Miller, S. M., Moore, M. J., Massey, V., Williams, C. H., Jr., Distefano, M. D., Ballou, D. P., & Walsh, C. T. (1989) *Biochemistry 28*, 1194–1205.
Moore, M. J., & Walsh, C. T. (1989) *Biochemistry 28*, 1183–1194.
Moore, M. J., Distefano, M. D., Zydowsky, L. D., Cummings, R. T., & Walsh, C. T. (1990) *Acc. Chem. Res. 23*, 301–308.
Ni'Bhriain, N. N., Silver, S., & Foster, T. J. (1983) *J. Bacteriol. 155*, 690–703.
Ross, W., Park, S. J., & Summers, A. O. (1989) *J. Bacteriol. 171*, 4009.
Sahlman, L., Lambeir, A.-M., Lindskog, S., & Dunford, H. B. (1984) *J. Biol. Chem. 259*, 12403–12408.
Sandstrom, A., & Lindskog, S. (1987) *Eur. J. Biochem. 164*, 243–249.
Schiering, N., Fritz-Wolf, K., Kabsch, W., Moore, M. J., Distefano, M. D., Walsh, C. T., & Pai, E. F. (1991a) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 615–625, Walter de Gruyter, Berlin.
Schiering, N., Kabsch, W., Moore, M. J., Distefano, M. D., Walsh, C. T., & Pai, E. F. (1991b) *Nature 352*, 168–172.
Schultz, P. G., Au, K. G., & Walsh, C. T. (1985) *Biochemistry 24*, 6840–6848.
Seidman, M. (1985) *BMBiochemica (Boehringer-Mannheim Biochemical) 2* (5), 10.
Summers, A. O. (1986) *Annu. Rev. Microbiol. 40*, 607–634.
Summers, A. O., & Silver, S. (1978) *Annu. Rev. Microbiol. 32*, 637–672.
United States Biochemicals (1988) *Sequenase: Step-By-Step Protocols for DNA Sequencing with Sequenase*, 2nd ed., Cleveland, OH.
Wang, Y., Moore, M., Levinson, H. S., Silver, S., Walsh, C., & Mahler, I. (1989) *J. Bacteriol. 169*, 4848–4851.
Williams, C. H., Jr., Arscott, D. L., Matthews, R. G., Thorpe, C., & Wilkinson, K. D. (1979) *Methods Enzymol. 62D*, 185–198.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.

A Low Molecular Weight Platelet Inhibitor of Factor XIa: Purification, Characterization, and Possible Role in Blood Coagulation†

Anne L. Cronlund‡ and Peter N. Walsh*,‡,§

Thrombosis Research Center and Departments of Medicine and Biochemistry, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, Pennsylvania 19140

Received June 18, 1991; Revised Manuscript Received November 6, 1991

ABSTRACT: A low molecular weight platelet inhibitor of factor XIa (PIXI) has been purified 250-fold from releasates of washed and stimulated human platelets. Molecular weight estimates of 8400 and 8500 were determined by gel filtration and SDS–polyacrylamide gel electrophoresis, respectively, although a second band of M_r 5000 was present upon electrophoresis. The inhibitor does not appear to be one of the platelet-specific, heparin-binding proteins, since it neither bound to nor was affected by heparin. An amount of PIXI which inhibited by 50% factor XIa cleavage of the chromogenic substrate S2366 (Pyr-Glu-Pro-Arg-pNA·$2H_2O$) only slightly inhibited (5–9%) factor XIIa, plasma kallikrein, plasmin, and activated protein C and did not inhibit factor Xa, thrombin, tPA, or trypsin, suggesting specificity for factor XIa. Kinetic analyses of the effect of PIXI on factor XIa activity demonstrated mixed-type, noncompetitive inhibition of S2366 cleavage and of factor IX activation with K_i's of 7×10^{-8} and 3.8×10^{-9} M, respectively. Immunoblot analysis showed that PIXI is not the inhibitory domain of protease nexin II, a potent inhibitor of factor XIa also secreted from platelets. Amino acid analysis showed that PIXI has no cysteine residues and, therefore, is not a Kunitz-type inhibitor. PIXI can prevent stable complex formation between α_1-protease inhibitor and factor XIa light chain as demonstrated by SDS–polyacrylamide gel electrophoresis. The inhibition by PIXI of factor XIa-catalyzed activation of factor IX and its capacity to prevent factor XIa inactivation by α_1-protease inhibitor, combined with the specificity of PIXI for factor XIa among serine proteases found in blood, suggest a role for PIXI in the regulation of intrinsic coagulation.

Control of intrinsic coagulation may be exerted most effectively by regulation of factor XIa activity, since factor XIa appears to be the first enzyme in this coagulation pathway required for normal hemostasis (Rosenthal et al., 1953; Ragni et al., 1985). Factor XIa, a serine protease produced in blood from zymogen factor XI, is a dimeric molecule of two identical, disulfide-linked, polypeptide chains (M_r 80000), each of which has a M_r 50000 heavy chain linked to a M_r 30000 light chain by disulfide bridges (Bouma & Griffin, 1977; Kurachi & Davie, 1977). The light chain contains the catalytic site (Kurachi & Davie, 1977; van der Graaf et al., 1983), whereas the heavy chain has sites for the binding surface (Mannhalter & Schiffman, 1980), a cofactor (high molecular weight kininogen) (van der Graaf et al., 1983; Sinha et al., 1985; Baglia et al., 1989, 1990), and its substrate factor IX (Sinha et al.,

† This research was supported by National Research Service Fellowship Award HL07832 (to A.L.C.), by National Institutes of Health Grants HL36579, HL46213, HL45486, and HL25661 (to P.N.W.), and by a grant from the W. W. Smith Charitable Trust (to P.N.W.). Portions of this study were previously reported in abstract form.

* To whom correspondence should be addressed at the Thrombosis Research Center.
‡ Thrombosis Research Center.
§ Departments of Medicine and Biochemistry.

0006-2960/92/0431-1685$03.00/0

1987; Baglia et al., 1989). Regulation of factor XIa activity may be accomplished by the extent of activation of factor XI, present in blood at a concentration of 25 nM (Bouma & Griffin, 1977), and thereafter by its inhibition by a variety of inhibitors.

The major plasma inhibitor of factor XIa is α_1-protease inhibitor (α1PI)[1] (Heck & Kaplan, 1974; Scott et al., 1982), a member of a protein superfamily containing serine protease inhibitors and called serpins (Hunt & Dayhoff, 1980; Carrell et al., 1987). Serpins are actually suicide substrates since, after their initial binding to the catalytic site and cleavage by the enzyme, they form a covalent product with the enzyme rendering it irreversibly inactivated (Carrell et al., 1987). Other known plasma inhibitors of factor XIa (C1-inhibitor, M_r 105 000; α_2-antiplasmin, M_r 70 000; and antithrombin III, M_r 62 000) are also serpins. Although stimulated platelets have been shown to promote the activation of factor XI via surface binding (Walsh, 1972; Walsh & Griffin, 1981) and to protect factor XIa from inactivation by α_1-protease inhibitor (Walsh et al., 1987), they have also been shown to secrete trace quantities of α_1-protease inhibitor (Nachman & Harpel, 1976; Bagdasarian & Colman, 1978), C1-inhibitor (Schmaier et al., 1985), and α_2-antiplasmin (Plow & Collen, 1981). The role of these inhibitors is unclear since they are secreted at very low levels; however, their close proximity to the region of platelet aggregation and surface activation of factor XI may result in locally high concentrations that modulate blood coagulation at the site of injury.

Releasates of human platelets have been shown to contain factor XIa inhibitory activity not attributable to these known serpins (Soons et al., 1986; Walsh et al., 1987) as well as the ability to "protect" factor XIa from covalent product formation with α1PI (Walsh et al., 1987). At physiologic concentrations of platelets, the releasate was shown to inhibit factor IX activation by factor XIa about 90% (Soons et al., 1986), which suggested that this inhibitor may be significant in control of the contact activation mechanism under physiologic conditions. Recently, other researchers have identified a high molecular weight inhibitor (M_r 110 000–130 000), secreted from α-granules (Van Nostrand et al., 1990a), as protease nexin II/amyloid precursor protein (PNII/APP) (Van Nostrand et al., 1990a; Smith et al., 1990; Bush et al., 1990). This protein, which contains one Kunitz-like inhibitory domain (Ponte et al., 1988; Tanzi et al., 1988; Kitaguchi et al., 1988; Smith et al., 1990), has been shown to be a potent inhibitor of factor XIa [K_i = (2.9–4.5) × 10^{-10} M] (Smith et al., 1990; Van Nostrand et al., 1990b) with significantly increased inhibitory activity in the presence of heparin [K_i = (2.5–5.5) × 10^{-11} M] (Smith et al., 1990; Van Nostrand et al., 1990b).

