Protease Nexin-2/Amyloid β-Protein Precursor Inhibits Factor Xa in the Prothrombinase Complex*

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Protease nexin-2/amyloid β-protein precursor (PN-2/AβPP) is a Kunitz-type protease inhibitor which has been shown to be a tight-binding inhibitor of coagulation factors Xla and IXa. Here we show that PN-2/AβPP and its KPI domain also inhibited isolated factor Xa with a Kᵢ of 10⁻⁸ m. On a solid phase assay, PN-2/AβPP formed a complex with factor Xa. Incubation of molar excess factor Xa to PN-2/AβPP produced a single cleavage within PN-2/AβPP’s heparin binding domain liberating a 8.2-kDa amino-terminal peptide. PN-2/AβPP and its KPI domain equally inhibited factor Xa in the prothrombinase complex with a Kᵢ of 1.9 × 10⁻⁸ m and 1.3 × 10⁻⁸ m, respectively. AβPP995 which does not contain the KPI domain was a substrate of factor Xa but did not inhibit it, indicating the PN-2/AβPP inhibition of factor Xa was not substrate inhibition. All of the factor Xa inhibition in the prothrombinase complex by PN-2/AβPP and its KPI domain on the chromogenic assay was accounted for by inhibition of release of prothrombin fragment F₁₋₂, as determined on immunochromatography. In the prothrombinase complex, PN-2/AβPP inhibited factor Xa with a kₚₒₒₛ = 1.8 ± 0.7 × 10⁶ m⁻¹ min⁻¹ similar to antithrombin III and heparin inhibition (kₒₒₛ of 3.0 ± 0.2 × 10⁶ m⁻¹ min⁻¹). These studies indicated that PN-2/AβPP inhibits factor Xa comparable to antithrombin III in the presence of heparin. PN-2/AβPP’s factor Xa inhibitory activity along with its known inhibition of factors Xla and IXa suggest that this protease inhibitor and related proteins could be regulators of hemostatic reactions on membranes of cells in the intravascular compartment.

Amyloid β-protein precursor (AβPP),¹ a multidomain protein, is the parent protein of amyloid β protein, a 39-42 amino acid peptide that is deposited in senile plaques and in the walls of cerebral blood vessels of patients with Alzheimer’s disease (Kang et al., 1987; Glenner and Wong, 1984). The single gene for AβPP found on chromosome 21 encodes at least three distinct mRNAs produced by alternative splicing that result in three different sized proteins (AβPP995, AβPP752, and AβPP770) (Ponte et al., 1988; Tanzi et al., 1988; Kitaguchi et al., 1988). Two of these mRNAs code for proteins (AβPP995 and AβPP770) which contain a domain homologous to Kunitz-type protease inhibitors (KPI) (Ponte et al., 1988; Tanzi et al., 1988; Kitaguchi et al., 1988). The secreted isoforms of AβPP containing the KPI domain are identical to protease nexin-2 (PN-2) (Van Nostrand et al., 1989; Oltersdorf et al., 1989).

PN-2/AβPP and its KPI domain have been recognized to be potent inhibitors of trypsin, chymotrypsin, epidermal growth factor binding protein, and the γ subunit of nerve growth factor (Van Nostrand et al., 1989, 1990b; Oltersdorf et al., 1989). PN-2/AβPP which is present in high concentrations in platelets is a potent inhibitor of factor Xla (Van Nostrand et al., 1990a, 1990b; Smith et al., 1990). PN-2/AβPP also is an inhibitor of factor IXa (FIXa) in the assembly of the tenase complex on phospholipid vesicles (PSPC), platelets, and endothelial cells (Schmaier et al., 1993, 1995). Similarly, a homologue of PN-2/AβPP, amyloid β-protein precursor-like protein-2, has been shown to have inhibitory activity against hemostatic enzymes factors Xla, IXa, and Xa (Petersen et al., 1994; Sprecher et al., 1993; Van Nostrand et al., 1994). These studies suggest that this family of proteins may have a regulatory role in hemostasis. While examining the ability of PN-2/AβPP to inhibit factor Xa on non-biologic surfaces, we found in one assay that the degree of factor Xa inhibition could not be fully accounted for by its inactivation alone. The present investigation shows that PN-2/AβPP also is an inhibitor of factor Xa alone and when assembled on PSPC in the prothrombinase complex. Recognition that PN-2/AβPP’s inhibitory factor Xa enlarges its role as a regulator of hemostasis.

