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Membrane-dependent Interaction of Factor Xa and Prothrombin with Factor Va in the Prothrombinase Complex

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Abstract

Because all three protein components of prothrombinase, factors (f) Xa and Va and prothrombin, bind to negatively charged membrane phospholipids, the exact role of the membrane in the prothrombinase reaction has not been fully understood. In this study, we prepared deletion derivatives of fXa and prothrombin in which both the Gla and first EGF-like domains of the protease (E2-fXa) as well as the Gla and both kringle domains of the substrate (prethrombin-2) were deleted. The fVa-mediated catalytic activity of E2-fXa toward prethrombin-2 was analyzed in both the absence and presence of phospholipids composed of 80% phosphatidylcholine (PC) and 20% phosphatidylserine (PS). PCPS markedly accelerated the initial rate of prethrombin-2 activation by E2-fXa, with the cofactor exhibiting saturation only in the presence of phospholipids (apparent K_d ~60 nM). Competitive kinetic studies in the presence of the two exosite-1-specific ligands Tyr^{63}-sulfated $hirudin^{54-65}$ and TM456 suggested that while both peptides are highly effective inhibitors of the fVa-mediated activation of prethrombin-2 by E2-fXa in the absence of PCPS, they are ineffective competitors in the presence of phospholipids. Since neither E2-fXa nor prethrombin-2 can interact with membranes, these results suggest that fVa interaction with PCPS improves the affinity of the activation complex for the proexosite-1 of the substrate. Direct binding studies employing OG_{488}-EGR-labeled fXa and E2-fXa revealed that the interaction of the Gla-domain of fXa with PCPS also induces conformational changes in the protease to facilitate its high-affinity interaction with fVa.

Factor X is a vitamin K-dependent serine protease zymogen in plasma that upon activation to factor Xa (fXa)[1] binds with high affinity to other components of the prothrombinase complex (fVa, negatively charged phospholipid vesicles and calcium) in order to activate prothrombin to thrombin during the blood coagulation process (1-5). The complex formation improves the catalytic efficiency of fXa by greater than 5 orders of magnitude by decreasing the apparent K_m of prothrombin ~100-fold and increasing the k_{cat} ~1000-fold (2,6). The decrease in K_m is thought to be due to the γ-carboxyglutamic acid (Gla) dependent interaction of both fXa and prothrombin with negatively charged phospholipid vesicles which raises the local concentration of prothrombin near the enzyme complex, while the increase in k_{cat} is largely attributed to the cofactor effect of fVa in the reaction (7,8). The mechanism by which fVa improves k_{cat} of prothrombin activation by fXa in the prothrombinase complex is poorly understood, though it has been established that several protein-membrane and protein-protein interactions are the driving force behind the high catalytic efficiency of fXa in the prothrombinase complex (8-11). Thus, a recent study has postulated that fVa binding to fXa in the prothrombinase complex exposes a new binding exosite on the protease that is a recognition site for the substrate, prothrombin, in the activation complex (12). Other kinetic and mutagenesis studies have indicated that the interaction of fVa with the substrate also plays

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a key role in improving the catalytic efficiency of fXa in the prothrombinase complex (13-15). In support of these hypotheses, several recognition sites for fVa on the protease domains of both the enzyme fXa and the substrate prothrombin have been identified by both kinetic and direct binding studies (13,16-19). Thus, it has been demonstrated that the mutagenesis of the basic residues of the heparin binding-site on the protease domain [particularly Arg-165 in the chymotrypsinogen numbering system (20)] dramatically impairs the ability of fXa to interact with fVa in the prothrombinase complex, suggesting that selected basic residues of the heparin-binding exosite of fXa interact with the cofactor (16). In support of a binding site for fVa on the substrate, it has been demonstrated that the exosite-1-specific ligand Tyr^{63}-sulfated $hirudin^{54-65}$ competitively inhibits the fVa-mediated activation of prothrombin by fXa (13). Moreover, the mutagenesis of the basic residues of exosite-1 on the substrate prothrombin (proexosite-1) has been shown to dramatically impair the rate of substrate activation by fXa in the prothrombinase complex (14), supporting the hypothesis that this basic proexosite-1 of the substrate constitutes a recognition site for fVa in the prothrombinase complex.

While recent studies have indicated that both the Gla and kringle-2 domains of prothrombin have binding sites for fVa (5,21,22), the mutagenesis and kinetic studies investigating such a role for either the Gla or the EGF-1 domain of fXa in the prothrombinase complex have not yielded consistent results. Thus, a recent kinetic study utilizing a chimeric fXa/fIXa, in which both the Gla and EGF-1 domains of fXa were replaced with the corresponding domains of fIXa, concluded that neither the Gla nor the EGF-1 domain of fXa contributes to the high-affinity of the fXa-fVa interaction on phospholipid vesicles composed of 80% phosphatidylcholine (PC) and 20% phosphatidylserine (PS) (23). Nevertheless, another study,

[1]Abbreviations

fVa activated factor V

fXa activated factor X

Gla γ-carboxyglutamic acid

EGF epidermal growth factor

GD-fXa Gla-domainless fXa in which residues 1-45 have been deleted by recombinant DNA methods

E2-fXa fXa in which both the Gla and first EGF domains (residues 1-84) have been deleted

fXa-R165A fXa mutant in which Arg-165 in the chymotrypsin numbering (20) has been substituted with an Ala

fXa-des-Gla recombinant fXa in which the Gla-domain is not γ-carboxylated

prethrombin-2 prothrombin derivative in which Gla, kringle-1 and kringle-2 domains have been deleted by recombinant DNA methods

OG_{488} $Oregon\ Green_{488}$

PEG polyethylene glycol

BSA bovine serum albumin

using an identical fXa/fIXa chimeric construct, investigated the affinity of the chimeric fXa for the cofactor by a similar prothrombinase assay and concluded that the Gla and/or EGF-1 domain of fXa contributes ~10-fold to the affinity of the protease-cofactor interaction (24). Our own kinetic studies with a deletion derivative of fXa lacking the first EGF domain did not assign a specific role for EGF-1 domain in the prothrombinase complex assembly (25), however, the removal of the Gla domain (GD-fXa) or both the Gla and EGF-1 domains (E2-fXa) dramatically impaired the rate of substrate activation and the affinity of the mutant proteases for interaction with fVa in both the absence and presence of PCPS vesicles, though with a considerably higher degree of impairment in the absence of the phospholipids (26). These results indicate an apparent role for the interaction of the Gla domain of fXa with PCPS, fVa or both for the high specificity of the protease assembly into the prothrombinase complex. Alternatively, the incorporation of fVa into PCPS vesicles or binding of the negatively charged PS to a regulatory site on fVa may modulate the specificity and affinity of fXa and/or prothrombin for interaction with the cofactor in the activation complex (27-29). However, the investigation of these important questions has been confounded by the observation that all three protein components of the prothrombinase reaction can interact with the PCPS vesicles.