Here we report on the purification and characterization of a low molecular weight platelet inhibitor of factor XIa (PIXI) which appears to be distinct from other known factor XIa inhibitors. We have investigated the possible physiologic role of PIXI in regulation of factor XIa activity by examination of its specificity, its effect on factor IX activation by factor XIa, and its effect on covalent product formation between factor XIa and α1PI. Our results suggest that both PIXI and PNII may be important regulators of factor XIa and, therefore, of intrinsic coagulation.

[1] Abbreviations: α1PI, α_1-protease inhibitor; PNII/APP, protease nexin II/amyloid precursor protein; PIXI, low molecular weight platelet inhibitor of factor XIa; 5HT, 5-hydroxytryptamine; pNA, *p*-nitroanilide; EDTA, ethylenediaminetetraacetic acid; IBMX, isobutylmethylxanthine; PMSF, phenylmethanesulfonyl fluoride; IA, iodoacetamide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NBT, nitro blue tetrazolium; DMF, dimethylformamide; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography; BPTI, bovine pancreatic trypsin inhibitor; APC, activated protein C.

Experimental Procedures

Materials. Bovine factor XIIa was kindly provided by Dr. Edward P. Kirby, and human tissue plasminogen activator (tPA) was donated by Drs. Ahmed A. K. Hasan and Andrei Z. Budzynski, all of the Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA. Human α-thrombin was a gift of Dr. John Fenton of the New York State Department of Health, Albany, NY. Activated protein C (APC) was donated by Drs. Mary Jo Heeb and John H. Griffin of the Scripps Research Institute, La Jolla, CA. The monoclonal antibody 7H5 directed against the Kunitz domain of protease nexin II (PNII) was donated by Dr. Sukanto Sinha of Athena Neurosciences, South San Francisco, CA. Mouse hybridoma culture medium containing a monoclonal antibody directed against the N-terminal region of PNII and a rabbit polyclonal antibody against determinants in the PNII molecule were donated by Dr. William Van Nostrand of the Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, CA. Factor Xa and plasma kallikrein were purchased from KabiVitrum, Molndal, Sweden. [^{14}C]-Labeled 5-hydroxytryptamine (5HT) was purchased from New England Nuclear Corp., Boston, MA. Chromogenic substrates, pyro-Glu-Pro-Arg-pNA (S2366), H-D-Pro-Phe-Arg-pNA (S2302), Bz-Ile-Glu-pip-Gly-Arg-pNA (S2337), H-D-Phe-pip-Arg-pNA (S2238), tosyl-Gly-Pro-Lys-pNA (S2251), H-D-Ile-Pro-Arg-pNA (S2288), and succinyl-Ala-Ala-Pro-Phe-pNA (S2586), were purchased from Helena Laboratories, Beaumont, TX. Native collagen fibrils were purchased from Hormon-Chemie, Munchen, Germany (Collagenreagent Horm), or from Chronolog Corp., Havertown, PA. Sephadex G-50, heparin–agarose, protein molecular weight standards, ethylenediaminetetraacetic acid (EDTA), isobutylmethylxanthine (IBMX), phenylmethanesulfonyl fluoride (PMSF), iodoacetamide (IA), *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), Tris, nitro blue tetrazolium (NBT), dimethylformamide (DMF), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), Tween 20, bovine serum albumin (BSA, A-7030), trypsin, chymotrypsin, plasmin, aprotinin, imipramine, and sheep anti-mouse IgG conjugated to alkaline phosphatase were obtained from Sigma Chemical Co., St. Louis, MO. α_1-Protease inhibitor was purchased from CalBiochem, La Jolla, CA. Reagents for protein determinations and electrophoresis, goat anti-rabbit IgG conjugated to alkaline phosphatase, and BioGel P-10 resin were purchased from Bio-Rad Laboratories, Richmond, CA. Thiourea was purchased from Eastman Kodak Co., Rochester, NY, and ZK36374 (now called iloprost) from Berlex Laboratories, Inc., Cedar Knolls, NJ. Porcine intestinal heparin was purchased from Abbott Laboratories, North Chicago, IL. The YM-10 ultrafiltration membrane was obtained from Amicon Corp., Lexington, MA. The MonoQ and MonoS columns were products of Pharmacia Fine Chemicals, Piscataway, NJ. BetaPhase scintillation fluid was obtained from WestChem Products, San Diego, CA.

Preparation of Platelet Releasate. Blood was collected from healthy donors into plastic tubes containing 0.1 volume of 3.8% trisodium citrate. Platelet-rich plasma was obtained by centrifugation of the anticoagulated blood at 170*g* for 15 min. Platelets were washed by a modification of the procedure of Mustard et al. (1972) in which HEPES-buffered Tyrode's solutions contained BSA (1 mg/mL) and no heparin. Suspended washed platelets were stimulated by the combined

addition of human α-thrombin (0.1 unit/mL) and microfibrillar type I collagen (10 μg/mL), or collagen alone, at 37 °C and incubation without stirring for 10 min, after which EDTA, PMSF, and IA were each added to final concentrations of 1 mM. Platelets were removed from the releasate by centrifugation at 12000*g* and 4 °C for 30 min. Larger quantities of factor XIa inhibitor were prepared as above by washing and stimulating outdated platelet preparations from the American Red Cross (Penn Jersey Region, Philadelphia, PA).

Preparation of Proteins. Human factor XI, purified by affinity chromatography according to the method of Sinha et al. (1985), was activated by incubation with bovine factor XIIa as described by Sinha et al. (1984). SDS–polyacrylamide gel electrophoresis of the reduced factor XIa preparation showed only two bands, which corresponded to the heavy chain (M_r 50000) and light chain (M_r 30000) of factor XIa. The preparation had a specific activity of approximately 250 units/mg as determined using a coagulant assay for factor XIa activity (Scott et al., 1984). For use in detection of covalent products between factor XIa and α1PI, factor XI was labeled with ^{125}I by the Iodogen method (Fraker & Speck, 1978) prior to its activation. ^{125}I-Labeled factor XI retained 100% of its clotting activity as determined by the kaolin-activated partial thromboplastin time (Procter & Rapaport, 1961) measured in factor XI deficient plasma (Walsh et al., 1987). After being activated, the labeled factor XIa exhibited 100% of its expected amidolytic activity as determined using the chromogenic assay described below.

Factor IX was purified from human plasma by a modification (Walsh et al., 1984) of the methods described by DiScipio et al. (1977) and Miletich et al. (1980) and had a specific activity of 225 units/mg. Labeling of factor IX with tritium for the peptide release assay was accomplished by a modification (Walsh et al., 1984) of the method described by Van Lenten and Ashwell (1971). Additional unlabeled factor IX having a specific activity of 162 units/mg was purchased from Enzyme Research Laboratories, Inc., South Bend, IN.

Platelet PNII was separated from PIXI and other low molecular weight contaminants during the ultrafiltration step for PIXI purification using an Amicon YM-10, M_r 10000 cutoff, ultrafiltration membrane. Further purification of PNII was accomplished by ion-exchange and gel filtration procedures. The concentrated retentate after ultrafiltration was half-diluted with H_2O and applied to a MonoQ column equilibrated with 20 mM Tris, pH 7.5. All of the bound protein absorbing at 280 nm eluted between 0.0 and 0.5 M NaCl of the linear salt gradient, whereas the factor XIa inhibitory activity (PNII) eluted between 0.8 and 1.0 M NaCl. A silver-stained SDS–polyacrylamide gel of this inhibitory pool demonstrated a major band of protein with $M_r \simeq 113000$ and a minor band of protein ($M_r \simeq 58000$ when not reduced and $M_r \simeq 68000$ when reduced). The PNII was separated from this contaminating protein by gel exclusion chromatography using Sephacryl S-200 (Pharmacia).

Protein Analyses. The protein concentration of enzyme-containing solutions was estimated by the Bio-Rad dye-binding protein assay as originally described by Bradford (1976). Proteins eluting from chromatographic procedures were detected by the absorbance at 280 nm in a spectrophotometer, and estimates of concentration were made assuming an average extinction coefficient of 1.0 mg mL^{-1} per absorbance unit for a 1-cm path length. Amino acid analysis of purified inhibitor was performed at the Wistar Protein Microchemistry Core Facility, Philadelphia, PA, using an amino acid analyzer and the Pico-Tag method (Waters, Milford, MA) after vapor-phase hydrolysis of the protein in 6 N HCl containing 1% phenol at 160 °C for 1 h. Total cysteine content was determined in separate samples by the addition of dimethyl sulfoxide to a final concentration of 4% in the 6 N HCl prior to hydrolysis for quantitative conversion of cysteines and cystines to cysteic acid (Spencer & Wold, 1969).

Polyacrylamide Gel Electrophoresis. Electrophoresis of inhibitor samples was performed by a modification of the method of Laemmli (1970), using 20% acrylamide and 0.5 M Tris, pH 8.8 in the separating gel polymerized with thiourea (2.0% of the total acrylamide concentration) and H_2O_2 (1.3% of the total acrylamide concentration) (Basch et al., 1985). Peptides in the gels were fixed with glutaraldehyde and stained with silver according to the method of Morrissey (1981).