EXPERIMENTAL PROCEDURES

Proteins—PN-2/AβPP (AβPP752) was purified from fibroblast culture media using techniques of heparin affinity chromatography and immunoaffinity chromatography as described previously (Van Nostrand et al., 1990b). The KPI domain of PN-2/AβPP, which was provided by Dr. Steven Wagner, Salk Institute Biotechnology/Industrial Associates, La Jolla, CA, was produced in a recombinant yeast expression system and purified as described previously (Wagner et al., 1992). The protease inhibitory activities of purified PN-2/AβPP and KPI domain were determined by neutralization with active-site titrated trypsin (Van Nostrand et al., 1990b; Wagner et al., 1992). AβPP995 was obtained and purified like PN-2/AβPP from culture media from human glioblastoma U-138 cells stably transfected to overexpress it (Davis-Salinas et al., 1994). Human factors Xla (FIXa), Xa (FXa), and XI (FII) were purchased from Enzyme Research Laboratories, South Bend, IN. Human FIXa on nonreduced sodium dodeyl sulfate-13% polyacrylamide gel electrophoresis (SDS-PAGE) showed two bands at 52 and 33 kDa, and when reduced with 2% β-mercaptoethanol, four bands at 29, 25, 14, and 12 kDa. The 25- and 12-kDa bands seen on reduced SDS-PAGE represented only 5–10% of the total protein in all preparations. Human FXa was activated with Russel viper venom. On reduced 15% SDS-PAGE, FXa consisted of three major bands at 36, 34, and 24 kDa. These bands
account for 90% of the protein present. Prothrombin on nonreduced 8% SDS-PAGE was a single band at 70 kDa; upon reduction, the apparent molecular mass increased to 80 kDa. All FIXa and Fxa used in these investigations were active-site titrated with antithrombin III (American Diagnostica, Greenwich, CT) using a modified procedure of Griffith et al. (1985) as previously reported (Schmaier et al., 1993). Purified thrombin-activated bovine factor Va (FVa) was purchased from Haemagen Diagnostics, Greenwich, CT. Thrombin-activated bovine factor V (FV) was purchased from Haemagen Diagnostics, Greenwich, CT, using a modified procedure of Griffith et al. (1985). Of the proteins present at 51 and 33 kDa. Human a-thrombin (3250 units/mg) was generously provided by Dr. John W. Fenton III, N.Y. State Department of Health, Albany, NY.

Phospholipid Vesicle Preparation—Phospholipid vesicles were prepared from a mixture of D-α-phosphatidylserine (Sigma) and D-α-phosphatidylcholine (Sigma) (25/75, mol/mol) that were dried in a glass test tube under a stream of nitrogen (Rawala-Sheikh et al., 1990). The dried material was resuspended in 0.05 M Tris-HCl, 0.175 M NaCl, pH 7.5, and sonicated for 30 s multiple times on ice over 60 min (Rawala-Sheikh et al., 1990). After sonication, some preparations were ultra centrifuged at 100,000 g to produce a homogenous suspension free of large particles and multilamellar liposomes (Barenholz et al., 1977). No difference in the cofactor activity of the phosphatidylserine/phosphatidylcholine vesicles (PSPC) was noted whether they were ultracentrifuged or not, and hence the two preparations were used interchangeably.

Measurement of Factor Va Formation—The enzymatic activity of human FIXa was measured by its ability to activate human factor X using polylysine as an artificial surface (Schmaier et al., 1993, 1995; Lundblad and Roberts, 1982; Griffith et al., 1985; McCard et al., 1990). Factor IXa (4.45 nm) was incubated with factor X (400 nm) in 1 ml triethanolamine, 0.1 M NaCl, pH 8.0, containing 0.1% polyethylene glycol (Mn = 8000), 0.2% bovine serum albumin, and 60 nm polylsine for 40 min at 20–25°C. At the end of the incubation, an aliquot of the activated factor X solution was added to a solution of 0.4 nm tosyl-Gly-Pro-Arg-P-nitroanilide (Sigma). Hydrolysis proceeded for 60 min at 20–25°C and the reaction was terminated by the addition of 50% acetic acid and the optical density was measured at 280 nm. The FIXa alone had no amidolytic activity on the chromatographic substrate. The FIXa used in this assay activated ~0.5% of the added factor X. When inhibition studies were performed with PN-2AβPP and its KPI domain, the inhibitor was incubated with FIXa for 5 min at room temperature prior to the addition of factor X. All inhibition constants determined from the results of the chromogenic assay were calculated from the residual activity at end point.

