Factor IXa Inhibition by Protease Nexin-2/Amyloid β-Protein Precursor on Phospholipid Vesicles and Cell Membranes†

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ABSTRACT: 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 enzymes, factors XIa and IXa (FIXa), suggesting a role for this protein in hemostasis. Since coagulant reactions are modulated on biologic surfaces, we investigated how 25:75 (mol/mol) phosphatidylserine/phosphatidylcholine vesicles (PSPC), thrombin-activated platelets, or umbilical vein endothelial cells influence inactivation of FIXa by PN-2/AβPP. The K_m of human or porcine FIXa activation of human factor X in the presence of PSPC, activated platelets, or endothelial cells in the absence or presence of thrombin-activated factor VIII (FVIIIa) was similar, (0.05−0.39 μM). The presence of FVIIIa increased the catalytic efficiency (k_{cat}/K_m ratio) of human and porcine factor IXa's activation of factor X 4952−406-fold, respectively. In the presence of PSPC, the K_i of human and porcine FIXa inhibition by PN-2/AβPP was $K_i = 1.9 \times 10^{-9}$ M and 5.8×10^{-9} M, respectively. After the addition of FVIIIa to the reaction, the K_i for both human and porcine FIXa inhibition by PN-2/AβPP on PSPC increased 13- and 4-fold to $K_i = 2.5 \times 10^{-8}$ M and 2.4×10^{-8} M, respectively. These K_i for inhibition of human FIXa on phospholipid vesicles by PN-2/AβPP were similar when factor X activation was measured by chromogenic or activation peptide release assays. FVIIIa reduced the inhibition of FIXa by PN-2/AβPP only in the presence of PSPC. Inhibition of human and porcine FIXa on PSPC by the isolated KPI domain of PN-2/AβPP was not influenced by FVIIIa. Activated human platelets provide further protection of human or porcine FIXa from inhibition by PN-2/AβPP over that seen with PSPC and FVIIIa or endothelial cells. PN-2/AβPP is an inhibitor of FIXa in the presence of the assembled tenase complex.

Amyloid β-protein precursor (AβPP)[1] is a multidomain protein which is best known for being the parent protein to the 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 & Wong, 1984). Although AβPP has a single gene found on chromosome 21, it encodes at least three distinct mRNA species produced by alternative splicing (Ponte et al., 1988; Tanzi et al., 1988; Kitaguchi et al., 1988). Two of these mRNAs encode proteins 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). The KPI domain of PN-2/AβPP has been recognized to be a potent serine protease inhibitor 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 also was found to be a potent inhibitor of factor XIa, suggesting that PN-2/AβPP could function as an anticoagulant (Van Nostrand et al., 1990b; Smith et al., 1990). This assessment was further bolstered by the finding of high concentrations of PN-2/AβPP in platelets (Smith et al., 1990; Van Nostrand et al., 1990a). A recent finding from our laboratory indicates that PN-2/AβPP also is an inhibitor of factor IXa (FIXa) (Schmaier et al., 1993). Since a deficiency in factor IX leads to severe bleeding problems compared with a deficiency of factor XI, the finding that PN-2/AβPP is an inhibitor of FIXa suggested that this protein may be an important anticoagulant (Schmaier et al., 1993). This notion has particular significance in the brain where there is decreased thrombomodulin and high levels of PN-2/AβPP (Maruyama & Majerus, 1985; Van Nostrand et al., 1991; Wagner et al., 1993). PN-2/AβPP also appears to be the first recognized protein of a family of Kunitz-type protease inhibitors of hemostasis (Sprecher et al., 1993; Peterson et al., 1994; Slunt et al., 1994; Van Nostrand et

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[1] Abbreviations: AβPP, amyloid β-protein precursor; PN-2, protease nexin-2; KPI, Kunitz protease inhibitor; FIXa, activated coagulation factor IX; FVIIIa, thrombin-activated coagulation factor VIII; PSPC, phosphatidylserine phosphatidylcholine vesicles; HUVEC, human umbilical vein endothelial cells.

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al., 1994). Since coagulant reactions occur on and are modified by interaction with cell surfaces, investigations were initiated to characterize the influence of phospholipid vesicles, thrombin-activated factor VIII (FVIIIa), and cells of the intravascular compartment (platelets and endothelial cells) on the inhibition of human and porcine FIXa by PN-2/AβPP.

EXPERIMENTAL PROCEDURES

Proteins. PN-2/AβPP was purified from fibroblast culture media using techniques of heparin affinity chromatography and immunoaffinity chromatography as previously described (Van Nostrand et al., 1990b). The KPI domain of PN-2/AβPP, which was provided by Dr. Steven Wagner, SIBIA, La Jolla CA, was produced in a recombinant yeast expression system and purified as previously described (Wagner et al., 1992). The protease inhibitory activities of purified PN-2/AβPP and KPI domain were determined by titration with active-site titrated trypsin (Van Nostrand et al., 1990b; Wagner et al., 1992). Purified porcine factors IXa and VIII were generously provided by Dr. Pete Lollar, Emory University, Atlanta, GA. Human factors IXa (FIXa), X, Xa, and XIa were purchased from Enzyme Research Laboratories, South Bend, IN. Human FIXa on nonreduced sodium dodecyl sulfate−13% polyacrylamide gel electrophoresis 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 sodium dodecyl sulfate−polyacrylamide gel electrophoresis represented only 5−10% of the total protein in all preparations. This protein corrected the coagulant activity of factor IX deficient plasma and was detected by antibodies to factor IX. All FIXa used in these investigations was 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). The factor X was activated with Russell's viper venom. Purified human plasma factor VIII:C (FVIII) concentrate (240 units/mL) was generously provided by Dr. Garrett Bergman, Armour Pharmaceuticals, Collegeville, PA. Dr. Andre Bagdasarian of Alpha Therapeutic Corporation, Los Angeles, CA, also gave purified human plasma factor VIII concentrate for these investigations. Recombinant human factor VIII was given by Dr. Mirella Ezban of Novo Nordisk A/S, Gentofte, Denmark; Dr. Mark Cochran, Miles Inc., Berkeley, CA; and Mr. David P. Graham, Genetics Institute, Cambridge, MA. Purified human von Willebrand factor was generously provided by Dr. Zaverio Ruggeri of the Scripps Research Institute, La Jolla, CA, and Mr. Mike Morris of Enzyme Research Laboratories, South Bend, IN. Human α-thrombin (3250 units/mg) was generously provided by Dr. John W. Fenton III, Division of Laboratories and Research, N.Y. State Department of Health, Albany, NY. Bovine trypsin was bought from Sigma Chemical Corp., St. Louis, MO.

