Inhibition of Prothrombinase by Human Secretory Phospholipase A2 Involves Binding to Factor Xa

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Carine M. Mounier‡‡, Tilman M. Hackeng††, Francis Schaeffer‡‡, Grazyna Faure‡, Cassian Bon††††, and John H. Griffin§§§

From the §Unité des Venins, Institut Pasteur, 75724 Paris, France, the ‡Departments of Molecular and Experimental Medicine and of Vascular Biology, The Scripps Research Institute, La Jolla, California 92037, the Laboratory de Résonance Magnétique Nucléaire, Institut Pasteur, 75724 Paris, France, and the †Unité de Cervy-Pontoise, Department of Biology, 95011 Cergy-Pontoise, France

Human group II secretory phospholipase A2 (hsPLA2) exhibits significant anticoagulant activity that does not require its enzymatic activity. We examined which coagulation factor was targeted by hsPLA2 and analyzed which region of the protein may be involved in this inhibition. Prothrombin time coagulation assays indicated that hsPLA2 did not inhibit activated factor V (FVa) activity, whereas activated factor X (FXa) one-stage coagulation assays suggested that FVa was inhibited. The inhibitory effect of hsPLA2 on prothrombinase activity of FXa, FVa, phospholipids, and Ca2+ complex was markedly enhanced upon preincubation of hsPLA2 with FXa but not with FX. Prothrombinase activity was also strongly inhibited by hsPLA2 in the absence of PL. High concentrations of FVAs in the prothrombinase generation assay reversed the inhibitory effect of hsPLA2. By using isothermal titration calorimetry, we demonstrated that hsPLA2 binds to FXa in solution with a 1:1 stoichiometry and a $K_a$ of 230 nM. By using surface plasmon resonance we determined the rate constants, $k_{on}$ and $k_{off}$ of the FVa/hSPLA2 interaction and analyzed the Ca2+ effect on these constants. When preincubated with FXa, synthetic peptides comprising residues 51–74 and 51–62 of hsPLA2 inhibited prothrombinase assays, providing evidence that this part of the molecule, which shares similarities with a region of FVa that binds to FXa, is likely involved in the anticoagulant interaction of hsPLA2 with FXa. In conclusion, we propose that residues 51–62 of hsPLA2 bind to FXa at a FVa-binding site and that hsPLA2 decreases the prothrombinase generation by preventing FXaFVa complex formation.

The human group II secretory phospholipase A2 (hsPLA2) has been detected in various cellular types including macrophages, eosinophils, and blood platelets (1). hsPLA2 is associated with the α-granules of platelets and is released into the extracellular medium upon activation (2). The hsPLA2 shares common characteristics with other group II secretory phospholipase A2 (sPLA2), including polypeptide sequence homology, and its catalytic mechanism of action is identical (3–6). The hsPLA2 has attracted particular attention with respect to inflammatory diseases since its level in body fluids correlated with the severity of the pathological states (7, 8). hsPLA2 may be involved in the degradation of bacteria (9), in exocytosis/degranulation processes (10, 11), and in the production of eicosanoids by stimulated inflammatory cells (12–14).

The hsPLA2 does not participate in the production of eicosanoids during platelet activation and does not interfere with platelet stimulation once secreted (15, 16). Platelet activation plays a central role during hemostasis, leading to primary plug formation and increasing the efficiency of the coagulation process (17–19). Blood platelets are also a source of factor V (20). It was thus tempting to suggest a role for hsPLA2 on blood coagulation, once it is secreted by activated platelets. The prothrombinase complex, composed of FVa, FXa, phospholipids (PL), and Ca2+, plays a central role in the coagulation cascade (17, 21). hsPLA2 exerts a moderate anticoagulant effect on plasma (22) and inhibits prothrombinase activity (23, 24). The anticoagulant effect of hsPLA2 is phospholipid-independent and not observed when FV-deficient plasma is used for coagulation assays (24). This observation leads to the hypotheses that the molecular target of hsPLA2 is either the FV/FVa protein, the FXa protein, or both proteins.

All sPLA2s have a common active site but may possess distinct pharmacological sites, explaining the diversity of pharmacological effects of venom sPLA2s, such as neurotoxicity, myotoxicity, cardiotoxicity, inhibition or potentiation of platelet aggregation, and anticoagulant action (25, 26). In particular, all venom anticoagulant sPLA2s have a basic pI correlated with the presence of basic amino acids located between residues 50 and 80 which may be involved in the anticoagulant effect of venom sPLA2 in addition to the lipolytic activity (27, 28).