Measurement of Factor Xa Activity—Factor Xa activity (1–2.5 nm) was measured in 0.1 M triethanolamine, 0.1 M NaCl, pH 8.0, containing 0.1% polyethylene glycol (Mn = 8000), 0.2% bovine serum albumin, and 60 nm polylsine or 0.02 M Heps, 0.15 M NaCl, pH 7.4, containing 0.5 mg/ml bovine serum albumin, 2 mM Ca2+, and 0.1% polyethylene glycol using the chromogenic substrate, Pro-Arg-Gly-Pro-Arg-P-nitroanilide (Sigma) for 35 min at 20–25°C. In certain experiments, the polylsine was removed from the buffer. In other experiments 25 mM PSSC, 4.8 units/ml thrombin-activated factor VIII (FVIIa), or 50 mM thrombin-activated bovine factor Va (FVa) were added to the reaction mixture in the presence of 2 mM Ca2+. The reaction was terminated by the addition of an equal volume of 50% acetic acid after which the optical density was obtained at 405 nm. Hydrolysis of the substrate was linear over the time of the reaction. When inhibition studies were performed with PN-2AβPP, its KPI domain, or antithrombin III, the inhibitor (2–10 nm) was incubated with FIXa (1 nm) for 5 min at room temperature prior to the addition of the chromogenic substrate. All inhibition constants determined from the results of the chromogenic assay were calculated from the residual activity at end point. When investigations with antithrombin III were performed, 1 unit/ml heparin (Elkins-Sinn, Cherry Hill, NJ) was included in the reaction mixture.

Measurement of Factor X Activation Peptide—Simultaneous samples of human FIXa activation of factor X in the presence of polylsine were prepared for both chromogenic and immunochemical determination of factor X activation. Immunochemical determination of activation of factor X by FIXa was measured as nanomoles of factor X activation peptide liberated as detected by radioimmun assay using an antiserum directed to the factor X activation peptide (Bauer et al., 1989). These assays were generously performed by Dr. Kenneth A. Bauer, Beth Israel Hospital, Boston, MA. The percent liberation of factor X activation peptide was determined by comparing the level of hydrolysis seen in the present assay with the level of hydrolysis measured by known concentrations of human a-thrombin under identical assay conditions. The turnover numbers for factor Xa formation (kcat) were determined by the ratio of the maximum concentration of factor Xa formed (Vmax) divided by the concentration of the forming enzyme (FAx). The stoichiometry of FXa inhibition by PN-2AβPP and its KPI domain was determined by non-linear regression as previously reported (Schmaier et al., 1993). Briefly, FXa at 1 nm was added to 2–100 nm inhibitor (PN-2AβPP or its KPI domain). Activity was determined by the release of the chromogenic substrate, and the x-intercept of the inhibitor concentration versus the inhibition of FXa activity indicated the concentration of added inhibitor to the known amount of added FXa.

The equilibrium inhibition constants (K) present for PN-2AβPP and its KPI domain were calculated as previously reported (Van Nos trand et al., 1990b) by the procedure of Bieth (1984) for tight-binding inhibitors using the following equation:

\[ K_{eq} = \frac{I}{I(1-I)} - E(1-I)/(I + E) \]

where (I) is the inhibitor concentration, (E) is the factor Xa concentration, and a is the residual factor Xa activity after incubation with the inhibitor. The actual K was calculated using the subsequent equation:

\[ K = K_{eq} + K_{i} \]

where K is the concentration of the inhibitor and K is the intrinsic constant for the factor Xa-factor II (protease-substrate) reaction (Bieth, 1984). The second-order association rate constants (Kassoc) for each of the inhibitors were calculated using the integrated second-order rate equation:

\[ k = \frac{d[I]}{dt} = \frac{K_{assoc}}{K_{eq} + K_{i}} \cdot [I] \]

where K is the concentration of the substrate and K is the second-order association rate constant.
TABLE I  

| Conditions | KPI | PN2AβPP |
|------------|-----|---------|
| Polysine*  | 1.6 ± 0.4 × 10⁻⁶ | 3.3 ± 1.5 × 10⁻⁶ |
| No polysine* | 4.5 ± 2.3 × 10⁻³ | 1.3 ± 1.1 × 10⁻³ |
| PSPC, FVII alc | 7.2 ± 3.8 × 10⁻⁸ | 2.7 ± 1.3 × 10⁻⁸ |
| PSPC, FVa alc | 5.1 ± 1.6 × 10⁻⁸ | 1.6 ± 1.6 × 10⁻⁷ |
| PSPC, FV alc | 5.7 ± 1.9 × 10⁻⁸ | 3.1 ± 1.1 × 10⁻⁸ |

* FXa (1 nM) was incubated in 0.1 M triethanolamine, 0.1 M NaCl, pH 8.0, containing 0.1% polyethylene glycol and 0.2% bovine serum albumin in the absence or presence of 60 nM polysine as indicated. After a 5-min incubation with PN2AβPP (2–10 nM), the reaction was started by the addition of substrate (see “Experimental Procedures”).

