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Research Article

Previous studies have demonstrated the binding of Factors IX and IXa to cultured bovine aortic endothelial cells. The present study examines the interaction of Factors IX, IXa, and Xa with the luminal surface of calf aortas, shown by microscopic examination to have a continuous layer of endothelium. Radioimmunoassay of Factor IX showed that 74 fmol/10(6) cells of Factor IX could be eluted from freshly prepared aortic segments. Binding of 3H-Factors IX and IXa to aortic segments was saturable, and comparable to binding in previous studies using cultured endothelial cells. Preincubation of aortic segments with 3H-Factor IXa and von Willebrand factor (VWF)/Factor VIII, followed by washing and addition of Factor X, resulted in formation of Factor Xa. The addition of prothrombin to these activation mixtures resulted in formation of thrombin. Exogenous phospholipid and Factor V were not required for Factor X and prothrombin activation on the intact native endothelium. Incubation of 125I-Factor Xa with the vessel segments resulted in most of the tracer being complexed with antithrombin III originally present on the aortic segment (3.8 pmol antithrombin III/10(6) cells). The Factor Xa-antithrombin III complex was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis exclusively in the supernatants. 125I-Factor Xa not complexed with antithrombin III bound specifically to the vessel segment. The time course of binding was biphasic, consisting of an initial more […]

A Coagulation Pathway on Bovine Aortic Segments Leading to Generation of Factor X_a and Thrombin

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Abstract. Previous studies have demonstrated the binding of Factors IX and IX_a to cultured bovine aortic endothelial cells. The present study examines the interaction of Factors IX, IX_a, and X_a with the luminal surface of calf aortas, shown by microscopic examination to have a continuous layer of endothelium. Radioimmunoassay of Factor IX showed that 74 fmol/10^6 cells of Factor IX could be eluted from freshly prepared aortic segments. Binding of ^{3}H-Factors IX and IX_a to aortic segments was saturable, and comparable to binding in previous studies using cultured endothelial cells. Preincubation of aortic segments with ^{3}H-Factor IX_a and von Willebrand factor (VWF)/Factor VIII, followed by washing and addition of Factor X, resulted in formation of Factor X_a. The addition of prothrombin to these activation mixtures resulted in formation of thrombin. Exogenous phospholipid and Factor V were not required for Factor X and prothrombin activation on the intact native endothelium.

Incubation of ^{125}I-Factor X_a with the vessel segments resulted in most of the tracer being complexed with antithrombin III originally present on the aortic segment (3.8 pmol antithrombin III/10^6 cells). The Factor X_a-antithrombin III complex was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis exclusively in the supernatants. ^{125}I-Factor X_a not complexed with antithrombin III bound specifically to the vessel segment. The time course of binding was biphasic, consisting of an initial more rapid reversible phase followed by a slower irreversible phase. The latter phase correlated with the formation of a covalent complex (M_r, 76,000) between ^{125}I-Factor X_a and a vessel-localized protein presumably distinct from antithrombin III. The activation of prothrombin by vessel-bound Factor X_a was inhibited by anti-bovine Factor V IgG, suggesting that there is interaction of Factor X_a with a Factor V-like molecule provided by the endothelial cell surface. Addition of antibody to antithrombin III prevented formation of Factor X_a-antithrombin III and thrombin-antithrombin III complexes in the supernatant and increased apparent thrombin activity 30–50-fold. These studies demonstrate that freshly obtained vessels with a continuous layer of native endothelium can support activation of Factor X and prothrombin: vessel-bound Factor IX_a can activate Factor X in the presence of VWF/Factor VIII. Factor X_a can also bind to the vessel and participate in the activation of prothrombin. The apparent efficiency of prothrombin activation, however, is dampened by the presence of functional antithrombin III on the vessel wall.

Introduction

Localized blood coagulation is important in both hemostasis and thrombosis. Endothelial cells inhibit thrombosis by synthesis of prostacyclin (1), plasminogen activator (2), and thrombomodulin (3), and by the presence of antithrombin III cofactor activity (4); they have the potential to support coagulation by generation of tissue factor (5), von Willebrand factor (VWF)[1] (6), and thromboxane (7). Recently, there has

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1. *Abbreviations used in this paper:* buffer A, 10 mM Hepes (pH 7.45) containing 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM $CaCl_2$, and 5 mg/ml bovine serum albumin, fatty acid free; buffer B, buffer A with 10 mM EDTA in place of $CaCl_2$; buffer C, 50 mM Tris-

been increased interest in clot-promoting activities of endothelium, and we and other groups reported that Factors IX, IX_a, X, and X_a bind to bovine aortic endothelial cells in culture (8–13). Cell-bound Factors IX and IX_a retain their coagulant properties: cell-bound Factor IX can be activated by either the intrinsic or extrinsic pathways, and cell-bound Factor IX_a can participate in Factor X activation (10). Factor X_a incubated with nonconfluent cultured endothelial cells can activate prothrombin (14). Conclusions from these studies rely on the assumption that cultured endothelial cells are a model for native endothelium. In the present study we have examined the interaction of Factors IX, IX_a, and X_a with intact native bovine aortic endothelium to determine whether these factors could bind specifically to the surface and whether the bound factors could lead to generation of thrombin. The results of the present study indicate that the native endothelium can support the activation of Factor X and prothrombin. These reactions are influenced by the presence of antithrombin III on the vessel wall.