The aim of this study was to identify the human plasma coagulation factor target for the anticoagulant effect of hsPLA2 in biochemical studies using purified proteins. Isothermal titration calorimetry affords simultaneous determination of all thermodynamic parameters relevant to protein-protein binding, including $N$, the stoichiometry of the association reaction; $K_a$, the association constant ($K_a = 1/K_d$); and the enthalpic ($\Delta H^\circ$) and entropic ($\Delta S^\circ$) contributions to the Gibbs free energy of association ($\Delta G^\circ$) (29, 30). By using this technique, we demonstrated here that hsPLA2 binds to FXa in solution. By using

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‡‡ To whom correspondence should be addressed: Unité des Venins, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France; Tel.: 33-1 45 68 86 85; Fax: 33-1 40 61 30 57; E-mail: cbon@pasteur.fr.

§§ To whom requests should be addressed: Unité des Venins, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France; or Depts. of Molecular and Experimental Medicine and of Vascular Biology, The Scripps Research Institute, 10555 North Torrey Pines Rd, SBR-5, La Jolla, CA 92037.

The abbreviations used are: hsPLA2, human group II secretory phospholipase A2; sPLA2, secretory phospholipase A2; BSA, bovine serum albumin; FV/FVa, factor V/activated factor V; FXa, activated factor X; ITC, isothermal titration calorimetry; PL, phospholipid; RU, resonance unit; SPR, surface plasmon resonance.
surface plasmon resonance, we investigated the effect of calcium ion on the kinetic rate constants of the FXa/hsPLA₂ interaction. Furthermore, by using overlapping synthetic peptides corresponding to residues 51–74, we determined which part of the hsPLA₂ is likely to be involved in its anticoagulant action.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human prothrombin was purified according to diScipio and Davie (31). Human FV was purified as described (32) with minor modifications (33). Activation of human FV was performed as described previously (33). FIXa, FXa, and FVa were from Enzyme Research Laboratories (South Bend, IN). Recombinant FVIII was a kind gift of Dr. Roger Lundblad of Baxter Hyland, Duarte, CA. The chromogenic substrate for thrombin amidolytic activity, CBS 34.47, and the chromogenic substrate for FXa amidolytic activity, S-2222, were from Diagnostica Stago (Asnières, France) and Chromogenix (Cincinnati, OH). FV-deficient plasma was from George King Bio-Medical (Overland Park, KS). Innovin (recombinant tissue factor reagent) was from Dade Diagnostics (Miami, FL); BSA (fraction V) was from Sigma. The synthetic scrambled peptide containing hsPLA₂ residues 51–74 as well as peptides 51–62, 59–70, 63–74, 62–51 (reverse) and 51–62 (all residues in the D-configuration) were from NeoSystem, Isochem SA (Strasbourg, France). The synthetic peptide containing residues 51–74 of the hsPLA₂ was synthesized by the organic chemistry unit of Pasteur Institute (Paris, France). The phospholipase A2 species is referred to as hsPLA₂ (Escherichia coli).

**Methods**

**Preparation of Recombinant hsPLA₂**

The recombinant Leu₈-hsPLA₂ was expressed as a fusion protein in E. coli. The pepstatin A-containing hsPLA₂ was purified by high pressure liquid chromatography (purity ≈95%), and the predicted sequence was verified by the observed mass using mass spectroscopy. All these peptides have an N-terminal acetyl group and a C-terminal amide group.

**Prothrombinase Assay**

Phospholipid vesicles (ratio of 1.9, phosphatidylcholine:phosphatidylserine) (PL) were prepared essentially as described by de Kruijff et al. (35). Prothrombinase-purified components (the concentrations, which are given below) were mixed and subsequently assayed at 37 °C in Tris-buffered saline (0.1 M Tris-HCl, 0.05 M NaCl, 0.5% BSA, 5 mM CaCl₂, pH 7.4) in the following various combinations.

**FV, FXa, and PL**—In FVa/FXa/PL preincubation conditions, 20 pm FXa was incubated with 120 pm FVa for 4 min, and then the reaction was started with 5 µM PL and 200 nM prothrombin. In FV preincubation conditions, 10 pm FVa was purified by high pressure liquid chromatography (purity ≈95%), and the predicted sequence was verified by the observed mass using mass spectroscopy. All these peptides have an N-terminal acetyl group and a C-terminal amide group.