In this study, we investigated the role of negatively charged phospholipids in the recognition specificity of the components of the prothrombinase complex by analyzing the kinetics of fVa-mediated prethrombin-2 activation by E2-fXa in both the absence and presence of PCPS vesicles. Competitive kinetic studies using the proexosite-1 specific ligands revealed that the interaction of fVa with PCPS improves the affinity of prothrombinase for the substrate. Furthermore, direct binding studies employing OG_{488}-EGR-labeled fXa and E2-fXa indicated that PCPS may also induce conformational changes in fXa, thereby facilitating its high-affinity interaction with fVa.

MATERIALS AND METHODS

Recombinant and plasma proteins

The expression and purification of wild-type fX and its deletion derivative lacking both the Gla and the first EGF-like domains (E2-fX) in human embryonic kidney- (HEK) 293 cells have been described (26,30). The deletion mutants of prothrombin lacking both the Gla and kringle-1 domains (prethrombin-1) or Gla, kringles-1 and kringle-2 domains (prethrombin-2) were expressed in baby hamster kidney (BHK) cells and purified to homogeneity as described (14). Thrombomodulin fragment 456 (TM456) was expressed in HEK-293 cells and purified to homogeneity as described (31). Human plasma proteins including fVa, fXa, prothrombin, and the factor X-activating enzyme from Russell's viper venom (RVV-X), were purchased from Haematologic Technologies Inc. (Essex Junction, VT).

Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PCPS) were prepared as described (32). The chromogenic substrates, Spectrozyme FXa (SpFXa) was purchased from American Diagnostica (Greenwich, CT) and S2238 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). Tyr^{63}-sulfated $hirudin^{54\text{-}65}$ ($Hir^{54\text{-}65}(SO^{-}_{3})$) was purchased from Sigma (St. Louis, MO). The N^{α}-[(acetylthio) acetyl] derivative of EGR-ck (ATA-EGR-ck) was a generous gift from Dr. Paul Bock (Vanderbilt University, Nashville, TN). Oregon $Green_{488}$ (OG_{488}) was purchase from Invitrogen (CA).

Activation of factor X derivatives by RVV-X

Both wild-type fX and E2-fX were converted to active forms by RVV-X as described (26, 30). Briefly, each factor X derivative (~1 mg) was incubated with RVV-X (10 nM for wild-type and 100 nM for the mutant) at 37 °C for 2 h in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 containing 5 mM Ca^{2+} (TBS/Ca^{2+}). Time course analysis of the activation reactions indicated

that both fX zymogens were converted to their active forms under these experimental conditions. Wild-type fXa was purified on a Mono Q ion exchange column as described (30). The fully γ-carboxylated proteins were eluted from the ion exchange column at ~0.45 M NaCl as described previously (30). Activated E2-fX (E2-fXa) was separated from the snake venom on SBTI (soy bean trypsin inhibitor) coupled to Affigel-10 as described (33). Active-site concentrations were determined by an amidolytic activity assay and titrations with human antithrombin assuming a 1:1 stoichiometry as described (30). These concentrations were within 80-100% of those expected based on zymogen concentrations as determined from the absorbance at 280 nm using a published absorption coefficient (34).

Prethrombin-2 activation

The initial rate of prethrombin-2 (5 μM) activation by E2-fXa (1-2 nM) was studied in the presence of varying concentrations of fVa (0-200 nM) in the absence or presence of PCPS vesicles (20 μM) in TBS/Ca^{2+} as described (35). Following 30 min incubation at room temperature, the activation reactions in 96-well assay plates were terminated by addition of 20 mM EDTA and the rate of thrombin generation was determined from the cleavage of S2238 (100 μM) at 405 nm by a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) using a standard curve prepared by known concentrations of thrombin. The ($K_{d(app)}$) value for interaction with fVa was calculated from the hyperbolic dependence of activation rates on the concentrations of the cofactor as described (35). In all reactions, it was ensured that less than 5% of prethrombin-2 was activated at all concentrations of the substrates.

Prethrombin-2 activation in the presence of $Hir^{54-65}(SO^{-}_{3})$

The inhibitory effects of TM456 and the hirudin peptide on the kinetics of fVa-mediated (50 nM) prethrombin-2 (1 μM) activation by E2-fXa (5 nM) in the absence and presence of PCPS vesicles (20 μM) was monitored in the presence of increasing concentrations of TM456 (0-10 μM) or the hirudin C-terminal peptide (0-20 μM) in TBS/Ca^{2+} as described (14). The concentration of thrombin generated in each reaction was calculated from a standard curve as described above. To simplify comparisons of the competitor dependence of the activation reactions, the data for all activation reactions were normalized to maximal thrombin generation in the absence of the competitor. The dissociation constant ($K_{d(app)}$) for the interaction of the competitors with prethrombin-2 was calculated by nonlinear least-squares computer fitting of the data by the quadratic competitive binding equation as described (13).

Oregon $Green_{488}$ labeling

The methods developed by Bock were used to conjugate OG_{488} to the active sites of the ATA-EGR-ck inhibited fXa derivatives (36). Briefly, fXa derivatives were incubated with 10-fold molar excess of ATA-EGR-ck and the extent of active-site labeling was monitored by the loss of amidolytic activities using the chromogenic substrate SpFXa. The incubation was continued for 30 min till more than 99.9% of the enzyme activity was inhibited. The ATA-EGR-ck labeled proteins were then incubated with 20-fold molar excess of OG_{488}-iodoacetamide in the presence of 0.1 M NH_2OH for 2 hr at room temperature and then chromatographed on Sephadex G-25 column in order to remove un-reacted reagents. The OG_{488}-labeled proteins, eluted in the void volume, and any potential remaining free dyes was separated by extensive dialysis in TBS/Ca^{2+} at 4 °C. The extent of fluorescence labeling and protein concentrations were determined as described by Bock using an extinction coefficient of 81,000 M^{-1} cm^{-1} at 490 nm for OG_{488} and a correction factor of $\varepsilon_{280nm}/\varepsilon_{498nm}$ = 0.19 for the contribution from the dye to 280-nm absorbance of the labeled proteins (36).

Anisotropy and fluorescent measurements

Steady-state anisotropy and fluorescence measurements were made using an Aminco-Bowman series 2 Spectrophotometer (Spectronic Unicam, Rochester, NY) equipped with an automatic polarizer interfaced to a PC computer for data acquisition and analysis as described (37). The equilibrium dissociation constants (K_D) for the interaction of OG_{488}-EGR-fXa with fVa on PCPS vesicles were determined from the increase in the anisotropy of OG_{488} in the active-sites of fXa derivatives upon interaction with the cofactor as described (37). This was done by titrating increasing concentrations of fVa (0.1-200 nM) with a fixed concentration of each labeled fXa derivative (10 nM) on PCPS vesicles (10 μM) in TBS/Ca^{2+}. The excitation and emission wavelengths were set at 490 and 520 nm, respectively. The bandwidths were set at 8 nm for both excitation and emission. The titration was carried out by the addition of 1-2 μL of a stock solution of fVa (1-4 μM) to the labeled fXa in a 4 × 4 mm quartz cuvette in 0.5 mL volume at 25 °C. The increase in the anisotropy of OG_{488}-EGR-fXa upon interaction with fVa was recorded and plotted as a function of cofactor concentrations as described (37). The KD for the interaction of the labeled fXa with fVa was calculated by nonlinear least-squares computer fitting of the data by the quadratic binding equation. In fluorescent experiments, similar experimental procedures were employed to monitor changes in the emission intensity of the fluorescence probe in the active-site pocket of OG_{488}-EGR-fXa derivatives upon their interaction with PCPS vesicles at excitation and emission wavelengths of 490 nm and 520 nm, respectively as described above.

