Phospholipid Barrier to Fibrinolysis

ROLE FOR THE ANIONIC POLAR HEAD CHARGE AND THE GEL PHASE CRYSTALLINE STRUCTURE*

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The massive presence of phospholipids is demonstrated in frozen sections of human arterial thrombi. Purified platelet phospholipids and synthetic phospholipids retard *in vitro* tissue-type plasminogen activator (tPA)-induced fibrinolysis through effects on plasminogen activation and plasmin function. The inhibition of plasminogen activation on the surface of fibrin correlates with the fraction of anionic phospholipid. The phospholipids decrease the amount of tPA penetrating into the clot by 75% and the depth of the reactive surface layer occupied by the activator by up to 30%, whereas for plasmin both of these parameters decrease by ~50%. The phospholipids are not only a diffusion barrier, they also bind the components of the fibrinolytic system. Isothermal titration calorimetry shows binding characterized with dissociation constants in the range 0.35–7.64 μM for plasmin and tPA (lower values with more negative phospholipids). The interactions are endothermic and thermodynamically driven by an increase in entropy, probably caused by the rearrangements in the ordered gel structure of the phospholipids (in line with the stronger inhibition at gel phase temperatures compared with liquid crystalline phase temperatures). These findings show a phospholipid barrier, which should be overcome during lysis of arterial thrombi.

The basis of the current thrombolytic therapy in acute myocardial infarction or ischemic stroke is the administration of plasminogen activators (*e.g.* tPA[1] and its recombinant variants), which convert the plasminogen in blood plasma or on the surface of the thrombi to plasmin and the latter dissolves fibrin, the solid matrix of thrombi (1). However, persistent recanalization of the occluded blood vessels often fails (in 15–40% of patients), and efficient doses of thrombolytics have significant bleeding side effects (2). The limitations of existing fibrinolytic therapy indicate that thrombolysis is not identical to fibrinolysis and direct attention to the variety of cellular and molecular factors present in thrombi that modulate the function of the basal fibrinolytic machinery (recently reviewed in Ref. 3).

Platelets render thrombi resistant to lysis with tPA (4), which can be partially explained by their PAI-1 content (5), but evidence for PAI-1-independent inhibition of fibrinolysis also exists (6). When arterial thrombi are formed, the platelet content of 10 ml of whole blood is compacted in a volume of 400 μl, whereas the fibrin content of the same thrombi corresponds to the fibrinogen concentration in blood plasma (7), the intra-thrombus plasminogen (0.4 μM), and α_2-PI (0.25 μM) concentrations are just fractions of the respective plasma values (8). After activation, the platelets release their cytosolic content and lose the majority of their phospholipid (9). By taking into account the average amount of phospholipids in platelets (10), the phospholipid content of arterial thrombi (7.5 g/liter) is expected to be higher than their fibrin content (1.7 g/liter). Phospholipids are known to bind fibrinogen and to modify the proteolytic susceptibility of fibrin(ogen) to thrombin and plasmin (11–16). The contribution of the platelet phospholipids to discrete steps of blood coagulation is well characterized (reviewed in Refs. 17 and 18), but data on the functional consequences with respect to fibrinolysis are rather controversial. Higher proteolytic susceptibility to plasmin has been reported for monomeric fibrinogen layers on phospholipid surfaces (11), but data for inhibited degradation of fibrinogen (19) and fibrin (12) are also available. In addition to the controversies at the substrate end of fibrinolysis, the phospholipid modulation of the enzymes involved in this process is scarcely investigated. The only definite observation in this respect is the phospholipid-induced enhancement of tPA inactivation by PAI-1 (20).

Generally the interaction of a protein and a phospholipid bilayer depends on the electric charge of the polar head in the lipid molecule (varying electrostatic attraction/repulsion) and the lamellar phase structure of the lipid layer (varying hydrophobic interactions related to membrane fluidity and rearrangements) (21). The known phospholipid composition of the human platelet membrane (22) indicates high heterogeneity considering both of these factors. The platelet membranes are dominated by the zwitterionic phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine (83 mol % of total phospholipid) with a significant fraction of anionic phosphatidylserine and phosphatidylinositol (17 mol %). The differently charged phospholipids are

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[1] The abbreviations used are: tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; α_2-PI, α_2-plasmin inhibitor; PC, dipalmitoylphosphatidylcholine; PS, dipalmitoylphosphatidylserine; poPC, palmitoyloleoylphosphatidylcholine; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; DPH, 1,6-diphenyl-1,3,5-hexatriene; DHPS, dihexanoylphosphatidylserine; LUV, large unilamellar phospholipid vesicle; PCPS3:1, LUV containing PC and PS mixed at 3:1 weight ratio; PCPS1:1, LUV containing PC and PS mixed at 1:1 weight ratio; poPCPS, LUV containing poPC, PC and PS mixed at 2:3:5 weight ratio; poPCPS1:1, LUV containing poPC and PS mixed at 1:1 weight ratio; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry.

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localized asymmetrically in the inner and outer layers of the platelet membrane (negatively charged polar heads predominantly in the inner leaflet at rest). Upon activation, however, extensive anionic patches are formed on the outer surface (reviewed in Refs. 17 and 18). The type of lamellar phase structure adopted by phospholipid membranes at a given temperature is basically determined by the type of their fatty acids (reviewed in Ref. 23), and considering the 40% contribution of unsaturated fatty acids to the total acyl moieties in platelet phospholipids (22), the platelet membrane is expected to be in the fluid lamellar phase. The presence of proteins, however, and their dynamic changes in the course of cellular function allow for the formation of lateral phospholipid domains in the distinct phase state, which has been shown in thrombin-activated platelets (24) and other cells (25, 26), but similar lateral domains can be reproduced *in vitro* in pure phospholipid membranes with variations of composition and temperature (27). Because of this lateral heterogeneity of platelet membranes with respect to both electric charge and phase state, any *in vitro* approach designed to analyze their interaction with proteins should utilize multiple experimental settings suitable to model each discrete structural aspect of the interaction. In our present study we model the contribution of polar head charge using gel phase mixtures (at 37 °C) of PC and PS, the T_m of which is in the range 41–45 °C (where T_m is melting temperature of the phospholipid, defined as the average value of the temperatures at the beginning and the completion of the transition from gel to the liquid-like phase) (27). We evaluate the role of the phospholipid crystalline phase in interactions with members of the fibrinolytic system at a defined temperature (25 or 37 °C) by using variations in the unsaturated fatty acid content of the applied phospholipid. The presence of 8–24% poPC decreases the T_m of pure PC membranes from 41 to 35–32 °C, and thus the membrane with such a ratio of poPC is in the gel phase at 25 °C and in the liquid-crystalline phase at 37 °C (23). One advantage of work with binary mixtures of phospholipids (zwitterionic/anionic, saturated/unsaturated acyl derivatives) is that these contain lipid clusters resembling the domains in natural membranes (reviewed in Refs. 28 and 29). The boundary regions of such clusters (order-disorder boundaries) give rise to some pre-melting phenomena even at temperatures far from the T_m, *e.g.* increased permeability (28), increased rate of protein binding (30), and increased enthalpy of binding (31). The binary systems, of course, are simplified models of the natural complex membranes, but they allow the dissection of the discrete effects of the separate phospholipid components.