**Prothrombinase Inhibition by hsPLA₂**

The recombinant Leu₈-hsPLA₂ was expressed as a fusion protein in E. coli. The peak activity of hsPLA₂ was referred to as hsPLA₂ throughout this paper. Active hsPLA₂ was obtained following tryptic cleavage of the fusion protein and then purified as described (24, 34).

**FXa Activity Measured in a Prothrombin Time Coagulation Assay**

FVA (1 nm) was incubated for 2 min at 37 °C in Hepes-buffered saline containing 5 mM CaCl₂ in the presence of various concentrations of hsPLA₂. Then, a prothrombin time assay was performed as follows. 5 µl of the incubation mixture was added to a pre-warmed mixture of 25 µl of FVa/FXa-PL, and then the activation was started by the addition of 100 µl of CaCl₂ (20 mM). The coagulation time was recorded using an ST4 coagulometer (Diagnostica Stago, Asnières, France).

**Isothermal Titration Calorimetry (ITC)**

Experiments were carried out on the MicroCal MCS ultrasonic titration calorimeter (MicroCal Inc., Northampton, MA) using the ORIGIN software provided by the manufacturer for instrument control and data acquisition (36). To improve base-line stability, the temperature of the system was kept at 5 °C below the temperature of the actual experiment with a water bath, and temperature was equilibrated for 12 h. During a titration experiment, the FVVa sample was thermostatted at 37.0 ± 0.1 °C in a stirred (410 rpm) reaction cell (1.3514 ml), and 31 injections, each of 8-µl volume and 5-s duration, with a 3.5-min interval between injections, were carried out using a 250-µl syringe filled with hsPLA₂ solution. An injection series was preceded by a 2-µl calibration injection. The reference cell of the calorimeter contained water plus 0.01% sodium azide. Data points were averaged and stored at 2-µl intervals. All buffer solutions (Tris-HCl 20 mM, pH 7.4, NaCl 0.125 M, and CaCl₂ 5 mM) were thoroughly degassed by stirring under vacuum before use. Protein samples were prepared in the same batch to avoid any artifacts due to differences in buffer composition. Titration experiments were performed with 1.5, 3.5, and 4.1 µM FXa and corresponding concentrations of hsPLA₂ in the syringe, ensuring a final hsPLA₂:Fxa mole ratio of 2:1 in the reaction cell. Raw calorimetric data, i.e., heats absorbed or released accompanying the addition of aliquots of the hsPLA₂ solution into the FVVa solution, were processed using the software package ORIGIN (36, 37). The area
under the resulting peak following each injection is proportional to the heat of interaction $Q$. When corrected for the titrant dilution heat and normalized to the concentration of added titrant, $Q$ is equal to the binding enthalpy $\Delta H^\circ$ at that particular degree of binding. The calorimetric binding isotherm was fitted by an iterative nonlinear least squares within (Marquardt method) to a binding model employing a single set of independent sites. The association ($K_a$) and dissociation ($K_d$) constants, molar binding stoichiometry ($N$), and molar binding enthalpy ($\Delta H^\circ$) were determined directly from the fitted curve. The Gibbs free energy and molar entropy of binding were calculated using the equations $\Delta G^\circ = -RT \ln K_a$ and $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$, respectively, where $R$ is the gas constant, and $T$ is the absolute temperature in degrees Kelvin.

**Surface Plasmon Resonance (SPR) Experiments**

Studies were performed using a BIACORE™ 2000 system (Biacore AB, Uppsala, Sweden). Reagents, including surfactant P20, the amine coupling kit containing N-hydroxysuccinimide, N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide, ethanalamine hydrochloride, and Sensor Chips CM5 were supplied by Biacore. The immobilization of FXa on the sensor chip surface was performed as follows: 30 μl of FXa (14 μg/ml in 10 mM sodium acetate, pH 4.8) was covalently coupled via primary amine groups on a CM5 sensor chip surface according to the manufacturer's description (Biacore). The immobilization run was performed at a flow of 5 μl/min at 25 °C. The SPR signals for immobilized FXa (three different flow cells with three different quantities of FXa) were found to be 4,400 resonance units (RU), 2,000 RU, and 1,000 RU, where 1 RU correspond to an immobilized protein concentration of 1 pg/mm². Unreacted moieties on the surface were blocked by ethanolamine. One independent flow cell of the same sensor chip, used as a control flow cell, was subjected to a “blank immobilization,” i.e. with no FXa added. All experiments were carried out in 10 mM Hepes, pH 7.4, 0.005% surfactant P20, and 150 mM NaCl. hsPLA2, varying from 0 to 25 μg/ml, was injected in the same buffer in the presence or in the absence of CaCl2 at 5 mM, with a flow of 10 μl/min. Between each injection, surfaces were regenerated with 10 μl of 1 mM NaCl. Analyses were performed at 25 °C. Kinetic constants, $k_{on}$ (association rate constant) and $k_{off}$ (dissociation rate constant), for the interaction of hsPLA2 with immobilized FXa were calculated using BIA evaluation 2.1 software using curve fitting to a simple two-component model of interaction ($A + B = AB$) for a titration of the solution hsPLA2-immobilized FXa (38). Values, for a series of FXa-hsPLA2 complexes, were determined after subtraction of control signals obtained from the injection of various hsPLA2 concentrations on the control flow cell.