**FXa (1 nM) was incubated in 0.02 M Hepes, 0.15 M NaCl, pH 7.4, containing 0.1% polyethylene glycol and 0.5 mM bovine serum albumin in the presence of 2 mM Ca²⁺ and PSPC. After a 5-min incubation with PN2AβPP or its KPI domain (2–10 nM), the reaction was started by the addition of substrate (see “Experimental Procedures”).

***PSPC were used at 25 μM; FVIIa was used at 4.8 units/ml; and FVa was used at 5 nM.

rabbit anti-mouse antibody conjugated with alkaline phosphatase (Sigma number 2429 at 1/10000) was added. The color reaction was initiated by the addition of p-nitrophenyl phosphate disodium (1 mg/ml) in 0.05 M Na₂CO₃, 1 mM MgCl₂, pH 9.8. An additional solid phase binding assay for complex determination was performed by linking FXa (50 ng) in 0.1 M Na₂CO₃, pH 9.6, to the microtiter plate. After blocking the cuvette wells with bovine serum albumin, asassay was successively incubated with substrate (see “Experimental Procedures”).

**FXa (1 nM) was incubated in 0.02 M Hepes, 0.15 M NaCl, pH 7.4, containing 0.1% polyethylene glycol and 0.5 mM bovine serum albumin in the presence of 2 mM Ca²⁺ and PSPC. After a 5-min incubation with PN2AβPP or its KPI domain (2–10 nM), the reaction was started by the addition of substrate (see “Experimental Procedures”).

***PSPC were used at 25 μM; FVIIa was used at 4.8 units/ml; and FVa was used at 5 nM.

Additional investigations showed that PN2AβPP was a substrate of FXa (Fig. 2). When PN2AβPP was incubated with increasing concentrations of FXa (1-16-fold molar excess), there was a decrease in the large, dark 124-kDa band of the starting material on an immunoblot of a nonreduced 6% SDS-PAGE and the appearance of 3 new bands at 116, 97, and 90 kDa, respectively, that migrated further into the gel (Fig. 2A). At a presumed 1:1 molar ratio of FXa to PN2AβPP, the presence of PSPC, FVIIa, and/or FVa did not influence the degree of inhibition of FXa by PN2AβPP and its KPI domain under various conditions. Similar to the results seen with isolated FXa and PN2AβPP, the presence of PSPC, FVIIa, and/or FVa did not influence the degree of inhibition of FXa by PN2AβPP and its KPI domain (Table I). These data indicated PN2AβPP and its KPI domain were equipotent inhibitors of FXa. Furthermore, the stoichiometry of FXa inhibition by PN2AβPP was 1:1. However, at 4 orders of magnitude molar excess KPI domain to FXa, FXa activity was not reduced to zero.

**PN2AβPP and Factor Xa Interactions—Investigations next were performed to determine if FXa and PN2AβPP formed a complex as determined by solid phase binding assay (Fig. 1). When PN2AβPP was coupled to microtiter plate wells, FXa specifically bound to the PN2AβPP as detected by an antibody to FXa followed by a second antibody conjugated with alkaline phosphatase (Fig. 1, top). Likewise, when FXa was linked to microtiter plate wells, PN2AβPP specifically bound to the FXa as detected by an antibody to PN2AβPP followed by a secondary antibody conjugated with alkaline phosphatase (Fig. 1, bottom). These studies indicated that FXa and PN2AβPP formed a complex characteristic of Kunitz-type inhibitors.