EXPERIMENTAL PROCEDURES

Materials—Human plasma and platelet concentrates were collected from healthy volunteers (Hungarian Blood Supply Service, Budapest, Hungary). Human fibrinogen (plasminogen-free) and FITC on Celite were from Calbiochem. The chromogenic plasmin substrate Spectrozyme-PL (*H*-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide) and the tPA were products of American Diagnostica (Hartford, CT) and Boehringer Ingelheim, Germany, respectively. Human thrombin (1000 NIH units·mg^{-1}), α_2-PI, PC, PS, poPC, *N*-octyl-β-D-glucopyranoside, BSA, DPH, Nile Blue A, and *p*-nitrophenyl *p'*-guanidinobenzoate were from Sigma. DHPS was from Avanti Polar Lipids Inc. (Alabaster, AL). 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF, Pefabloc®) was from Roche Applied Science. Published procedures were used for the isolation of Glu-plasminogen from citrated human plasma (32) as well as for the generation of plasmin and determination of its active concentration (33). Proteins were labeled with Eu^{3+} chelate (N^1-(*p*-isothiocyanatobenzyl)-diethylenetriamine-N^1,N^2,N^3,N^4-tetraacetic acid chelated with Eu^{3+}) according to the manufacturer's instructions (Wallac, Turku, Finland) with efficiency of 1–2 Eu^{3+}/molecule of protein (determined by using the europium standard solution from Wallac). The Eu^{3+} label binds covalently to primary amino groups of the proteins. The detection sensitivity of the label is similar to that of radioisotopes (34). Proteins were labeled with FITC using a published procedure (35) yielding ~2 FITC labels/molecule of protein (on the basis of the A_{495}/

A_{280} ratio). Neither of the two labels altered the functional properties of the plasminogen, tPA, and plasmin as evidenced by plasminogen activation and turbidimetric fibrinolytic assays performed according to published procedures (36, 37).

Preparation of LUVs—PC, PS, and poPC were weighed, mixed at the desired mass ratio, and dissolved in chloroform/methanol (95:5 volume ratio). Following evaporation of the solvent, the phospholipids were suspended with brief sonication (three times for 30 s with a 50-watt, 20-kHz, Branson Sonifier 250, Branson Ultrasonics Corp., Danbury, CT) in 10 mM HEPES, pH 7.4, buffer containing 150 mM NaCl (Buffer A, in which all reactions were carried out unless otherwise stated), and LUVs were prepared with extrusion through a 50-nm pore diameter polycarbonate filter in a Liposofast mini-extruder (Avestin Inc., Ottawa, Canada) (38). The concentration of phospholipids in the LUV suspension was determined with the fluorescent probe DPH (39).

Preparation of Platelet Membrane Lipids—Platelet membranes were prepared from human platelet concentrates according to the glycerol lysis procedure (40). Following the hyperosmotic/hypo-osmotic shock, the unlysed platelets were sonicated three times for 30 s (50 watts, 20 kHz), and this fraction was used as a crude platelet homogenate. The phospholipids were extracted from the membranes with chloroform with continuous rotation (40 rpm) for 6 h at 40 °C. The chloroform fraction was separated from the water phase. Following evaporation of the chloroform, the lipids were suspended in Buffer A; LUVs were prepared, and their phospholipid content was determined as described for the synthetic phospholipids (with this procedure 1 unit of platelet concentrate yielded 1 ml of protein-free lipid suspension containing ~0.6 g/liter phospholipid).

Turbidimetric Fibrinolytic Assay—This was carried out as described previously (37). Briefly, 100 μl of 6 μM fibrinogen containing 0.25 or 0.5 μM plasminogen and the examined LUVs at various concentrations was clotted with 0.5 NIH units/ml thrombin in microplate wells at 37 °C for 45 min. The dissolution of the clots was induced by 100 μl of 3–140 nM tPA applied on the surface of the clot, and 60 μl of mineral oil was layered over the solution to prevent evaporation. The course of clot formation and dissolution was monitored by measuring the light attenuance at 340 nm at 25 or 37 °C with a Zenyth 200rt microplate spectrophotometer (Anthos Labtec Instruments GmbH, Salzburg, Austria). The lysis time ($t_{1/2}$) defined as the time needed to reduce the turbidity of the clot to half-maximal value was used as a quantitative parameter of fibrinolytic activity.

Plasminogen Activation Assays—Plasminogen activation in homogeneous solution was evaluated as described previously (36). Briefly, 3 μM plasminogen containing the examined LUVs at various concentrations was mixed with 70 nM tPA. Samples were taken at intervals, and the amidolytic activity of the generated plasmin was measured on 0.1 mM Spectrozyme-PL. Plasminogen activation in the presence of fibrin was measured on the surface of clear fibrin clots (A_{405} <0.1) (41) prepared in standard 96-well microtiter plates from 20 μl of 10 NIH units/ml thrombin and 60 μl of 4.4 μM fibrinogen in 10 mM HEPES, pH 7.4, buffer containing 200 mM NaCl, 0.1 μM plasminogen, and the examined LUVs at various concentrations. After 30 min of incubation at 37 °C 60 μl of 14 nM tPA in Buffer A and 0.6 mM Spectrozyme-PL were layered on the clot surface followed by 60 μl of mineral oil. The light attenuance at 405 nm (A_{405}) indicating the release of *p*-nitroaniline by the generated plasmin was continuously recorded with a Zenyth 200rt microplate spectrophotometer at 37 °C. The measured A_{405} values are plotted *versus* time-squared yielding a linear relationship with a slope directly proportional to the rate of plasminogen activation (42).

Determination of the Second-order Rate Constant for the Inactivation of Plasmin by α_2-PI—This was carried out as described previously by using equimolar concentrations of titrated plasmin and α_2-PI (33) in the absence and presence of 1 g/liter poPCPS.

Penetration of Proteins into Fibrin Clots—To measure the depth of the reactive layer, fibrin was prepared in 0.5-mm high chambers constructed from glass slides by clotting 2 g/liter fibrinogen in Buffer A, 3 mM CaCl$_2$, and 1 g/liter PCPS1:1 or PCPS3:1 with 2 NIH units/ml thrombin for 30 min. Thereafter, FITC-tPA or FITC-plasmin (in the same buffer containing 5 mM Pefabloc) was added to the edge of the clot, and the FITC label was monitored with the confocal laser scanning system LSM510 (Carl Zeiss GmbH, Jena, Germany) taking sequential images of the fluid-fibrin interface at a distance of ~50 μm from the glass surface with identical exposures and laser intensities (attenuated to 7% of the full intensity) for all examined conditions. The size of the layer, into which the labeled proteins penetrate, was measured on the digital images with SigmaGel 1.0 software (Jandel Scientific, Erkrath, Germany).

