Protease Nexin-2/Amyloid β Protein Precursor
A Tight-binding Inhibitor of Coagulation Factor IXa

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Abstract

Protease nexin-2/amylloid β protein precursor (PN-2/AβPP) is an abundant, secreted platelet protein which is a potent inhibitor of coagulation Factor Xa. We examined other potential anticoagulant activities of PN-2/AβPP. Purified Kunitz protease inhibitor domain of PN-2/AβPP and PN-2/AβPP itself were found to prolong the coagulation time of plasma and pure Factor IXa. The Kunitz protease inhibitor domain also inhibited the ability of Factor IXa to activate Factor X. PN-2/AβPP inhibited Factor IXa with a K_i of 7.9 to 3.9 × 10^{-11} M in the absence and presence of heparin, respectively. When the second-order rate constant of PN-2/AβPP's inhibition of Factor IXa (2.7 × 10^4 M^{-1} min^{-1}) was compared to that of antithrombin III (3.8 × 10^6 M^{-1} min^{-1}), PN-2/AβPP was at least a 71-fold more potent inhibitor of Factor IXa than antithrombin III. PN-2/AβPP formed a complex with Factor IXa as detected by gel filtration and ELISA. The finding that PN-2/AβPP is a potent inhibitor of Factor IXa could help to explain the spontaneous intracerebral hemorrhages seen in patients with hereditary cerebral hemorrhage with amyloidosis Dutch-type where there is an extensive accumulation of PN-2/AβPP in their cerebral blood vessels. (J. Clin. Invest. 1993. 92:2540–2545.) Key words: amyloid β protein precursor • protease nexin-2 • Factor IXa • Kunitz protease inhibitor

Introduction

The amyloid β protein precursor (AβPP)1 (1) is the parent protein to the amyloid β-protein (Aβ) that is deposited in senile plaques and in the walls of cerebral blood vessels of patients with Alzheimer's disease (2), and patients with hereditary cerebral hemorrhage with amyloidosis Dutch-type (HCHWA-D) (3, 4). There are three major isoforms of AβPP, two of which contain a domain homologous to Kunitz-type protease inhibitors (KPI) (5). The secreted isoforms of AβPP containing the KPI domain are analogous to protease nexin-2 (PN-2) (6, 7). Although the physiologic functions of PN-2/AβPP are not yet fully understood, several studies show that PN-2/AβPP is an abundant platelet α-granule protein that is secreted upon platelet activation (8, 9) and is a potent inhibitor of intrinsic blood coagulation Factor Xla (9, 10). These findings suggest that PN-2/AβPP may function as an anticoagulant. Although Factor XI is the first protein of the hemostatic system, clinical states associated with Factor XI abnormalities are usually mild bleeding conditions that occur after trauma but are not spontaneous (11–13). If PN-2/AβPP were a physiologically important anticoagulant, it would be expected to interact with other proteins of the hemostatic system. Therefore, we sought to determine if PN-2/AβPP influences other proteins of the hemostatic system.