**Statistical Analyses**

The significance of the data was evaluated with the Student’s t test for unpaired data.

**RESULTS**

**Effect of hsPLA2 on FXa and FVa Activities Measured in Coagulation Assays**—FXa one-stage coagulation assays, performed with FV-deficient plasma and fixed amounts of FXa that had been preincubated with varying amounts of hsPLA2, showed a dose-dependent inhibition of FXa activity by hsPLA2 (Fig. 1). The inhibition was strongly reduced in the absence of Ca$^{2+}$ in the preincubation mixture, even though Ca$^{2+}$ was later present during FXa one-stage assays (Fig. 1). This implies that the inhibitory effect of hsPLA2 does not involve a Ca$^{2+}$-dependent action on PL, but rather an interaction with FXa that requires the presence of Ca$^{2+}$. The effect of hsPLA2 on FXa activity was investigated with a prothrombin time coagulation assay using FV-deficient plasma. Fixed amounts of FXa were preincubated with hsPLA2 before the assay, in which coagulation efficiency was related to the activity of FXa. Fig. 2A shows that preincubation of hsPLA2 with FXa did not reduce the activity of FXa. Thus, hsPLA2 did not inhibit FXa activity, whereas FXa was inhibited by hsPLA2.

**Inhibition of Prothrombinase Activity by hsPLA2**—Prothrombinase complex formation was first analyzed under different conditions that were suboptimal for prothrombinase activity. Using 20 μM FXa, 120 μM FV, 5 μM PL, and 5 mM Ca$^{2+}$, we observed a strong inhibition of generation of prothrombinase activity by low concentrations of hsPLA2 when hsPLA2 was preincubated with FXa and FV (Fig. 2A). When the PL concentration was decreased to 1 μM, or increased to 50 μM, the inhibitory activity of hsPLA2 was essentially the same (data not shown). When hsPLA2 was preincubated with FXa (Fig. 2B), the inhibitory action of hsPLA2 was much stronger than for preincubation with FV (Fig. 2C), consistent with the above observations made using coagulation assays.

The effect of hsPLA2 on prothrombinase activity was then tested in the absence of PL. We observed a potent inhibitory effect of hsPLA2 in the presence of FVa (Fig. 3A and B). In contrast, the action of FXa on prothrombin in the absence of both PL and FVa was unaffected by hsPLA2 (Fig. 3A and B), suggesting that hsPLA2 does not interfere with direct prothrombin activation by FXa. Moreover, the amidolytic activity of FXa on its chromogenic substrate, S-2222, was not inhibited by hsPLA2 (data not shown). The inhibition of prothrombinase (20 μM FXa, 120 μM FV, and 5 mM Ca$^{2+}$) by hsPLA2 was never complete in the absence of PL, even at high concentrations of hsPLA2 (10 μM), and we observed that the rate of prothrombin activation decreased until a value corresponding to the same as that obtained with FXa alone, i.e. 0.16 ± 0.008 nM thrombin/min compared with 0.14 ± 0.004 nM thrombin/min (Fig. 3A). Under these suboptimal conditions, only a fraction of FXa is bound to FVa, and the prothrombinase activity of free FXa is not inhibited by hsPLA2. When assays were performed in the absence of PL under conditions that increase the ratio of FXa bound to FVa (1 nM FXa, 1 nM FVa, and 5 mM Ca$^{2+}$), the percent of inhibition by hsPLA2 was much higher, and at high hsPLA2 levels the prothrombin activation rate approached that seen for FXa alone (Fig. 3B).