Additional investigations showed that PN2AβPP was a substrate of FXa (Fig. 2). When PN2AβPP was incubated with increasing concentrations of FXa (1-16-fold molar excess), there was a decrease in the large, dark 124-kDa band of the starting material on an immunoblot of a nonreduced 6% SDS-PAGE and the appearance of 3 new bands at 116, 97, and 90 kDa, respectively, that migrated further into the gel (Fig. 2A). At a presumed 1:1 molar ratio of FXa to PN2AβPP, some cleavage in PN2AβPP occurred (Fig. 2A). As the concentration of FXa to PN2AβPP increased from 4:1 to 16:1, all of the 124-kDa starting material was converted into lower molecular mass bands at 116, 97, and 90 kDa. Four-fold molar excess FXa to PN2AβPP liberated a single 8.2-kDa peptide which was detected on a reduced 18% SDS-PAGE (data not shown). The amino terminus sequence of this peptide was LEVPTDG-NAG (the same is the known amino-terminal sequence of PN2AβPP after cleavage of its signal peptide at alanine 17 (Fig. 3). The liberated peptide was a single peptide because on multiple gel electrophoreses using different percentage acrylamide gels (15-22%), only this single amino-terminal sequence was obtained. These data indicated that FXa liberated an amino-terminal peptide from PN2AβPP.

Further investigations sought the FXa cleavage site on the amino-terminal side of PN2AβPP. When PN2AβPP (a major band at 124 kDa and two minor bands at 105 and 98 kDa) was cleaved by 4-fold molar excess FXa, three new corresponding lower molecular mass bands were detected (a major one at 115 kDa and two minor bands at 97 and 90 kDa) when the sample was reduced and electrophoresed on a 6% SDS-PAGE, as detected by a Comassie Blue staining (Fig. 2B). The amino-terminals of each of these three bands were sequenced and a single amino acid sequence (KCKTHPHFV... ) was found for all three of these bands (Fig. 3). These data indicated that molar excess FXa to PN2AβPP cleaved PN2AβPP at a single site after arginine 102. Additional investigations showed that AβPP₆₉₅ a
form of AβPP which does not contain the KPI domain and does
not inhibit Fxa, also was a substrate of the enzyme (Fig. 2C).
These data indicated that inhibition of Fxa was independent of
the inhibitor being a Fxa substrate. Further isolated KPI do-
main was not cleaved by Fxa (data not shown).

PN-2/AβPP Inhibits Factor Xa in the Prothrombinase Complex—The possible importance of PN-2/AβPP and its KPI do-
main to inhibit Fxa is dependent upon whether these proteins
produce inhibition in biologic assays. Studies were performed
to determine if PN-2/AβPP and its KPI domain could inhibit
Fxa in the prothrombinase complex. Initial experiments deter-
mined the Km and kcat of prothrombin activation on phospho-
lipid vesicles in the presence of FVa. By double reciprocal plot,
Fxa activation of FII in the presence of PSPC and FVa was
shown to have a mean Km of 0.62 μM (range 0.23–1.6 μM) with
a Vmax of 7 nm α-thrombin formed min⁻¹ (range 3.6–19.1 nm
min⁻¹) (Fig. 4). These values calculated to a kcat of 7 min⁻¹ and
kcat/Km of 10.2 μM⁻¹ min⁻¹. Both PN-2/AβPP and its KPI
domain blocked Fxa activity in the prothrombinase complex
(Table II). When Fxa and PN-2/AβPP or its KPI domain were

FIG. 2. PN-2/AβPP is a substrate of Fxa. Panel A, PN-2/AβPP (1.0
μg or 8.3 pmol) were incubated with equal to 16-fold molar excess Fxa
in 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4, in the presence of 2 mM Ca2⁺
for 1 h at room temperature. The reactions were stopped with sample
buffer and applied nonreduced to a 6% SDS-PAGE. The samples were
electrophoresed onto nitrocellulose and an immunoblot was performed
using monoclonal antibody P2–1. The immunoblot was detected by
chemiluminescence. The figure is a photograph of an autoradiogram.
PN-2 represents the immunoblot of 8.3 pmol of PN-2/AβPP being ap-
plied to the SDS-PAGE. Ratio 1:1 to 1:16 represent the ratio of PN-2/
AβPP to Fxa (mol/mol) in the incubation mixture. Panel B, the figure
represents a Coomassie-stained 6% SDS-PAGE of reduced 83 pmol of
PN-2/AβPP alone (PN2) or 332 pmol of Fxa and 83 pmol of PN-2/AβPP,
ratio 4:1 of Fxa to PN-2/AβPP (Xa PN2). The numbers and stained
bands to the right of the figure represent molecular mass standards (M,
standards) in kilodaltons. Panel C, the figure represents a photograph
of a Coomassie-stained reduced 6% SDS-PAGE of 83 pmol of AβPP (apo
PP alone (695) or 332 pmol of Fxa and 83 pmol of AβPP, ratio 4:1 of
Fxa to AβPP, (Xa 695). The numbers and stained bands to the right
of the figure represent molecular mass standards (M, standards) in
kilodaltons.