To measure the amount of diffusing protein, fibrin clots of 100 μl
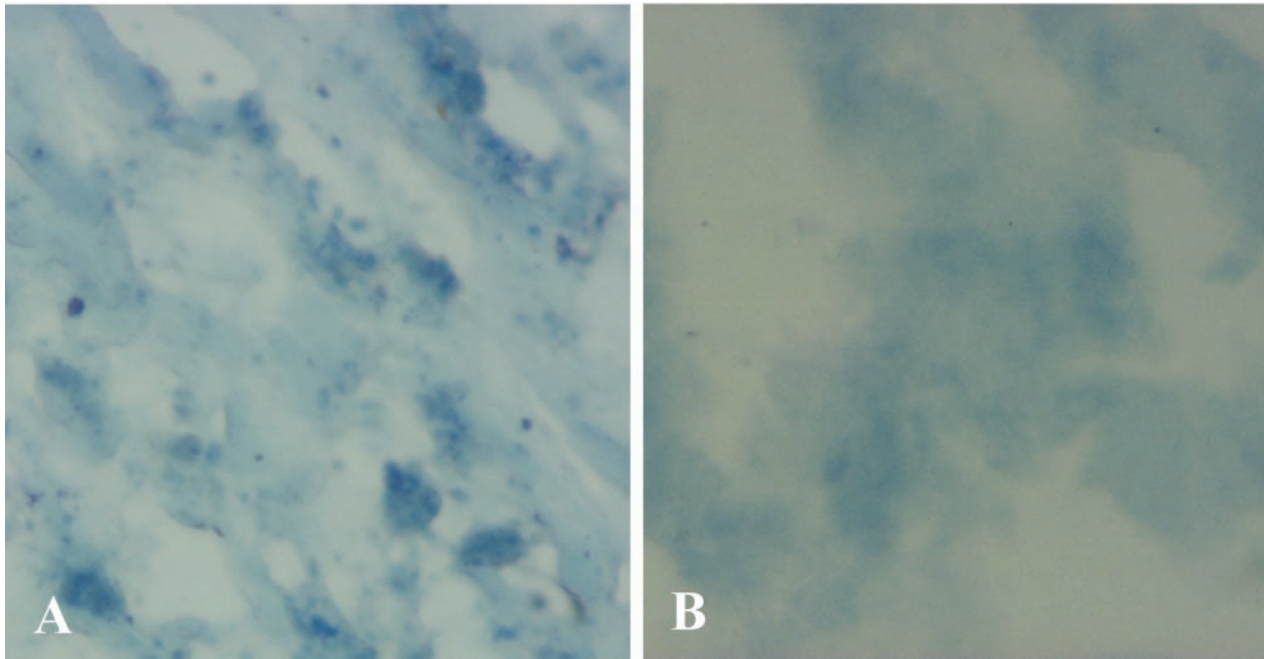
FIG. 1. **Phospholipids in arterial thrombi.** Frozen sections of thrombi from femoral artery (*A*) or fibrin containing 5 g/liter PCPS3:1 (*B*) have been stained with Nile Blue for phospholipids as described under "Experimental Procedures." Small *blue* groups of platelets are seen on *A*, whereas *B* shows a inhomogeneous *light blue* reaction according to the distribution of phospholipid. Original magnification, ×40.

volume and the same composition as described above were prepared in microtiter plates. Thereafter, Eu^{3+}-labeled protein (in 50 μl of Buffer A containing 5 mM Pefabloc) was added to the surface of the clots, and various intervals were allowed for diffusion and binding, at the end of which the fluid phase over the clot was thoroughly removed. Following washing (less than 5 s) of the fibrin surface with 100 μl of Buffer A, 200 μl of Enhancement Solution (Wallac, Turku, Finland), which releases the Eu^{3+} from the proteins and forms the fluorescent chelate, was added. The emission fluorescence of Eu^{3+} was measured after 4 h with a time-resolved microplate fluorimeter Victor2 (Wallac, Turku, Finland) (excitation wavelength 340 nm, emission wavelength 615 nm, counting delay 400 μs). By using this fluorescent data and the known specific label in the added protein, the amount of tPA or plasmin retained in the clot was calculated.

Binding of Eu^{3+}-labeled Proteins to Fibrin Monomers—Standard 96-well microplates were coated with fibrin monomers and BSA as described previously (33). 100 μl of 10 nM Eu^{3+}-plasminogen, Eu^{3+}-plasmin (active site blocked with Pefabloc), or Eu^{3+}-tPA was applied to the protein-coated surface in the absence and the presence of varying concentrations PCPS1:1 LUVs. After 10 min of incubation at 25 °C, the solution was removed, and the plates were washed three times (in less than 10 s) with 200 μl of Buffer A. 200 μl of Enhancement Solution was added, and the Eu^{3+} fluorescence was measured as above.

Sedimentation Analysis of LUV-Protein Complexes—LUVs (0.5 g/liter) were mixed with Eu^{3+}-plasminogen, Eu^{3+}-plasmin, or Eu^{3+}-tPA (1 μM) in Buffer A and 5 mM Pefabloc and incubated for 15 min at 25 °C. Thereafter, 100 μl of each mixture were centrifuged at $100,000 \times g$ in an Airfuge (Beckman Instruments Inc., Palo Alto, CA) for 15 min. The amount of Eu^{3+} label in the upper 40 μl of the supernatant was measured (no phospholipid could be detected with the DPH probe in this fraction).

Biomolecular Interaction Analysis—The interaction of phospholipids and proteins (plasmin(ogen), tPA) was evaluated with SPR technology on Biacore X system (Biacore AB, Uppsala, Sweden) (43). Sensor chip L1 (carboxymethylated dextran matrix modified with lipophilic substance) was used to immobilize phospholipid LUVs, whereas Sensor chip HPA (long chain alkanethiol surface) was used to form a phospholipid monolayer according to the manufacturer's instructions.

Isothermal Titration Calorimetry (ITC)—The enthalpy changes accompanying the interaction of phospholipids and proteins were measured using isothermal titration method on VP-ITC microcalorimeter (Microcal Inc., Northampton, MA) (44, 45). The proteins were injected in a series of 25 aliquots (10 μl each) into the cell of the calorimeter containing LUVs, and the heat increment of each addition was recorded by the instrument. Dilutions of protein into buffer were carried out in a separate series of injections, and these heat increments were subtracted

from the raw data (these background peaks were significantly larger than the titration heats of buffer into buffer only for the injection of 0.14 mM plasmin at 37 °C). The heat data from the injection of proteins into LUV suspension were evaluated according to the single-site algorithm (46) with ITC Data Analysis version 7.0 software (Microcal).

Staining of Phospholipids with Nile Blue—Frozen sections of thrombi removed with surgery (kindly collected by Dr. András Nagy at the 1st Department of Surgery, Semmelweis University, Budapest, Hungary) from human femoral artery were stained for phospholipids (47). 1 h of pretreatment with 1 N HCl followed by 1 h of incubation in acetone was used to eliminate the triglycerides and sterols. Thereafter, the sections were immersed in 10 g/liter Nile Blue in water for 5 min, rinsed with water, and destained with 0.1 N HCl. The phospholipids stain blue with this procedure. For comparison, fibrin clots were prepared from 4 g/liter fibrinogen containing 5 g/liter PCPS3:1 clotted with 10 NIH units/ml thrombin for 24 h at 37 °C, and these were sectioned and stained with the same procedure (control clots prepared without phospholipids did not yield any color reaction).

RESULTS

Nile Blue staining of thrombi from human femoral artery shows massive presence of phospholipids (Fig. 1). The intensity of the phospholipid stain in the thrombus sections is comparable with that in the fibrin clots containing 5 g/liter phospholipid. The dimensions of the phospholipid clusters correspond to the size of platelets.