Methods

Proteins. PN-2/AβPP was purified from fibroblast culture media using techniques of heparin affinity chromatography and immunoaffinity chromatography as previously described (10). On nonreduced sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis PN-2/AβPP is a single band at 116 kDa; upon reduction, the molecular mass of the protein increased slightly. The KPI domain of PN-2/AβPP was produced in a recombinant yeast expression system and purified as previously described (14). The protease inhibitory activities of purified PN-2/AβPP and KPI domain were determined by titration with active-site titrated trypsin (10, 14). Purified human Factor IXa was purchased from Enzyme Research Laboratories (South Bend, IN). This protein 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. This protein corrected the coagulant activity of Factor IX–deficient plasma and was detected on immunoblot with primary antibody to Factor IX. Factor IXa was also generously provided by Dr. Steven Olson, Henry Ford Hospital (Detroit, MI). Factor X was also purchased from Enzyme Research Laboratories. Antithrombin III was purchased from Ameri- 2540 Schmaier et al.
Assays. An activated partial thromboplastin time (APTT) was performed by mixing 0.05 ml plasma with 0.05 ml of a kaolin (Fisher Scientific, King of Prussia, PA) suspension (10 mg/ml) in buffer, 0.01 M Tris, 0.15 M NaCl, pH 7.4, and 0.05 ml automated APTT reagent (Organon Teknika, Research Triangle Park, NC) diluted 1:3 with buffer. After 5 min incubation at 37°C, the material was recalcified (0.05 ml of 30 mM CaCl₂), and the time necessary to clot formation was measured. Factors XI and IX coagulant assays were performed by mixing 0.05 ml of a 1:2 dilution of pooled normal human plasma (George King Inc., Overland Park, KS) or Factor IXa with 0.05 ml APTT reagent, 0.05 ml of a kaolin suspension, and 0.05 ml of Factor XI or IX deficient plasmas, respectively (Organon Teknika). After 5 min incubation at 37°C, the material was recalcified with 0.05 ml 30 mM CaCl₂ and the time to clot formation was measured. Plasma Factors X and VII coagulant activity were determined by mixing a 1:20 dilution of pooled normal human plasma (0.05 ml) with Factors X or VII deficient plasmas (0.05 ml) (Organon Teknika). After a 3-min incubation at 37°C, clotting was initiated by the simultaneous addition of 0.05 ml Simplastin (Organon Teknika) and 0.05 ml of 30 mM CaCl₂. Thrombin’s ability to clot plasma fibrinogen was performed with 0.1 ml human α-thrombin (4 U/ml) added to 0.2 ml pooled normal plasma made 6 mM with CaCl₂.

Factor IXa enzymatic activity was measured by its ability to activate Factor X in the presence of polystyrene and polyethylene glycol (15-17). Factor IXa (4.5 nM) was incubated with Factor X (400 nM) in 0.1 M triethanolamine, 0.1 M NaCl, pH 8.0 containing 0.1% polyethylene glycol (8,000 M₉) and 60 mM 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 Chemical Co. St. Louis, MO). 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. The $K_a$ of Factor IXa activation of Factor X under the conditions of this assay was determined by the rate of 10-1,000 nM Factor X activation by 4.5 nM Factor IXa. These data were analyzed by double reciprocal plot. Factor IXa had no direct activity on the chromogenic substrate used in the Factor X activation assay.

Kinetics of Factor IXa inactivation. All Factor IXa used in kinetic studies was active-site titrated with antithrombin III (16). Factor IXa at an assumed concentration of 250 nM was added to various solutions containing 50-250 nM antithrombin III in 0.1 M triethanolamine, 0.1 M NaCl, pH 8.0, containing 1 U/ml heparin and 0.1% polyethylene glycol and incubated for 20 min at 20°-25°C. At the end of the incubation, aliquots were removed and added to a solution of 400 nM Factor X in 60 mM polylysine, 0.1 M triethanolamine, 0.1 M NaCl, pH 8.0 containing 0.1% polyethylene glycol and 0.3 mM Np-Tosyl-Gly-Pro-Arg-p-nitroanilide. After incubation for 20 min at 20°-25°C, the reaction was stopped by the addition of an equal volume of 50% acetic acid, and the samples were read at 405 nm. Assuming that the stoichiometry of Factor IXa inhibition by antithrombin III is 1:1, the antithrombin III concentration at the x-intercept of the linear regression analysis of a plot of Factor X activation versus antithrombin III concentration yielded the corresponding concentration of Factor IXa. The stoichiometry of the Factor IXa and PN-2/AβPP interaction was determined by similar means as described above for the active-site titration of Factor IXa by antithrombin III.