![Fig. 1. Effect of hsPLA2 on FXa and FVa activities in coagulation assays.](image-url)
The effect of hsPLA₂ on intrinsic tenase activity in the absence of PL was examined. Intrinsic tenase-purified components included 25 nM FIXa, 1 nM FVIII, and 5 mM Ca²⁺. The substrate, FX, was used at 1 mM. We did not observe any inhibitory effect of the hsPLA₂ on the ability of these components to activate FX under these conditions, even at hsPLA₂ concentrations up to 10 μM (data not shown). Thus, the PL-independent anticoagulant action of hsPLA₂ was specific for the prothrombinase complex.

We observed an inhibition of the prothrombinase generation in the absence of FVa but in the presence of PL. This may result from an inhibition of the binding of FXa to PL vesicles due to an interaction of hsPLA₂ with FXa, or PL vesicles, or both (data not shown). Thus, the PL-independent anticoagulant action of hsPLA₂ was specific for the prothrombinase complex.

To investigate whether the observed inhibition of prothrombinase complex formation by hsPLA₂ was caused by an effect on the activation of FV by α-thrombin or by FXa, several studies of FV activation were performed. First, 300 nM FV was preincubated for 30 min with or without 3.5 μM hsPLA₂, and then activation was started by the addition of 1 nM thrombin. Second, 3 nM FV was incubated for 30 min with or without 3.5 μM hsPLA₂ in the presence of 25 μM PL, and activation was started by the addition of 6 nM FXa. In both cases, the formation of FVa was determined as a function of time in a prothrombin time assay using FV-deficient plasma. We observed that hsPLA₂ was unable to inhibit FV activation by either thrombin or FXa/PL (data not shown). This suggested that hsPLA₂ may specifically inhibit prothrombin activation by inhibiting the formation of the prothrombinase complex.

Reversal of hsPLA₂ Inhibition of Prothrombinase Generation by Addition of FVa—When conditions were optimal for prothrombinase assembly (i.e., 20 pM FXa, 200 μM FVa, and 5 mM Ca²⁺), we observed a loss of the inhibitory effect of hsPLA₂ on prothrombinase activity in the presence of FVa and PL, we reconstituted the prothrombinase complex with FV instead of FVa (200 nM FXa, 120 μM FV, and 5 mM Ca²⁺). 3.5 μM hsPLA₂ was preincubated with FV, FXa, and PL for 4 min, and then prothrombin (200 nM) was added, and prothrombin activation was followed for 6 min. At this time, various amounts of FVa (4, 20, or 200 pm) were added to the prothrombinase reaction mixture. Consistent with previous experiments (Fig. 2A), in the absence of added FVa, a strong inhibition of prothrombinase generation by hsPLA₂ was seen during the first 6 min (Fig. 4). However, addition of increasing amounts of FVa at 6 min led to a dose-dependent reversal of the anticoagulant effect of hsPLA₂ (Fig. 4).
It should be noted in Fig. 4 that in the presence of hsPLA₂ and FVa but in the absence of additional FVa, the maximal rate of thrombin generation indicated by the slope of the prothrombinase activity curve eventually reached that of the prothrombinase complex obtained in the absence of hsPLA₂ after a prolonged lag time. Thus, in the presence of hsPLA₂, the thrombin activation curve was shifted to the right along the x axis, leading to the suggestion that the assembly of the prothrombinase complex was delaying but eventually was formed and yielded the same final activity. Using the same rationale as described above, this observation can be easily explained as follows: whereas hsPLA₂ is effectively able to inhibit prothrombinase activity in the presence of FV, traces of prothrombin activation will provide enough thrombin to activate FV, yielding high enough levels of FVa to subsequently counteract the inhibitory effect of hsPLA₂. To rule out the possibility that the lag was due to proteolytic destruction of hsPLA₂, we used SDS-polyacrylamide gel electrophoresis to show that the hsPLA₂ was not cleaved by either FXa or thrombin during the prothrombinase complex activity measurements (data not shown).

The hsPLA₂ was thus able to inhibit the prothrombinase activity under conditions that were suboptimal for prothrombinase complex assembly, even when FVa was present. However, when prothrombinase complex assembly conditions were optimal, the presence of FVa reversed the inhibitory effect of hsPLA₂. These results suggest that hsPLA₂ could either bind to FXa at the same site(s) as FVa or that binding of FVa to FXa may modify the structure of FXa leading to the dissociation of hsPLA₂.