FIG. 3. The Fxa cleavage site in PN-2/AβPP. The figure is the
amino-terminal sequence of PN-2/AβPP using the single letter code for
each amino acid. The arrow after alanine 17 represents the cleavage
site for the single peptide for PN-2/AβPP (Ponte et al., 1988). The arrow
after arginine 102 represents the Fxa cleavage site in PN-2/AβPP.
preincubated for 5 min with PSPC and FVa prior to the addition of FII and chromogenic substrate, the $K_i$ determined was 1.9 (SD 0.1) $10^{-8}$ M and 1.3 (SD 0.8) $10^{-8}$ M, respectively (Table II). These results only were 2.8- and 4.9-fold more inhibition for PN-2/A PP and its KPI domain than the $K_i$ for its KPIdomain on chromogenic and immunochemical assays

| Conditions | KPI (M) | PN-2/A PP (M) |
|------------|---------|---------------|
| Preincubation | $1.3 \pm 0.8 \times 10^{-8}$ (15) | $1.9 \pm 1.5 \times 10^{-8}$ (18) |
| No preincubation | $6.4 \pm 2.3 \times 10^{-8}$ (7) | $5.3 \pm 2.5 \times 10^{-8}$ (11) |

* The following abbreviations are used in Table II: Kunitz protease inhibitor domain of PN-2/A PP (KPI); protease nexin-2/amyloid $\beta$-protein precursor (PN-2/A PP).

* Human factor Xa (FXa) was used at 1.0 nM in all studies; phospholipid vesicles (PSPC) were used at 25 nM; bovine Factor Va (FVa) was used at 5 nM; human Factor II (FII) was used at 1 nM; and both the KPI domain and PN-2/A PP were used at 2-10 nM.

* Preincubation means that the FXa and inhibitor (KPI domain or PN-2/A PP) were preincubated with PSPC, FVa, and chromogenic substrate for 5 min prior to the addition of FII. No preincubation means that inhibitor (KPI domain or PN-2/A PP), PSPC, FVa, chromogenic substrate, and FII were mixed together and the reaction was started by the addition of FXa.

* The values in parentheses represent the number of independent determinations performed for each of the assay conditions. Each value is the mean $\pm$ S.D. of all the independent determinations.

* $p < 0.0000007$ between the two values.

* $p < 0.0000008$ between the two values.

The data indicated that polylysine had opposite effects on both PN-2/A PP and antithrombin III. In the prothrombinase complex, the biologically relevant assay, the $K_{assoc}$ was 3.0 $10^{-9}$ M$^{-1}$ min$^{-1}$ for the inhibition of PN-2/A PP and 0.7 $10^{-9}$ M$^{-1}$ min$^{-1}$ for antithrombin III. If heparin were present in the reaction mixture with PN-2/A PP, no inhibition of FXa was detected (data not shown). Alternatively, if polylysine was absent from the reaction mixture, the inhibitory abilities of PN-2/A PP and antithrombin III were reversed. PN-2/A PP inhibited FXa with a $k_{assoc}$ of $3.0 \pm 0.2 \times 10^{-9}$ M$^{-1}$ min$^{-1}$; antithrombin III and heparin blocked FXa with a $k_{assoc}$ of $1.3 \pm 0.3 \times 10^{-9}$ M$^{-1}$ min$^{-1}$. The data indicated that polylysine had opposite effects on both PN-2/A PP and antithrombin III. In the prothrombinase complex, the biologically relevant assay, the $K_{assoc}$ was $1.8 \pm 0.7 \times 10^{-9}$ M$^{-1}$ min$^{-1}$ for PN-2/A PP and essentially the same as the $K_{assoc}$ was $3.0 \pm 0.2 \times 10^{-9}$ M$^{-1}$ min$^{-1}$ seen with antithrombin III and heparin. These data indicated that in the prothrombinase complex, PN-2/A PP in the absence of heparin and antithrombin III and heparin were equipotent inhibitors of FXa.