Methods

Coagulation factors. Bovine coagulation factors were used throughout these studies. Bovine Factor IX was purified to homogeneity (260 U/mg) by the method of Fujikawa et al. (5). Factor IX was tritiated (3.26×10^6 cpm/μg) as described previously (9) and its coagulant activity was unaffected by the radiolabeling procedure. Tritiated Factor IX was activated by incubation with Factor XI_a bound to CNBr-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at 37°C in Tris-buffered saline (0.05 M, Tris-HCl, pH 7.5, 0.1 M NaCl) containing 5 mM $CaCl_2$ as described previously (9). A radioimmunoassay was developed to quantify the amount of Factor IX eluted from vessel segments. For the radioimmunoassay Factor IX was iodinated to achieve a higher specific radioactivity. Iodination was carried out by the lactoperoxidase method (16) using the Enzymobead Reagent (Bio-Rad Laboratories, Sacramento, CA). After the radioiodination procedure, Factor IX retained full coagulation activity, migrated as a single chain on reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a molecular weight of 55,000, and had a specific radioactivity of $0.8–1.2 \times 10^4$ cpm/ng.

Bovine Factor X_1, purified to homogeneity by the method of Fujikawa et al. (17), was activated by incubation with the Factor X activator from Russell's viper venom (18) previously coupled to CNBr-Sepharose in Tris-buffered saline containing 5mM $CaCl_2$ (19). Activation reached a plateau by 10 min as judged by coagulation assay (100 U/mg) performed by the method of Bajaj and Mann (20), and SDS-PAGE, which showed complete cleavage of the zymogen. Active site-blocked Factor X_a (Factor X_{ai}) was prepared by reacting Factor X_a in 5 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.0, 0.1 M NaCl for 60 min with a 10-fold molar excess of (*p*-amidinophenyl)-methanesulfonyl-fluoride (Calbiochem-Behring Corp., San Diego, CA) as described by Laura et al. (21). Unreacted inhibitor was removed by dialysis at 4°C against Tris-buffered saline. Factor X_{ai} had no detectable activity in a clotting assay and did not hydrolyze Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S2222) (Helena Laboratories, Beaumont, TX) in the Factor X_a chromogenic substrate assay (see below). Factors X_a and X_{ai} were iodinated by the lactoperoxidase method as described for Factor IX. After the iodination procedure Factor X_a retained coagulant activity and had a specific radioactivity of $0.6–1.3 \times 10^4$ cpm/ng. ^{125}I-Factor IX_{ai} had a specific radioactivity of $0.7–1.3 \times 10^4$ cpm/ng. The radioactivity profile of reduced ^{125}I-Factor X_a (Fig. 5 *A*) showed that the heavy chain, light chain, and activation peptide had been labeled. Although immediately after activation Factor X_a was predominantly in the α-form, by the time it was used in these studies Factor X_a was principally in the β-form (19). ^{125}I-Factors X_a and X_{ai} appeared identical by SDS-PAGE. SDS-PAGE of ^{125}I-Factor X_a after gel filtration on Sephadex G75 in Tris-buffered saline demonstrated noncovalent association of the activation peptide with Factor X_a as previously reported by Furie et al. (22).

Bovine factor VWF/Factor VIII was prepared by the method of Newman et al. (23). It was then chromatographed on an antifibrinogen and antifibronectin affinity column. The final material was homogenous on reduced 5% SDS-PAGE, showing one band with a molecular weight about 200,000 corresponding to the VWF. The VWF/Factor VIII preparations used in this work had a protein concentration of 1.1 mg/ml and a Factor VIII coagulant activity of 70 U/ml. Factor VIII coagulant activity in this preparation was stable over 6 mo of storage at −80°C, and no other coagulant activities (including Factors VII, IX, X, XI, and XII) were detected by clotting assay. VWF/Factor VIII (10 U/ml) was activated by incubation with bovine α-thrombin (0.01 U/ml) in Tris-buffered saline for 3 min and was used immediately (10, 24). Bovine α-thrombin (2.5 NIH U/μg), prothrombin (13 U/mg), and Factor VII (7,100 U/mg) were purified to homogeneity as described previously (25–27). Thrombin was iodinated by the lactoperoxidase technique (see above); the radiolabeled protein had a specific radioactivity of $4–8 \times 10^3$ cpm/ng and the coagulant activity was unaffected by radiolabeling. Factor VII, radioiodinated by the method of Fraker and Speck (28) as modified for Factor VII by Broze (29), had a specific radioactivity of $2–4 \times 10^3$ cpm/ng and was homogeneous on SDS-PAGE, migrating as a single band of radioactivity at a molecular weight of 54,000.