**Binding of hsPLA₂ on FXa**—The direct association of hsPLA₂ and FXa was demonstrated using isothermal titration calorimetry (ITC). Fig. 5 (top panel) shows original data from the calorimetric titration of a 1.4-ml solution containing 4.1 μM FXa with a solution containing 45.7 μM hsPLA₂ at 37 °C. Two kinetic phases were associated with each injection. Immediately following injection, an initial exothermic phase (negative numbers) which we will refer to as site binding was observed. This was followed by a smaller and slower endothermic phase (positive numbers) suggesting that a slow conformational rearrangement takes place after binding. As the number of injections increases, binding sites become saturated since the final exothermic peaks decrease in size and remain nearly constant with further injections of hsPLA₂. The corresponding binding isotherm in Fig. 5 (filled squares, bottom panel) shows the association transition. A least squares estimate of association parameters using a single-site binding model gave N = 0.95 ± 0.10, ΔH° = -4.56 ± 0.08 kcal/mol, ΔS° = 15.8 ± 0.8 cal/mol/K (ΔH°/ΔS° = 48%), and Kd = 230 ± 30 nM. These results (Fig. 5) were confirmed by titration experiments performed at lower concentrations of FXa (1.5 and 3.5 μM; data not shown). Therefore, these experiments show that hsPLA₂ and FXa bind with a small change in enthalpy (ΔH°) in a 1:1 stoichiometry. The Kd value is between the Kd values reported for the FVa-FXa complex in the absence of PL (0.8 μM) and in the presence of PL (1 nm) (39, 40).

**Effect of Ca²⁺ on k_on and k_off Rate Constants of FXa/hsPLA₂ Interaction**—The inhibitory effect of hsPLA₂ on FXa activity, measured in FXa one-stage assays, was mainly observed in the presence of Ca²⁺ (Fig. 1). Based on this observation, the influence of Ca²⁺ on FXa/hsPLA₂ binding affinity was tested using surface plasmon resonance (SPR), studies that require less protein material than ITC. SPR allows measurements of the association rate constant (k_on) and of the dissociation rate constant (k_off) of hsPLA₂ to immobilized FXa. The ratio of the rate constants measured by SPR provides an apparent dissociation constant (K_d^app = k_off/k_on) that is an estimation of the equilibrium dissociation constant in solution (K_d) measured by ITC. The K_d^app value is usually smaller than the equilibrium K_d value as SPR rate constant measurements are performed far from equilibrium, and with one immobilized protein which decreases the overall entropy of the association reaction with respect to free protein association in solution.

Effects of Ca²⁺ on k_on and k_off for FXa/hsPLA₂ association are given in Table I. Table I shows that hsPLA₂ binds to FXa immobilized onto the sensor chip surface in the absence of
Ca\(^{2+}\). However, 5 mM Ca\(^{2+}\) in the screening buffer decreases \(k_{\text{off}}\) nearly 10-fold and increases \(k_{\text{on}}\) nearly 3-fold, leading to a 30-fold increase in \(K_{\text{app}}\). Therefore, data in Table I demonstrate that Ca\(^{2+}\) allows the formation a higher affinity FXa-hsPLA\(_2\) complex.

**Inhibition of Prothrombinase Activity by Peptides Related to Residues 51–74 of hsPLA\(_2\).**—We investigated the anticoagulant effect of the synthetic peptide 51–74, corresponding to the basic region of the hsPLA\(_2\) (see Table II for the amino acid sequence of the whole molecule and Table III for three smaller 12-mer peptides related to peptide 51–74). A scrambled peptide containing a randomized sequence comprising the amino acids of residues 51–74, as well as a reversed peptide 62–51 and a control peptide 51–62 that contained all amino acids in the D-configuration were used to characterize the specificity of the inhibitory effects observed with peptides 51–75 and 51–62, respectively.