The equilibrium inhibition constants ($K_i$) presented for PN-2/AβPP and its KPI domain were calculated by the procedure for tight-binding inhibitors of Beth (18) using the following equation to yield an apparent $K_i$: $K_	ext{app} = [(1/(1-a)) - E]/(1/a)$, where $l$ 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: $K_i = K_	ext{app}/1 + (S/K_m)$, 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 (18). The second-order association rate constants ($K_	ext{assoc}$) for each of the inhibitors were calculated using the integrated second-order rate equation: $K^* = ((1/l) - E·ln(E(l - E))/l(E - E))/(t)$. Where $E$ is the Factor IXa concentration, $l$ is the inhibitor concentration, $E$ is the concentration of the enzyme-inhibitor complex, and $t$ is the time of incubation in minutes (19).

Determination of complex formation between Factor IXa and PN-2/AβPP. Sephadex G-100 (Sigma) gel filtration of Factor IXa (5 μg) or a mixture of Factor IXa (5 μg) and PN-2/AβPP (11 μg) was performed on a 1 × 100 cm column equilibrated in 0.01 M Tris-HCl, 0.15 M NaCl, pH 8.0. The column that was packed freshly upon each use was run on gravity at room temperature at a flow rate of 30 ml/h. Sample size was < 0.03 ml, and 1-ml fractions were collected. The void volume was determined by gel filtration of blue dextran (Sigma Chemical Co.). Molecular mass markers of human immunoglobulin G (160 kD) and bovine serum albumin (68 kD) were used to characterize the column. Column fractions were assayed for Factor IXa antigen by ELISA. 0.5 ml of each fraction was freeze dried in a concentrator (Speed-Vac; Savant Instruments, Farmingdale, NY). Each lyophilized column fraction was resuspended in 100 μl of 0.1 M Na₂CO₃, pH 9.6, and applied to a well of a microtiter plate. After incubation overnight at 37°C, the wells were washed with 0.01 M sodium phosphate 0.15 M NaCl, pH 7.4, containing 0.05% Tween 20. After blocking the wells with 1% radioimmunoassay grade bovine serum albumin, the wells were washed again and then incubated with a goat anti-human Factor IX antibody (Enzyme Research Laboratories) diluted 1:800 followed by a rabbit anti-goat antibody conjugated with alkaline phosphate (Sigma Chemical Co.) diluted 1:500. The color reaction was initiated by the addition of p-nitrophenylphosphate sodium (1 mg/ml) in 0.05 M Na₂CO₃, 1 mM MgCl₂, pH 9.8.

Complex formation between PN-2/AβPP and Factor IXa was also demonstrated by ELISA. Microtiter plates were coated with PN-2/AβPP (50 ng) in 0.1 M Na₂CO₃ pH 9.6 and then blocked with 1% radioimmunoassay grade bovine serum albumin (Sigma Chemical Co.). After washing, the wells were incubated with Factor IXa (20 ng) followed by a 1:800 dilution of a goat anti-human Factor IX antibody in 0.01 M sodium phosphate 0.15 M NaCl, pH 7.4 containing 0.05% Tween-20 and 10% fetal calf serum (Flow Laboratories, Springfield, VA). After further incubation and washing, a rabbit anti-goat antibody conjugated with alkaline phosphatase was added. The color reaction was initiated by the addition of p-nitrophenyl phosphate sodium (1 mg/ml) in 0.05 M Na₂CO₃, 1 mM MgCl₂, pH 9.8.

Results

Initial investigations were performed to determine if a purified, recombinant KPI domain of PN-2/AβPP inhibited the coagulant activity of normal human plasma (Fig. 1 A). KPI domain at 1 μM prolonged the APTT more than twofold over pooled normal plasma. When the ability of recombinant KPI domain to inhibit the coagulant activity of pooled normal human plasma was compared to purified whole PN-2/AβPP, which was only available at concentrations ≤ 200 nM, KPI domain was found to be a more potent inhibitor of the coagulant activity of plasma than PN-2/AβPP (Fig. 1 A). Since PN-2/AβPP and its KPI domain are potent inhibitors of Factor Xla, the prolongation of the APTT could simply be explained by inhibition of this enzyme alone.