Bovine antithrombin III was prepared according to Mahoney et al. (30) and was homogeneous as judged by SDS-PAGE. Radiolabeling of antithrombin III was accomplished by the lactoperoxidase method as described for Factor IX and the specific radioactivity achieved was $0.5–1.1 \times 10^4$ cpm/ng. Iodinated antithrombin III comigrated with unlabeled material on SDS-PAGE.

Monospecific antisera against Factor IX, Factor VII, and antithrombin III were raised in rabbits by standard methods (31). IgG from rabbit serum was purified by use of protein A-Sepharose CL-6B (Pharmacia Fine Chemicals) according to the manufacturer's recommendations. Burro anti-bovine Factor V IgG (32) and normal burro serum were generously provided by Drs. P. Tracy and K. Mann (Mayo Clinic, Rochester, MN). Normal burro IgG was prepared from nonimmune serum as described (31). Purified IgG preparations had no detectable Factor XI, VII, IX, or X, or thrombin activity by clotting assay. Protein concentrations were determined colorimetrically (33). The concentrations of activated factors were also determined by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (34). Clotting assays were done as described previously (35) and calcium concentrations were determined with murexide (36). SDS-PAGE was carried out as

HCl (pH 7.8), 175 mM NaCl, 10 mM, EDTA, 0.5 mg/ml ovalbumin; Factor X_{ai}, active site-blocked Factor X_a; FAF, fatty acid free; PAGE, polyacrylamide gel electrophoresis; S-2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide; VWF, von Willebrand factor.

described previously (9) except that samples of vessel bound ^{125}I-Factors X_a and X_{ai} were prepared as follows: vessel segments with bound tracer were incubated for 20 min at 4°C with Tris-buffered saline containing 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), 10 mM EDTA, 0.5 mM phenylmethyl sulfonyl fluoride, 2 mM diisopropyl fluorophosphate. The vessel eluate was then prepared for SDS-PAGE as described previously. Electrophoresis standards included calibration proteins obtained from Pharmacia Fine Chemicals and complexes of ^{125}I-Factor X_a or ^{125}I-thrombin with antithrombin III (37, 38) prepared by incubation of ^{125}I-Factor X_a (5 nM) or ^{125}I-thrombin (5 nM) with antithrombin III (10 nM) and heparin (1 U/ml) in a final volume of 25 μl for 3 min.

Bovine aortas. Thoracic aortas, a generous gift of the Great American Veal Co. (Newark, NJ), were obtained from 1-yr-old calves within 5 min postmortem. Killing and exsanguination of the animals occurred simultaneously. Experiments were performed either immediately or after transport of the aortas to the laboratory (about 40 min later). During transport, aortas were stored at 21°C immersed in Hank's balanced salt solution (Gibco Laboratories, Gibco Div., Grand Island, NY) containing 25 mg/ml bovine serum albumin, fatty acid free (BSA-FAF). For binding and activation studies the surrounding fat was removed and aortas were cut longitudinally between the intercostal vessels into 4.5 × 21 cm segments, spread flat, and placed within a Lucite template. The Lucite template consisted of two sheets of half-inch-thick Lucite. The lower sheet was solid, and the upper sheet was prepared by drilling 0.79, 3.8, or 10 cm^2 holes at regular intervals. Aortic segments were placed on the lower sheet and covered with the upper sheet. The sheets fit closely together so that wells were formed by the holes in the upper sheet. These wells did not leak during the incubation period and >98% of the added counts could be recovered. Control experiments were carried out in natural depressions in the vessel formed by resting the aorta on top of the upper sheet of the Lucite template; natural depressions of roughly the same volume as those generated by the 3.8 cm^2 well area were selected. Other experiments were performed directly on lucite sheets without aortas to exclude possible artifacts due to lucite-coagulation factor interaction. Some aortas were treated with collagenase (0.1%, CLSII; Worthington Biochemical Corp., Freehold, NJ) in Dulbecco's phosphate buffered saline by incubating the luminal surface of the vessel with the enzyme preparation for 10 min at 37°C. Soybean trypsin inhibitor (0.5 mg/ml; Sigma Chemical Co.) was added for 5 min at 21°C to neutralize tryptic activity in the collagenese preparation, and aortas were washed extensively with 10 ml of buffer A (10 mM Hepes [pH 7.45] containing 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM $CaCl_2$, and 5 mg/ml BSA-FAF). Binding and activation studies were then carried out as described below.

To determine if Factors IX and VII could be eluted from the aortas, the intercostal vessels and distal part of the aortic segment were clamped off, and the aorta was washed seven times at 21°C with buffer A. Aortas were then incubated with 5 ml buffer B (which is buffer A with 10 mM EDTA in place of $CaCl_2$) for 1 min. After incubation with buffer B the vessel was washed again with buffer B. Where indicated, EDTA eluates were concentrated by ultrafiltration using a PM10 membrane (Amicon Corp., Lexington, MA). The amount of vessel-associated antithrombin III was determined similarly by first washing the vessel with buffer A, and subsequently eluting the antithrombin III with Tris-buffered saline containing 1% Nonidet P-40 for 30 min.