RESULTS

Effect of PCPS vesicles on prethrombin-2 activation and interaction with fVa

The initial rate of prethrombin-2 activation by E2-fXa in the presence of increasing concentrations of human fVa is presented in Figure 1. The activation curve exhibited a saturable dependence on the concentrations of fVa on PCPS vesicles with an apparent dissociation constant ($K_{d(app)}$) of ~60 nM, however, no saturation kinetics for fVa was observed in the absence of phospholipids at up to 200 nM, the highest concentration of the cofactor used in the reaction. Since neither the protease nor the substrate can interact with PCPS vesicles, these results suggest that the interaction of fVa with the negatively charged phospholipids is associated with structural changes in the cofactor that promotes its interaction with the protease and/or the substrate in the prothrombinase complex.

Based on several recent studies, it has been hypothesized that fVa may provide a binding site for direct interaction with the proexosite-1 of prothrombin in the prothrombinase complex (13,14). In support of this hypothesis, it has been demonstrated that the fVa-mediated acceleration of prethrombin-1 activation by the prothrombinase complex can be specifically and competitively inhibited by the exosite-1 and proexosite-1 specific peptide ligands derived from either thrombomodulin (TM456) or the C-terminal domain of the leech inhibitor, hirudin (13,14). To investigate the possibility that the interaction of fVa with PCPS modulates the structure of the cofactor, thereby influencing its interaction with the substrate, we took advantage of these observations and thus monitored the fVa-mediated activation of prethrombin-2 by E2-fXa in the absence and presence of PCPS vesicles as a function of the increasing concentrations of either TM456 or the hirudin C-terminal peptide $Hir^{54\text{-}65}(SO^{-}_{3})$. Interestingly, while TM456 had no effect on the activation of prethrombin-2 by E2-fXa in the absence of fVa, the peptide effectively inhibited the fVa-mediated activation of the deletion mutant of prothrombin by the deletion mutant of fXa in the absence, but not in the presence of PCPS vesicles (Figure 2A). The Tyr^{63}-sulfated hirudin peptide also exhibited a similar competition profile, thus efficiently inhibiting the substrate activation in the absence of PCPS vesicles, while being a poor inhibitor of the substrate activation in the presence of phospholipids (Figure 2B). Non-linear regression analysis of kinetic data by a competitive binding equation

(13) yielded apparent dissociation constants of 0.6-1 μM for the interaction of competitors with proexosite-1 of prethrombin-2 in the absence of PCPS vesicles, however, no reliable value for interaction of either peptide with the substrate could be calculated in the presence of phospholipids. These results strongly suggest that the affinity of the substrate for fVa is lower in the absence of PCPS, thus the exosite-1 specific ligands are capable of effectively competing with the cofactor for interaction with the substrate. On the other hand, the incorporation of fVa into phospholipid vesicles markedly improves the affinity of the proexosite-1 interactive-site on the cofactor for interaction with the substrate, thus rendering both inhibitory peptides ineffective competitors of the substrate activation by E2-fXa. It should be noted that neither TM456 nor the hirudin C-terminal peptide has a binding site for the protease.

Analysis of fXa-fVa interactions by equilibrium binding measurements

Previous studies have indicated that the fluorescence anisotropy of OG_{488}-EGR, tethered into the catalytic pocket of fXa, is increased upon interaction with fVa on PCPS vesicles (12). Furthermore, it has been demonstrated that the affinity of the Arg-165 to Ala substitution mutant of fXa (R165A) for interaction with fVa has been impaired at least 10-fold, suggesting that this basic residue is an important recognition site for the cofactor in the prothrombinase complex (17,37). We utilized this direct-binding approach to compare the affinity of the OG_{488}-EGR-labeled fXa-R165A and OG_{488}-EGR E2-fXa with that of wild-type fXa in the presence and absence of PCPS vesicles. Since no change in anisotropy for any one of the OG_{488}-EGR-labeled fXa derivatives was observed at up to 200 nM fVa in the absence of PCPS vesicles, the investigation had to be limited to the analysis of the interactions only in the presence of phospholipids. The results presented in Figure 3A suggest that wild-type fXa labeled with the fluorescent probe binds to fVa with a K_D of ~1.5 nM, a value consistent with previous results reported for the interaction of the two proteins using the same approach (12, 38). On the other hand, the R165A mutant of fXa exhibited a markedly weaker K_D of ~ 10.5 nM, suggesting that Arg-165 is a residue on fXa that may directly interact with fVa incorporated into PCPS vesicles. Interestingly, the enhancement in the anisotropy of the labeled probe in the active-site of E2-fXa was approximately twice as high as that of wild-type fXa, whereas the K_D (~60 nM) for the interaction of the fXa deletion mutant with the cofactor was dramatically weakened (Figure 3B). To further investigate this question, we labeled the active-site of a non-γ-carboxylated (fXa-des-Gla) fraction of recombinant fXa which elutes from the Mono Q column at a much lower concentration of NaCl (39). We previously demonstrated that this fraction of fXa cannot bind PCPS vesicles and its proteolytic and clotting activity has been impaired greater than 100-fold, however, this fXa fraction exhibits a normal amidolytic activity (39). Analysis of the binding data suggested that OG_{488}-EGR-fXa-des-Gla interacts with fVa with a K_D of ~60 nM that is similar to the same value obtained for the labeled E2-fXa interaction with the cofactor (Figure 3C). In agreement with the direct binding data, the apparent affinity of fXa-des-Gla for interaction with fVa was dramatically impaired in the prothrombinase assay, as the wild-type and des-Gla derivative of fXa exhibited $K_{d(app)}$ values of 0.5 nM and 50 nM, respectively, in a prothrombinase assay using the prethrombin-1 derivative as the substrate (Figure 4). Essentially similar results were obtained when prethrombin-2 was used as the substrate, except that the rate of activation was reduced ~5-fold for both proteases (data not shown). Taken together, these results support the hypothesis that the interaction of the Gla domain of fXa with PCPS contributes to the high affinity interaction of the protease with the cofactor on phospholipid vesicles.