When the effect of crude platelet homogenates on tPA-induced fibrin dissolution is evaluated *in vitro*, significant retardation is seen (Fig. 2), which at first glance can be explained with the known PAI-1 content of platelets (5). However, at equivalent concentrations, the platelet homogenate does not inhibit the generation of plasmin by tPA in a homogeneous, fibrin-free assay system (not shown). This inhibiting effect in the fibrinolytic assay is partially reproduced by LUVs prepared from protein-free platelet membranes or from synthetic poPCPS at identical phospholipid concentrations and is completely reconstituted with higher phospholipid contents of the clot (Fig. 2*A*). The degree of inhibition markedly decreases at 37 °C (Fig. 2*B*). The maximal inhibiting effect is achieved at phospholipid concentration of 1 g/liter (Fig. 2*B, inset*), but if water-soluble DHPS, which does not form membrane structures, is used in the fibrinolytic assay instead of LUVs, no
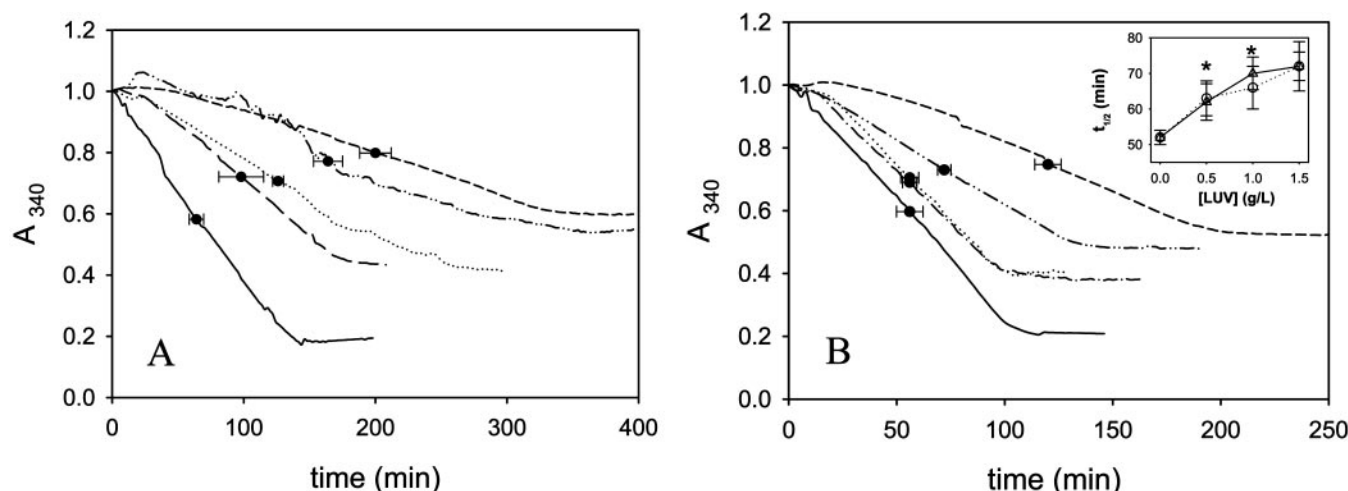
FIG. 2. **Effect of phospholipids on tPA-induced fibrinolysis.** Fibrin clots containing 0.25 μM plasminogen have been prepared as described under "Experimental Procedures" with the following additives: none (*solid line*), platelet homogenate (0.5 g/liter final phospholipid concentration, *short dashed line*), platelet membrane phospholipid (0.5 g/liter final concentration, *dotted line*), poPCPS (0.5 g/liter final concentration, *long dashed line* or 1 g/liter final concentration, *dashed, double-dotted line*), poPCPS1:1 (1 g/liter final concentration, *dashed, dotted line*). tPA (140 nM) is layered on the surface and the light attenuation at 340 nm (A_{340}) is continuously recorded at 25 (*A*) or 37 °C (*B*). Depending on the LUV content, the initial A_{340} values vary in the range 0.8–1.2; in the figure all curves have been normalized (dividing all A_{340} values by the initial value for each curve) for presentation purposes. *Symbols* and *bars* represent the mean and S.D. of lysis times from four independent measurements. *Inset,* concentration dependence of lysis time on phospholipid concentration (*circles*, PCPS1:1; *triangles*, PC). *Asterisk* indicates significant ($p < 0.01$) difference compared with the preceding concentration value.

inhibition of the dissolution process is observed at concentrations up to 4.5 g/liter (not shown). The inhibiting effect of 1 g/liter poPCPS is equivalent to the effect of 440 nM α_2-PI present in the fibrin (Fig. 3), and these two independent actions are additive in the fibrinolytic assay despite the reduced rate of plasmin inactivation by α_2-PI in the presence of phospholipids in a fibrin-free system (Fig. 3, *inset*).

The background of the observed effects could be inhibition of either plasminogen activation or plasmin activity. When plasminogen activation is examined in homogeneous solutions (Fig. 4, *inset*) or hydrolysis of synthetic substrate (Spectrozyme-PL) by plasmin is monitored (not shown), the presence of LUVs (up to 1.5 g/liter) does not affect the rate of the reactions. However, if the LUVs are dispersed in the solid phase fibrin and plasmin is generated on its surface, the rate of plasminogen activation decreases (Fig. 4). The degree of inhibition correlates with the fraction of anionic phospholipid in the LUV. If the water-soluble DHPS is used in this plasminogen activation assay instead of LUVs, no inhibition of the reaction is observed (not shown).

Analysis of the sequential images taken with a confocal microscope in the course of FITC-tPA penetration into fibrin shows that an interface reactive layer of constant size is formed within 30 min after the external application of the tPA independently of the presence of phospholipid (Fig. 5). When 1 g/liter PCPS1:1 is present in the fibrin clot, the final depth of layer, into which the tPA penetrates, decreases significantly (21.53 ± 2.05 μm *versus* 30.84 ± 2.98 μm in the absence of phospholipid, $p < 0.001$). 1 g/liter PCPS3:1, however, does not affect the size of the penetration layer (29.44 ± 1.42 μm). If FITC-plasmin is applied to the surface of fibrin, both PCPS1:1 and PCPS3:1 reduce the size of the reactive layer from 74.56 ± 9.19 to 34.28 ± 6.25 and 39.76 ± 4.28 μm, respectively. Although the size of the zone occupied by tPA or plasmin is an essential determinant of the fibrinolytic efficiency, the rate of the process depends primarily on their concentration in the interfacial reactive layer. Measurement of the fibrin-entrapped tPA and plasmin (Fig. 6) indicates that phospholipids affect the amount of penetrating enzymes to a greater extent than the size of the reactive zone with a definite dependence on the anionic charge of their polar head. The penetration of plasmin-

ogen is affected significantly only by the LUVs with 50% anionic polar heads. The time course of this diffusion is essentially the same in all examined systems with maximal retention of proteins achieved within 40 min (not shown).

If 1 pmol of Eu^{3+}-plasminogen, Eu^{3+}-plasmin, or Eu^{3+}-tPA is applied to fibrin monomers immobilized on a plastic surface, following 10 min of incubation and thorough washing, 146, 324, and 212 fmol of the respective protein remain bound to the surface. If the binding is evaluated in the presence of increasing concentrations of PCPS1:1 LUVs, a progressive decrease in the fraction of bound protein is observed with a maximal degree of inhibition achieved at 0.5 g/liter PCPS1:1 (9, 42, and 31 fmol of bound protein). A similar trend is seen on the BSA-coated surface, where the PCPS1:1 reduces the amount of BSA-bound ligand from 41 fmol of Eu^{3+}-plasminogen, 52 fmol of Eu^{3+}-plasmin, and 45 fmol of Eu^{3+}-tPA to 4, 3, and 3 fmol, respectively. The similar competing effects of the phospholipid on the two different surfaces indicate interference with the soluble phase ligands (Eu^{3+}-labeled proteins) rather than with the surface-immobilized fibrin or BSA. Consequently, more direct procedures have been applied to evaluate these interactions.

Sedimentation of LUVs at $100,000 \times g$ yields a phospholipid-free supernatant. If protein/LUV mixtures are centrifuged under the same conditions, the amount of protein in this supernatant reflects the fraction of protein that has not bound the LUVs (the LUVs and their complexes with proteins are sedimented). PCPS3:1 LUVs remove 56% of plasmin out of this supernatant (relative to the sedimentation fraction in the absence of phospholipid), whereas PCPS1:1 removes 86% plasmin. Both types of LUVs remove ~30% plasminogen from the supernatant fraction. This type of evaluation could not be performed with tPA, because of the high background sedimentation of tPA in the absence of LUV.