When peptide 51–74 was preincubated with FXa and FV in the presence of PL, we observed a dose-dependent inhibition of prothrombinase generation that progressively disappeared as described in Fig. 4 for the inhibitory action of hsPLA\(_2\) (data not shown). The \(I_{50}\) value (where \(I_{50}\) is the concentration of inhibitor producing 50% inhibition) for the peptide 51–74 was 8 ± 1 \(\mu M\) (Table III). Among the three smaller overlapping 12-mer peptides, only peptide 51–62 inhibited prothrombinase activity with an \(I_{50}\) value of 22 ± 3 \(\mu M\), close to that observed for the larger peptide 51–74 (Table III). The control scrambled peptide, which did not inhibit in 0.22 mM NaCl, was weakly inhibitory in 0.1 mM NaCl (\(I_{50}\) of 70 ± 5 \(\mu M\)) (Table III). This supports the idea that the inhibitory effect of the scrambled peptide in 0.1 mM NaCl concentration was nonspecific due to its basic nature (net charge +6) rather than due to a specific sequence of residues. In contrast, the inhibition of prothrombinase activity by peptides 51–74 and 51–62 was maintained with similar efficiency in 0.22 mM NaCl (Table III). A 12-mer reversed peptide 62–51 was devoid of inhibitory effect, as was the control peptide 51–62 with all amino acid residues in a D-configuration. These results indicate that the basic region (residues 51–74) of hsPLA\(_2\), and particularly residues 51–62, specifically inhibits prothrombinase activity and is most likely responsible for the inhibition of FXa-FVa complex formation. Furthermore, the stereospecific l-conformation of amino acids in peptide 51–62 was required for the inhibition of FXa-FVa complex formation, since control peptides with either a reversed sequence or with d residues were inactive.

**DISCUSSION**

hsPLA\(_{2b}\), secreted during platelet activation, exhibits anticoagulant activity and may exert a negative feedback regulation on coagulation, which would prevent an excessive procoagulant effect of activated platelets (24). This inhibitory effect does not require the enzymatic activity of the enzyme, indicating that hsPLA\(_2\) may interact with non-phospholipid targets (24). It was suggested that some venom sPLA\(_{2}\)s are able to affect blood coagulation by mechanisms that do not involve their catalytic activity, although the targets of the venom enzymes were not identified (41–43). We investigated which plasma coagulation factors may be affected by hsPLA\(_2\). Prothrombin time coagulation assays indicated that hsPLA\(_2\) did not inhibit FVb activity, whereas FXa one-stage coagulation assays suggested an inhibition of FXa by hsPLA\(_2\) particularly in the presence of Ca\(^{2+}\). An inhibitory action of hsPLA\(_2\) on prothrombinase activity has also been observed (25), although the molecular mechanism was not investigated.

Our results indicate that hsPLA\(_2\) is able to inhibit prothrombinase activity, especially when the conditions are not optimal for prothrombinase complex assembly. We demonstrated that, in the absence of PL and in the presence of FV, preincubation of hsPLA\(_2\) with FXa effectively down-regulated prothrombinase generation (Fig. 3). When FV was present instead of FVa in the presence of PL, hsPLA\(_2\) inhibited generation of prothrombinase activity only until there was enough FVa generated by traces of thrombin/FXa to overcome hsPLA\(_2\) (Fig. 4). The activity of the intrinsic tenase complex (FXa, FVIII, and Ca\(^{2+}\)) was unaffected by hsPLA\(_2\), although this coagulation complex shares common characteristics with the prothrombinase complex (associated with the structural homology of FV and FVIIIa, and of FXa and Fxa). Moreover, activation of FV by either FXa or thrombin was not inhibited by hsPLA\(_2\). These observations indicate that the inhibitory action of hsPLA\(_2\) is likely to be specific for the prothrombinase complex or for its formation.

Our results suggest that hsPLA\(_2\) may bind to FXa at the same site(s) as FVa, but with a lower affinity, and thereby may decrease the prothrombinase activity by inhibiting the formation of a FXa-FVa complex. This was supported by the demonstration that hsPLA\(_2\) binds to FXa with a 1:1 stoichiometry and a \(K_{d}\) value of 230 nM (Fig. 5). Although not necessary for the interaction, the Ca\(^{2+}\) increases the \(k_{\text{on}}\) rate constant and decreases the \(k_{\text{off}}\) rate constant, leading to a higher affinity of hsPLA\(_2\) for FXa (Table I). The prothrombinase complex has a catalytic efficiency in the activation of prothrombin that is several orders of magnitude higher than FXa acting alone. In the absence of PL, the association of FV with FXa is governed by a \(K_{d}\) of 0.8 \(\mu M\) and is dependent on the presence of Ca\(^{2+}\) (39). In the presence of PL vesicles and Ca\(^{2+}\), the \(K_{d}\) of FVa for the PL-FXa complex decreases to approximately 1 nM (40). This may explain why, under suboptimal conditions where FVa was added in the absence of PL, FVa was unable to eliminate the hsPLA\(_2\) inhibitory effect, since in this case the affinity of FVa for FXa is lower than that of hsPLA\(_2\) for FVa.