Unless otherwise stated, purified human factor VIII from Armour Pharmaceuticals was used for all experiments. This preparation of purified human factor VIII contained 0.72 unit/mL human von Willebrand factor antigen. Human von Willebrand factor antigen was quantified by electroimmunodiffusion using Helena plates (Beaumont, TX) performed according to the manufacturer's instructions. Upon reconstitution, human factor VIII (Armour Pharmaceuticals) was aliquoted into small volumes at ~240 units/mL and frozen at −70 °C until use. Immediately prior to addition in the assay, a small aliquot of the purified human factor VIII was activated with human α-thrombin (0.01 unit/mL) for 1 min at 37 °C. Investigations revealed that the final concentration of α-thrombin for FVIII activation did not hydrolyze the factor Xa substrate in the assay.

Phospholipid Vesicle Preparation. Phospholipid vesicles were prepared from a mixture of L-α-phosphatidylserine (Sigma) and L-α-phosphatidylcholine (Sigma) (25:75, mol/mol) that was 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 and 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 ultracentrifuged at 100000*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. The PSPC were stored at 4 °C. Phospholipid vesicles were also generously supplied by Dr. Pete Lollar, Emory University, Atlanta, GA.

Preparation of Platelets and Endothelial cells. Fresh human blood was drawn into sodium citrate (0.013 M final concentration), and platelet-rich plasma was prepared as previous published (Schmaier et al., 1980). The platelet-rich plasma was gel filtered on a Sepharose 2B equilibrated in Hepes Tyrode's buffer (14.7 mM Hepes, 0.135 M NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, pH 7.35) containing BSA (1 mg/mL) and dextrose (3.5 mg/mL) (Gustafson et al., 1986). Cultures of human umbilical vein endothelial cells (HUVEC) were established as described previously (Schmaier et al., 1988; Zini et al., 1993; Jaffe et al., 1973; Cines et al., 1984). HUVEC were passaged two to four times in Medium 199 (GIBCO, Grand Islands, NY) supplemented with 10% heat-inactivated fetal calf-serum (Flow Laboratories, McLean, VA), penicillin-streptomycin (GIBCO, Grand Islands, NY), and endothelial cell growth factor (Maciag et al., 1979) and grown to confluence on fibronectin coated 96-well microtiter plates (Nunclon, product of Nunc Intermed, Denmark, and procured from Thomas Scientific, Swedsbord, NJ). HUVEC were always used within 24 h of reaching confluence.

Measurement of Factor Xa Formation. Activation of human factor X (400 nM) by human or porcine FIXa (2.5−4.45 nM) in the presence of 25 μM PSPC vesicles, platelets, or endothelial cells in the absence or presence of 4.8 units/mL factor VIIIa (FVIIIa) was performed at 20−25 °C in 0.15 M NaCl, 0.2 M Hepes, and 0.1% poly(ethylene glycol), pH 7.4 containing 5 mM $CaCl_2$ and 0.5 mg/mL bovine serum albumin (Sigma). After an activation time which varied as to the reagent mixture, the reaction was made 6 mM with EDTA and an aliquot was added to a solution of 0.3 mM tosyl-Gly-Pro-Arg-*p*-nitroanilide (Sigma). On PSPC in the presence of FVIIIa, the activation time for human and porcine FIXa (2.5−4.45 nM) was 3 min. When 0.25 nM FIXa was employed on PSPC and FVIIIa, the activation time was 40 min. A 40 min activation time also was used for factor X activation by human and porcine FIXa on activated platelets. A 60 min activation time was employed for porcine FIXa on PSPC and human FIXa on HUVEC. Last, a 90 min activation time was used for human FIXa on PSPC alone. Hydrolysis of the chromogenic substrate at 20−25 °C

proceeded for 10 min in all assays that included human and porcine FIXa and FVIIIa and 90−120 min in all assays of human FIXa in the absence of FVIIIa after which the reaction was terminated by adding acetic acid (16.6% final concentration). The substrate hydrolysis was linear during the factor X activation time in the absence of FVIIIa. In the presence of FVIIIa, the assay was not linear over 10 min. However, calculation of kinetic parameters determined on the linear portion of the assay (4 min) versus at the endpoint (10 min) showed no significant difference. Calculations of the inhibition constants from residual activity values obtained on the linear portion of the curve were within 10% of those obtained at the endpoint. In other experiments gel filtered platelets (2×10^8 platelets/mL) were activated with 0.5 unit/mL human α-thrombin and used in a final concentration of 5×10^7 platelets/mL in the reaction mixture. The amount of α-thrombin (0.1 unit/mL) in the final platelet suspension had no significant hydrolysis on the factor Xa substrate used in the investigation. With endothelial cells, factor X activation was performed directly on confluent monolayers of endothelial cells at about 4×10^4 cells/well. Using a linear calibration curve made with known amounts of factor Xa, the degree of hydrolysis of the chromogenic substrate detected at 405 nm was converted to nM factor Xa formed/min. The rate of factor Xa formation/min in the reaction mixtures was then calculated from the nM factor Xa formed/min divided by the number of minutes of factor Xa activation prior to the addition of the chromogenic substrate. These latter values were used to calculate the kinetic parameters. In the presence of PSPC, the FIXa used in these assays activated ~0.25% of the added factor X in the absence of FVIIIa and ~12.5−25% in the presence of FVIIIa.