The strength of interaction of phospholipids with members of the fibrinolytic system was evaluated with two techniques, SPR and microcalorimetry. The SPR response indicates plasmin binding to both forms of immobilized PCPS1:1 (monolayer formed directly on the surface of the HPA chip and vesicles on the short dextran chains of the L1 biosensor chip) with equilibrium dissociation constants (K_d) of 80.32 ± 15.86 and $312 \pm$

FIG. 3. **Effect of phospholipids on the inhibition of fibrinolysis by α_2-PI.** Fibrin clots containing 0.5 μM plasminogen have been prepared as described under "Experimental Procedures" with the following additives: none (*solid line*), 440 nM α_2-PI (*dashed line*), 1 g/liter poPCPS (*dotted line*), 440 nM α_2-PI and 1 g/liter poPCPS (*dashed, double-dotted line*). tPA (140 nM) is layered on the surface, and data are collected at 25 °C and presented as in Fig. 2. *Inset,* inhibition of plasmin by α_2-PI in the absence of fibrin. Plasmin and α_2-PI are mixed at equimolar concentrations (133 nM) at 25 °C in a total volume of 20 μl containing no additive (*filled symbols*) or 1 g/liter poPCPS (*open symbols*). At the indicated times, 200 μl of 0.1 mM Spectrozyme-PL is added, and the residual plasmin [*Pln*] activity measured. *Symbols* represent the mean and S.D. of four measurements; *lines* show the best fits of the equation, $[\text{Pln}] = ([\text{Pln}]_0)/(1 + [\text{Pln}]_0 \cdot k'' \cdot t)$ (33), with apparent second-order rate constant k'' of 0.87×10^6 M^{-1} s^{-1} in the absence of phospholipid and 0.38×10^6 M^{-1} s^{-1} in the presence of poPCPS.
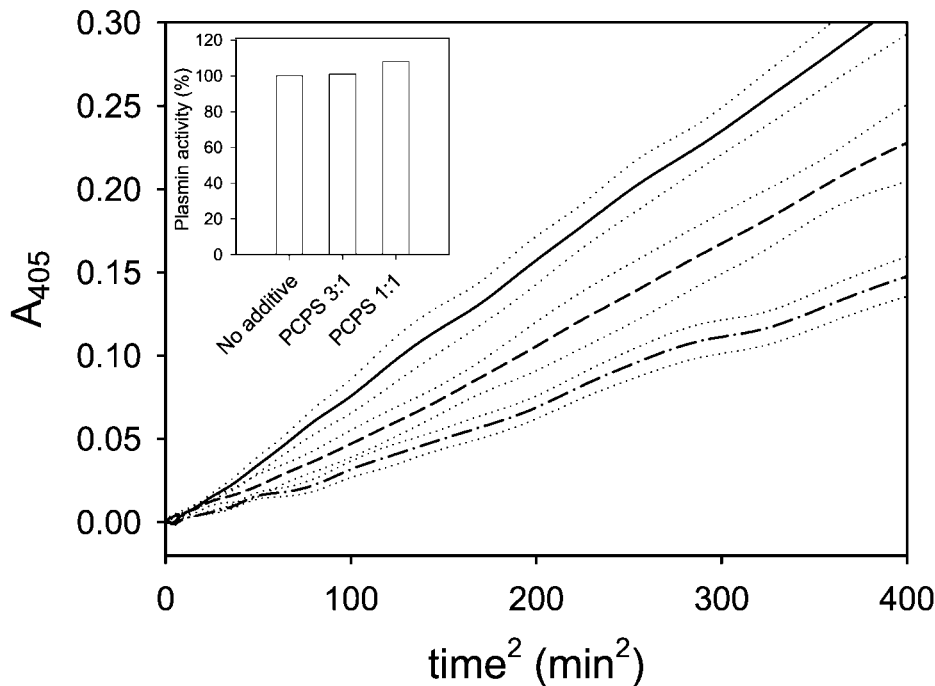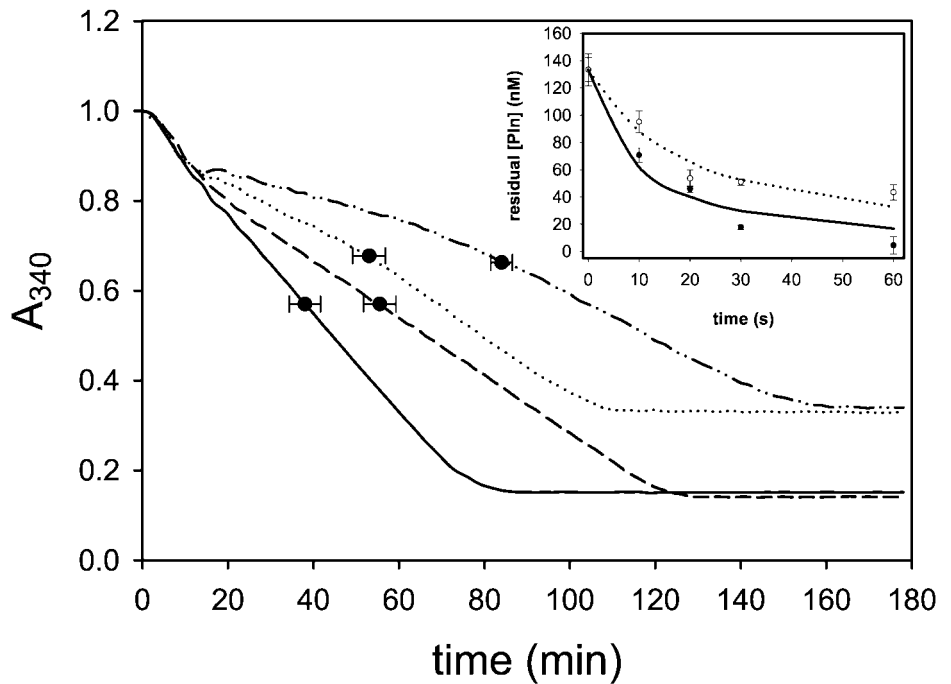
FIG. 4. **Plasminogen activation by tPA in the presence of phospholipids.** Clear fibrin clots containing plasminogen and various phospholipids have been prepared as described under "Experimental Procedures." tPA is layered on the clot surface together with the chromogenic plasmin substrate Spectrozyme-PL, and the A_{405} is recorded continuously. Mean values of three independent measurements are represented by the *solid line* for fibrin without any phospholipid, the *dashed line* for fibrin with 1 g/liter PCPS3:1, the *dashed, dotted line* for fibrin with 1 g/liter PCPS1:1, and the *dotted lines* represent the mean ± S.D. values for each type of experiment. *Inset,* plasmin activity generated in a homogeneous mixture (in the absence of fibrin) of plasminogen, tPA, and the indicated LUVs (1 g/liter total phospholipid concentration) after 4 min of incubation (plasmin activity in the absence of phospholipid is taken as 100%, mean of two measurements are presented).