Equilibrium binding measurements of fXa interaction with PCPS vesicles

Previous studies monitoring the interaction of the fluorescein-labeled EGR-inhibited fXa with PCPS vesicles have reported ~15% quenching of the fluorescence intensity of the labeled fXa upon its interaction with saturating concentrations of negatively charged phospholipids (40, 41). To investigate the hypothesis that the interaction of fXa with PCPS vesicles is associated

with a conformational change in the fVa interactive site of the protease, the fluorescence of the OG_{488}-EGR labeled fXa was monitored in the presence of increasing concentrations of PCPS vesicles. The results presented in Figure 5 indicate that, similar to the fluorescein-labeled EGR-fXa, the fluorescence of OG_{488}-EGR-fXa is decreased ~10% upon interaction with PCPS vesicles yielding a K_D of ~400-500 nM. Interestingly, however, the fluorescence of the OG_{488}-EGR labeled fXa-R165A was not affected by interaction with PCPS, clearly suggesting that Arg-165 of the 170-helix in fXa is part of a structural motif sensitive to the PCPS-mediated conformational change in the active site groove of the protease.

DISCUSSION

Previous results have indicated that the interaction of proexosite-1 of prothrombin with fVa contributes to the recognition mechanism of the substrate by the prothrombinase complex (13-15). We have demonstrated in this study that the incorporation of fVa into the PCPS vesicles improves the affinity of the cofactor for interaction with the substrate/protease. This hypothesis is derived from the observation that PCPS vesicles markedly improved the initial rate of prethrombin-2 activation by E2-fXa in the presence of fVa. Furthermore, the exosite-1 specific ligands TM456 and the hirudin C-terminal peptide were effective competitive inhibitors of the fVa-mediated prethrombin-2 activation by E2-fXa in the absence of PCPS vesicles, however, both peptides were poor inhibitors of the substrate activation in the presence of the negatively charged phospholipid vesicles. Since neither the protease nor the substrate can interact with the membrane, these results suggest that the interactive site of the substrate on fVa in the solution phase may be of low-affinity type, thus explaining the ability of both competitors to effectively inhibit the interaction of the substrate with the cofactor. On the other hand, the substrate binding site of the membrane-bound fVa becomes a high-affinity type, thus rendering the peptides poor competitors of the substrate interaction with the cofactor in the prothrombinase complex. It was interesting to note that TM456 exhibited no inhibitory effect on the substrate activation in the presence of PCPS vesicles, suggesting that the affinity of fVa for interaction with prethrombin-2 is dramatically enhanced upon interaction of the cofactor with the negatively charged phospholipids. Noting that membrane is required for the fVa-mediated thrombin generation by fXa via the meizothrombin intermediate pathway (first cleavage at Arg-320 site), it is likely that such a PCPS-induced structural change in the cofactor is responsible for binding and channeling the substrate into this preferred thrombin generation pathway, as has been previously hypothesized (8). The cofactor interactive site on the substrate is most likely proexosite-1 as evidenced by previous direct-binding and kinetic studies which have established that the hirudin C-terminal peptide and TM456 can specifically interact with both proexosite-1 on prothrombin and exosite-1 on thrombin (13,14,42) and that no binding site for these peptides have been identified on the protease fXa. Based on several mutagenesis and fVa-derived peptide binding studies, an acidic hirudin-like region on the C-terminal end of the A2 domain of fVa has been identified as the potential proexosite-1 interactive on the cofactor (43). However, another similar mutagenesis study with fVa deletion mutants lacking the same C-terminal sequence of the A2 domain has reported no significant role for this region of the cofactor in interaction with the substrate in the prothrombinase complex (44). Thus, further studies may be required to fully characterize the identity of the membrane-dependent proexosite-1 interactive site on the cofactor.

A negatively charged phospholipid-dependent structural change in fVa leading to an improvement in its affinity for the protease fXa has also been reported (7,18,45). Thus, it has been demonstrated that amino acid residues 307-348 of fVa provide an interactive site for fXa (18,46) and that selected Lys/Arg residues of the same sequence are susceptible to inactivation cleavage by plasmin in the presence, but not in the absence of PCPS vesicles (47). It is also known that the cleavage at Arg-306 of fVa by activated protein C requires the interaction of the cofactor with anionic phospholipids, further supporting the hypothesis that this region of

the cofactor may undergo structural changes upon incorporation into PCPS vesicles (48,49). The site on fXa that interacts with this region of fVa on PCPS vesicles has not been identified, though we and others have demonstrated that basic residues of the heparin-binding exosite of fXa contribute to the high-affinity interaction of the protease with fVa in the prothrombinase complex (16,17). In particular, Arg-165 of the 170-helix in the catalytic domain of fXa has been demonstrated to be critical for the high affinity interaction of the protease with fVa on PCPS vesicles (16,17,37). With the exception of the catalytic domain on the heavy chain of fXa, no interactive site for fVa has been identified on the light chain of the protease, though kinetic studies have indicated that the removal of the Gla-domain of fXa dramatically impairs the affinity of the deletion mutant for interaction with fVa, with the mutant protease exhibiting ~1000-fold lower K_D for interaction with the cofactor (26). This finding is in agreement with other kinetic and direct binding studies demonstrating that negatively charged phospholipids improve the affinity of fXa for fVa in the prothrombinase complex by three orders of magnitude (7). Nevertheless, understanding the mechanism and the extent of the contribution of the Gla-domain for the high affinity interaction of fXa with fVa has been confounded by the observation that two different studies using identical chimeric constructs in which the Gla and EGF-1 domains of factor IXa had replaced the same regions of fXa reported inconsistent results, with one study finding a critical role for the Gla and/or EGF-1 domain of fXa in interaction with fVa (24) and the other observing no significant role for either domain in interaction with the cofactor (23). Since both studies used prothrombinase assays to assess the role of these domains in the complex assembly, differences in the assay conditions most likely account for the inconsistent results, underscoring the unreliability of the functional kinetic assays for the quantitative evaluation of the contribution of Gla and/or EGF-1 domains of fXa to specificity of the protease interaction with fVa. Thus, we labeled the active-site of wild-type fXa, the R165A mutant and the Gla-EGF-1 deletion mutant of fXa (E2-fXa) with the fluorescent probe OG_{488} tethered to the tripeptidyl inhibitor EGR-ck and directly measured the K_D values for the interaction of these mutants with fVa at equilibrium. The analysis of the binding data suggested that fVa interacts with the OG_{488}-labeled EGR-fXa with a K_D of 1.5 ± 0.6 nM on PCPS vesicles, which is consistent with the results reported for the fXa-fVa interaction using the same experimental approach by others (12,38). On the other hand, the OG_{488}-labeled EGR-fXa-R165A interacted with fVa with the markedly higher K_D of 10.3 ± 3.5 nM, suggesting that Arg-165 of fXa contributes to the high affinity interaction of the protease with the cofactor. The same studies with the OG_{488}-labeled EGR-E2-fXa suggested that the affinity of the deletion mutant for interaction with fVa on PCPS vesicles (K_D = 62 ± 4 nM) was also dramatically impaired, indicating that the interaction of the Gla-domain of fXa with PCPS makes a significant contribution to the high affinity of the protease interaction with the cofactor. No binding for the fVa-E2-fXa interaction was detected in the absence of PCPS at up to 200 nM cofactor, further suggesting that fVa interaction with the negatively charged phospholipids is also required for the interaction. To understand whether loss of the interaction of the Gla and/or EGF-1 domain of fXa in E2-fXa with fVa or if the loss of its Gla domain interaction with PCPS vesicles accounts for the low affinity interaction of the mutant protease with the cofactor, we also monitored the binding of the OG_{488}-labeled EGR-fXa-des-Gla in which the Gla domain of the full-length protease is not γ-carboxylated and thus unable to interact with the membrane (39). The observation that the affinity of this fXa derivative for interaction with fVa (K_D = 58 ± 9 nM) was weakened to an extent similar to that of the OG_{488}-labeled E2-fXa supports the previous modeling and mutagenesis data that neither the Gla nor the EGF-1 domain of fXa has a direct interactive site for fVa (23,50), however, the reduced affinity of OG_{488}-labeled EGR-fXa-des-Gla for fVa further suggests that the interaction of the protease with PCPS vesicles is required for its high affinity interaction with the cofactor. This would be possible if fXa makes simultaneous interactions with both PCPS and fVa in the prothrombinase complex.

