9], suggesting that the phospholipid surface formed on the L1 chip behaves similarly to membranes of intact phospholipid vesicles. In a control experiment, factor VIII did not bind to 100% PC surfaces formed both on HPA and L1 chips (Fig. 1, curve 4), consistent with the ultimate requirement of the PS component in the membrane for the formation of factor VIII binding sites [8,9].

3.2. Effect of PE on factor VIII binding to PS-containing surfaces formed on HPA and L1 chips

Since it has been previously shown that inclusion of PE into vesicles containing 4% PS induces formation of additional factor VIII binding sites [10], we examined whether inclusion of PE to phospholipid surfaces formed on HPA and L1 chips will have a similar effect on factor VIII binding. As seen from Fig. 2, a significant (5.5-fold) increase in the number of factor VIII binding sites (R_{max}) upon inclusion of 20% PE was observed only for the lipid surface formed on the L1 chip. Noteworthy is that

Table 1
Parameters of factor VIII interaction with PS- and PE-containing surfaces formed on HPA and L1 chips[a]

Composition of the phospholipid surface	Kinetic constants					
	HPA chip			LI chip		
	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (n*M*)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (n*M*)
PS/PC (25/75)	$(4.36\pm0.2)\times10^5$	$(0.96\pm0.08)\times10^{-3}$	2.2±0.1	$(4.7\pm0.37)\times10^5$	$(1.03\pm0.02)\times10^{-3}$	2.2±0.17
PS/PC (10/90)	$(3.94\pm0.12)\times10^5$	$(1.3\pm0.03)\times10^{-3}$	3.26±0.13	$(4.2\pm0.22)\times10^5$	$(1.5\pm0.02)\times10^{-3}$	3.7±0.2
PS/PC (4/96)	No binding	$(2.3\pm0.18)\times10^5$	$(1.8\pm0.06)\times10^{-3}$	7.8±0.7		
PS/PC/PE (4/76/20)	No binding	$(2.7\pm0.15)\times10^5$	$(1.64\pm0.02)\times10^{-3}$	6.0±0.4		

[a] The parameters are derived from the kinetic curves shown in Figs. 1 and 2 as described in Section 2.

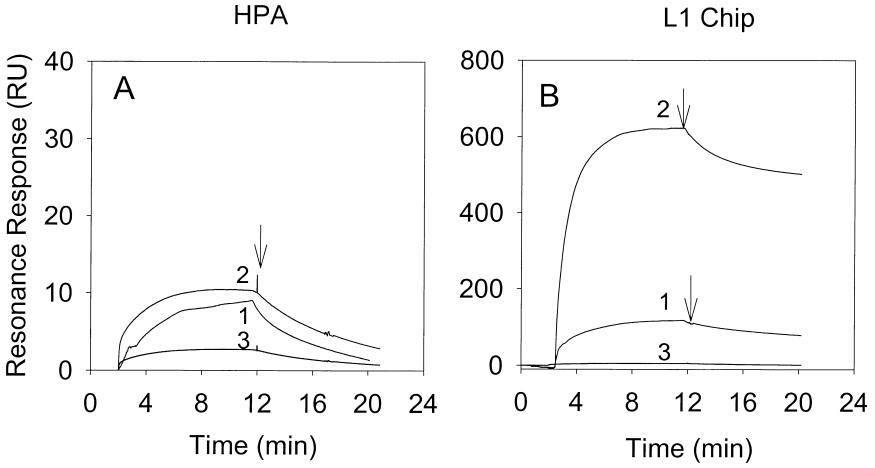

Fig. 2. Effect of inclusion of PE in the composition of PS/PC phospholipid surfaces formed on HPA and L1 chips on factor VIII binding. Interaction of factor VIII (20 n*M*) with phospholipid surfaces formed on HPA (A) and L1 (B) chips was studied as described in Fig. 1. Curves 1 and 2 represent factor VIII binding to phospholipid surfaces containing PS/PC (4/96) and PS/PC/PE (4/76/20), respectively. In the control experiment (curve 3), the binding of factor VIII to PC/PE (80/20) surfaces was measured as above. The kinetic parameters were derived from association and dissociation kinetic curves as described in Section 2 and are presented in Table 1.

this addition of PE did not affect K_d for factor VIII interaction with the phospholipid surface (Table 1). In the control experiment, no binding of factor VIII to the PC/PE (80/20) surface was observed (Fig. 2, curve 3).