We (24) and others (22) have previously reported that hsPLA\(_2\) possesses an anticoagulant activity in whole plasma, as demonstrated by the increase of its recalcification time, and that this anticoagulant activity in plasma is even observed when activated platelets are present. hsPLA\(_2\) is thus able to produce an inhibitory effect on blood coagulation under experimental conditions occurring during clot formation. Moreover, it is well established that the level of hsPLA\(_2\) in serum is strongly increased, from 0.35 nM up to 0.6 \(\mu M\), under various pathological states associated with inflammation, as in the case of acute pancreatitis, multiple organ failure, septic shock, or rheumatoid arthritis (44–47). Blood platelets secrete large amounts of hsPLA\(_2\) upon activation (2, 15), and it is likely that high local concentrations of this enzyme are reached during stasis platelet activation and secretion or during local thrombolytic events, even if hsPLA\(_2\) levels are more difficult to assess than in serum (48). We demonstrate in this study that the hsPLA\(_2\)/FXa interaction is governed by a \(K_{d}\) of 230 nM, a value

**TABLE I**

|          | \(k_{\text{on}}\) | \(k_{\text{off}}\) | \(K_{d\text{app}} = k_{\text{off}}/k_{\text{on}}\) |
|----------|------------------|-------------------|-----------------------------------------------|
| hsPLA\(_2\)_ | 190,000 ± 40,000 | 0.017 ± 0.001 | 86                                            |
| hsPLA\(_2\)Ca\(^{2+}\_\) | 530,000 ± 120,000 | 0.0022 ± 0.0003 | 4.2                                           |
Table II

| Peptide 51–74 | AcGFYSKGSLSRTFYGNKL
|-------------|---------------------|
|             | (net charge -16)    |

The synthetic peptide 51–74 represents the basic region of the hsPLA2 (residues 51–74) with the cysteine 59 replaced by a serine (indicated by an asterisk), to avoid disulfide formation between peptide molecules. For the two peptides, basic residues are shown in bold, and acidic residues are underlined.

The importance of the 51–62 region in the binding of hsPLA2 on FXa points out the presence of basic clustered residues that might be critical for the interaction. In agreement, it has been shown that substitution of Lys^{56} by Gln in hsPLA2 reduced the antiprothrombinase activity of the enzyme, whereas substituting Asp^{59} by Arg in porcine pancreatic sPLA2 increased this activity (23). The thermodynamic characterization of the hsPLA2-FXa association reaction reported here also agrees with this conclusion. hsPLA2 binding to FXa in solution presents a favorable but small binding enthalpy (ΔH^\circ = -4.56 kcal/mol) and a favorable and significant entropic contribution to binding (TΔS = +4.90 kcal/mol). The small enthalpic contribution may account for the short amino acid sequence size at the binding site, whereas the favorable entropic contribution to
Protegrinase Inhibition by hsPLA₂

Protegrinase activity assays were performed as described under “Experimental Procedures.” Reactants (20 pm FXa, 120 pm FV, 5 µm PL, and 5 mM CaCl₂) were preincubated in the presence of various concentrations of the indicated peptides. After 5-min preincubations, the protegrinase activity was determined and expressed in terms of the protegrinase activity measured in the absence of peptide. Then the percent of residual protegrinase activity was analyzed as a function of the peptide concentration, and the IC₅₀ value was calculated. ND, not determined.

| Peptide name | Sequences | NaCl | IC₅₀  |
|--------------|-----------|------|------|
| Peptide 51–74 | YKRLEKRGSGTKFLSYKFSNSGR | 0.10 | 8 ± 1 |
| Scrambled peptide 51–74 | GFKSGSLSRTRFYKGNKESLRS | 0.22 | 8 ± 2 |
| Peptide 51–62 | YKRLEKRGSGTK | 0.10 | 22 ± 3 |
| Peptide 59–70 | SGTKFLSYKFSN | 0.10 | 22 ± 4 |
| Peptide 63–74 | FLSYKFSNSGR | 0.22 | ND |
| Peptide reverse 62–51 | KTSGGRKELRKY | 0.10 | >200 |
| Peptide d-51–62 | (all d) YKRLEKRGSGTK | 0.10 | >200 |

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