Electrophoresis of radiolabeled factor XIa samples was performed with 10% acrylamide and 0.5 M Tris, pH 8.8 in the separating gel which was also polymerized with thiourea and H_2O_2.

Measurement of Factor XIa Activity and Factor XIa Inhibitory Activity. For the purpose of locating the factor XIa inhibitor in fractions produced during its purification and for determination of equilibrium inhibition constants, factor XIa activity and the decrease of this activity caused by the inhibitor were measured by a modification of the amidolytic assay of Scott et al. (1984). The substrate S2366 (1 mM) was cleaved in microtiter wells (no. 3075 Falcon plates from Beckton Dickinson, Oxnard, CA) by purified factor XIa (0.120 pmol) in PBS/BSA (10 mM phosphate, 0.15 M NaCl, and 0.1% BSA, pH 7.4) in the absence or presence of inhibitor in a total volume of 150 μL. The formation of product absorbing at 405 nm was followed over time using a Vmax microplate reader (Molecular Devices Corp., Palo Alto, CA) and was compared to the rate of product formation by known concentrations of factor XIa (0.11–1.1 nM). In order to compare inhibitor samples to each other, the inhibitory activity of serial 2-fold dilutions of the samples was measured, and units of inhibitor were determined. One unit of inhibitory activity was defined as that amount which inhibits 1 unit (25 pmol) of factor XIa activity 50% in the described chromogenic assay performed at 23 °C.

Secretion of Substances from Stimulated Platelets. To examine whether or not the factor XIa inhibitor is secreted from platelets, platelets preloaded with [^{14}C]-labeled 5HT and in buffer containing 5.0 μM imipramine (to prevent reuptake of released 5HT) were stimulated in the absence or presence of a mixture of inhibitors of secretion, i.e., IBMX (200 μM), ZK36374 (100 nM), and EDTA (10 mM). Thrombin (0.1 unit/mL) and collagen (10 μg/mL) were added together (7.5 μL) to 0.5 mL of stirring, washed human platelets (3×10^8/mL) at 37 °C in aggregometer cuvettes. Aggregation was followed using a Payton dual-channel aggregometer complete with chart recorder. At timed intervals after agonist addition, the cuvettes were removed from the aggregometer, and the contents were quickly poured into ice-cold microfuge tubes containing 38 μL of the inhibitor mix to terminate secretion. Control samples contained inhibitor mix prior to agonist addition and were processed immediately. After centrifugation for 30 s in an Eppendorf microfuge, 400 μL of the supernatant was placed into scintillation vials with 5.0 mL of BetaPhase scintillation fluid for labeled 5HT counting. Another aliquot (110 μL) was stored at −20 °C to await analysis for platelet factor 4 and factor XIa inhibitor concentrations. Platelet factor 4 concentration was determined by Dr. Boguslaw Rucinski (Temple University School of Medicine, Philadelphia,

PA) by the radioimmunoassay method of Rucinski et al. (1979).

Immunoblot Analysis of Secreted Factor XIa Inhibitors. Samples of crude platelet releasate, purified high molecular weight inhibitor or PNII (positive control), PIXI pools purified from releasate, α1PI (negative control), and aprotinin at concentrations sufficient to give equal levels of factor XIa inhibition (40% inhibition in the chromogenic assay) were spotted onto Immobilon P membranes (Millipore Corp., Waltham, MA). The membranes were then gently agitated in TBS (20 mM Tris/0.15 M NaCl, pH 7.5) containing 5% nonfat dry milk for 2 h at room temperature to block free membrane sites. The membranes were then incubated for 20 h at 4 °C with anti-PNII antibody (7H5 monoclonal antibody at 10 μg/mL, mouse monoclonal hybridoma culture medium at 1:5 dilution, and rabbit polyclonal antiserum at 1:200 dilution) in TBS. After three 10-min washes of membranes at room temperature with 20 mM Tris/0.5 M NaCl buffer (pH 7.5) containing 0.05% Tween 20, the membranes were incubated for 2 h at room temperature with the enzyme-conjugated goat anti-rabbit or sheep anti-mouse IgG (diluted 1:1000 in TBS). Membranes were washed 3 times with TBS and once with substrate buffer (0.05 M sodium carbonate/1 mM $MgCl_2$, pH 9.8) prior to development with the substrate (300 μg/mL NBT, 150 μg/mL BCIP, and 1.4% DMF in substrate buffer). Spots positive for bound antibody became gray-purple in 5–15 min.

Specificity of PIXI. A preliminary analysis of specificity was carried out using PIXI at a concentration (300 nM) which inhibited 0.8 nM factor XIa (1.6 nM active-site concentration) 50% in the chromogenic assay. Various serine proteases of the coagulation and fibrinolytic systems, as well as trypsin and chymotrypsin, at 1–2 nM concentrations were similarly tested for inhibition by PIXI with appropriate chromogenic substrates at 1 mM [which was (3–4)K_m for each substrate] in triplicate amidolytic assays and compared to triplicate controls of enzyme and substrate alone. Initial rates of cleavage of the substrates over 5–10 min at room temperature were measured on a Vmax microplate reader (Molecular Devices, Menlo Park, CA), and percent activity was determined using standard curves of initial rates of known concentrations of the enzymes which were generated at the same time.

Kinetic Analysis of the PIXI Effect on Factor XIa Activation of Factor IX. Factor IX activation by factor XIa in the absence and presence of varying concentrations of PIXI was measured by the peptide release assay described by Walsh et al. (1984) in which the ^{3}H-labeled, TCA-soluble, activation peptide of factor IX formed by proteolytic activation was counted in a scintillation counter and picomoles of released peptide was determined. Briefly, varying concentrations of factor IX (0.25–2.0 μM) containing a constant concentration of labeled factor IX (9 nM) were incubated with 1 nM factor XIa in TBS/BSA (20 mM Tris, 0.15 M NaCl, and 0.1% BSA, pH 7.4) containing 5 mM $CaCl_2$ in the absence or presence of PIXI. The mixture (50 μL) was incubated at 37 °C for 10 min after which 110 μL of ice-cold EDTA (15-fold excess compared with Ca^{2+}) in TBS/BSA was added to stop the reaction and the samples were placed on ice. Eighty microliters of cold 15% TCA was then added; the samples were mixed well and centrifuged in a microfuge (13000g) for 3 min. Aliquots (200 μL) of the supernatants were placed in 5.0 mL of BetaPhase scintillation fluid and were counted in a Beckman LS 8000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA) to measure the released activation peptide. Least-squares linear regression lines of the data were examined in $1/v$ vs $1/S$ double-reciprocal plots using the equation for noncompetitive inhibition:

$$1/v = (K_s/V_{max})(1 + I/K_i)(1/S) + (1/V_{max})(1+ I/\alpha K_i)$$

and the resulting slopes and y-axis intercepts were replotted against inhibitor concentration to determine the kinetic constants of PIXI inhibition of factor XIa.

Analysis of the PIXI Effect on the Interaction of α1PI with Factor XIa. Determination of the percent loss of free factor XIa light chain to covalent product formed between α1PI and the light chain in the absence and presence of platelet inhibitors of factor XIa was accomplished by a modification of the method of Walsh et al. (1987). ^{125}I-Factor XIa (5.5 nM and 3.0×10^4 cpm) was incubated alone or with α1PI (5.9 μM) in the absence or presence of various sources of platelet inhibitor: either crude releasate (4.0×10^{-3} unit of factor XIa inhibitory activity), PNII (1.8×10^{-3} unit of factor XIa inhibitory activity), or PIXI (6.4×10^{-4} unit of factor XIa inhibitory activity for a 300 nM final concentration) in a total volume of 25 μL at 37 °C for 15 min. An equal volume of 2× gel electrophoresis sample buffer (0.13 M Tris, 5% SDS, 2% β-mercaptoethanol, and 20% glycerol, pH 6.8) was then added, and samples were heated in boiling water for 5 min. After the samples cooled, they were separated by electrophoresis on a 10% acrylamide slab gel. At the completion of the electrophoresis, the gel was dried between sheets of cellophane; then each lane of the gel was cut into 2-mm bands, placed individually into plastic tubes, and counted in an NE1600 γ counter (Nuclear Enterprises, Ltd., Edinburgh, Scotland). The percentages of counts corresponding to different chains of reduced factor XIa were determined. Standard proteins were included in separate lanes of each gel and were stained with Coomassie brilliant blue at the completion of electrophoresis to establish molecular weight positions of the radioactive factor IXa bands.