Morphological study of the vessels was carried out by scanning electron microscopy using standard procedures (39).

Binding and activation studies on aortic segments. Binding studies were carried out at 21°C. After the aorta was opened and placed beneath the template, each well was washed five times with buffer A without calcium, incubated with buffer B for 30 s, and finally washed twice with buffer A in order to remove plasma and Factor IX bound in vivo from the endothelium. The integrity of the endothelium was assessed microscopically (see below). Next, 1 ml buffer A was added to each 3.8 cm^2 well, and 0.5 ml was added to each 0.79 cm^2 well. Tracer and other components of the reaction mixture, each in a volume of 10–25 μl, were added, and aortas were incubated at 21°C for the indicated time on an orbital shaker at 30 oscillations/min. At the end of the incubation period, wells were washed five times with 1 ml of buffer A for a total of 10 s per well. Bound ^{3}H-Factors IX/IX_a or ^{125}I-Factors X_a/X_{ai} were eluted by the addition of 0.5 ml of 0.2 M NaOH/1% SDS/0.01 M EDTA for 5 min. Where indicated, after the initial washing of the vessel segment, wells were pre-incubated for 30–40 min at 21°C with either anti-antithrombin III, anti-Factor V, or nonimmune IgG before the addition of purified proteins. Neither nonimmune rabbit IgG nor nonimmune burro IgG affected ^{125}I-Factor X_a binding. Dissociation of bound ^{125}I-Factors X_a and X_{ai} was measured as described by Lollar et al. (40): after the aortas were incubated with ^{125}I-Factor X_a and the vessel was washed free of unbound ligand, buffer A (0.5 ml/well) was added. At the specified times, buffer A was removed, the wells were washed, and more buffer A (0.5 ml) was added. Then the wells were washed with buffer A again and the cells solubilized as described above. Activation studies were carried out at 21°C in wells (0.79 cm^2) prepared with buffers A and B as for binding studies. To study activation of Factor X and prothrombin by Factors IX_a and VWF/Factor VIII, ^{3}H-Factor IX_a (1–5 nM) and VWF/Factor VIII (2 U/ml) in 0.5 ml of buffer A were equilibrated with the aorta for 30 min. The wells were then washed five times with buffer A (0.5 ml/wash) and finally filled with 0.5 ml of buffer A. Factor X (0.21 μM) with or without prothrombin (1.4 μM) was then added to the well. Thrombin (0.1 nM) was added to effect rapid activation of VWF/Factor VIII. When prothrombin activation by Factor X_a was studied, wells were incubated with ^{125}I-Factor X_a (3–60 nM), washed, and then prothrombin (1.4 μM) was added. In the experiments using antibodies, either nonimmune burro or rabbit IgG, or anti-Factor V IgG or anti-antithrombin III IgG was added to 0.5 ml of buffer A and equilibrated with the aortic wells for 40 min at 21°C before the addition of clotting proteins. Samples for Factor X_a and thrombin assay were obtained by the addition of 0.5 ml buffer C (50 mM Tris-HCl [pH 7.8], 175 mM NaCl, 10 mM EDTA, 0.5 mg/ml ovalbumin) to each well and then withdrawing 0.2 ml for assay. Where specified, 0.1-ml aliquots were withdrawn from reaction wells and placed in glass tubes. Buffer C (0.2 ml) was then added either immediately or after 10–20 min. Samples were assayed for Factor X_a by monitoring hydrolysis of the chromogenic peptide, S-2222 (24). Thrombin was assayed (41) by the same protocol as for Factor X_a, with H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238) (Helena Laboratories) at 0.1 mM final concentration. Factor X_a inactivation studies were carried out with the S2222 assay by the method of Odegard et al. (42). Control wells incubated with 0.1 nM of thrombin (as used for activation of VWF/Factor VIII) or with buffer alone showed no significant hydrolysis of either synthetic substrate under these conditions. The amounts of Factor X_a and thrombin formed were determined from the linear portion of a standard curve in which known amounts of Factor X_a or thrombin were assayed under conditions identical to the experimental samples. Although qualitatively the results were similar in each binding and activation experiment and yielded similar dose-response relationships, the base line varied by as much as

two- to threefold from one experiment to another. Accordingly, the figures present results from representative experiments.

Radioimmunoassays for Factor IX, Factor VII, and antithrombin III. Radioimmunoassay for Factor IX antigen was carried out using a specific rabbit antiserum for bovine Factor IX generously provided by Dr. K. Fujikawa (University of Washington, Seattle, WA). The assay was carried out by the method of Suzuki and Thompson (43). The limit of detection in this assay was 1.1 nM Factor IX antigen, which corresponded to 80% binding on the standard curve. This assay did not distinguish between Factor IX and Factor IX_a. The assay for Factor VII was carried out by the same protocol described for Factor IX, using a monospecific rabbit anti-bovine Factor VII antiserum. The limit of detection was 2 nM. The assay for antithrombin III using a monospecific rabbit antibody was also carried out by the same protocol except that all tubes (including the standards) had a final concentration of 0.5% Nonidet P-40. The limit of detection in this assay was 1 nM.