The enzymatic activity of human and porcine FIXa also was measured by its ability to activate human factor X using polylysine as an artifical surface (Schmaier et al., 1993; Lundblad & Roberts, 1982; Griffith et al., 1985; McCord et al., 1990). Factor IXa (4.45 nM) was incubated with factor X (400 nM) in 0.1 M triethanolamine and 0.1 M NaCl, pH 8.0, containing 0.1% poly(ethylene glycol) ($M_r = 8000$) and 60 nM polylysine 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 0.3 mM 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 after which the optical density reading was obtained at 405 nm. Factor IXa alone had no amidolytic activity on the chromogenic substrate. The FIXa used in this assay activated ~0.5% of the added factor X. Factor XIa and trypsin activity were measured enzymatically as previously reported (Van Nostrand et al., 1990b; Wagner et al., 1992).

When inhibition studies were performed with PN-2/AβPP 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 endpoint.

Measurement of Factor X Activation Peptide. Simultaneous samples of human FIXa activation of factor X in the presence of PSPC in the presence or absence of FVIIIa were prepared for both chromogenic and immunochemical determination of factor X activation. Immunochemical detection of activation of factor X by FIXa was measured as nanomoles of factor X activation peptide liberated by radioimmunoassy using an antisera directed to the factor X activation peptide (Bauer et al., 1989). The percent liberation of factor X activation peptide in PN-2/AβPP- or its KPI domain-treated samples was calculated from the ratio of nanomoles of activation peptide released when the inhibitor was present versus the nanomoles of activation peptide released when no inhibitor was present times 100. The percent liberated peptide in the inhibitor-treated sample was utilized as a measure of residual FIXa activity to calculate inhibition constants.

Calculation of Kinetic Parameters and Constants. The K_m and V_{max} of human and porcine FIXa activation of human factor X in the presence of PSPC vesicles, activated human platelets, or endothelial cells in the absence or presence of FVIIIa were determined by measuring the rate of factor X activation (10−800 nM) by 0.25−4.45 nM FIXa in three to four independent experiments. The mean ± SEM of each point performed in triplicate in three to four individual experiments was analyzed on a double-reciprocal plot by linear regression. The K_m and V_{max} were determined from the negative reciprocal of the x- and y-intercepts of the double-reciprocal plots, respectively. The turnover numbers for factor Xa formation (k_{cat}) were determined by the ratio of the maximum concentration of factor Xa formed (V_{max}) divided by the concentration of the forming enzyme (FIXa).

The equilibrium inhibition constants (K_i) presented for PN-2/AβPP and its KPI domain were calculated as previously reported (Van Nostrand et al., 1990b) by the procedure of Bieth (1984) for tight-binding inhibitors using

$$K_{i,app} = \{[(I)/(1 - a)] - (E)\}/(1/a)$$

to yield an apparent K_i where (I) is the inhibitor concentration, (E) is the factor IXa concentration, and a is the residual factor IXa activity after incubation with the inhibitor. The actual K_i was calculated using the subsequent equation:

$$K_i = K_{i,app}/1 + ([S]/K_m)$$

where [S] is the concentration of the substrate, factor X, and K_m is the Michaelis constant for the factor IXa−factor X (protease−substrate) reaction (Bieth, 1984).

Statistics. The significance of difference in results between groups was measured by t test for groups of unpaired data.

RESULTS

Kinetics of Factor X Activation. Investigations were performed to determine the conditions for factor X activation by FIXa on PSPC, thrombin-activated platelets, or confluent endothelial cells (Figure 1 and Table 1). The conditions used for the kinetic investigations were those which were found optimal on our previous studies to detect FIXa inhibition by PN-2/AβPP and its KPI domain (Schmaier et al., 1993). Preliminary results indicated that calcium ion was an absolute requirement for FIXa catalyzed factor X activation on phospholipid vesicles. Best results were seen in the presence of ≥3 mM calcium. Saturable kinetics with hyperbolic plots were seen for each of the conditions of human factor X activation by FIXa (data not shown). The kinetic parameters obtained for the activation of factor X by human FIXa (4.45 nM) in the presence of PSPC, but in the absence of FVIIIa, activated platelets, or endothelial cells, showed a K_m of 0.09