56.20 nM, respectively (Fig. 7A) These data are in line with the results from the titration calorimetry (Fig. 7B and Table I) for comparable conditions (PCPS1:1 vesicles at 25 °C). It seems that the monolayer form of phospholipid immobilization improves the binding conditions for plasmin. Such a conclusion is supported by the data for PCPS3:1. The SPR response indicates plasmin binding only to the monolayer form of PCPS3:1 with a K_d of 41.03 ± 23.53 nM, whereas microcalorimetry (Fig. 8) provides evidence for interaction with its vesicular form, but with K_d values an order of magnitude higher than with PCPS1:1 (Table I). Thus, the lower sensitivity threshold of the microcalorimetric titration allows the detection of interactions that yield detectable SPR response only on the phospholipid monolayer attached directly to the chip. The data on the interactions of tPA and plasminogen with phospholipids are in line

with this interpretation. tPA produces SPR response only on the PCPS1:1 monolayer ($K_d = 27.39 ± 7.89$ nM), whereas the ITC indicates binding to PCPS1:1 with a K_d of 0.62 ± 0.14 μM and to PCPS3:1 with a K_d of 7.64 ± 1.64 μM. Plasminogen does not display saturable SPR response on any of the examined phospholipid surfaces, but microcalorimetry detects very weak interactions as follows: K_d values of 74.9 ± 16.8 μM for PCPS1:1 and 117.1 ± 14.2 μM for PCPS3:1. The two methods applied for evaluation of the binding (SPR and ITC) give consistent results only for the interactions with the highest affinity (plasmin and PCPS1:1 LUVs). The discrepancies seen for the weaker interactions can be attributed to the immobilization-related specifics of the SPR technology. When vesicles of 50nm diameter are immobilized to the L1 chip surface, the interaction layer is shifted away from the detection surface, but the measured

FIG. 5. **Penetration of FITC-tPA into fibrin containing phospholipids.** Fibrin with no phospholipid (*A*), 1 g/liter PCPS1:1 (*B*), or 1 g/liter PCPS3:1 (*C*) has been prepared as described under "Experimental Procedures," and 12 nM FITC-tPA is added to the surface. The fluid-gel interface is monitored with a confocal microscope as described under "Experimental Procedures." The images show the surface layer (*green band*) occupied by FITC-tPA 30 min after its application.
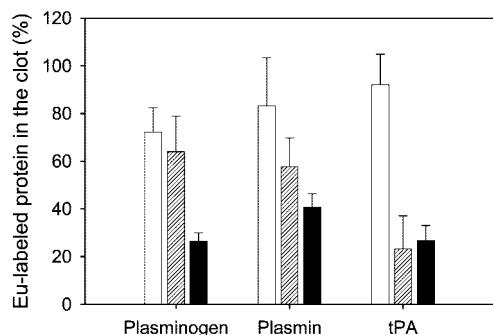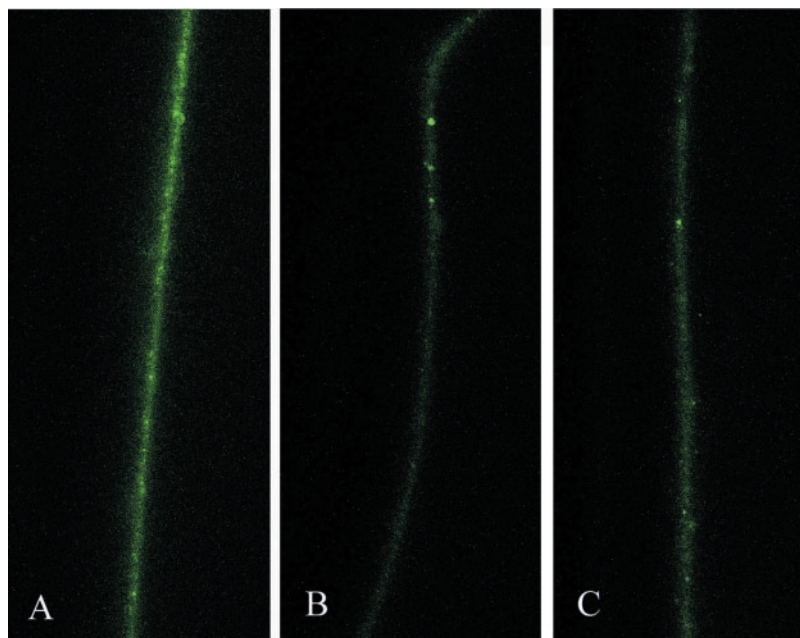

FIG. 6. **Penetration of Eu^{3+}-labeled proteins into fibrin-containing phospholipids.** Fibrin with PC (*open bar*), PCPS3:1 (*shaded bar*), or PCPS1:1 (*black bar*) LUVs dispersed within the clots at 1 g/liter final phospholipid concentration have been prepared as described under "Experimental Procedures." Thereafter, 50 μl of 16 nmol/liter Eu^{3+}-plasminogen, Eu^{3+}-plasmin, or Eu^{3+}-tPA (all in buffer containing 5 mM Pefabloc) are layered on the surface of the clot. After 40 min the fluid layer is removed; the surface is washed, and the fibrin-retained Eu^{3+} label is measured. The *bars* represent the mean and S.D. values of three independent measurements (100% is the amount of respective protein retained in clots without any phospholipid).

evanescent wave decays exponentially with increasing distance. If the interface layer is brought nearer the detection surface (phospholipid monolayer on HPA chip), this problem is overcome, but the changed geometry of the contact surface results in different parameters.

DISCUSSION

Because histological examination of arterial thrombi indicates the presence of phospholipids yielding color reaction with intensity comparable with that of 5 g/liter phospholipid in fibrin clots (Fig. 1), we examined the overall impact of phospholipids on fibrinolysis in an assay system that matches maximally the composition of arterial thrombi with respect to fibrin (6 μM), plasminogen (0.5 μM), and α_2-PI (0.44 μM) content (7, 8). The applied tPA concentrations (3–140 nM) are definitely higher than the typical basal levels in human plasma (0.06 nM) (8), but estimation of the plasma levels of tPA in the course of thrombolytic therapy predicts concentrations as high as 350 nM (49). The evaluated concentration range (0.5–1.5 g/liter) of phospholipids is severalfold lower than the values predicted from the platelet content (7) or phospholipid staining of arterial

thrombi (Fig. 1), but this amount of phospholipids is sufficient to achieve the maximal inhibiting effect on fibrinolysis (Fig. 2*B*, *inset*). Thus, the concentrations of our fibrinolytic assay are relevant to the conditions of arterial thrombolysis. At equivalent phospholipid concentrations, the crude platelet homogenate results in inhibition of the tPA-induced fibrinolysis comparable with the effect of LUVs prepared from purified platelet phospholipids and identical to the inhibition effected by the synthetic poPCPS (at 1 g/liter concentration) (Fig. 2*A*). This finding precludes the role of platelet proteins in the inhibition. Indeed, the tPA concentration used (140 nM) is sufficiently high to overtitrate the expected PAI-1 introduced in the assay with the homogenate ($\sim$3 nM) (8). The inhibiting effect of 1 g/liter poPCPS on tPA-induced fibrinolysis is equivalent to the effect of 440 nM α_2-PI present in the clot (Fig. 3), a concentration higher than the average value for α_2-PI content in arterial thrombi (8). Although phospholipids slow down the inhibition of plasmin by α_2-PI (Fig. 3, *inset*), the combined presence of 1 g/liter poPCPS and 440 nM α_2-PI exerts stronger inhibition in the fibrinolytic assay than each of the inhibitors alone (Fig. 3). This result suggests that the phospholipids impair the well known protection of plasmin against its inhibitors on the fibrin surface (33). In line with earlier findings (12, 19), phospholipids also retard the dissolution of fibrin with plasmin layered on the surface of the clot (not shown).

The application of a plasminogen activation assay that detects plasmin generation on the surface of fibrin clots indicated that the observed retardation of fibrinolysis is at least partially based on the inhibition of plasminogen activation (Fig. 4). With conventional activation assays in homogeneous solution (Fig. 4, *inset*), the effect of phospholipids would have remained unrevealed. This finding raises the important methodological issue that the efficiency of thrombolytics should be tested in experimental settings that adequately model the *in vivo* thrombi.