Results

Microscopic study of calf aortas. A continuous layer of endothelium was present on the vessels at the end of the incubation period (data not shown). Examination by scanning electron microscopy showed no evidence of cytoplasmic rupture, endothelial loss, exposure of the internal elastic laminae, or adherent platelets. The cells did, however, appear protuberant as a result of a lack of transmural pressure. Direct cell counts showed $1\pm0.04 \times 10^6$ cells/cm^2 (mean±1 SD) vessel surface area. Aortas treated with collagenase showed total loss of endothelium.

Factor IX/IXa studies

Binding. Elution of Factor IX from bovine aortic segments was studied to assess binding of endogenous Factor IX at the time of slaughter. Eluates obtained from aortas 5 min postmortem were tested for Factor IX antigen by radioimmunoassay (Table I). A small amount of Factor IX antigen was observed

Table I. Elution of Coagulation Factors from Aortas

	Factor IX antigen*	Factor VII antigen*	Antithrombin III antigen*
	fmol/10^6 cells	*fmol/10^6 cells*	*pmol/10^6 cells*
Buffer A wash			
1	26±2	19±2	1.2±0.2
2	14±2	<10	0.5
5	<11	<10	<0.5
7	<11	<10	<0.5
Elution‡			
1	74±8	<10	3.8±0.5
2	<11	<10	<0.5

* Each value is the mean of triplicates from a single experiment and the SEM is given. The experiment was repeated three times.
‡ Factors IX and VII were eluted with buffer B, which contains EDTA. Antithrombin III was eluted with Tris-buffered saline containing 1% Nonidet P-40.

only in the early washes with buffer A, reflecting the presence of plasma on the vessel surface. EDTA elution yielded 74 fmol/10^6 cells, corresponding to ~45,000 molecules of Factor IX bound per cell. The Factor IX previously eluted from 30 aortas was pooled, concentrated by ultrafiltration, recalcified, and assayed for Factor IX_a activity in a clotting assay where the lowest detectable Factor IX_a concentration was 100 pM. At a Factor IX antigen concentration of 15 nM, no Factor IX_a clotting activity could be detected. As a control for the specificity of Factor IX binding, the EDTA eluates were tested for the presence of Factor VII antigen. Although this antigen could be detected in the initial wash, no Factor VII could be detected in the EDTA eluate (Table I) or in the 50-fold concentrated eluate pooled from 30 aortas.

Binding of Factors IX and IX_a to vessel segments, washed with calcium-free buffer to remove endogenously bound Factor IX, was then examined. The parameters of Factor IX and IX_a binding were quite comparable to those previously observed using cultured bovine aortic endothelial cells (9). Binding was specific for Factors IX and IX_a, reversible (50% of bound Factor IX eluted in 10 min), calcium dependent (reaching a plateau by 2.5 mM), and saturable (half-maximal at a total added Factor IX concentration of 2.6 nM). Binding of ^{3}H-Factor IX IX_a could be inhibited by a 100-fold molar excess of either unlabeled Factor IX_a or Factor IX. At an added ^{3}H-Factor IX concentration of 2.6 nM, binding was half-maximal and total vessel binding was 2.2–2.9% of the added tracer. The nonspecific binding was 16–19% of the total binding (0.4–0.5% of the added tracer).

Activation. Vessel-bound ^{3}H-Factor IXa in the presence of VWF/Factor VIII activated added Factor X (Fig. 1 *A*). If unactivated VWF/Factor VIII was added in place of the thrombin-treated VWF/Factor VIII, Factor X activation occurred though there was a slightly longer lag (2–3 min with unactivated Factor VIII as compared with 1–1.5 min with activated Factor VIII) before Factor X_a activity was detected. When prothrombin was added along with Factors VIII and X, vessel-bound ^{3}H-Factor IX_a initiated a series of reactions leading to thrombin formation (Fig. 1 *C*). Unlabeled Factor IXa promoted activation of Factor X and prothrombin under the same conditions. Interaction of coagulation factors with the endothelium was essential for Factor X_a and thrombin formation, as no activation was observed when the proteins were mixed on a Lucite plate in the absence of an aortic segment or when collagenase-treated vessel segments devoid of endothelium replaced the intact segments. Furthermore, when an aliquot of the reaction mixture was removed from the reaction well and incubated in a glass tube, no additional activation of Factor X or prothrombin occurred. The amount of Factor X_a formed in 10 min was correlated with the amount of ^{3}H-Factor IX_a specifically bound to the aorta (Fig. 1 *A*). Omission of Factor IX_a, VWF/Factor VIII substitution of Factor IX for Factor IX_a, or elution of specifically bound ^{3}H-Factor IX_a with calcium-free buffer prevented Factor X_a