To determine if the prolongation of the APTT was simply caused by the KPI domain of PN-2/AβPP inhibiting the coagulant activity of Factor Xla, specific coagulation factor assays were performed for each of the coagulation enzymes below the level of Factor Xla in the hemostatic cascade (Fig. 1 B). As previously reported for an enzymatic assay (9, 10), the KPI domain in mM concentrations had no influence on the ability of α-thrombin to clot plasma. The KPI domain at 100 μM independently inhibited the coagulant activity of both Factors Xa and VIIa in plasma more than twofold over control.
Figure 1. Inhibition of plasma coagulation by recombinant KPI domain or purified PN-2/AβPP. (A) Pooled normal human plasma was incubated with increasing concentrations of purified recombinant KPI domain of PN-2/AβPP (1 nM–6 μM) (c) or purified PN-2/AβPP (1–200 nM) (e) for 5 min at 37°C. At the conclusion of the incubation time, the APTT was measured (see Methods). The data represent the means of three or more experiments and are presented as fold prolongation of the clotting time over a simultaneously performed uninhibited sample. (B) The ability of the KPI domain to inhibit the coagulant activity of various specific coagulation factors in plasma was determined. Factor Xla activity (FXla) was determined on pooled normal human plasma incubated with 1–500 nM KPI domain for 30 min at 37°C. At the conclusion of the incubation, Factor Xla coagulant activity (a) in the mixture was measured as the time necessary for clot formation. Factor Xla coagulant activity (FXla) (a) was determined on plasma preincubated with 1 nM–10 μM KPI domain. Factor Xa (FXa) (c) and Factor VIIa (FVIIa) (Δ) coagulant activities were determined on plasma preincubated with 1 nM–100 μM KPI domain. Thrombin’s activity (FIIa) (Δ) was determined on α-thrombin (4 U/ml) preincubated with 1 nM–1 nM KPI domain for 30 min at 37°C. The data represent the means of three or more experiments and are presented as fold prolongation of the clotting time over a simultaneously performed uninhibited sample.

However, this inhibition was at least two orders of magnitude weaker than the inhibition of Factor Xla by the KPI domain, which at ~0.5 μM resulted in a twofold prolongation of the Factor Xla coagulant assay. Unexpectedly, the KPI domain of PN-2/AβPP at about 1 μM also prolonged the Factor Xa coagulant activity twofold over control. Provided that excess KPI domain was not available to inhibit the coagulant activity of Factor Xla in this plasma, these investigations with Factor IX–deficient plasma suggested for the first time that the KPI domain of PN-2/AβPP could also be a potent inhibitor of Factor IXa.

To confirm that the KPI domain of PN-2/AβPP inhibited Factor IXa, studies were performed with pure Factor IXa. The KPI domain inhibited pure Factor IXa's (0.2 nM) ability to clot Factor IX–deficient plasma with an IC₅₀ of 70 nM (Fig. 2A). To circumvent the possibility that excess KPI domain in a coagulant assay could be inhibiting Factor Xla in the substrate plasma, an assay for Factor X activation by Factor IXa was...
Table I. Factor IXa Inhibition Constants

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<tr>
<th>Inhibitor</th>
<th>No heparin</th>
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<td></td>
<td>$K_i$</td>
<td>$K_{assoc}$</td>
<td>$K_i$</td>
<td>$K_{assoc}$</td>
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<tr>
<td>KPI domain$^+$</td>
<td>$1.9\pm0.3 \times 10^{-7}$</td>
<td>—</td>
<td>$6.9\pm1.3 \times 10^{-5}$</td>
<td>—</td>
</tr>
<tr>
<td>PN-2/AbβPP$^2$</td>
<td>$7.9\pm1.1 \times 10^{-11}$</td>
<td>$3.9\pm1.3 \times 10^{-11}$</td>
<td>$2.7\pm0.3 \times 10^{-6}$</td>
<td>$3.6\pm1.2 \times 10^{-6}$</td>
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* Each inhibitor was incubated with purified Factor IXa (4.5 nM) for 5–10 min at 20–25°C. The KPI domain was used at 22.5 and 44.5 nM and PN-2/AbβPP was used at 5 and 9 nM. At the end of the incubation, the inhibitor-treated Factor IXa was used to activate Factor X (400 nM) (see Methods). The data presented are the mean±SEM of five or more experiments. $^*$ Heparin was added at 1 U/ml final concentration.

established to study the effect of KPI domain on Factor IXa activity. KPI domain inhibited the ability of Factor IXa (4.5 nM) to activate Factor X (400 nM) with an IC$_{50}$ of 280 nM (Fig. 2 B). These studies indicated that the KPI domain of PN-2/AbβPP is an inhibitor of Factor IXa, as well as Factor Xla.