3.3. Inclusion of PE increases affinity of thrombin-activated factor VIII for PS-containing surface formed on the L1 chip

The above experiments suggested that factor VIII-binding properties of phospholipid surfaces formed on the L1 chip resemble those of intact vesicles. Previously we found that factor VIIIa binds to intact PS/PC/PE (4/76/20) vesicles with an affinity (K_d = 0.7 n*M*) that is approximately 10-fold higher than that of non-activated factor VIII (K_d = 7.4 n*M*) [11]. To examine whether the PS/PC/PE surface formed on the L1 chip behaves similarly to intact vesicles in the binding of activated factor VIII, we determined factor VIIIa-binding parameters for immobilized PS/PC/PE (4/76/20) surface. Since heterotrimeric factor VIIIa (A1/A2/A3–C1–C2) is inactivated due to rapid dissociation of the A2 subunit, the measurements of factor VIIIa binding were performed under conditions preventing the loss of A2. Since the affinity of A2 for A1/A3–C1–C2 dimer is significantly higher at pH 6.0 than at pH 7.4 [22,23], and addition of the exogenous A2 subunit also suppresses dissociation, we studied the interaction of factor VIIIa with phospholipid surface at pH 6.0 and in the presence of 200 n*M* A2. It was previously demonstrated that, under these conditions, >85% of initial factor VIIIa activity is preserved within 90 min [11]. Thus, inactivation of factor VIIIa during the course of binding measurements was not significant and therefore the resonance response curves shown in Fig. 3 reflect interaction of integral heterotrimeric factor VIIIa with phospholipid surfaces formed on the L1 chip. The parameters of these interactions are shown in Table 2. The affinity of factor VIIIa binding to PS/PC/PE (4/76/20) surface (K_d = 0.7 n*M*) proved to be approximately 9 times higher than that of non-activated factor VIII (K_d = 6 n*M*, see Table 1). Since the effect of factor VIII activation on its affinity for intact PS/PC/PE (4/76/20) vesicles in solution was similar (10-fold [11]), we conclude that the factor VIIIa binding properties of the phospholipid surface formed on the L1 chip are similar to those of intact vesicles.

Noteworthy, that in the absence of PE component

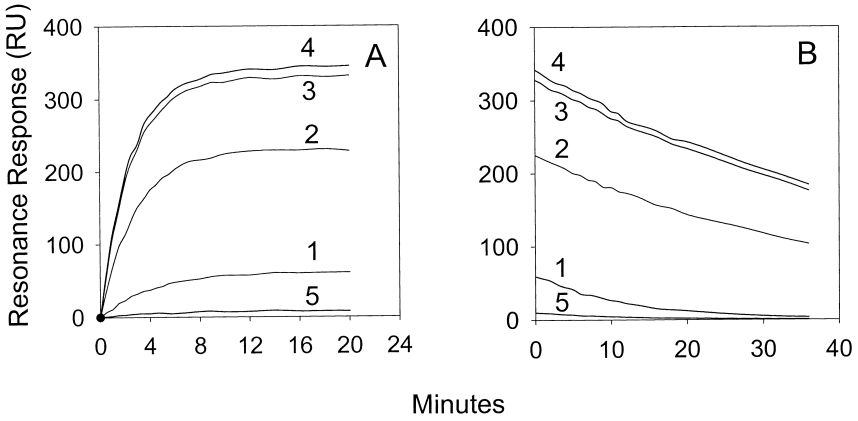

Fig. 3. Effect of PE on the interaction of thrombin-activated factor VIII (factor VIIIa) with the PS-containing surface formed on L1 chip. Association of factor VIIIa (20 n*M*) with the phospholipid surfaces (A) and its dissociation (B) was studied in the presence of exogenous A2 (200 n*M*) in 0.02 *M* MES, pH 6.0, 0.1 *M* NaCl, and 5 m*M* $CaCl_2$. The curves 1, 2 and 3 correspond to factor VIIIa binding to the surfaces containing PS/PC (4/96), PS/PC/PE (4/86/10), PS/PC/PE (4/76/20) and PS/PC/PE (4/56/40), respectively. In the control experiment (curve 5), binding of factor VIIIa to PC/PE (80/20) surface was measured. The kinetic parameters were derived from association and dissociation kinetic curves as described in Section 2 and are presented in Table 2.