Results

Measurements of factor XIa inhibitory activity in supernatants of human platelets after stimulation by thrombin and collagen demonstrated that an inhibitor appeared within 30 s and increased in concentration with a time course similar to that of platelet factor 4 and 5HT secretion (Figure 1). Factor XIa inhibitory activity was absent in supernatants of platelets treated with thrombin and collagen in the presence of inhibitors of secretion, showing that the factor XIa inhibitor, like platelet factor 4 and 5HT, is contained in platelet secretory storage pools. Since this work was completed, Van Nostrand and co-workers have demonstrated that the location of the PNII/APP factor XIa inhibitor is in platelet α-granules (Van Nostrand et al., 1990a).

Purification of PIXI. Separation of crude platelet releasate on Sephadex G-50 demonstrated two peaks of inhibitory activity (Figure 2). The earlier eluting peak, comprising approximately 46% of the factor XIa inhibitory material, coeluted with the majority of the material absorbing at 280 nm at the void volume of the column. (When this peak was pooled, concentrated, and rechromatographed on a calibrated Sephacryl S-300 column, a molecular weight of 125 000–130 000 was demonstrated.) The later eluting inhibitor peak from the Sephadex G-50 column (54% of the total inhibitory material) eluted near the position of aprotinin (M_r 6500), suggesting that, contrary to an earlier report (Soons et al., 1986), there exists in platelets a factor XIa inhibitor having a very low molecular weight. Thereafter, platelet releasate was filtered through a M_r 10 000 cutoff membrane (Amicon YM-10) to separate PIXI from most of the contaminating proteins and

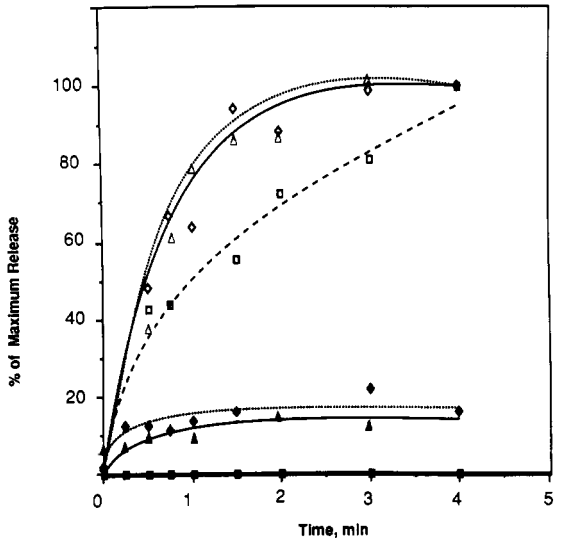

FIGURE 1: Release of platelet factor 4, [^{14}C]-labeled 5HT, and platelet factor XIa inhibitory activity from stimulated platelets in the absence and presence of a mixture of inhibitors of platelet secretion. The concentrations of platelet factor 4 (△, ▲), 5HT (◇, ◆), and factor XIa inhibitory activity (□, ■) were determined in the releasates of platelets stimulated in the absence (open symbols) and presence (closed symbols) of ZK36374 (100 nM), IBMX (200 μM), and EDTA (10 mM) as described under Experimental Procedures.

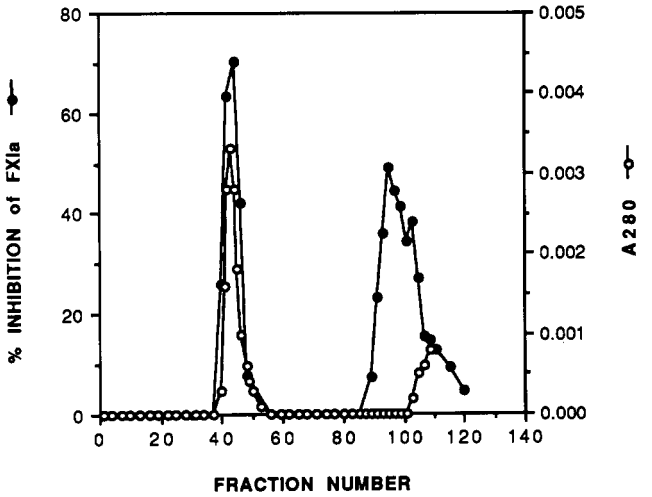

FIGURE 2: Chromatography of platelet releasate on Sephadex G-50. Column dimensions were 1.2 × 86.0 cm, and the fraction volume was 800 μL. Ten percent of the releasate of approximately 3.5 × 10^{10} platelets was applied to the column equilibrated in 15 mM HEPES buffer, pH 7.5. Aliquots (60 μL) of fractions were tested for factor XIa inhibition using the chromogenic assay described under Experimental Procedures.

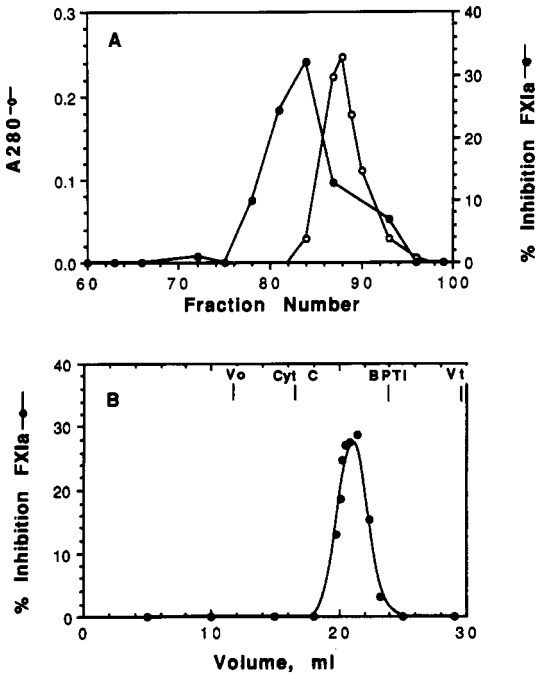

FIGURE 3: Chromatography of the YM-10 filtrate of platelet releasate on BioGel P-10. (A) Concentrated filtrate was applied to a 5.0 × 38 cm column equilibrated in 15 mM HEPES, pH 7.5. Fractions of 5.7 mL were collected after an initial 150-mL pool. (B) Elution of concentrated filtrate on a calibrated column (1.4 × 19 cm) in 15 mM HEPES, pH 7.5. Fractions of 300 μL were collected. Aliquots (60 μL) of fractions from each chromatography were tested for factor XIa inhibition using the chromogenic assay as described under Experimental Procedures. Abbreviations: Vo, void volume; Cyt C, cytochrome *c* (M_r 12400); BPTI, bovine pancreatic trypsin inhibitor (M_r 6500); Vt, totally included volume.

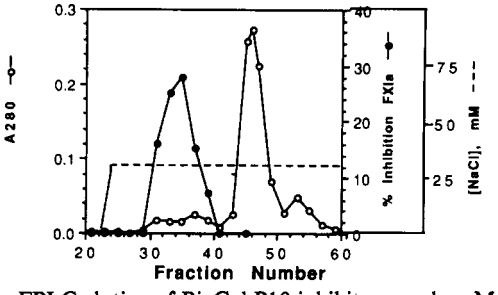

FIGURE 4: FPLC elution of BioGel P10 inhibitory pool on MonoS. The lyophilized and then dialyzed BioGel P10 inhibitory pool was applied to a MonoS column (1-mL bed volume) equilibrated in 50 mM citrate, pH 2.0. After a 10-min wash at 1 mL/min, the inhibitory activity was eluted with 50 mM citrate/0.03 M NaCl, pH 2.0. The fraction size was 0.5 mL. Aliquots (60 μL) of fractions titrated to pH 7.5 with 1 M K_3PO_4 were tested for factor XIa inhibitory activity as described under Experimental Procedures.

the high molecular weight inhibitory activity. No factor XIa inhibitory activity was detected in YM-10 filtrates of platelet-poor plasma.

Chromatography of concentrated (by lyophilization) YM-10 filtrate on BioGel P-10 separated PIXI from lower molecular weight contaminants (Figure 3A). A molecular weight for PIXI of 8400 was estimated from chromatography of an aliquot of concentrated YM-10 filtrate on a calibrated Biogel P-10 column (Figure 3B).

Final purification of PIXI was accomplished by ion-exchange chromatography of the dialyzed inhibitor pool eluted from BioGel P-10 using a MonoS column on a Pharmacia FPLC system (Figure 4). Inhibitory activity was eluted in a peak which contained proteins with molecular weights of approximately 8500 and 5000 as seen with both reduced and nonreduced samples on silver-stained SDS–polyacrylamide gels (Figure 5). Since the peak of inhibitory activity was shown to elute at the position of a M_r 8400 protein in gel exclusion chromatography, the M_r 8500 band is presumed to be the inhibitor. However, the pooled material from that column may contain a lower molecular weight inhibitory species since it is usually a broad peak eluting from a large column. In addition, some breakdown of the M_r 8500 protein may have occurred during the extended sample dialysis prior to ion-exchange chromatography.