Both the KPI domain and PN-2/AbβPP inhibited Factor IXa's ability to activate Factor X in concentrations less than 10-fold molar excess within 10 min. The stoichiometry of active-site titrated Factor IXa to PN-2/AbβPP was 1.3 to 1.0. The $K_m$ of Factor IXa activation of Factor X in this assay was 2.08 μM as determined by a double reciprocal plot. Equilibrium inhibition constants ($K_i$) were determined to be $7.9 \times 10^{-11}$ and $3.9 \times 10^{-11}$ M for the inhibition of Factor IXa by PN-2/AbβPP in the absence and presence of heparin, respectively (Table I). The present findings with Factor IXa are similar to the equilibrium inhibition constants previously described for inhibition of Factor Xla by PN-2/AbβPP (9, 10). Alternatively, a $K_i$ of $1.9 \times 10^{-7}$ M was obtained for the inhibition of Factor IXa by the purified KPI domain suggesting that regions outside the KPI domain may be important for optimum inhibition of Factor IXa by PN-2/AbβPP. The second-order association rate constants ($K_{assoc}$) for Factor IXa and the KPI domain or PN-2/AbβPP were determined for comparison to the $K_{assoc}$ for Factor IXa and antithrombin III, the major plasma inhibitor of Factor IXa (20). The KPI domain itself had a $K_{assoc}$ of $6.9 \times 10^5$ M$^{-1}$min$^{-1}$; however, in this assay purified PN-2/AbβPP was a 400-fold faster acting inhibitor of Factor IXa with $K_{assoc}$ of $2.7 \times 10^6$ M$^{-1}$min$^{-1}$ (Table I). The addition of heparin (1 U/ml) increased the rate of Factor IXa inhibition less than twofold with a $K_{assoc}$ of $3.6 \times 10^6$ M$^{-1}$min$^{-1}$. PN-2/AbβPP in the absence or presence of heparin was a 71-fold or 95-fold, respectively, more potent inhibitor of Factor IXa than antithrombin III in the presence of heparin ($K_{assoc} = 3.8 \times 10^6$ M$^{-1}$min$^{-1}$).

To confirm that PN-2/AbβPP associated with Factor IXa to inactivate it, investigations were performed to determine if complex formation occurred between Factor IXa and PN-2/AbβPP (Fig. 3). Purified immunoglobulin G and blue dextran (data not shown) gel filtered identically at fraction 32, characterizing the void volume of the column (Fig. 3 A). Bovine serum albumin gel filtered at fraction 38. Purified Factor IXa antigen gel filtered as a broad peak between fractions 40 and 58. This finding indicated that Factor IXa alone did not gel filter as a void volume protein on this column. When Factor IXa was incubated with purified PN-2/AbβPP, a portion of the total Factor IXa antigen appeared in a new broad peak between fractions 32 and 39 (Fig. 3 B). These data indicated that some of the Factor IXa formed a higher molecular mass complex with the purified PN-2/AbβPP. Further studies were performed to determine if Factor IXa forms a complex with PN-2/AbβPP as detected by ELISA (Fig. 4). When Factor IXa was incubated with PN-2/AbβPP linked to microtiter plate wells, Factor IXa was detected specifically bound to the PN-2/AbβPP.