in the bilayer, non-activated and activated factor VIII had similar affinities for PS/PC (4/96) surface formed on the L1 chip (see Tables 1 and 2). Progressive increase of the PE content up to 20% resulted in an increase of factor VIIIa affinity for the phospholipid surface (Table 2). Thus, it is the presence of PE that is responsible for the higher affinity of factor VIIIa for the PS/PC/PE surfaces in comparison with non-activated factor VIII. In a control experiment, only a negligible binding of factor VIIIa to the PC/PE (80/20) surface was observed. This implies that although PE increases the factor VIIIa affinity for the membrane, the presence of PS in the membrane composition is an ultimate requirement for the formation of factor VIIIa binding sites.

4. Discussion

The similarity of factor VIII binding characteristics of synthetic phospholipid vesicles and physiological membranes of activated platelets [10,12] justifies the use of synthetic vesicles to model factor VIII interaction with physiological membranes. Membranes of activated platelets contain 4–10% PS and 10–30% PE [6,7], both of which are required for the formation of factor VIII binding sites [10,12]. A wide use of surface plasmon resonance technique and its convenience for quantitative measurements of protein–phospholipid interactions initiated our search for the immobilized phospholipid surface with factor VIII-binding properties closely mimicking those of intact vesicles. In the present study, we compared

Table 2
Parameters of interaction of (factor VIIIa) with phospholipid surfaces with different PE content formed on the L1 chip[a]

Composition of the phospholipid surface	Kinetic constants for factor VIIIa interaction with the phospholipid surface formed on the L1 chip		
	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (n*M*)
PS/PC (4/96)	$(1.8\pm0.12)\times10^5$	$(1.4\pm0.01)\times10^{-3}$	7.8 ± 0.52
PS/PC/PE (4/86/10)	$(2.4\pm0.12)\times10^5$	$(3.6\pm0.02)\times10^{-4}$	1.5 ± 0.08
PS/PC/PE (4/76/20)	$(3.4\pm0.16)\times10^5$	$(2.4\pm0.014)\times10^{-4}$	0.7 ± 0.03
PS/PC/PE (4/56/40)	$(3.6\pm0.22)\times10^5$	$(2.6\pm0.02)\times10^{-4}$	0.72 ± 0.04

[a] The parameters are derived from the kinetic curves shown in Fig. 3 as described in Section 2.

factor VIII-binding properties of supported phospholipid surfaces formed on HPA and L1 biosensor chips with different surface chemistries. We found that factor VIII was able to bind to a flexible phospholipid bilayer with a physiologically low content of PS (4%) immobilized on the dextran-modified surface of the L1 chip. In contrast, no factor VIII binding was observed for the rigid monolayer with the same PS content formed on the hydrophobic alkanethiol-coated surface of the HPA chip. Since the parameters for factor VIII binding to the immobilized PS/PC (4/96) surface formed on the L1 chip (Table 1) were similar to those for binding to intact phospholipid vesicles of the same composition in solution [9], we concluded that the surface formed on the L1 chip represents a suitable model of the membrane with a physiologically low content of PS. The resemblance of the phospholipid bilayer formed on L1 chip with membranes of intact vesicles may be due to its higher mechanical flexibility in comparison with the surface formed to HPA chip.

The factor VIII-binding characteristics of the PS/PC surfaces formed on HPA and L1 chips were similar, when PS concentration was higher than physiological ($\geq$10%). Indeed, as seen from Table 1, the kinetic and equilibrium parameters for factor VIII binding to PS/PC (10/90) and PS/PC (25/75) surfaces formed on either the HPA or the L1 chip are close. The K_d values for the immobilized surfaces containing 10 and 25% PS (Table 1) are also similar to the K_d values previously determined for factor VIII binding to the PS/PC vesicles of the same composition (2.2 and 4.2 n*M* [9], respectively). This implies that surfaces with PS content $\geq$10% formed on either the HPA or the L1 chip represent an adequate model of vesicles in solution.