Recently, a membrane-independent role for a soluble phosphatidylserine (C6PS) in regulating the structure and function of both the cofactor and the protease of prothrombinase has been reported (27,28,45,51,52). Thus, it has been demonstrated that C6PS can bind to two sites on C1 and C2 domains of fVa light chain, with the former binding site playing a key role in regulating the prothrombinase complex assembly (27,45). Furthermore, the same group has demonstrated that C6PS can also bind to a regulatory site on Gla-EGF1 domains of fXa light chain to modulate the catalytic activity of the protease in the activation complex (29). Although these previous observations were made with soluble C6PS, nevertheless, the regulation of the prothrombinase activity by this molecule has been shown to be nearly identical to regulation by PCPS vesicles (27,28,51,52). Since E2-fXa lacks both the Gla and EGF1 domains, it is unlikely that the binding of PS to a regulatory site on the mutant protease plays a role in mediating the prothrombinase complex assembly. However, it is possible that the interaction of PS with the regulatory site on the C1 domain of fVa would improve the affinity of the cofactor for E2-fXa and/or prethrombin-2, thus accounting for the PCPS-mediated improvement in the catalytic activity of the mutant prothrombinase as observed in this study. In this model of the prothrombinase complex assembly, the reduced affinity of OG_{488}-labeled EGR-fXa-des-Gla for the PCPS-bound fVa could also reflect the inability of PS to bind to the regulatory site on Gla-EGF1 domains of the fXa variant (29). Further studies with C6PS will be required to test these possibilities.

Spectral data has indicated that the interaction of the fluorescein-labeled EGR-fXa with PCPS vesicles is associated with ~15% decrease in the emission intensity of the fluorophore in the active-site groove of the protein (40,41). A similar PCPS-induced decrease in the fluorescent intensity of the OG_{488}-labeled EGR-fXa was observed in this study. In a recent study, by using a fluorescein labeled Cys-195 mutant of fXa, we demonstrated that the PCPS-induced change in the spectral properties of the fluorescent probe in the catalytic groove does not coincide with a conformational change in the active-site of fXa since the emission intensity of the fluorescein labeled Cys-195 mutant of fXa was not altered upon the interaction of the labeled protease with PCPS vesicles (41). Noting that the fluorescent probe in the EGR-labeled protease is tethered to the P3-Glu residue of the tripeptidyl inhibitor and that the inhibitor through a chloromethylketone group (attached to P1-Arg) covalently binds to His-57 of the protease, it follows that this labeling strategy would position the fluorescent dye somewhere in the vicinity of the S4 subsite of the protease ~15-20 Å away from Ser-195 of the activesite as has been proposed previously (53). It is interesting to note that in the crystal structure of fXIa catalytic domain in complex with ecotin, the basic residues of the conserved 170-helix have been found to constitute part of the S4 loop, thus influencing the specificity of the P4-binding pocket of the catalytic groove (54). In this context, the observation that the loss of the affinity of fXa-R165A for interaction with fVa on PCPS vesicles was associated with a lack of sensitivity in the emission intensity of the fluorescent probe in the catalytic pocket of the mutant suggests that a conformational change in the environment of the positively charged 170-helix of fXa is responsible for the quenching of the fluorescent emission intensity of the probe in the active-site pocket of the protease upon its interaction with PCPS vesicles. It follows therefore that substituting Arg-165 with a hydrophobic residue (Ala) eliminates the decrease in the emission intensity of the fluorophore upon interaction of the protease with negatively charged phospholipids. These results strongly suggest that the interaction of fXa with PCPS vesicles is associated with a conformational change in the 170-helix of fXa, possibly making the residues of this loop accessible for the high affinity interaction with fVa. Further support for this hypothesis is provided by the observation that the mutagenesis of other residues of the 170-helix (in particular Val-163 and Ser-167) is also associated with impairment in the interaction of the mutant proteases with fVa (55). Such a role for the membrane in prothrombinase can explain the basis for the mutagenesis and modeling studies where no role for the Gla-domain of fXa for interaction with fVa on PCPS vesicles has been observed (23, 50), yet the non-membrane interacting fXa mutants E2-fXa and fXa-des-Gla of this study

exhibit dramatic impairment in their affinity for interaction with fVa on PCPS vesicles. In the context of this model, it cannot be ascertained whether the loss of affinity of the R165A mutant of fXa for interaction with fVa is due to a loss of direct interaction of this residue with fVa or if it is the result of a mutagenesis-induced conformational change in the 170-helix of the protease. Whatever the case, it is clear that the 170-helix of fXa plays a critical role in the PCPS- dependent interaction of the protease with the cofactor.

In summary, our results suggest that the role of negatively charged phospholipids in the prothrombinase complex is more than merely co-condensation of fXa with its substrate prothrombin to decrease the K_m of the catalytic reaction. Based on the kinetic and the direct binding data presented above, it appears that protein-phospholipid interactions are associated with key structural changes in the protein components of the prothrombinase complex that facilitate their recognition and high-affinity interaction with one another on the membrane surface. Such a PCPS-mediated recognition mechanism in the prothrombinase complex must play an important regulatory role in coagulation since it ensures that physiological concentrations of fXa and fVa will not form activation complexes to recruit prothrombin unless negatively charged PS containing membrane surfaces become available due to injury and/or inflammation.