**DISCUSSION**

The present investigations expand the coagulation protease inhibitory spectrum of PN-2/A PP. Although first recognized as a hemostatic inhibitor to factor Xa (Smith et al., 1990; Van Nostrand et al., 1990b), recent investigations have shown that PN-2/A PP also is a potent inhibitor of factor IXa (Schmaier et al., 1993, 1995) and its KPI domain has some inhibitory activ-
PN-2/ AβPP Inhibits Factor Xa

Comparison of PN-2/ AβPP and antithrombin III inhibition of Factor Xa

| Condition                  | k<sup>a</sup> (µM<sup>-1</sup> min<sup>-1</sup>) |
|----------------------------|-------------------------------------------------|
| Factor Xa 1N polylysine<sup>b</sup> | 3.0 ± 3.3 x 10<sup>6</sup> (9)±<sup>c</sup> 1.4 ± 0.4 x 10<sup>7</sup> (10)<sup>d</sup> |
| Factor Xa NO polylysine<sup>b</sup> | 1.3 ± 0.3 x 10<sup>7</sup> (7) 3.0 ± 2.0 x 10<sup>7</sup> (6) |
| Prothrombinase complex     | 3.0 ± 0.2 x 10<sup>6</sup> (4) 1.8 ± 0.7 x 10<sup>6</sup> (4) |

<sup>a</sup> FXa was added at 1 nM; PN-2/AβPP and antithrombin III were added at 2–10 nM. When using antithrombin III, the reaction mixture was made 1 Unit/ml with heparin. The FXa and PN-2/AβPP or antithrombin III were incubated 5 min prior to adding the chromogenic substrate for FXa. When polylysine was present, it was added at 60 nM.

<sup>b</sup> Each value is the mean ± S.D. of all determinations. The values in parentheses represent the number of determinations performed for the individual assay conditions.

<sup>c</sup> p < 0.000013 between the two values.

<sup>d</sup> FXa (1 nM) was incubated with 10 nM PN-2/AβPP or 10 nM antithrombin III and heparin (1 Unit/ml) for 5 min in the presence of PSPC (25 µM) and FVa (5 nM) at 37°C. The enzymatic reaction was initiated by the addition of FII (1 µM) and chromogenic substrate for α-thrombin.

ity to tissue factor-factor VIIa (Dennis and Lazarus, 1994a, 1994b). Initial reports suggested that PN-2/AβPP was not an inhibitor of FXa to any great extent (Smith et al., 1990; Van Nostrand et al., 1990b); however, other reports, consistent with coagulant assays, suggest otherwise (Kitaguchi et al., 1990; Petersen et al., 1994; Schmaier et al., 1993). Our investigations indicate that PN-2/AβPP is a direct inhibitor of FXa both as an isolated protein and in the prothrombinase complex. The inhibitory activity of PN-2/AβPP resides completely in its KPI domain because both the parent protein and its isolated KPI domain inhibit FXa to the same degree. Although the stoichiometry of inhibition appears to be 1:1, inhibition by the KPI domain does not appear to be active site-directed. Four orders of magnitude for the KPI domain to FXa does not reduce the FXa activity to zero, both in assays of isolated FXa and FXa in the prothrombinase complex. These data are different from those found with FIIa. Infinite concentrations of KPI domain abolished FIIa activity in the tenase complex (Schmaier et al., 1995). Nonactive site-directed FXa inhibitors also recently have been described for the hookworm-derived inhibitor of human FXa (Cappello et al., 1995).

We found that when using a polylysine-based FX activation assay, some of the measured inhibition of FIIa by PN-2/AβPP can be accounted for by PN-2/AβPP inhibiting generating FIIa. Since the degree of FXa generated in this assay is small (1–2 nM), the concentration of PN-2/AβPP or KPI domain present in the assays would have been sufficient to inhibit both FIIa and the generated FVa (Schmaier et al., 1993, 1995). It also was of interest to learn that polylysine itself potentiated the degree of inhibition of FIIa by PN-2/AβPP and reduced that of antithrombin III/heparin. The mechanism for this independent activity of polylysine is not known. Since polylysine itself can be an independent variable contributing to PN-2/AβPP's inhibitory ability, it should probably be avoided in assays of FIIa.