Purification of PIXI from fresh platelet releasate is summarized in Table I and indicates a maximum release of 0.75 unit per 3 × 10^{10} platelets (normal for 100 mL of blood) for a molar concentration of approximately 88 nM. As indicated in the footnote of Table I, 3 inhibitory units out of the 7 total

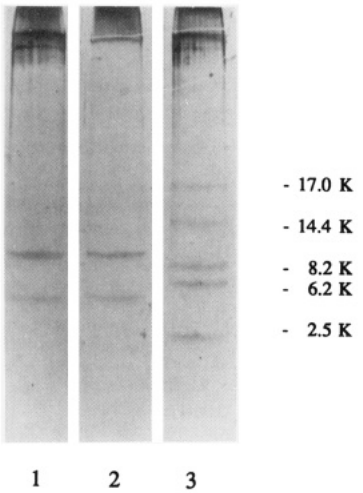

FIGURE 5: SDS–polyacrylamide gel electrophoresis of purified PIXI. Samples of dialyzed and lyophilized pooled PIXI from MonoS chromatography were electrophoresed on a polyacrylamide slab gel and stained with silver as described under Experimental Procedures. Unreduced (lane 1) and reduced (lane 2) PIXI samples were compared to myoglobin CNBr peptide standards (lane 3).

Table I: Purification of the Low Molecular Weight Platelet Inhibitor of Factor XIa (PIXI)

step	protein (mg)	units	sp act. (units/mg)	purification (*x*-fold)	yield (%)
releasate	75	3[a]	0.040	1	100
YM10 filtrate	46	3	0.065	1.6	100
BioGel P-10	2	2	1.0	25	67
MonoS	~0.01	0.1	10	250	3

[a] Based on the first measure of the low molecular weight species in the filtrate and assuming no loss in filtration; total activity which includes high molecular weight inhibitor was 7 units.

inhibitory units or 42% of the factor XIa inhibitory activity in the platelet releasate is due to PIXI. The PIXI portion of the total secreted factor XIa inhibitory activity was found to range from 18 to 54% if prepared from freshly drawn blood and from 8 to 20% if prepared from outdated platelets.

Cation-exchange chromatography, as the final step of purification, increases the specific activity of the inhibitor 10-fold. The inhibitory activity could not be detected after reverse-phase chromatography and did not bind to factor XIa affinity resins, hydrophobic media, or anion-exchange media under various conditions of buffer, pH, and ionic strength.

Inhibition of Factor XIa by PIXI. Kinetic analysis of the effect of PIXI on factor XIa cleavage of S2366 showed mixed-type noncompetitive inhibition with an approximate K_i of 7×10^{-8} M. Since the physiological substrate for factor XIa is factor IX, the kinetics of factor XIa inhibition by PIXI were analyzed using factor IX as the substrate and the generation of the factor IX activation peptide as an assay for factor IX activation. Analysis of the data for the effect of PIXI on factor XIa-catalyzed factor IX activation also showed linear, mixed-type, noncompetitive inhibition with an apparent K_i of 3.8×10^{-9} M and an αK_i of 1.7×10^{-8} M. The K_m of 0.26 μM determined for factor IX is consistent with published values of 0.37 and 0.49 μM (Walsh et al., 1984; Sinha et al., 1987).

The reversibility of factor XIa inhibition by PIXI was examined using the chromogenic assay previously described. In this case, factor XIa was mixed either with PIXI or with buffer (control) such that an aliquot (100 μL) could be directly tested in the assay by addition of 50 μL of S2366 to give final assay concentrations of factor XIa active site and PIXI of 1.6 and 370 nM, respectively. The activity of factor XIa present in dilutions of these mixes was also measured, and the percent inhibition for each dilution was determined by comparison of the factor XIa/PIXI mix with the correspondingly diluted factor XIa/buffer control. Preincubation of factor XIa with PIXI was not required since it was previously noted that the level of factor XIa inhibition is achieved by PIXI within 10 s and is stable over at least 30 min. PIXI was shown to be a reversible inhibitor of factor XIa since the level of inhibition decreased with dilution of the enzyme/inhibitor (Table II), whereas an irreversible inhibitor would maintain a constant level of inhibition with dilution.

Table II: Effect of Dilution on Inhibition of Factor XIa by PIXI

dilution factor	enzyme act. (units $\times 10^{-3}$)[a] factor XIa	factor XIa + PIXI	% inhibn[b]
0	5.0	2.9	42
2	2.5	1.9	24
4	1.3	1.2	8
5	1.1	1.2	0

[a] Measured in the chromogenic assay described under Experimental Procedures with samples prepared as described under Results. [b] Calculated for each dilution as (activity of factor XIa + PIXI)/(activity of factor XIa) $\times$ 100.

Specificity of PIXI. To determine whether PIXI has inhibitory activity against serine proteases other than factor XIa, the purified inhibitor was incubated with trypsin, chymotrypsin, and a variety of coagulation and fibrinolytic enzymes, and the residual enzymatic activity was determined. Since it is difficult to obtain enough PIXI for K_i determinations of all these enzymes, the concentration of inhibitor protein (300 nM) employed in these experiments was that required to cause 50% inhibition of 1.6 nM factor XIa (active site concentration) in the chromogenic assay. This concentration was 3.4-fold higher than the concentration (88 nM) estimated to result from maximal secretion from platelets in whole blood. Thus, the experimental design is likely to yield physiologically relevant information. Chromogenic assays of residual enzymatic activity under these experimental conditions demonstrated no significant inhibition of thrombin, tissue plasminogen activator, factor Xa, or trypsin, whereas significant but minimal inhibitor of factor XIIa (7%), plasma kallikrein (5%), plasmin (5%), activated protein C (9%), and chymotrypsin (13%) was observed (Table III). Although a definitive analyis of the specificity of PIXI will require formal determinations of K_i for each enzyme tested using both chromogenic and normal macromolecular substrates, the data presented in Table III provide strong evidence of a high degree of specificity of PIXI as an inhibitor of factor XIa under physiologically relevant experimental conditions.

Comparison of PIXI with Known Platelet Proteins. Reported molecular weights of proteins known to be secreted by platelets show that the heparin-binding proteins (high- and low-affinity platelet factor 4, β-thromboglobulin, and platelet basic protein) are the only proteins with molecular weights near that of PIXI (Niewiarowski & Holt, 1987). When a small amount of inhibitor was mixed with 1 mL of heparin–agarose in a test tube under conditions which allow those proteins to bind heparin (low ionic strength buffer at pH 7.5), all of the factor XIa inhibitory activity remained in the supernatant above the settled resin. In an additional test, factor XIa activity was assayed in the presence of BioGel P10 purified PIXI with 0–44 units/mL USP-standardized porcine intestinal heparin. Factor XIa activities in all samples containing heparin and PIXI were 70% inhibited, whereas the sample of PIXI alone was 68% inhibited. These data, showing no binding of PIXI to heparin and no effect of heparin on the activity of

Table III: Effect of PIXI on the Activity of Serine Proteases

enzyme (1–2 nM)	substrate (1 mM)	% act.[a] ± SEM	significance[b] level
factor XIa	S2366 pyro-Glu-Pro-Arg-pNA	49 ± 100	$p < 0.00$
plasma kallikrein	S2302 H-D-Pro-Phe-Arg-pNA	95 ± 1.64	$p < 0.05$
factor XIIa	S2302 H-D-Pro-Phe-Arg-pNA	93 ± 0.69	$p < 0.025$
factor Xa	S2337 Bz-Ile-Glu-pip-Gly-Arg-pNA	102 ± 0.30	NS ($p = 0.080$)
thrombin	S2238 H-D-Phe-pip-Arg-pNA	105 ± 5.57	NS ($p = 0.458$)
plasmin	S2251 tosyl-Gly-Pro-Lys-pNA	95 ± 1.47	$p < 0.05$
tissue plasminogen activator	S2288 H-D-Ile-Pro-Arg-pNA	101 ± 6.21	NS ($p = 0.848$)
activated protein C	S2238 H-D-Phe-pip-Arg-pNA	91 ± 0.15	$p < 0.01$
chymotrypsin	S2586 succinyl-Ala-Ala-Pro-Phe-pNA	83 ± 3.72	$p < 0.025$
trypsin	S2238 H-D-Phe-pip-Arg-pNA	141 ± 5.33	$p < 0.005$

[a] The activity of each protease, measured in triplicate assays with chromogenic substrate in the presence of 300 nM PIXI, was compared with the activity of that protease measured in triplicate assays in the absence of PIXI, which represented 100% activity. The SEM for the 100% activity controls was 0.49. [b] Determined using the two-tailed Student's *t*-test