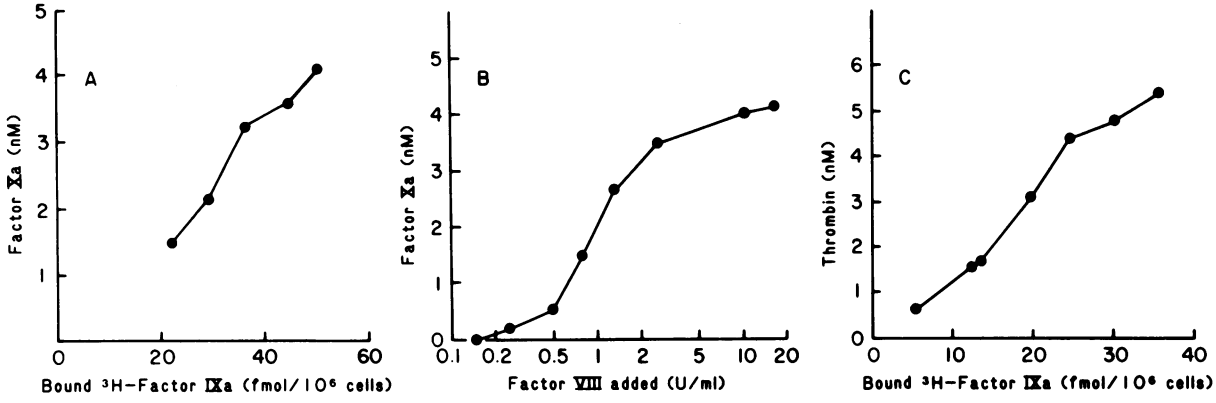

Figure 1. Activation of Factor X and prothrombin by Factor IX_a and VWF/Factor VIII on aortic segments. (*A*) Dependence of Factor X_a formation on bound Factor IX_a. ^{3}H-Factor IX_a (1–5 nM) and VWF/Factor VIII (2 U/ml), 10 μl each, were added to wells containing 0.5 ml buffer A and incubated for 10 min. After aspiration and washing, 0.5 ml buffer A and 10 μl each of thrombin (0.1 nM) and Factor X (0.21 μM) were added and followed by a 10-min incubation. Then 0.5 ml buffer C was added to each well, and a 0.2-ml aliquot of the supernatant was removed for the S-2222 synthetic substrate assay. Specific ^{3}H-Factor IX_a binding was determined from the difference between the binding observed in wells incubated with ^{3}H-Factor IX_a alone and the binding observed in wells incubated with ^{3}H-Factor IX_a in the presence of a 100-fold molar excess of unlabeled factor IX_a. The concentration of Factor X_a formed is plotted against specifically bound ^{3}H-Factor IX_a. (*B*) Dependence of Factor X_a formation on VWF/Factor VIII concentration. ^{3}H-Factor IX_a (3.8 nM) and varying amounts of VWF/Factor VIII (0.15–17 U/ml) each in a volume of 15 μl were added to 0.5 ml of buffer A in 0.79 cm^2 wells and incubated for 10 min at 21°C. After aspiration and washing, Factor X and thrombin were added and the procedure was carried out as in *A*. (*C*) Activation of prothrombin. Aortic segments were incubated with ^{3}H-Factor IX_a and VWF/Factor VIII (2 U/ml), then washed as described above. Next, 10 μl of prothrombin (1.4 μM) was added along with Factor X and thrombin (0.1 nM). The remainder of the procedure was identical to that described in *A* except that S-2238 was used in the synthetic substrate assay. The amount of specifically bound ^{3}H-Factor IX_a is plotted against thrombin formed. In *A–C* above each point is the mean of duplicate wells from a single representative experiment. The experiments were repeated four times and in each case not more than 3% of the available substrate was activated.

formation. In addition, the inclusion of a 500-fold molar excess of Factor IX in each initial incubation mixture in Fig. 1 *A* blocked Factor X activation and specific ^{3}H-Factor IX_a binding by >90%. The amount of Factor X_a formed could also be correlated with the concentration of exogenous VWF/Factor VIII (Fig. 1 *B*) added. The retention of Factor VIII activity on the aorta after washing of the vessel suggests that Factor VIII bound to the vessel wall. Thrombin formation initiated by cell-bound ^{3}H-Factor IX_a occurred only in presence of VWF/Factors VIII, X, and prothrombin and correlated well with the amount of specifically bound ^{3}H-Factor IX_a (Fig. *C*).