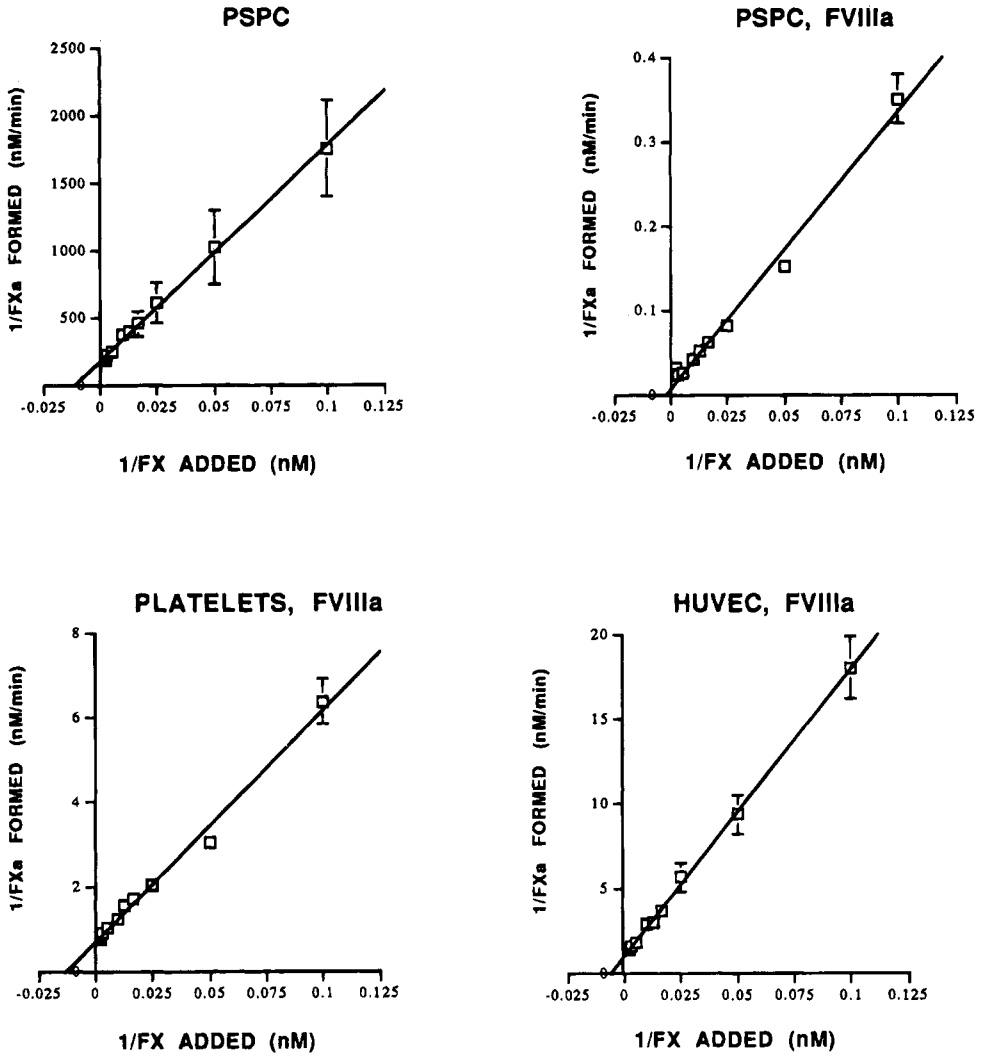

FIGURE 1: Double-reciprocal graphs of human factor X activation by human factor IXa. Endpoint rates of factor X activation (mean ± SEM) were determined as described under Experimental Procedures at each of the various concentrations of added factor X as indicated in the graphs. The upper left panel (PSPC) is the results of factor X activation by human factor IXa (4.45 nM) in the presence of phospholipid vesicles (25 μM) alone. The upper right panel (PSPC, FVIIIa) shows factor X activation by human factor IXa (4.45 nM) in the presence of phospholipid vesicles and thrombin-activated factor VIII (4.8 units/mL). The lower left panel (PLATELETS, FVIIIa) shows factor X activation by human factor IXa (2.5 nM) in the presence of thrombin-activated platelets (5×10^7 platelets/reaction) and factor VIIIa. The lower right panel (HUVEC, FVIIIa) shows factor X activation by human factor IXa (2.5 nM) in the presence of a confluent endothelial cell monolayer (4×10^4 cells/well) and factor VIIIa. The plotted results at each point are the mean ± SEM of three to four independent experiments.

μM and a k_{cat} of 0.0013 mol of factor Xa formed per minute per mole of FIXa added (Table 1 and Figure 1). When less human FIXa (0.25 nM) was used under identical conditions for activation, both the K_m (0.38 μM) and the k_{cat} (0.008 min^{-1}) increased to a similar degree (Table 1). However, under conditions of the assay with both concentrations of FIXa, the catalytic efficiency as indicated by the ratio of k_{cat}/K_m was calculated to be similar (Table 1). The addition of FVIIIa (4.8 units/mL) led to a proportionate decrease in the K_m (0.39−0.05 μM) and k_{cat} (27−5.2 min^{-1}) of human factor X activation at the lower of the two concentrations of human FIXa (4.45 and 0.25 nM, respectively). However in the presence of FVIIIa, there was a marked increase the ratio of k_{cat}/K_m (i.e., the catalytic efficiency of factor X activation). These increases in the k_{cat}/K_m ratios were 4929- and 4952-fold, respectively, over that seen in the absence of FVIIIa (Table 1). Increasing the concentration of added FVIIIa in the assay from 4.8 to 38.4 unit/mL did not increase the catalytic efficiency of factor X activation by 0.1−0.25 nM FIXa as measured by the ratio of k_{cat}/K_m over that seen with 4.45 nM FIXa. Qualitatively similar results were obtained when thrombin-activated platelets and endothelial cells were substituted for phospholipid vesicles in the activation of factor X by human FIXa in the presence of FVIIIa (Table 1 and Figure 1). The K_m for activated platelets and endothelial cells were 0.08 and 0.17 μM, respectively, and their k_{cat}/K_m ratios were 7.4 and 2.4, respectively. Activated platelets and endothelial cells in the presence of FVIIIa increased the activation of factor X 528- and 171-fold, respectively, over PSPC alone but were a 9- and 29-fold weaker activating surface, respectively, than PSPC in the presence of FVIIIa (Table 1).