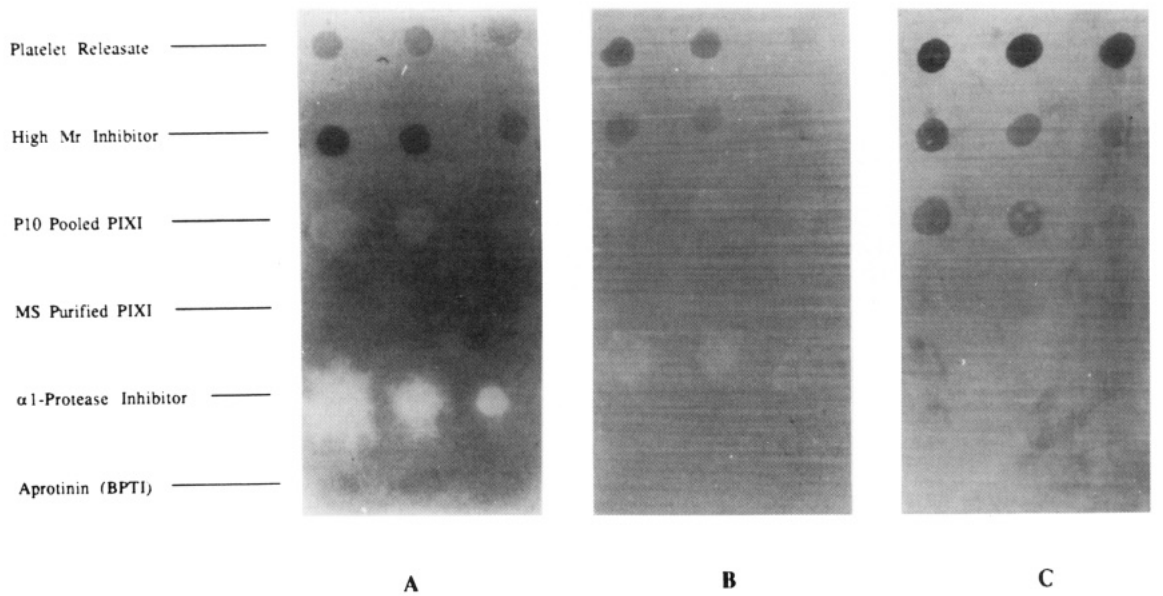

FIGURE 6: Immunoblots of PNII antibodies binding to factor XIa inhibitors. Samples of platelet releasate and high molecular weight inhibitor (PNII), BioGel P10 pool, and MonoS PIXI pool purified from platelet releasate were blotted at equal levels of factor XIa inhibition and then at serial 1:2 dilutions (to the right) onto Immobilon P membranes and compared with similarly blotted α_1-protease inhibitor and aprotinin (BPTI) for binding to antibodies made against (A) the Kunitz domain of PNII, (B) the amino terminus of PNII, and (C) the PNII molecule as described under Experimental Procedures.

PIXI, indicate that PIXI is not one of the heparin-binding proteins.

Because there are two separate sizes of inhibitors of factor XIa activity in platelet releasates, as shown by gel exclusion chromatography, it was necessary to determine if the later eluting inhibitor (PIXI) is a degradation fragment of the earlier eluting protein (PNII/APP). Since sequence analysis of PIXI has yielded no data, another approach to answering this question was applied. Protease nexin II/APP has a Kunitz-like inhibitory domain beginning around residue 288 which is inhibitory to proteases without the remainder of the molecule (Kitaguchi et al., 1990; Sinha et al., 1990). To determine if PIXI is a portion of PNII/APP, especially the Kunitz domain of PNII/APP, immunoblots were performed with PIXI and antibodies directed against the whole PNII/APP molecule, its amino terminus, and the Kunitz domain. The monoclonal antibodies used bound to platelet releasate and to the high molecular weight inhibitory fraction in a dose-dependent manner (Figure 6A,B), but they did not bind to samples of PIXI, suggesting that PIXI is not the Kunitz domain or amino-terminal portion of PNII/APP. The increased level of staining of the purified PNII over that of the releasate itself (Figure 6A) suggests that a protein other than PNII is responsible for a portion of the factor XIa inhibitory property. The Kunitz domain of PNII cannot be present in the PIXI preparation since this antibody is able to detect the PNII Kunitz domain on a dot blot at levels well below those which inhibit factor XIa activity (data not shown). Moreover, PIXI does not appear to be distantly genetically related to the Kunitz inhibitor (aprotinin or BPTI, bovine pancreatic trypsin inhibitor) since BPTI, which has 47% sequence homology with the Kunitz domain of PNII/APP (Ponte et al., 1988; Tanzi et al., 1988; Kitaguchi et al., 1988), reacted slightly with monoclonal antibody 7H5 and PIXI did not. These obser-

Table IV: Comparison of the Amino Acid Composition of PIXI with the Kunitz Domain of PNII/APP

amino acid	no. of residues		amino acid	no. of residues	
	PNII/APP[a]	PIXI		PNII/APP[a]	PIXI
Asx	5	5	Val	3	2
Glx	7	9	Met	2	1
Ser	3	12	Cys	6	0
Gly	7	9	Ile	1	2
His	0	3	Leu	0	3
Arg	3	3	Phe	4	3
Thr	3	7	Lys	1	4
Ala	5	4	Trp	1	ND[b]
Pro	2	2	total	56	71
Tyr	3	2			

[a] Calculated from the sequence (Kitaguchi et al., 1990). [b] Not determined.

Table V: Effect of Platelet Inhibitors on Complex Formation of Factor XIa Light Chain with α1PI

		addition			
	α1PI	none	releasate, 4.0×10^{-3} unit	PIXI, 6.4 $\times 10^{-4}$ unit	PNII, 1.8 $\times 10^{-3}$ unit
% of total cpm, recovered as free light chain	−	52	55	48	53
	+	16	34	36	46
% of light chain complexed with α1PI		69	27	25	13

vations are further supported by comparison of the amino acid compositions of the Kunitz domain of PNII/APP and purified PIXI, shown in Table IV. Although the PIXI sample appears to be a mixture of proteins of M_r 8500 and 5000, the total absence of cysteine residues, which are characteristic of Kunitz inhibitors (Laskowski & Kato, 1980) and present in the PNII Kunitz domain, is strong evidence that PIXI is not the Kunitz domain of PNII/APP.

The anti-PNII rabbit polyclonal antibody, which also recognized material in the crude releasate and the high molecular weight inhibitory fraction (Figure 6C), bound less well to the BioGel P10 PIXI pool but did not bind to purified PIXI or cross-react with aprotinin. These data show that the P10 pool contains protein which reacts with antibodies directed against PNII but which is distinct from PIXI. PIXI, then, does not appear to be any portion of the PNII molecule. Sequence analysis of PIXI has been attempted and may confirm this conclusion, but so far these efforts have been unsuccessful due to apparent blockage of the amino terminus.

Effects of Platelet Inhibitors on Factor XIa Complex Formation with α_1-Protease Inhibitor. The major plasma inhibitor of factor XIa is α1PI (Scott et al., 1982; Heck & Kaplan, 1974), which is a "suicide substrate" that forms a covalent product with the catalytic domain of factor XIa (Walsh et al., 1987). Previous studies have demonstrated that substances secreted from activated platelets can prevent complex formation between factor XIa and α1PI (Walsh et al., 1987). The capacity of PIXI and PNII to interfere with complex formation between factor XIa and α1PI was, therefore, examined. Less covalent product was formed between factor XIa light chain and α1PI in the presence of PIXI (Figure 7D compared to Figure 7A) in a manner similar to that seen with the crude releasate (Figure 7B) and with the high molecular weight inhibitor PNII (Figure 7C). The summary of the percent loss of free factor XIa light chain in each incubation mixture (Table V) shows that both factor XIa inhibitors in the platelet releasate can prevent covalent product formation.

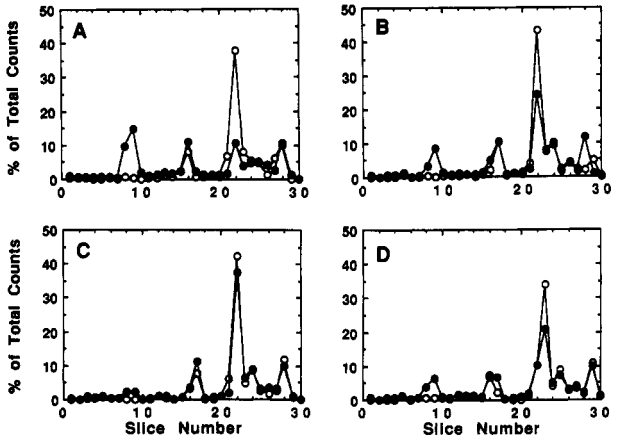

FIGURE 7: Effect of PIXI and PNII on complex formation between factor XIa light chain and α_1-protease inhibitor. ^{125}I-Factor XIa was incubated at 37 °C with (●) or without (○) α1PI and in the absence of platelet inhibitors (A) or the presence of (B) platelet releasate (4.0×10^{-3} unit of factor XIa inhibitory activity), (C) PNII (1.8×10^{-3} unit of factor XIa inhibitory activity), or (D) PIXI (6.4×10^{-4} unit of factor XIa inhibitory activity) as described under Experimental Procedures. Incubation mixtures were subjected to SDS–polyacrylamide gel electrophoresis, and gel slices were counted in a γ counter to locate the positions and determine the relative amounts of factor XIa light chain/α1PI complex, heavy chain, and free light chain. The percent of total counts in each gel slice is shown such that the cathode position is on the left and the anode position is on the right of each figure. The four peak positions seen from left to right, therefore, represent factor XIa light chain cross-linked to α1PI, factor XIa heavy chain (constant at 12 ± 2%), factor XIa light chain, and degraded ^{125}I-factor XIa at the end of the gel.