Discussion

This investigation indicates that PN-2/AbβPP is a newly recognized inhibitor of Factor IXa. PN-2/AbβPP and its KPI domain

Figure 3. Sephadex G-100 in 0.01 M Tris, 0.15 M NaCl, pH 8.0 was used for the gel filtration of (A) Factor IXa (5 μg) alone (c) (A) or (B) a Factor IXa (5 μg) and PN-2/AbβPP (11 μg) mixture (v). The fractions where human gG and BSA gel filtered are indicated by the arrows. The data presented represents the mean of two similar experiments.

![Figure 3](image-url)
inhibited Factor IXa’s coagulant and enzymatic activity. The inactivation of Factor IXa was associated with complex formation between the enzyme and PN-2/\(\alpha\beta\)PP. The broad peaks seen in the gel filtration experiment probably result from heterogeneity in the size of the Factor IXa used. The interaction between PN-2/\(\alpha\beta\)PP and Factor IXa appears to involve more than a complex between the inhibitor and the enzyme’s active site. KPI domain is a better inhibitor of plasma coagulant activity than PN-2/\(\alpha\beta\)PP suggesting that various plasma proteins may modify the whole inhibitor’s interaction with plasma coagulation enzymes. Alternatively, using the enzymatic assay for Factor IXa described in this study, PN-2/\(\alpha\beta\)PP is a much tighter inhibitor of Factor IXa than KPI domain. These combined findings suggest that PN-2/\(\alpha\beta\)PP may interact with Factor IXa at its enzymatic site and, perhaps, another region. Under the conditions of the assay performed for Factor X activation, PN-2/\(\alpha\beta\)PP also appears to be a more potent inhibitor for Factor IXa than is antithrombin III, the recognized plasma inhibitor of this protein (21). Since the relative potency of an inhibitor in plasma can be determined by multiplying its \(K_{\text{ass}}\) by the inhibitor’s plasma concentration and comparing this value with other known inhibitors (22), antithrombin III, which has a plasma concentration of 2.7 \(\mu\)M (20), must account for 99% of the plasma inhibition of Factor IXa since the normal plasma concentration of PN-2/\(\alpha\beta\)PP is only 60 pM (23). However, large amounts of PN-2/\(\alpha\beta\)PP are secreted by activated platelets (8, 9), an event that occurs in parallel to coagulation at sites of vascular damage. If physiologically significant activation of Factor IX occurs on endothelial cell and platelet surfaces (24, 25), then PN-2/\(\alpha\beta\)PP, a cell surface-associated protein, would be the most potent regulator of Factor IXa at this locus. This new property of PN-2/\(\alpha\beta\)PP to inhibit Factor IXa, coupled with its potent inhibition of Factor XIa, strongly suggests that PN-2/\(\alpha\beta\)PP influences the initiation of hemostatic reactions.

The importance of Factor IX in hemostasis is underscored by the finding that individuals with a deficiency in this protein have spontaneous bleeding states of whom 12% have intracerebral hemorrhage (26, 27). The finding that PN-2/\(\alpha\beta\)PP is an inhibitor of Factor IXa may be important in understanding the pathogenesis of HCHWA-D. HCHWA-D, a rare disorder that is associated with recurrent and often fatal spontaneous intracerebral hemorrhages in middle (3, 4), results from a point mutation in the \(\alpha\beta\)PP gene that substitutes glutamine for glutamic acid at residue 22 in the \(\alpha\) domain (28, 29). It has been postulated that this mutation contributes to the pathologic liberation of \(\alpha\) and its deposition in the brain parenchyma and in the cerebral blood vessels of patients with this disorder. In addition to \(\alpha\) deposition, immunohistochemical staining investigations showed that HCHWA-D patients also exhibited extensive deposits of PN-2/\(\alpha\beta\)PP in their cerebral vessels (30). The selective accumulation of PN-2/\(\alpha\beta\)PP in the cerebral vessels of HCHWA-D patients could result in decreased Factor IXa activity, leading to the spontaneous intracerebral hemorrhages characteristic of this disorder (3, 4).

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**References**


8. Van Nostrand, W. E., A. H. Schmaier, J. S. Farrow, and D. D. Cun-