Comparative studies were performed with porcine FIXa activation of factor X (Figure 2 and Table 1). Porcine FIXa, like human FIXa, achieved saturable kinetics with hyperbolic plots (data not shown). The K_m of human factor X activation by porcine FIXa in the absence and presence of FVIIIa was 0.42 and 0.38 μM, respectively (Table 1). Moreover, the

Table 1: Factor IXa-Catalyzed Factor X Activation: Kinetic Analysis[a]

additions[b]	K_m (μM)	V_{max} (nM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
HIXa, PSPC¶	0.09	0.006	0.0013	0.014
HIXa, PSPC, VIIIa¶	0.39	120	27	69
HIXa, PSPC§	0.38	0.002	0.008	0.021
HIXa, PSPC, VIIIa§	0.05	1.3	5.2	104
PIXa, PSPC	0.42	0.3	0.12	0.286
PIXa, PSPC, VIIIa	0.38	110	44	116
HIXa, PLTS, VIIIa†	0.08	1.5	0.59	7.4
PIXa, PLTS, VIIIa	0.18	3.3	1.3	7.2
HIXa, HUVEC, VIIIa†	0.17	1.0	0.4	2.4

[a] The following abbreviations are used in this table: human factor IXa (HIXa); phospholipid vesicles (PSPC); thrombin-activated factor VIII (VIIIa); porcine factor IXa (PIXa); thrombin-activated human platelets (PLTS); human umbilical vein endothelial cells (HUVEC). [b] Porcine factor IXa was used at 2.5 nM; human factor IXa was used at variable concentrations. In the experiments marked with the symbol ¶, 4.45 nM HIXa was used; §, 0.25 nM HIXa; and †, 2.5 nM HIXa.

presence of FVIIIa increased the catalytic efficiency of human factor X activation by porcine IXa 406-fold. Although activated platelets in the presence of FVIIIa were a 25-fold better activator of factor X by porcine FIXa over PSPC alone, the factor X turnover rate was 16-fold less on activated platelets than on PSPC in the presence of FVIIIa (Table 1). Similar to our study using human proteins, the substitution of activated platelets did not increase the catalytic efficiency of factor X activation by porcine FIXa above that seen with PSPC.

Inhibition Studies by PN-2/AβPP and Its KPI Domain. Investigations next determined the inhibitory ability of PN-2/AβPP and its KPI domain under assay conditions using biologic membranes. Factor Xa inhibition was first measured using PSPC. The KPI domain of PN-2/AβPP inhibited human FIXa in the absence or presence of FVIIIa with a K_i of 2.4×10^{-8} and 5.2×10^{-8} M, respectively (Table 2). These results indicated that in the presence of PSPC there was 5−12-fold more inhibition of human FIXa than that observed for the KPI domain on a polylysine-based factor X activation assay (Schmaier et al., 1993) (Table 4). These results were independent of FVIIIa (Table 4). Alternatively, the inhibition of human FIXa by PN-2/AβPP on PSPC in the absence or presence of FVIIIa produced a $K_i = 1.9 \times 10^{-9}$ and 2.5×10^{-8} M, respectively. Compared to the artifical polylysine-based factor X activation assay, these results show a 32- and 208-fold weaker inhibition, respectively (Schmaier et al., 1993) (Table 4). The results with PN-2/AβPP were influenced by the presence of FVIIIa.

In order to confirm that the degrees of FIXa inhibition by PN-2/AβPP and its KPI domain seen on the phospholipid-based factor X activation assays were due to FIXa inhibition, simultaneously prepared, identical samples were measured in both a chromogenic and immunochemical assay for activated factor X (Table 3). Inhibition of FIXa by KPI domain or PN-2/AβPP as measured simultaneously by either chromogenic assay or liberation of the factor Xa activation peptide gave similar results (Table 3). In the presence of PSPC alone, the KPI domain and PN-2/AβPP inhibited FIXa measured with the factor X activation peptide assay with a K_i of 2.4×10^{-8} and 11.3×10^{-9} M, respectively (Table 3). These inhibitory constants were similar to those obtained by the chromogenic assay performed under identical conditions (Tables 2 and 3). In the presence of PSPC and FVIIIa,