Discussion

The present work shows that platelets secrete PIXI which is distinct from other known inhibitors of factor XIa. Molecular size alone distinguishes it from α_1-protease inhibitor, C1-inhibitor, α_2-antiplasmin, and antithrombin III, as well as the cosecreted PNII. Although a fragmented Kunitz domain of PNII retains inhibitory activity (Kitaguchi et al., 1990; Sinha et al., 1990), as do Kunitz domains of inter-α-trypsin inhibitor (Hochstrasser & Wachter, 1983) and unfragmented Kunitz inhibitors which have been found in serum (Fioretti et al., 1987), PIXI was shown not to contain cysteines, six of which are present in all these inhibitors and are necessary for their inhibitory structure. Since PIXI was not found in plasma, it appears to be a platelet-specific protein, yet it does not share the anti-heparin property of other platelet-specific proteins of similar size. A definitive structural description of PIXI will require results from amino acid sequence analysis which will also establish its purity.

The role of PIXI in regulation of factor XIa activity must be examined due to the 10-fold higher K_i for PIXI compared to that of PNII with factor XIa and the chromogenic substrate S2366. Slow, tight-binding inhibition was demonstrated with PNII (Smith et al., 1990), which appeared to be competitive (W. E. Van Nostrand, personal communication), whereas PIXI inhibition appears to be noncompetitive. The differences in the kinetic mechanism of inhibition displayed by these inhibitors indicate separate interaction sites with factor XIa, suggesting different, and possibly complementary, roles for the two inhibitors. It is possible that PIXI, with a more rapid diffusion constant due to its lower molecular weight, may function at the site of platelet thrombus formation while the slower-binding, heparin-influenced PNII may be more important for factor XIa regulation at cell surfaces distant from the site of coagulation.

The data presented in this paper indicate that PIXI is a

highly specific inhibitor of factor XIa, since under the experimental conditions employed that are likely to yield physiologically relevant information, minimal or no significant inhibition of plasma serine proteases other than factor XIa was observed. Protease nexin II is also secreted from platelet granules and is a potent inhibitor [K_i = (2.9–4.5) × 10^{-10} M] of factor XIa (Smith et al., 1990; Van Nostrand et al., 1990b). Although PNII has some inhibitory activity (K_i = 2.9 × 10^{-8} M) against plasmin (Van Nostrand et al., 1990b), it was also shown not to be an inhibitor of the other serine proteases of coagulation and fibrinolysis. Several of the plasma inhibitors of factor XIa are present in platelets and can be secreted during platelet activation. These are the serpins α1PI (Nachman & Harpel, 1976; Bagdasarian & Colman, 1978), C1-inhibitor (Schmaier et al., 1985), α_2-antiplasmin (Plow & Collen, 1981), and plasminogen activator inhibitor 1 (Kruithof et al., 1986). Although all these serpins have been documented to inhibit factor XIa, they are unlikely to play a significant role in the regulation of factor XIa activity for several reasons. First, they are all present in platelet granules in concentrations unlikely to contribute to factor XIa inhibition when secreted into plasma where they are already present in vast molar excess relative to the concentration achievable upon platelet secretion alone. Second, the concentrations of these proteins required to produce significant inhibition of factor XIa are many orders of magnitude higher than those achievable in releasates of activated platelets. Third, gel filtration profiles of platelet releasates contain only two peaks of factor XIa inhibitory activity, one of which is entirely attributable to PIXI (18–54%), whereas the remainder (46–82%) is entirely attributable to PNII. These considerations suggest that the only two platelet inhibitors likely to have a role in the regulation of factor XIa are PIXI and PNII.

The K_i of 3.8 nM determined for inhibition by PIXI of factor XIa-catalyzed factor IX activation is about 100-fold lower than the K_m for factor IX (260–490 nM). This indicates that factor XIa is likely to be preferentially inhibited in the presence of both factor IX and PIXI, since the concentration (88 nM) of factor IX in plasma (Osterud et al., 1978) is equivalent to the maximal concentration (88 nM) of PIXI after secretion by platelets into plasma. Moreover, the maximum concentration of secreted PIXI is ~25-fold higher than the K_i for factor IX activation, suggesting the inhibitor is active under physiological conditions. By comparison, the amount of PNII present in platelet lysates is ~320 ng/10^8 platelets (Van Nostrand et al., 1990a). About 50% of the PNII is secreted (Van Nostrand et al., 1990a), yielding a concentration in the releasate of ~480 ng/mL or 3.7 nM, which is ~10-fold higher than its K_i (0.29–0.45 nM) for factor XIa inhibition (Smith et al., 1990; Van Nostrand et al., 1990b). It is, therefore, likely that factor XIa located near the newly stimulated platelets of the forming platelet plug would be inhibited by PIXI and/or PNII. As noted earlier, the diffusion coefficient of PIXI should be more rapid than that of PNII due to its lower molecular weight, so PIXI may be the major inhibitor at the site of thrombus formation. In addition, PNII is a slow, tight-binding inhibitor of factor XIa (Smith et al., 1990) whose K_i for factor XIa is decreased to 25–55 pM in the presence of heparin (Smith et al., 1990; Van Nostrand et al., 1990b). Thus, PNII may have a greater role at the surface of cells other than platelets, such as endothelial cells which produce heparin-like, anticoagulant molecules on their surface (Marcum et al., 1983; Marcum & Rosenberg, 1985), not only as an inhibitor of coagulation but also as a stimulator of tissue repair, since PNII has also been shown to have growth factor activity (Schubert et al., 1989; Saitoh et al., 1989).

The kinetic analysis of factor XIa inhibition using factor IX as the substrate demonstrated a linear, mixed-type, noncompetitive mechanism with a K_i of 3.8 nM. By comparison, when a chromogenic substrate was used, the apparent K_i was 70 nM. This difference in K_i's is not surprising due to the major differences in molecular weights and K_m's for these two substrates (FIX, M_r 57 000 and K_m 0.26 μM; S2366, M_r 539 and K_m 0.25 mM). Although the mechanistic reason for this difference is not elucidated here, several important points emerge from the analysis. First, PIXI is a reversible factor XIa inhibitor, in agreement with previous observations (Soons et al., 1986; Walsh et al., 1987) and in contrast to protease inhibitors of the serpin class (Carrell et al., 1987). Moreover, the site to which PIXI binds in factor XIa is unlikely to be solely the catalytic site, since such binding would be expected to produce a simple noncompetitive mechanism of inhibition (Segel, 1975; Sinha et al., 1987). Thus, previous studies have shown that a monoclonal antibody that binds to the catalytic domain of factor XIa inhibits factor IX activation noncompetitively (Sinha et al., 1987), whereas another monoclonal antibody that binds to a heavy-chain domain in factor XI inhibits factor XIa-catalyzed factor IX activation competitively and recognizes a substrate (factor IX) binding site (Sinha et al., 1987). Therefore, it follows that since PIXI displays a mixed-type, noncompetitive mechanism of inhibition of factor IX activation and since it also inhibits factor XIa amidolytic activity, it is unlikely to bind exclusively to the substrate (factor IX) binding site in the heavy chain of factor XIa or exclusively to the catalytic site. Further studies will clearly be required to elucidate the mechanism by which PIXI inhibits factor XIa.

Studies performed to date have measured the effect of PIXI and PNII on factor XIa free in solution. The significance of these studies for the regulation of intrinsic coagulation will require a detailed understanding of the complex interactions of factor XI and factor XIa with the proteins involved in its activation (high molecular weight kininogen and factor XIIa) and the expression of its activity (factor IX), with surfaces such as the platelet membrane and with the two factor XIa inhibitors released from platelets, PIXI and PNII. Platelets activated with ADP, collagen, or thrombin have previously been shown to have specific, high-affinity binding sites for high molecular weight kininogen (Greengard & Griffin, 1984), for factor XI (Greengard et al., 1986), and for factor XIa (Sinha et al., 1984) and to promote the activation of factor XI by factor XIIa (Walsh & Griffin, 1981). Factor IX activation also occurs on the platelet membrane, where factor XIa can be protected from complex formation with and inactivation by α1PI (Walsh et al., 1987), the major plasma inhibitor of factor XIa. Since both PIXI and PNII protect factor XIa from complex formation with α1PI (Figure 7), it is reasonable to conclude that these inhibitors can successfully compete with α1PI for binding to factor XIa. Since factor XIa also binds tightly to activated platelets, and since activated platelets release PIXI and PNII, it is possible that platelet-bound factor XIa is also protected from inactivation by PIXI and by PNII, thereby promoting the activation of factor IX preferentially on the platelet surface where factor IXa binding then promotes factor X activation (Rawala-Sheikh et al., 1990).