In addition to inhibition of enzymatic activity, we were able to demonstrate a physical interaction between PN-2/AβPP and FXa. On a solid phase binding assay, specific complex formation was detected between PN-2/AβPP and FXa. This information suggests that PN-2/AβPP is an inhibitor of FXa of the slow, tight class characteristic of Kunitz type inhibitors. PN-2/AβPP was also a substrate of FXa when the enzyme was in molar excess to inhibitor. It appears that FXa proteolyses the major band of PN-2/AβPP at 124 kDa and the two minor bands (105 and 98 kDa) into corresponding lower molecular mass species (116, 97, and 90 kDa, respectively), each with the same amino terminus as seen on immunoblot and Coomassie-stained gels. It is possible that FXa also degrades PN-2/AβPP at a single point on the carboxyl-terminal side of the protein liberating an approximate 30–34 kDa protein. This result would explain the intensification of the post-decayed 90 kDa band of PN-2/AβPP seen in Fig. 2, A and B. However, we have never found any evidence of such a band on our Coomassie-stained gels since it would be migrating with one of the subunits of FXa and thus be hidden. The fact that PN-2/AβPP is a substrate to molar excess FXa does not indicate that its mechanism of inhibition of the enzyme is substrate inhibition. First, AβPP <sub>PP</sub> is a substrate of FXa but it is not a FXa inhibitor. Second, the isolated KPI domain of PN-2/AβPP which does not contain the Fxa cleavage site inhibits FXa to the same degree as its parent protein. Third, the isolated KPI domain is not cleaved by FXa. It is of interest that FXa degrades PN-2/AβPP through its heparin binding domain. Since heparin neutralizes PN-2/AβPP's inhibitory activity on FXa, cleavage through this domain may preserve the inhibitory function of PN-2/AβPP for FXa.

PN-2/AβPP is a potent anticoagulant of FXa in the prothrombinase complex. In our laboratory the K<sub>m</sub> and K<sub>i</sub>/K<sub>m</sub> ratio of prothrombin activation by FXa is 0.62 µM and 10.2 µM<sup>-1</sup> min<sup>-1</sup>, respectively, results which are comparable to the findings of other investigators (Krishnaswamy et al., 1987) using a fluorescent marker instead of a chromogenic substrate to monitor prothrombin activation. The degree of inhibition of FXa by PN-2/AβPP and its KPI domain is to the same order of magnitude in the prothrombinase complex as with the isolated pure enzyme. Regardless of the order of addition of reactants, PN-2/AβPP and its KPI domain inhibit FXa on PSPC. PSPC in the presence of FVa must have oriented FXa such that it was susceptible to inhibition by PN-2/AβPP or its KPI domain even though the inhibitors were competing with 5 orders of magnitude more substrate. The finding that PN-2/AβPP and its KPI domain inhibit FXa in the prothrombinase complex make this class of inhibitors more important than what would be appreciated by just examining isolated FXa inhibition. Since PN-2/AβPP is not a plasma protein but rather a cell surface-associated protease inhibitor, influencing FXa activity in the prothrombinase complex suggests that this class of Kunitz-type protease inhibitors may be important regulators of various hemostatic enzymes. In fact, PN-2/AβPP and its homologue, amyloid β-protein precursor-like protein-2, may constitute a new class of serine protease inhibitors modulating hemostasis (Sprecher et al., 1993).

Although our investigations show that artificial agents can influence the degree of FXa inhibition by PN-2/AβPP or anti-thrombin III and heparin, in the prothrombinase complex assembly, PN-2/AβPP and antithrombin and heparin were comparable inhibitors. In the absence of added heparin, the degree of antithrombin III inhibition of FXa was orders of magnitude less potent than that seen with PN-2/AβPP. In plasma, antithrombin III would be the predominant inhibitor because its plasma concentration is 4 times higher than that reported by other investigators (Olson et al., 1992; Ellis et al., 1982). Differences in heparin preparations and concentrations and ionic strengths in the buffers may account for these variations. Alternatively, in the presence of heparin, PN-2/AβPP did not inhibit FXa. PN-2/AβPP is known to have a heparin binding domain which allows
heparin to potentiate its inhibition of factor Xa but not factor IXa (Smith et al., 1990; Van Nostrand et al., 1990b; Schmaier et al., 2003). It is of interest that the FXa cleavage site on PN-2/AbPP is at arginine 102 which is in the heparin binding region of the protein (Small et al., 1994). Excess FXa could be preventing PN-2/AbPP from associating with heparin on cell membranes. These investigations show that PN-2/AbPP is in the absence of heparin and antithrombin III in the presence of heparin are the naturally occurring inhibitors of FXa. Isolated KPI domain of PN-2/AbPP is a fragment from a naturally occurring human protein which may have potential use as an anticoagulant since its inhibitor activity is equal to the tick anticoagulant peptide (Waxman et al., 1990).

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