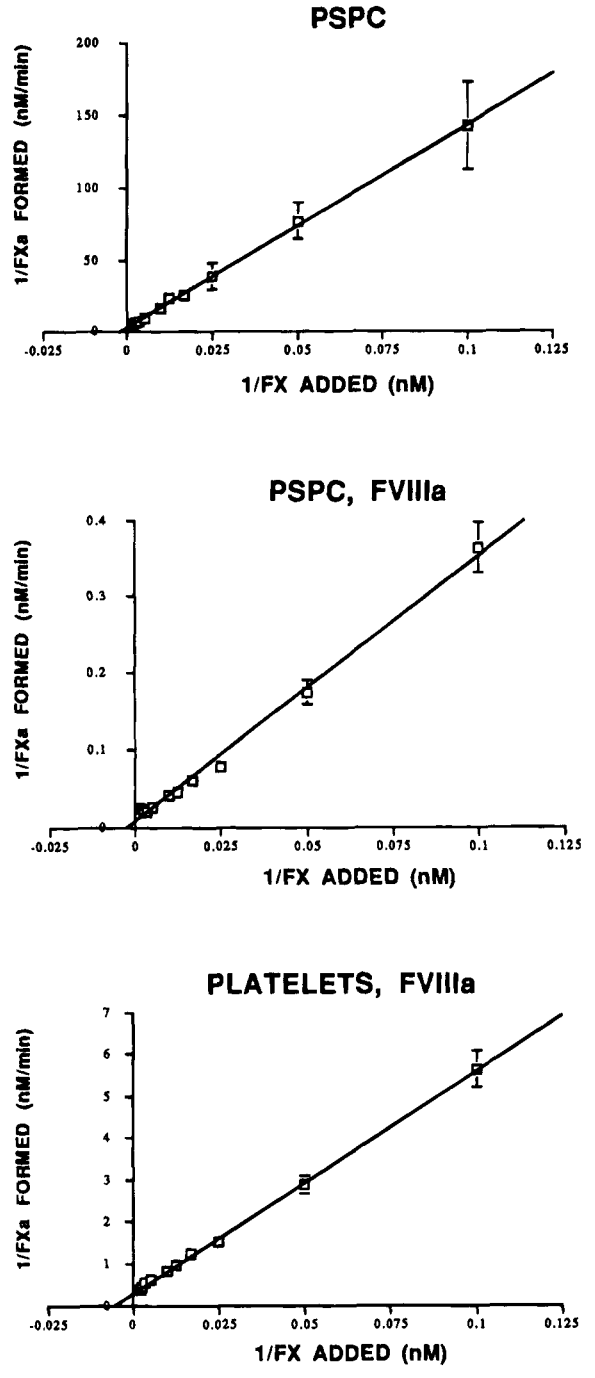

FIGURE 2: Double-reciprocal graphs of human factor X activation by porcine factor IXa. Endpoint rates of factor X activation (mean ± SEM) were determined as described under Experimental Procedures at each of the various concentrations of added factor X as indicated in the graphs. The upper panel (PSPC) is the results of factor X activation by porcine factor IXa (2.5 nM) in the presence of phospholipid vesicles (25 μM) alone. The middle panel (PSPC, FVIIIa) shows factor X activation by porcine factor IXa (2.5 nM) in the presence of phospholipid vesicles and thrombin-activated factor VIII (4.8 units/mL). The lower panel (PLATELETS, FVIIIa) shows factor X activation by porcine factor IXa (2.5 nM) in the presence of thrombin-activated platelets (5×10^7 platelets/reaction) and factor VIIIa. The plotted results at each point are the mean ± SEM of three to four independent experiments.

the KPI domain and PN-2/AβPP inhibited FIXa measured with the factor X activation peptide assay with a K_i of 3.4×10^{-8} and 2.1×10^{-8} M, respectively (Table 3), values which again were similar to those obtained using the chromogenic assay (Tables 2 and 3). These findings with an immu-

Table 2: Factor IXa Inhibition Constants with PN-2/AβPP and KPI Domain[a]

conditions[b]	K_i (M) KPI	K_i (M) PN2/AβPP
HIXa, PSPC	$(2.4 \pm 1.6) \times 10^{-8}$ (10)[c,d]	$(1.9 \pm 0.5) \times 10^{-9}$ (9)[e]
HIXa, PSPC, VIIIa	$(5.2 \pm 2.3) \times 10^{-8}$ (8)	$(2.5 \pm 1.7) \times 10^{-8}$ (12)[e]
PIXa, PSPC	$(7.1 \pm 5.8) \times 10^{-8}$ (9)	$(5.8 \pm 5.6) \times 10^{-9}$ (12)[f]
PIXa, PSPC, VIIIa	$(6.7 \pm 3.9) \times 10^{-8}$ (9)	$(2.4 \pm 1.6) \times 10^{-8}$ (6)[f]
HIXa, PLTS, VIIIa	$(1.8 \pm 0.5) \times 10^{-7}$ (4)	$(1.2 \pm 0.2) \times 10^{-7}$ (4)
PIXa, PLTS, VIIIa	no inhibition	$(1.3 \pm 0.7) \times 10^{-7}$ (6)
HIXa, HUVEC, VIIIa	$(5.1 \pm 3.4) \times 10^{-8}$ (5)	$(6.7 \pm 1.1) \times 10^{-8}$ (3)

[a] The following abbreviations are used in this table: human factor IXa (HIXa); phospholipid vesicles (PSPC); thrombin-activated factor VIII (VIIIa); porcine factor IXa (PIXa); thrombin-activated human platelets (PLTS); human umbilical vein endothelial cells (HUVEC). [b] Human factor IXa (HIXa) was used at 4.45 nM in all studies; porcine factor IXa (PIXa), 2.5 nM. For the determination of the kinetic constants, KPI domain and PN-2/AβPP were used in concentrations ≤10-fold molar excess. [c] The values in parentheses represent the number of independent determinations performed for each of the assay conditions. [d] Each value is the mean ± SD of all the independent determinations. [e] $p < 0.0009$ between the two values. [f] $p < 0.0006$ between the two values.

Table 3: Comparison of Inhibition of Factor X Activation by PN-2/AβPP and Its KPI Domain on Chromogenic and Immunochemical Assays

conditions	K_i (M) chromogenic assay[a]	K_i (M) immunochemical assay[b]
PSPC[c]		
KPI domain	1.4×10^{-8}	2.4×10^{-8}
PN-2/AβPP	3.7×10^{-9}	11.3×10^{-9}
PSPC, FVIIIa[d]		
KPI domain	7.6×10^{-8}	3.4×10^{-8}
PN-2/AβPP	4.3×10^{-8}	2.1×10^{-8}

[a] The K_i were calculated from residual activity determined at the endpoint of the reaction. The values are the mean of two experiments performed at different times. [b] The K_i were calculated from the percent liberated factor X activation peptide at the endpoint of the reaction, which was determined by the ratio of nmoles peptide liberated in an inhibited sample over nanomoles of peptide liberated in an uninhibited sample times 100. The values are the mean of two experiments performed at different times. [c] The results were obtained by factor IXa (4.45 nM) activation of factor X (400 nM) in the presence phospholipid vesicles (25 μM) in the presence of inhibitors (44.5 nM) (see Experimental Procedures). [d] The results were obtained by factor IXa (4.45 nM) activation of factor X (400 nM) in the presence phospholipid vesicles (25 μM) and factor VIIIa (4.8 U/ml) in the presence of inhibitors (44.5 nM) (see Experimental Procedures).

nochemical assay to determine factor X activation indicated that under the conditions of the phospholipid-based factor X activation assays, the majority of inhibition produced by the KPI domain and PN-2/AβPP as measured by a chromogenic assay is directed toward FIXa.