ACKNOWLEDGMENTS

We are grateful to Dr. Barrie Ashby for many helpful discussions, to Frances S. Seaman for preparation of factor XI, and to Patricia Pileggi for expert secretarial assistance.

Registry No. Factor XIa, 37203-61-5; factor XIIa, 37203-62-6; kallikrein, 9001-01-8; plasmin, 9001-90-5; activated protein C,

42617-41-4; factor IX, 9001-28-9; thrombin, 62046-50-8.

References

Bagdasarian, A., & Colman, R. W. (1978) *Blood 51*, 139–156.

Baglia, F. A., Sinha, D., & Walsh, P. N. (1989) *Blood 74*, 244–251.

Baglia, F. A., Jameson, B. A., & Walsh, P. N. (1990) *J. Biol. Chem. 265*, 4149–4154.

Basch, J. J., Douglas, F. W., Jr., Procino, L. G., Holsinger, V. H., & Farrell, H. M., Jr. (1985) *J. Dairy Sci. 68*, 23–31.

Bouma, B. N., & Griffin, J. H. (1977) *J. Biol. Chem. 252*, 6432–6437.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Bush, A. I., Martins, R. N., Rumble, B., Moir, R., Fuller, S., Milward, E., Currie, J., Ames, D., Weidemann, A., Fischer, P., Multhaup, G., Beyreuther, K., & Masters, C. L. (1990) *J. Biol. Chem. 265*, 15977–15983.

Carrell, R. W., Christy, P. B., & Boswell, D. R. (1987) in *International Society of Thrombosis & Haemostasis XIth Congress*, pp 1–15, Leuven University Press, Leuven, Belgium.

DiScipio, R. G., Hermondson, M. A., Yates, S. G., & Davie, E. W. (1977) *Biochemistry 16*, 698–706.

Fioretti, E., Angeletti, M., Citro, G., Barra, D., & Ascoli, F. (1987) *J. Biol. Chem. 262*, 3586–3589.

Fraker, P. J., & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.

Greengard, J. S., & Griffin, J. H. (1984) *Biochemistry 23*, 6863–6869.

Greengard, J. S., Heeb, M. J., Ersdal, E., Walsh, P. N., & Griffin, J. H. (1986) *Biochemistry 25*, 3884–3890.

Heck, L. W., & Kaplan, A. P. (1974) *J. Exp. Med. 140*, 1615–1630.

Hochstrasser, K., & Wachter, E. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1679–1687.

Hunt, L. T., & Dayhoff, M. O. (1980) *Biochem. Biophys. Res. Commun. 95*, 864–971.

Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., & Ito, H. (1988) *Nature 331*, 530–532.

Kitaguchi, N., Takahashi, Y., Oishi, K., Shiojiri, S., Tokushima, Y., Utsunomiya, T., & Ito, H. (1990) *Biochim. Biophys. Acta 1038*, 105–113.

Kruithof, E. K., Tran-Thang, C., & Bachmann, F. (1986) *Thromb. Haemostasis 55*, 201–205.

Kurachi, K., & Davie, E. W. (1977) *Biochemistry 16*, 5831–5839.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593–626.

Mannhalter, C., & Schiffman, S. (1980) *Thromb. Haemostasis 43*, 124–126.

Marcum, J. A., & Rosenberg, R. D. (1985) *Biochem. Biophys. Res. Commun. 126*, 365–372.

Marcum, J. A., Fritze, L., Galli, S. J., Karp, G., & Rosenberg, R. D. (1983) *Am. J. Physiol. 245*, H725–H733.

Miletech, J. P., Broze, G. J., Jr., & Majerus, P. W. (1980) *Anal. Biochem. 105*, 304–310.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.

Mustard, J. F., Perry, D. W., Ardlie, N. G., & Packham, M. A. (1972) *Br. J. Haematol. 22*, 193–204.

Nachman, R. L., & Harpel, P. C. (1976) *J. Biol. Chem. 251*, 4514–4521.

Niewiarowski, S., & Holt, J. C. (1987) in *Hemostasis & Thrombosis: Basic Principles & Clinical Practice* (Colman, R. W., Hirsch, J., Marder, V. J., & Salzman, E. W., Eds.) 2nd ed., pp 618–630, J. B. Lippincott, Philadelphia.

Osterud, B., Bouma, B. N., & Griffin, J. H. (1978) *J. Biol. Chem. 253*, 5946–5951.

Plow, E. F., & Collen, D. (1981) *Blood 58*, 1069–1074.

Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., & Cordell, B. (1988) *Nature 331*, 525–527.

Procter, R. R., & Rapaport, S. J. (1961) *Am. J. Clin. Pathol. 36*, 212–219.

Ragni, M. V., Sinha, D., Seaman, F., Lewis, J. H., Spero, J. A., & Walsh, P. N. (1985) *Blood 65*, 719–724.

Rawala-Sheikh, R., Ahmad, S. S., Ashby, B., & Walsh, P. N. (1990) *Biochemistry 29*, 2606–2611.

Rosenthal, R. L., Dreskin, O. H., & Rosenthal, N. (1953) *Proc. Soc. Exp. Biol. Med. 82*, 171–174.

Rucinski, B., Niewiarowski, S., James, P., Walz, D. A., & Budzynski, A. Z. (1979) *Blood 53*, 47–62.

Saitoh, T., Sundsmo, M., Roch, J.-M., Kimura, N., Cole, G., Schubert, D., Ottersdorf, T., & Schenk, D. B. (1989) *Cell 58*, 615–622.

Schmaier, A. H., Smith, P. M., & Colman, R. W. (1985) *J. Clin. Invest. 75*, 242–250.

Schubert, D., Cole, G., Saitoh, T., & Oltersdorf, T. (1989) *Biochem. Biophys. Res. Commun. 162*, 83–88.

Scott, C. F., Scapira, M., James, H. L., Cohen, A. B., & Colman, R. W. (1982) *J. Clin. Invest. 69*, 844–852.

Scott, C. F., Sinha, D., Seaman, F. S., Koshy, A., & Walsh, P. N. (1984) *Blood 63*, 42–50.

Segel, I. H. (1975) in *Enzyme Kinetics: Behavior & Analysis of Rapid Equilibrium & Steady-State Enzyme Systems*, pp 125–135, John Wiley & Sons, New York.

Sinha, D., Seaman, F. S., Koshy, A., & Walsh, P. N. (1984) *J. Clin. Invest. 73*, 1550–1556.

Sinha, D., Koshy, A., Seaman, F. S., & Walsh, P. N. (1985) *J. Biol. Chem. 260*, 10714–10719.

Sinha, D., Seaman, F. S., & Walsh, P. N. (1987) *Biochemistry 26*, 3768–3775.

Sinha, S., Dovey, H. F., Seubert, P., Ward, P. J., Blacker, P. W., Blaber, M., Bradshaw, R. A., Arici, M., Mobley, W. C., & Lieberburg, I. (1990) *J. Biol. Chem. 265*, 8983–8985.

Smith, R. P., Higuchi, D. A., & Broze, G. J., Jr. (1990) *Science 248*, 1126–1128.

Soons, H., Janssen-Claissen, T., Henaker, H. C., & Tans, G. (1986) *Blood 68*, 140–148.

Spencer, R. L., & Wold, F. (1969) *Anal. Biochem. 32*, 185–190.

Tanzi, R. E., McClatchey, A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F., & Neve, R. L. (1988) *Nature 331*, 528–530.

van der Graaf, F., Greengard, J. S., Bouma, B. N., Keribiriou, D. M., & Griffin, J. H. (1983) *J. Biol. Chem. 258*, 9669–9675.

Van Lenten, L., & Ashwell, G. (1971) *J. Biol. Chem. 246*, 1889–1894.

Van Nostrand, W. E., Schmaier, A. H., Farrow, J. S., & Cunningham, D. D. (1990a) *Science 248*, 745–748.

Van Nostrand, W. E., Wagner, S. L., Farrow, J. S., & Cunningham, D. D. (1990b) *J. Biol. Chem. 265*, 9591–9594.

Walsh, P. N. (1972) *Br. J. Haematol. 22*, 237–254.

Walsh, P. N., & Griffin, J. H. (1981) *Blood 57*, 106–118.

Walsh, P. N., Bradford, H., Sinha, D., Piperno, J. R., & Tuszynski, G. P. (1984) *J. Clin. Invest. 73*, 1392–1399.

Walsh, P. N., Sinha, D., Kueppers, F., Seaman, F. S., & Blankstein, K. B. (1987) *J. Clin. Invest. 80*, 1578–1586.