Our experiments identified two structural requirements for the inhibiting action of phospholipids on the fibrinolytic system as follows: the presence of anionic polar head and near-melting membrane structure. The plasminogen activation assay (Fig. 4) shows correlation between the degree of inhibition and the fraction of anionic phospholipid in the LUVs. The data on the binding affinities of plasmin and tPA to phospholipids (Table I) are in line with the role of the anionic charge. Sedimentation of PCPS1:1 LUVs removes 30% more plasmin from the solution
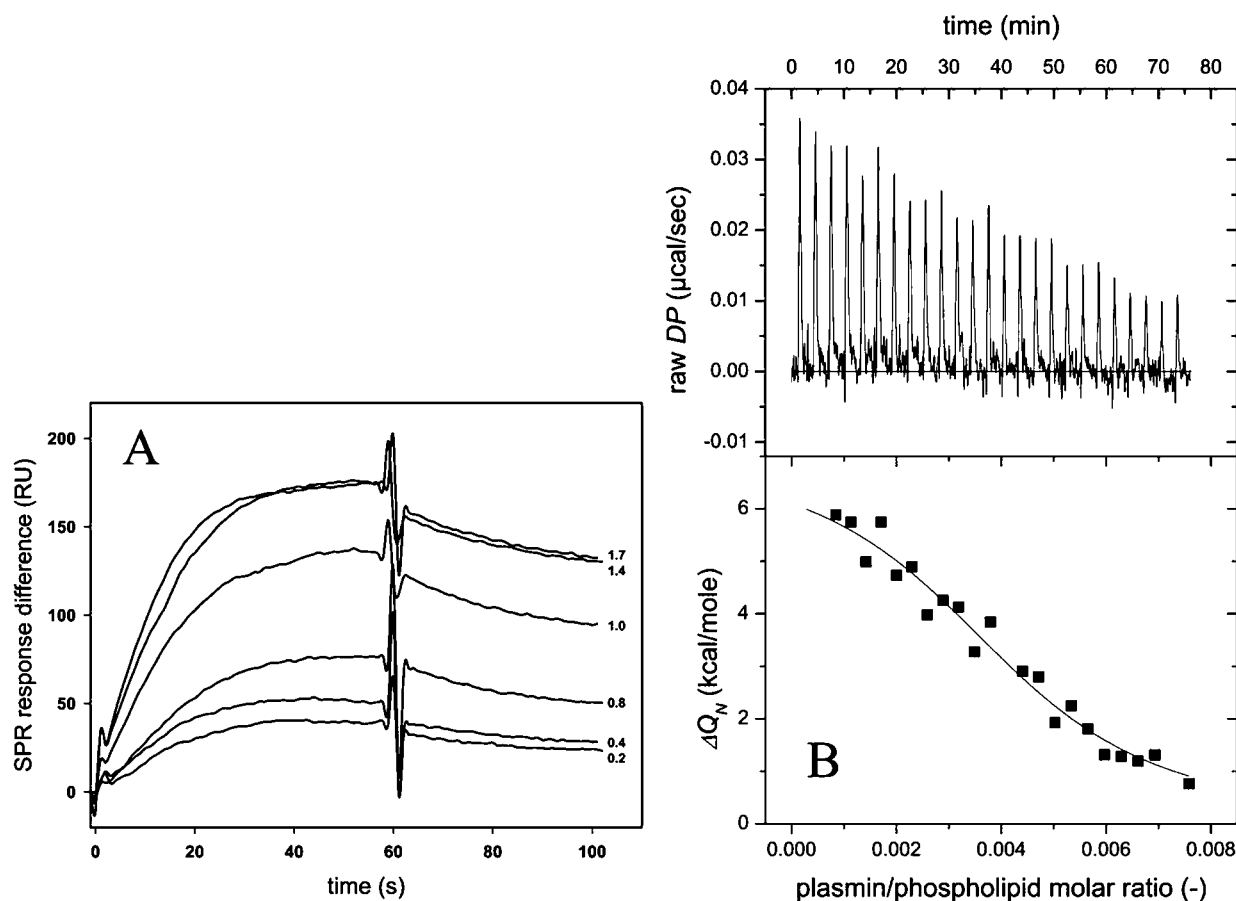
FIG. 7. **Interaction of plasmin with PCPS1:1.** *A*, PCPS1:1 and PC LUVs are immobilized to the surface of Fc1 and Fc2 of Sensor chip L1; plasmin is injected over the surface of the chip at 40 μl/min flow rate, and the SPR response is recorded. The figure shows the difference in the response of the PCPS1:1 and the PC surfaces for 6 different concentrations of plasmin in the range 0.2–1.7 μM (indicated next to the curves). *B*, the cell (1.43 ml) of the titration calorimeter is filled with 0.5 mM PCPS1:1, 25 successive aliquots (10 μl each) of 0.02 mM plasmin are injected into the cell at 25 °C, and the heat increments of each addition (raw differential power, *DP*) are measured (*top panel*). The base-line corrected, peak-integrated, and concentration-normalized enthalpy changes (ΔQ_N, symbols, *bottom panel*) are evaluated according to the single site algorithm. The *curve* shown in the *bottom panel* is the regression binding isotherm gained with the parameters reported in the Table I.

TABLE I

Binding and thermodynamic parameters of the interaction of plasmin with phospholipids

The parameters are gained from the experiments illustrated in Figs. 7 and 8. The ITC data analysis software reports the association equilibrium constant (K_a), the number of binding sites per molecule in the cell (N), the enthalpy (ΔH) and entropy (ΔS) changes accompanying binding, as well as the statistical measures of their variance. Because in our experiments the injected proteins bind to clusters of phospholipid molecules, the reciprocal value $1/N$ is more informative and has the physical meaning of size (in number of molecules) of clusters providing one binding site for the protein. For comparative purposes (BIA results, data in the literature), the dissociation equilibrium constant ($K_d = 1/K_a$) is reported instead of K_a. The S.D. of the two reciprocal values was estimated from the S.D. of N and K_a reported by the ITC software using a bootstrap procedure for normally distributed parameters (48).
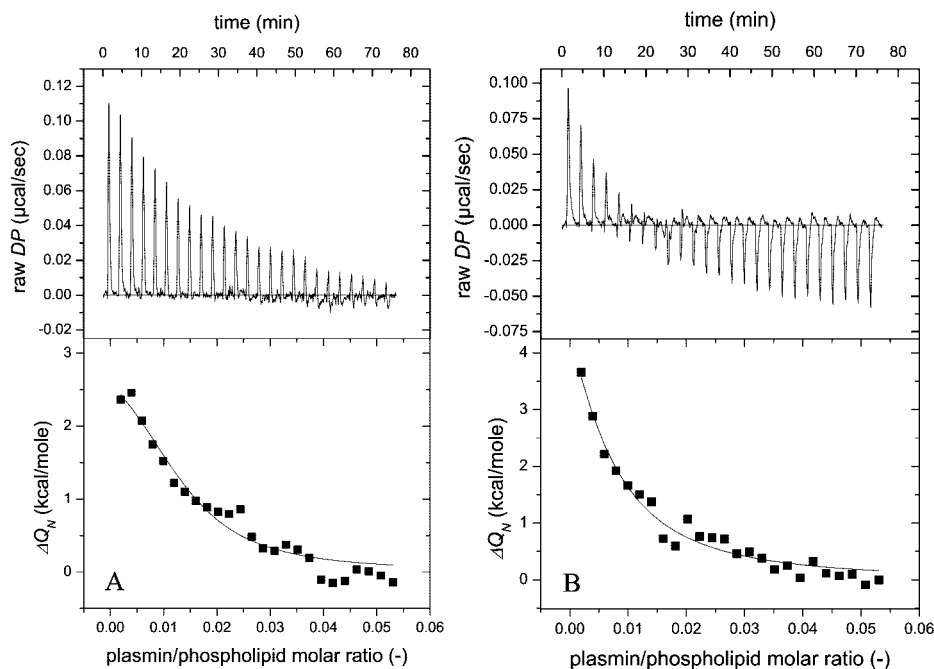
	Phospholipid			
Parameter	PCPS1:1		PCPS3:1	
	25 °C	37 °C	25 °C	37 °C
K_d (μM)	0.35 ± 0.08	0.57 ± 0.14	2.45 ± 0.50	5.50 ± 1.20
$1/N$ (−)	229.4 ± 10.6	224.2 ± 18.0	78.6 ± 9.4	234.6 ± 14.0
ΔH (kcal/mol)	7.03 ± 0.5	4.85 ± 0.69	3.42 ± 0.54	13.48 ± 9.09
ΔS (cal/K·mol)	53.1	44.2	37.2	67.6