Similar inhibitory constants to human FIXa were seen with the porcine enzyme. KPI domain of PN-2/AβPP inhibited porcine FIXa in the absence and presence of FVIIIa with a K_i of 7.1×10^{-8} and 6.7×10^{-8} M, respectively (Table 2). These results on the PSPC-based assay showed 3.5−3.7 greater inhibition than that seen for porcine FIXa by KPI domain in the absence or presence of FVIIIa using the polylysine-based assay ($K_i = 2.5 \times 10^{-7}$ and 2.4×10^{-7} M, respectively) (Table 4). Similar to our studies with human FIXa, a different pattern of inhibition was observed when intact PN-2/AβPP was investigated. In the presence of PSPC, either in the absence or presence of FVIIIa, PN-2/AβPP inhibited porcine FIXa with a K_i of 5.8×10^{-9} and 2.4×10^{-8} M, respectively (Table 2). These values for porcine FIXa, which are essentially the same as those seen with human FIXa, suggested 14- and 24-fold weaker inhibition in the more physiologically based factor X activation assay, respectively, than that seen with the artificial, polylysine-based FIXa inhibition assay (Table 4).

Table 4: Influence of Factor VIIIa on Factor IXa Inhibition Constants with PN-2/AβPP and KPI Domain Using the Polylysine-Based Assay

conditions[a,b]	K_i (M) KPI	K_i (M) PN2/AβPP
HIXa	$(3.0 \pm 2.2) \times 10^{-7}$ (7)[c]	$(6.0 \pm 3.7) \times 10^{-11}$ (5)[d]
HIXa, VIIIa	$(2.4 \pm 1.4) \times 10^{-7}$ (4)	$(1.2 \pm 0.6) \times 10^{-10}$ (6)[d]
PIXa	$(2.5 \pm 1.7) \times 10^{-7}$ (5)	$(4.1 \pm 2.2) \times 10^{-10}$ (4)[e]
PIXa, VIIIa	$(2.4 \pm 0.8) \times 10^{-7}$ (5)	$(9.7 \pm 5.0) \times 10^{-10}$ (4)[e]

[a] The following abbreviations are used in this table: human factor IXa (HIXa); thrombin-activated factor VIII (VIIIa); porcine factor IXa (PIXa). [b] Human factor IXa (HIXa) was used at 4.45 nM in all studies; porcine factor IXa (PIXa), 2.5 nM. For the determination of the kinetic constants KPI domain and PN-2/AβPP were used in concentrations ≤10-fold molar excess. [c] Each value is the mean ± SD of all determinations. The values in parentheses represent the number of determinations performed for the individual assay conditions. [d] $p > 0.112$ between the two values. [e] $p > 0.1$ between the two values.

The kinetics of inhibition of FIXa by KPI domain and PN-2/AβPP were investigated next using physiologic surfaces provided by activated platelets or cultured endothelial cells. Using thrombin-activated human platelets and confluent HUVEC in microtiter plates in the presence of FVIIIa, KPI domain inhibited human FIXa with a K_i of 1.8×10^{-7} and 5.1×10^{-8} M, respectively, (Table 2). Of interest, the isolated KPI domain did not inhibit porcine FIXa associated with platelets in the presence of FVIIIa even when concentrations up to 50-fold molar excess were employed (Table 2). When investigations were performed with human PN-2/AβPP, the results of inhibition studies indicated that platelets protected human and porcine FIXa from inhibition by PN-2/AβPP compared to PSPC alone (Table 2). The K_i of PN-2/AβPP inhibition of human and porcine FIXa on platelets were 1.2×10^{-7} and 1.3×10^{-7} M, respectively, which were 63- and 22-fold weaker inhibition, respectively, than the inhibition measured on PSPC alone (Table 2). Moreover, human or porcine FIXa inhibition by PN-2/AβPP associated with activated platelets was 5-fold less than that seen with PSPC in the presence of FVIIIa. The results with HUVEC and FVIIIa were similar to what was seen with phospholipid vesicles and FVIIIa. On confluent HUVEC, human FIXa was inhibited by PN-2/AβPP in the presence of FVIIIa with a K_i of 6.7×10^{-8} M (Table 2). These combined data suggested that the association of the various FIXa's with platelets conferred better protection from exogenously added PN-2/AβPP and its KPI domain than did binding to phospholipid vesicles or to the endothelial cell surface.