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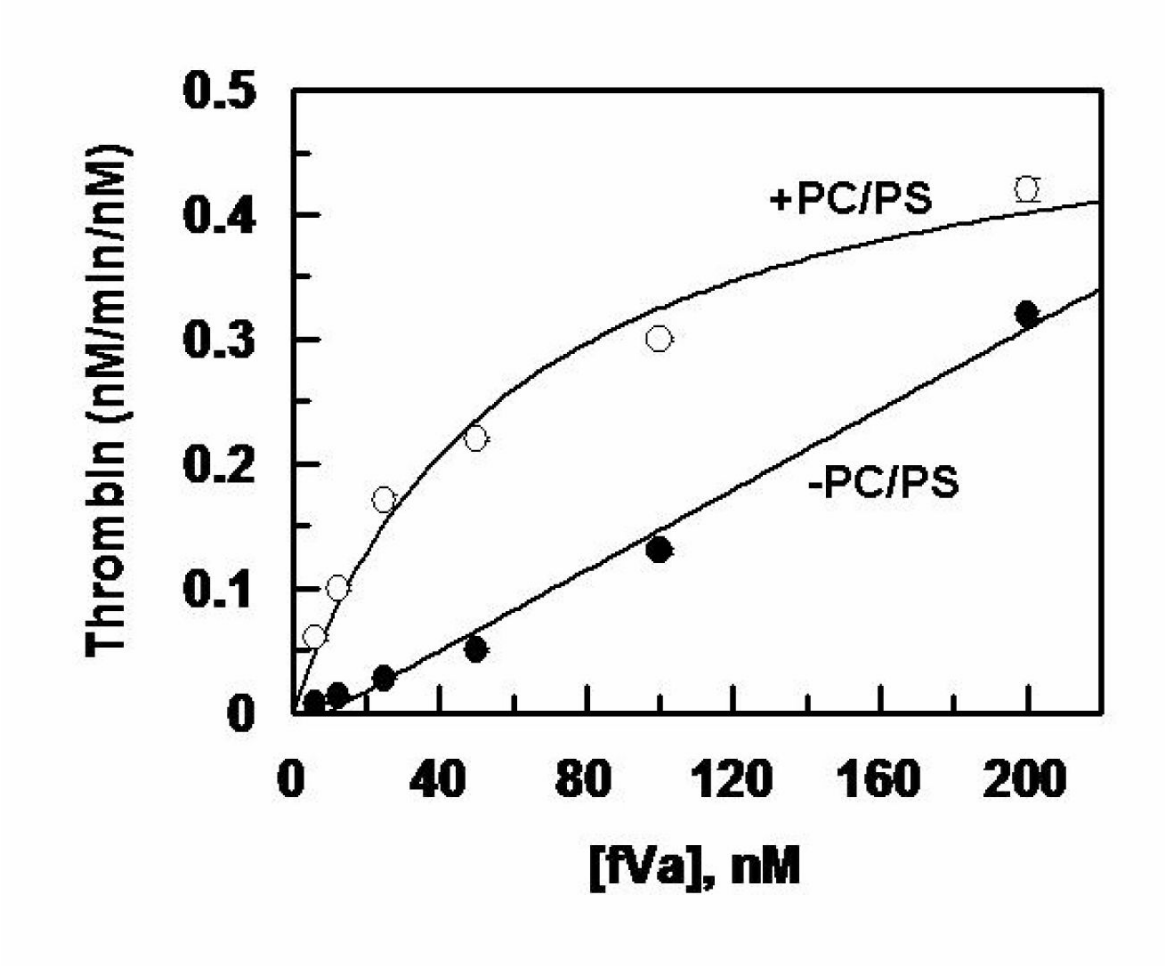

Figure 1.

Concentration dependence of fVa-mediated prethrombin-2 activation by E2-fXa in the absence and presence of PCPS vesicles. The activation of prethrombin-2 (5 μM) by E2-fXa (2 nM) was monitored as a function of increasing concentrations of fVa in the absence (●) or presence (○) of 20 μM PCPS vesicles in TBS/Ca^{2+}. Following 30 min activation at room temperature, EDTA was added to a final concentration of 20 mM and the rate of thrombin generation was measured from the cleavage rate of S2238 as described under "Materials and Methods". Solid lines in the presence of PCPS vesicles are nonlinear regression fits of kinetic data to a hyperbolic equation yielding apparent K_d of 61.7 ± 13.1 nM (n =3) for fVa in the prethrombin-2 activation reaction.

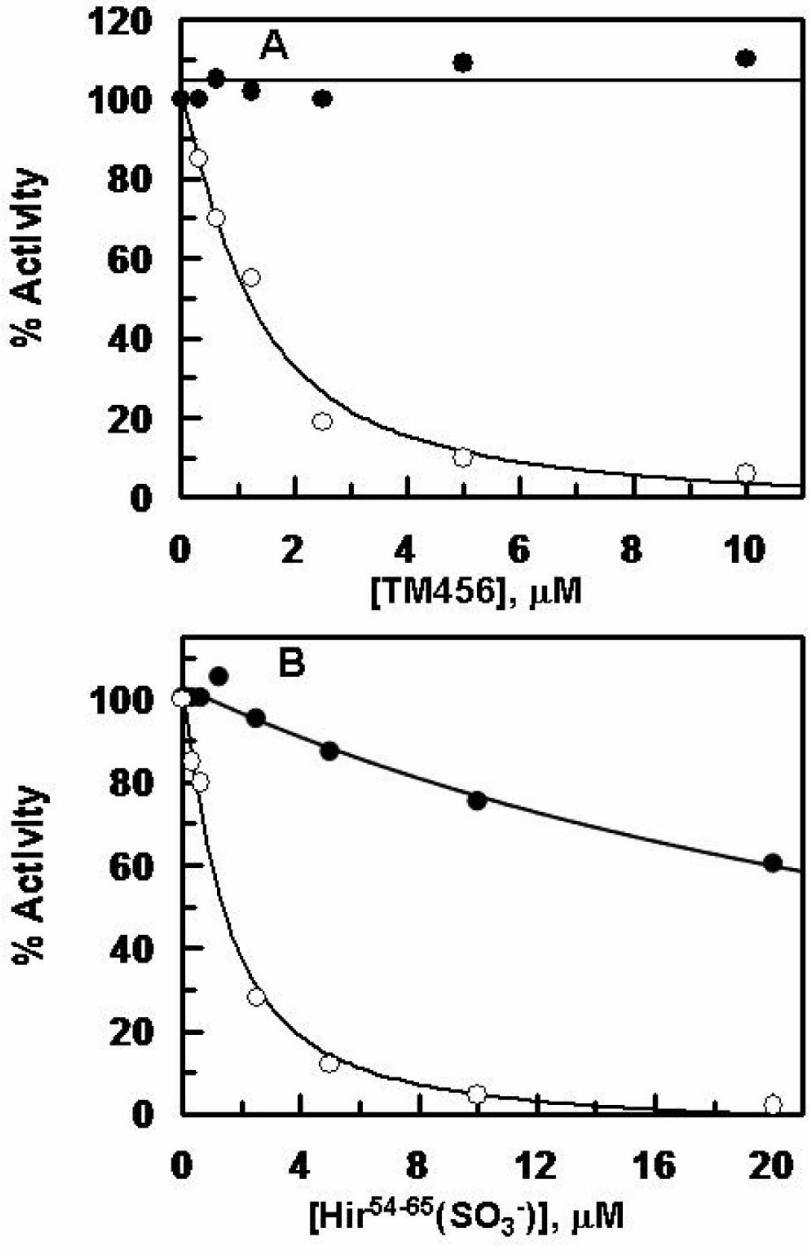

Figure 2.