than the PCPS3:1. The values of the dissociation equilibrium constants of both plasmin and tPA indicate an order of magnitude higher affinity to PCPS1:1 than to PCPS3:1.

The electric charge, however, is not a sufficient prerequisite for the phospholipid effects. If the water-soluble anionic dihexanoylphosphatidylserine is used in the functional assays even at 5-fold higher molar concentrations than the membrane-forming phospholipids, neither the fibrin dissolution nor the plasminogen activation is inhibited. Not only the existence but also the type of membrane arrangement is essential. The ordered gel structure PC and PS mixtures (with solely dipalmi-

toyl derivatives and a T_m of 41 °C) (27) inhibit the tPA-induced fibrinolysis at 37 °C. If the membrane is in the liquid-crystal-line structure at temperatures far from the melting point, the inhibiting effect is completely eliminated as demonstrated for poPCPS1:1 (with 50% palmitoyloleoyl component and a T_m less than 20 °C) (23) in Fig. 2*B*. The poPCPS mixture with 20% palmitoyloleoyl component and a T_m of 33 °C (23) inhibits fibrinolysis at near T_m temperatures: stronger on the gel phase side of the transition (25 °C) and weaker on the liquid crystal-line side (37 °C). In line with the gel phase requirement and as expected from the overall lipid composition of the platelet mem-

Fig. 8. **Interaction of plasmin with PCPS3:1.** The measurements were carried out as described in Fig. 6*B* using 0.5 mM PCPS3:1 in the cell and 0.14 mM plasmin in the syringe at 25 °C (*A*) and 37 °C (*B*). The last raw differential power (*DP*) peaks of *B* illustrate the size of the dilution heat that can be measured if 0.14 mM plasmin is diluted in buffer at 37 °C.

brane (22), the LUVs prepared from purified platelet phospholipids inhibit fibrinolysis mainly at 25 °C (Fig. 2). At 37 °C the maintenance of gel phase structures in the platelet membrane is probably related to the presence of proteins, which contribute to the formation of lateral membrane domains with different lipid composition (24–27). This explanation is in agreement with the results on the effects of the crude platelet homogenate, which inhibits fibrinolysis to a similar degree at 25 and 37 °C, but if the proteins are eliminated, the phospholipids reproduce the inhibiting effect only at the near-melting temperature (25 °C). The phase structure requirement explains why studies approaching the dissolution of alveolar fibrin deposits and modeling the action of lung surfactant with dipalmitoylphospholipids (gel phase) detected inhibition (12, 19), whereas others working with natural phospholipid mixtures (with abundant unsaturated fatty acyl derivatives) missed this effect (11). Because the gel phase formation at a given temperature is proportional to the fraction of saturated acyl moieties in the phospholipid membranes, the decreased frequency of such domains in platelet membranes could be an additional factor contributing to the beneficial effects of unsaturated fatty acids in atherothrombosis. The decreased anti-fibrinolytic potency of the more fluid membranes may counterbalance their favorable effects on the assembly of the prothrombinase complex (50).

The enthalpy data from our calorimetric experiments provide hints on the thermodynamic background of the gel phase structural requirement discussed above. Under the experimental conditions the interactions of plasmin, plasminogen, and tPA with LUVs are endothermic (Figs. 7 and 8), and consequently these are thermodynamically spontaneous, if entropy increases in parallel (Table I). The increase in entropy is probably caused by the protein-induced rearrangements in the ordered gel structure of the LUVs. However, if originally the phospholipid is in the disordered liquid crystalline state, the interactions are thermodynamically limited (in line with the behavior of poPCPS discussed above).

The necessity for membrane formation raises the possibility that the phospholipid structures fill in the pores of the fibrin gel and thus impair the penetration of exogenously applied proteins. This possibility is confirmed by the data on the size of the superficial reactive layer (Fig. 5) and the amount of fibrin-retained protein (Fig. 6). The anionic LUVs decrease the amount of tPA in the clot to 25% of the level in the absence of phospholipids, and the activator occupies a layer 70–100% (depending on the fraction of anionic phospholipid) the depth in lipid-free fibrin. The penetration of plasmin is also significantly reduced by anionic LUVs; both the amount of enzyme in the clot and the occupied layer decrease by ~50%.

The phospholipids are not only a diffusion barrier, they also bind the components of the fibrinolytic system (Figs. 7 and 8). The affinity of the binding is characterized by K_d values in the range 0.08–7.6 μM for plasmin and tPA (lower values for the more negative LUVs) and an order of magnitude higher dissociation constants for plasminogen. The differences in the binding parameters determined on phospholipid monolayers and vesicles suggest that the curvature of the surface might be an important factor in the interactions with proteins because of the large size of the single binding sites composed of 100–200 phospholipid molecules for all examined proteins (plasmin, tPA, and plasminogen) (Table I), which corresponds to an area of 150–300 nm^2 (51). At this point it should be emphasized that we have not performed any direct evaluation of the structural requirements for the binding of fibrinolytic factors to membranes, and thus, although plausible, the latter suggestion remains speculative until direct support is not provided. Furthermore, it can be a matter of debate which surface geometry (planar monolayer or spherical vesicle) approximates better the *in vivo* situation, where platelet membranes and their remnants are dispersed in thrombi. Most probably the parameters of the real interactions lie within the range limited by the values determined under these two extreme conditions. The binding of fibrinolytic components to phospholipids provides alternative location for them within the thrombi and may explain some apparently paradoxical observations, *e.g.* that thrombi-bound plasminogen is inversely related to thrombus lysibility (52). Within thrombi, phospholipids compete with fibrin for the binding fibrinolytic components and retard them in their lytic action. The affinity parameters determined in our study are comparable with the dissociation constants of fibrin with native plasminogen (38 μM), proteolytically modified plasminogen (0.32 μM), or tPA (0.02–1 μM) (see Ref. 53 and reviewed in Ref. 54). Considering the physiological plasma levels of plasminogen (around 2 μM) the "steal" effect of phospholipids may be of primary relevance for plasmin (Table I).

In conclusion, our study identifies phospholipids as platelet-derived components responsible for the PAI-1-independent inhibition of fibrinolysis (6), suggests some structural aspects of this inhibition, and points out the necessity to design thrombolytics that penetrate into the amphiphilic milieu created by the phospholipids or destroy the phospholipid barrier in arterial thrombi.

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