Investigations with both human and porcine FIXa associated with PSPC suggested that the presence of FVIIIa also significantly contributed to the decrease in inhibition seen with PN-2/AβPP (Table 2). The presence of FVIIIa caused a 13- and 4-fold weaker inhibition ($p \leq 0.0006$) of human and porcine FIXa, respectively, by PN-2/AβPP (Table 2). These differences were examined further. First, using the polylysine-based assay for factor X activation by FIXa, the

addition of FVIIIa to either human or porcine FIXa resulted in K_i values which were not significantly reduced ($p \geq 0.1$) from the values obtained in the absence of FVIIIa (Table 4). Second, FVIIIa had no effect on the inhibition of factor XIa or trypsin by PN-2/AβPP (data not shown). Third, the protection of FIXa from inhibition by PN-2/AβPP was not affected by von Willebrand factor (1 unit/mL) (data not shown). Last, all purified recombinant FVIIIa preparations when used in the phospholipid-based factor X activation assay altered the ability of PN-2/AβPP to inhibit FIXa to the same extent. These data suggested that diminution of PN-2/AβPP's inhibitory capacity on FIXa on PSPC in the presence of various preparations of FVIIIa cannot be simply attributable to a direct effect of factor VIIIa on PN-2/AβPP or a contaminant in the factor VIII preparations.

DISCUSSION

These investigations show that, on biologic membranes, PN-2/AβPP and its isolated KPI domain are inhibitors to factor IXa from two species. Initial investigations were performed to establish phospholipid-based measurements of factor X activation in the absence of inhibitors. The values obtained for the K_m of factor X activation by human FIXa in the absence and presence of FVIIIa are comparable to those of Rawala-Sheikh et al. (1990) and Nishimura et al. (1993). Similarly, our investigations on human factor X activation by porcine FIXa on PSPC in the absence or presence of FVIIIa yielded results in which the K_m of factor X activation was similar to that seen by Duffy and Lollar (1992) working exclusively with porcine proteins. Although the k_{cat} and calculated k_{cat}/K_m ratio for these reactions were similar to that reported by Sinha and Wolf (1993), they were somewhat less than that reported by other investigators (Rawala-Sheikh et al., 1990; Nishimura et al., 1993; Duffy & Lollar, 1992). The reasons for the differences are not entirely clear. These results were not dependent on the PSPC used since different preparations of vesicles from our laboratory and those provided by Dr. Pete Lollar of Emory University had no influence on the V_{max} and k_{cat}. The k_{cat}/K_m ratios were similar whether we were working with 0.25–4.45 nM human factor IXa, obviating the possibility that by using the higher concentration of activating enzyme we were underestimating the velocity of the reaction. However, in the absence of FVIIIa, we found that the K_m and k_{cat} increased with the lower concentration of FIXa; in the presence of FVIIIa, the opposite occurred at the lower concentration of FIXa. These combined changes could result in the stability of the k_{cat}/K_m ratio. Since the k_{cat} and K_m for factor X activation by factor IXa should not change when the concentration of factor IXa is decreased, it is not clear if the changes in K_m and k_{cat} seen represent a range of variation about these points or real changes in these parameters which would not be accounted for in the kinetic model. Further, the lowered catalytic efficiency cannot be explained by rate limitation due to the cofactor, factor VIIIa. Increasing concentrations of factor VIIIa in the presence of one concentration of factor IXa resulted in an increase in both the K_m and k_{cat} of the reaction leaving the resultant k_{cat}/K_m ratio unchanged. Although an alteration in the K_m is mainly due to phospholipid in these assays (van Dieijen et al., 1981), our data suggest that FVIIIa at increasing concentrations can also influence the K_m probably due to its own interaction with the PSPC membrane (Bardelle et al., 1993).

The finding that the equilibrium inhibition constants for FIXa by PN-2/AβPP and its KPI domain were similar on both a chromogenic factor X activation assay and the immunochemical factor X activation peptide release assay indicated that, on PSPC, FIXa was the predominant site of inhibition. Our investigations with both human and porcine FIXa indicated that the presence of FVIIIa with PSPC influenced the ability of intact PN-2/AβPP, but not its KPI domain, to inhibit FIXa. The role of FVIIIa in regulating the inhibitory activity of PN-2/AβPP is not completely understood. Since all our preparations of purified plasma or recombinant FVIIIa decreased the inhibition of FIXa by PN-2/AβPP, it is unlikely that the mechanism was due to a contaminant in the factor VIII preparations. Rather, the complex between FVIIIa and PSPC on a biologic surface must allow for a milieu whereby FIXa is protected from exogenous PN-2/AβPP. It was also of interest that activated human platelets created an even more protective milieu for FIXa activity than PSPC. This activity must be special to the platelet membrane itself because activated platelets themselves could have contributed their own PN-2/AβPP which would have been expected to potentiate rather reduce the level of FIXa inhibition (Smith et al., 1990; Van Nostrand et al., 1990a).

These studies show the degree of inhibition by PN-2/AβPP and its KPI domain of human and porcine FIXa on PSPC and biologic surfaces. The platelet surface in particular counteracts to the greatest extent the ability of PN-2/AβPP to inhibit FIXa activity. The fact that surface factors and FVIIIa modify inhibition of FIXa by PN-2/AβPP indicates that there may be another region on the protein that contributes to FIXa inhibition in addition to its KPI domain. Whether this region is a binding site for FIXa or causes a conformational change in the expression of the KPI domain that enhances its inhibitory capacity is as yet unknown. The importance of PN-2/AβPP as an anticoagulant probably cannot be appreciated fully by adding it to a solution of FIXa associated with a biologic membrane. PN-2/AβPP is not a plasma protein but one which exists as a transmembrane inhibitor. In a previous study we showed that PN-2/AβPP is overexpressed in the vasculature in individuals with hereditary cerebral hemorrhage with amyloidosis-Dutch type, a condition associated with spontaneous intracerebral hemorrhage (Schmaier et al., 1993; Rozemuller et al., 1993). Excess accumulation of this protein within the cell membrane may correlate with its *in vivo* anticoagulant function.

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