Factor X_a studies

Binding. Incubation of ^{125}I-Factor X_a with bovine aortic segments resulted in progressive loss of Factor X_a activity in the supernatant (Fig. 2 *A*). When aliquots of the incubation mixture were removed at various times and analyzed by SDS-PAGE, the radioactivity profile showed formation of a new slowly migrating peak (labeled X_a-ATIII in Fig. 2, *B* and *C*). After a 1-min incubation of Factor X_a with the vessel segment (Fig. 2 *B*), 49% of the initial Factor X_a activity was lost and 46% of the added Factor X_a was present in the higher molecular weight peak. After a 28-min incubation of ^{125}I-Factor X_a with the vessel segment (Fig. 2 *C*), 94% of the initial Factor X_a activity was lost and 90% of the added Factor X_a was present in the peak labeled X_a-ATIII. This high molecular weight peak represented ^{125}I-Factor X_a complexed with antithrombin III since it comigrated with the ^{125}I-Factor X_a-antithrombin III standard and its formation could be blocked by >90% when vessel segments were pre-incubated with anti-antithrombin III IgG (600 μg/ml). Since no antithrombin III was added to these incubation mixtures, it seemed possible that the source of this protein was vessel-associated antithrombin III. To confirm this, vessel segments were washed with buffer A until no further antithrombin III antigen could be detected and then eluted with Tris-buffered saline containing 1% Nonidet P-40. The amount of antithrombin III antigen eluted from the vessel wall corresponded to 3.8±0.5 pmol/10^6 cells (Table I).

Association of ^{125}I-Factor X_a with the vessel was then examined. ^{125}I-Factor X_a bound in a time-dependent manner to the vessel segment, and the amount of specifically bound ^{125}I-Factor X_a (Fig. 3) increased markedly in the presence of anti-antithrombin III IgG and decreased slightly in the presence of added antithrombin III. Binding of ^{125}I-Factor X_a in the absence of nonimmune IgG (not shown) was identical to that shown in the presence of nonimmune IgG. Furthermore, anti-antithrombin III IgG prevented complex formation between ^{125}I-Factor X_a and antithrombin III. These data suggested that complex formation between ^{125}I-Factor X_a and antithrombin III prevented binding of Factor X_a to the vessel. Vessel binding of ^{125}I-Factor X_{ai}, which cannot complex with antithrombin III, was substantially greater than binding of ^{125}I-Factor X_a

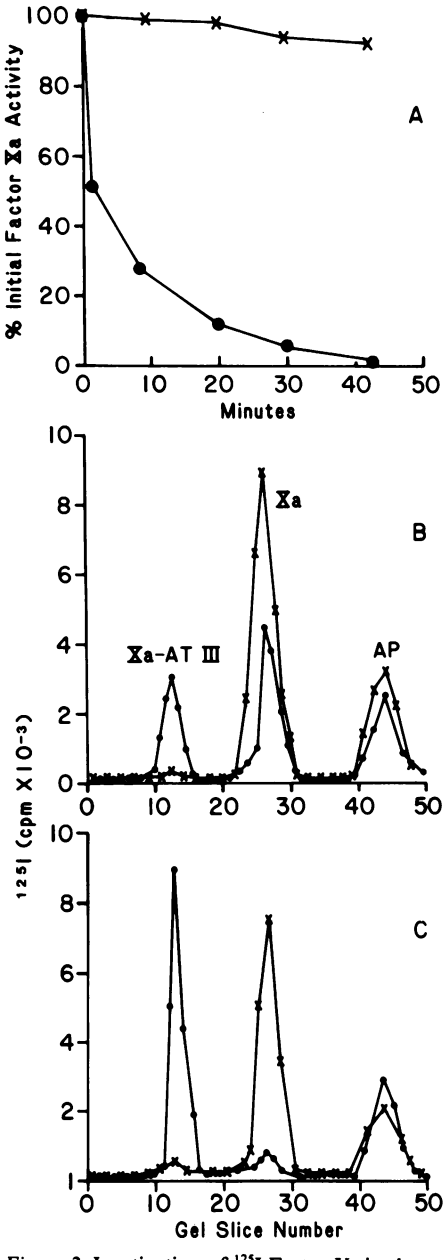

Figure 2. Inactivation of ^{125}I-Factor X_a in the supernatant incubated with bovine aortic segments. Aortic wells were incubated with anti-antithrombin III IgG (×) or nonimmune IgG (●), each at 600 μg/ml for 40 min. (*A*) ^{125}I-Factor X_a (11 nM) was added and Factor X_a inactivation was assessed by chromogenic substrate assay at the indicated times (see text). In *B* and *C*, aliquots of supernatant were subjected to nonreduced 7.5% SDS-PAGE. The incubation times were 1 and 28 min in *B* and *C*, respectively. Labeled peaks correspond to Factor X_a-antithrombin III complex (X_a-AT III), Factor X_a, and activation peptide (AP).

and was not affected by the presence of the antithrombin III antibody or addition of exogenous antithrombin III. In addition, when vessel segments were pre-incubated with thrombin, binding of ^{125}I-Factor X_a (Fig. 3), but not of ^{125}I-Factor X_{ai}, was increased. However, specific binding of Factor X_a remained somewhat less than in the presence of anti-antithrombin III IgG. The effect of thrombin on the binding of ^{125}I-Factor X_a was due to complex formation between thrombin and antithrombin III, since when thrombin was added in the presence of antithrombin III antibody (which could partially block thrombin-antithrombin III complex formation) there was no further enhancement of ^{125}I-Factor X_a binding. The inability of thrombin, even at higher concentrations, to enhance binding to as great an extent as antibody to antithrombin III may be due to multiple interactions of thrombin with the cells in addition to complexing with vessel-associated antithrombin III.