FVa-dependent inhibitory effects of exosite-1 specific ligands on the E2-fXa activation of prethrombin-2 in the absence and presence of PCPS vesicles. A. The inhibitory effect of increasing concentrations of TM456 (x-axis) on the fVa (50 nM)-mediated prethrombin-2 (1 μM) activation by E2-fXa (1 nM) in the absence (○) or presence (●) of PCPS vesicles (20 μM) was monitored in TBS/Ca^{2+} at room temperature. The initial rate of thrombin generation was measured by an amidolytic activity assay using S2238 and the data were normalized to % of activity at each concentration of the inhibitor (100% in the absence of the inhibitor) as described under "Materials and Methods". B. The same as A except that the inhibitory effect of $Hir^{54-65}(SO^{-}_{3})$ on the fVa-mediated prethrombin-2 activation by E2-fXa was monitored in

the absence (○) or presence (●) of PCPS vesicles. Nonlinear regression analysis of data in the absence of PCPS vesicles according to a competitive binding equation (13) yielded apparent K_d values of 0.7± 0.2 μM (n =3) for the TM456 (panel A) and 0.9 ± 0.2 μM (n =3) for the hirudin C-terminal peptide (panel B) inhibition of the activation reactions.

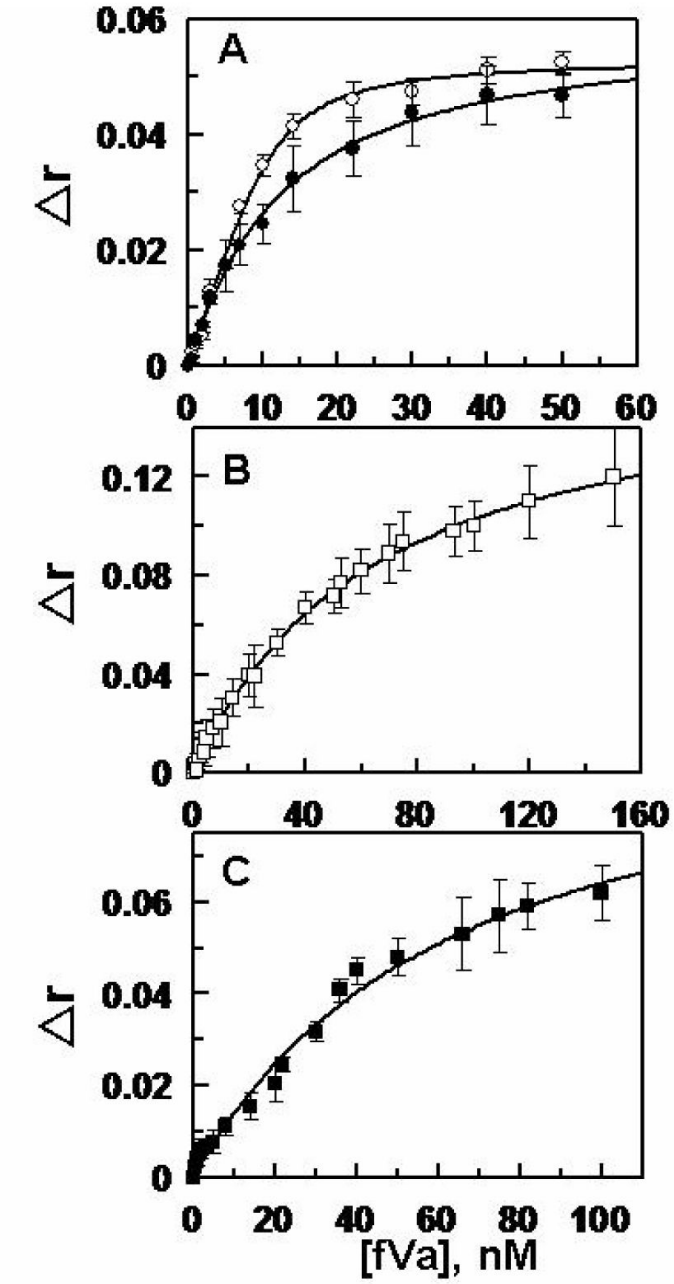

Figure 3.

Enhancement in the anisotropy of the OG_{488}-EGR labeled fXa derivatives upon interaction with fVa on PCPS vesicles. A. Fixed concentrations of the OG_{488}-EGR labeled wild-type fXa (○) (10 nM) or OG_{488}-EGR labeled fXa-R165A (●) (4 nM) were titrated with increasing concentrations of human fVa (0-50 nM) on PCPS vesicles (10 μM) in TBS/Ca^{2+} at 25 °C. K_D values of (1.5 ± 0.6 nM, n =3) and (10.3 ± 3.5 nM, n =3) for wild-type fXa and fXa-R165A, respectively, were calculated for fVa from the saturable changes in the anisotropy (Δr) of the labeled proteins according to a quadratic binding equation as described under "Materials and Methods". B. The same as A except that OG_{488}-EGR labeled E2-fXa (10 nM) was used in the fVa titration yielding a K_D of (62 ± 4 nM, n =3) for the interaction of the mutant protease with

the cofactor. C. The same as above except that OG_{488}-EGR labeled fXa-des-Gla (10 nM) was used in the fVa titration yielding a K_D of (58 ± 9 nM, n =3) for the interaction of the mutant protease with the cofactor.