To further validate our finding that factor VIII-binding properties of the 4% PS-containing surface formed on the L1 chip are similar to those of intact vesicles, we tested the effect of PE incorporation into this surface on factor VIII binding. Since inclusion of PE into the vesicles with 4% PS led to a several-fold increase in the number of factor VIII binding sites on intact vesicles [10], we tested whether this phenomenon can be reproduced for the immobilized phospholipid surface formed on the L1 chip. We found that inclusion of PE at the physiological concentration (20%) to the PS-containing lipid surface formed on the L1 (but not the HPA) chip-induced formation of additional factor VIII binding sites reflected by approximately 6-fold increase in the maximal factor VIII binding. Remarkably, the magnitude of the effect was similar to that previously observed for vesicles in solution [10].

At least two possible explanations for the effect of PE on factor VIII binding to surfaces with a low PS content have been proposed [10]: (i) bulky PC may hinder the access of factor VIII to PS, whereas smaller PE molecules do not; (ii) PE may induce aggregation of PS and PS clusters function as factor VIII binding sites. One might speculate that the phospholipid bilayer formed on the L1 chip provides sufficient lateral mobility of phospholipids, which is required for PE-mediated formation of factor VIII binding sites at the physiologically low concentration of PS. Within this assumption, the rigid monolayer formed on HPA chip does not provide this lateral mobility of phospholipids, thus preventing formation of factor VIII binding sites at low PS concentration.

Another line of evidence that the properties of the phospholipid surface formed on the L1 chip resemble those of intact vesicles was obtained from comparison of affinities of factor VIII and factor VIIIa to PS/PC/PE (4/76/20) surface formed on the L1 chip. We found that factor VIIIa affinity for PS/PC/PE (4/76/20) surface formed on the L1 chip (K_d = 0.7 n*M*, Table 2) was similar to that previously determined for intact PS/PC/PE vesicles (K_d = 0.6 n*M* [11]) and in both cases the affinity of activated factor VIII was 10 times higher than that of non-activated factor VIII. This finding confirms our conclusion that the L1-supported surface is identical to the membrane of intact vesicle.

Using PS/PC/PE surfaces formed on the L1 chip as a model of the membrane, we demonstrated for the first time that the presence of at least 20% PE is required for the maximal binding affinity of factor VIIIa. Indeed, in the absence of PE, non-activated and activated factor VIII had similar affinities for PS/PC membrane (see Tables 1 and 2), whereas inclusion of PE into the membrane composition led to a progressive increase of the affinity of activated factor VIII only. These data predict that phospholipid requirements for the optimal binding of factor VIII and factor VIIIa are different: while the affinity of

non-activated factor VIII is defined mainly by interaction with PS molecules [9,24], the formation of high affinity binding sites for factor VIIIa requires the presence of both PE and PS in the membrane.

The use of phospholipid surfaces formed on L1 chips may become advantageous for analytical applications. It was recently demonstrated by Raut et al. that the binding levels of chromatographically purified plasma-derived therapeutic factor VIII concentrates to phospholipid surfaces formed on HPA chips can serve as one of the criteria of the quality of concentrates. This test was shown to be sensitive to the changes in factor VIII molecule induced by heat-treatment and may predict the propensity of various factor VIII products to induce formation of anti-factor VIII antibodies (inhibitors) in hemophilia A patients [25,26]. It is tempting to speculate that the binding test employing phospholipid surfaces formed on L1 instead of HPA chips will be more sensitive and thus more prospective in monitoring undesirable structural changes in factor VIII products. Besides, the L1-based phospholipid binding assay may be advantageous for determination of factor VIII concentrations in chromatographic fractions in comparison with the previously described HPA-based assay [27]. Moreover, the demonstrated dependence of the maximal binding of both non-activated and thrombin-activated factor VIII on the PE concentration in the membrane composition together with a selective sensitivity of the factor VIIIa (but not factor VIII) affinity to this parameter may be useful in confirmation of the biochemical purity and functional activity of factor VIII preparations.

In summary, the current study demonstrates that immobilized phospholipid surfaces with the physiological contents of PS and PE formed on the L1 but not on the HPA chip closely mimic intact phospholipid vesicles and, likely, physiological membranes, in their factor VIII and factor VIIIa binding properties.

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