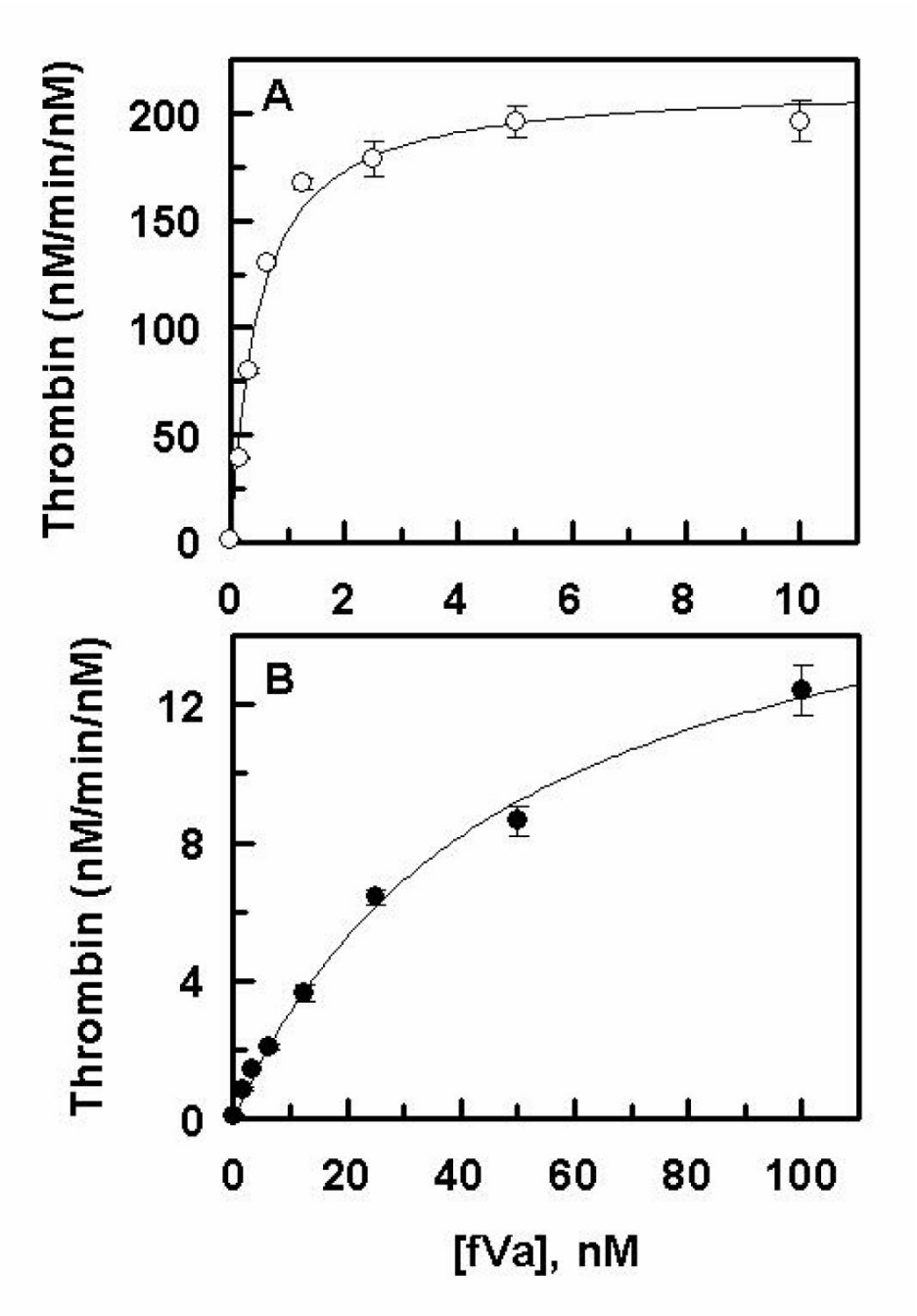

Figure 4.

Concentration dependence of the fVa-mediated prethrombin-1 activation by wild-type fXa and fXa-des-Gla on PCPS vesicles. A. The activation of prethrombin-1 (2 μM) by fXa (○) (0.1 nM) was monitored as a function of increasing concentrations of fVa on PCPS vesicles (20 μM) in TBS/Ca^{2+}. Following 2 min activation at room temperature, EDTA was added to a final concentration of 20 mM and the rate of thrombin generation was measured from the cleavage rate of S2238 as described under "Materials and Methods". B. The same as A except that fXa-des-Gla (2 nM) (●) was used as the protease in the substrate activation reaction. Solid lines in both panels are nonlinear regression fits of kinetic data to a hyperbolic equation yielding

apparent K_d values of 0.5 ± 0.06 nM (n =3) fVa for wild-type fXa and 48.6 ± 6.2 nM (n =3) fVa for fXa-des-Gla.

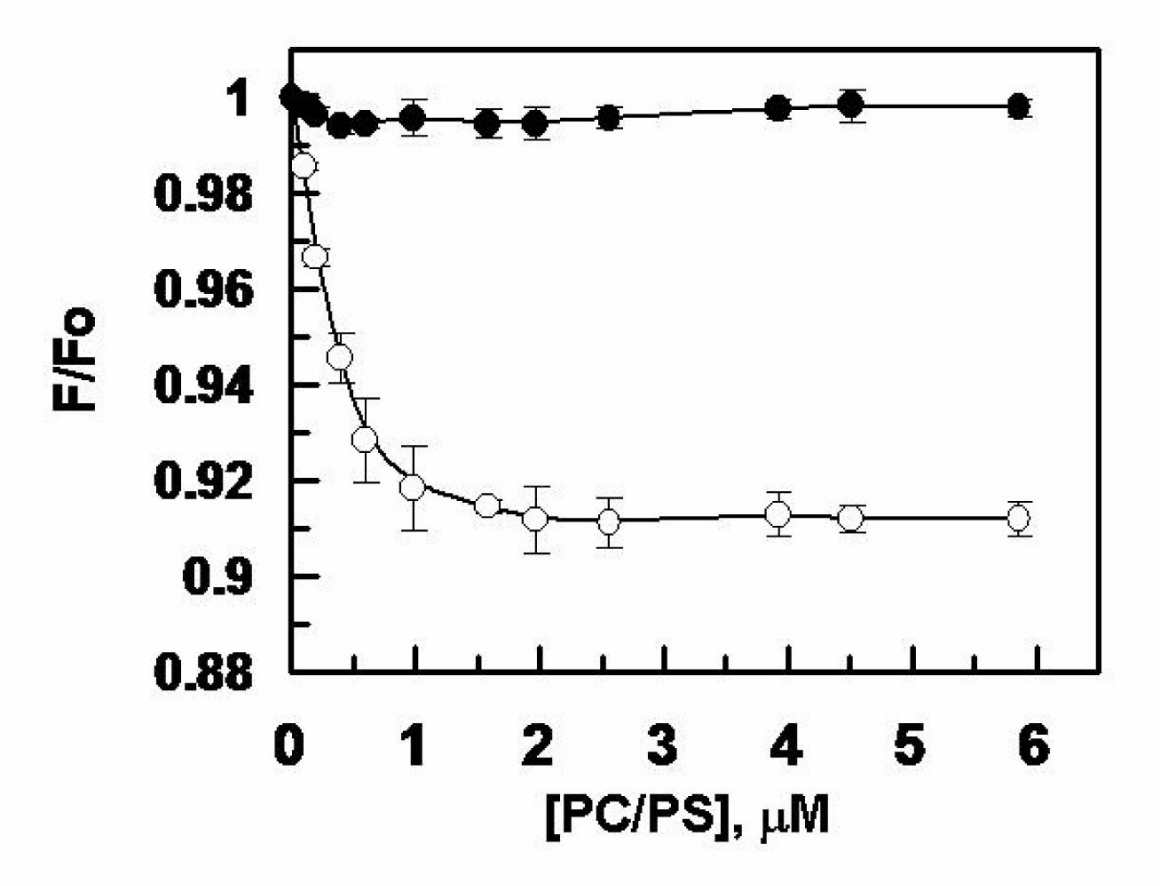

Figure 5.

PCPS-mediated changes in the fluorescence of OG_{488}-EGR labeled fXa derivatives. The OG_{488}-EGR labeled fXa (○) or fXa-R165A (●) (50 nM each) was titrated with increasing concentrations of PCPS vesicles (x-axis) in TBS/Ca^{2+} at 25 °C. The changes in the emission intensity of the fluorescence probe in the active-site pocket of both proteases, upon their interaction with PCPS vesicles, were recorded at excitation and emission wavelengths of 490 nm and 520 nm, respectively, as described under “Materials and Methods”.