Comparison of the vessel binding of ^{125}I-Factors X_a and X_{ai} (Fig. 4 *A*) showed that the time course of ^{125}I-Factor X_a (1 nM) binding appeared biphasic, consisting of a more rapid initial phase which was complete within 3–4 min and a steadily increasing slower component. The initial phase of ^{125}I-Factor X_{ai} binding was identical to Factor X_a binding. However, in contrast to ^{125}I-Factor X_a, the binding of ^{125}I-Factor X_{ai} (1 nM) reached an apparent maximum after 4 min and did not change later. Binding of ^{125}I-Factor X_a, which was allowed to proceed for only 3 min, was largely reversible (Fig. 4 *A*). The dissociation of bound ^{125}I-Factors X_a and X_{ai} was 50% complete by 7 min for each protein. After 18 min of incubation with the vessel the amount of dissociable ^{125}I-Factors X_a and X_{ai} remained the same, though the total amount of bound ^{125}I-Factor X_a had increased owing to formation of a pool of covalently bound ^{125}I-Factor X_a (see below). From Fig. 4 *A* it appeared that after 3–4 min of incubation, the binding of ^{125}I-Factor X_a in the presence of antibody to antithrombin III and ^{125}I-Factor X_{ai} were quite similar. Semilogarithmic plots

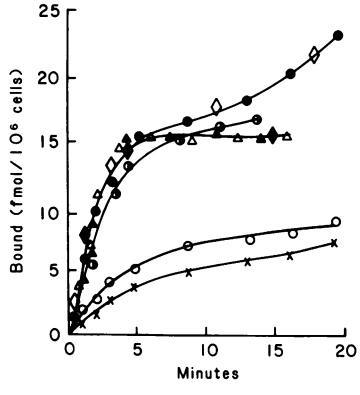

Figure 3. Time course of ^{125}I-Factors X_a and X_{ai} binding: the effect of exogenous antithrombin III, antibody to antithrombin III, and thrombin. Aortic wells were incubated with either anti-antithrombin III IgG (550 μg/ml; ●), nonimmune IgG (600 μg/ml; ○), buffer alone (◇), thrombin (1.80 nM; ⊙), or added antithrombin III (2.7 μM; ×) for 30 min. Then ^{125}I-Factor X_a (0.9 nM) was added alone (total binding), or with unlabeled Factor X_a (480 nM) (nonspecific binding). Other wells were pre-incubated with the same amount of anti-antithrombin III IgG (△), or buffer alone (◆), or nonimmune IgG (▲) for 30 min, and then ^{125}I-Factor X_{ai} (1 nM) alone or with unlabeled Factor X_{ai} (360 nM) was added. Nonspecific binding was 19–22% of the total binding. Each point is the mean of duplicate measurements, and the experiment was repeated three times.

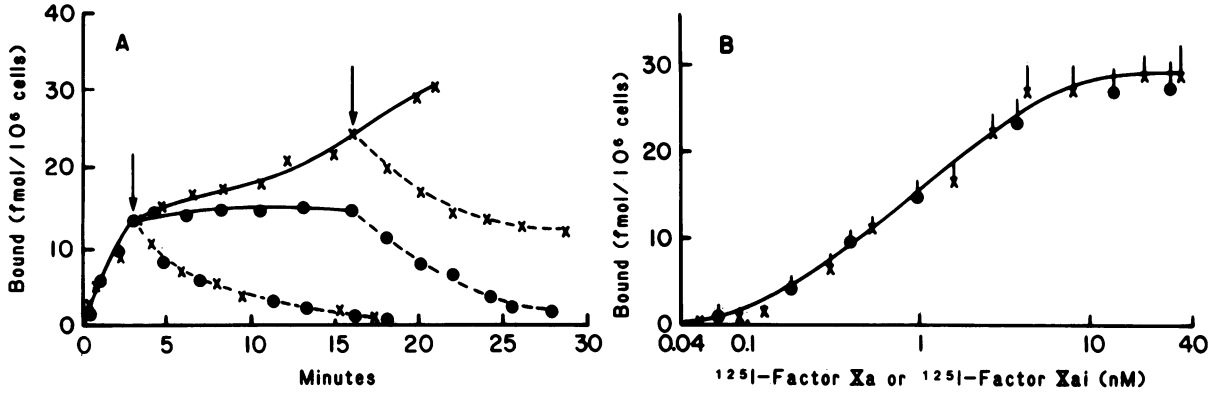

Figure 4. (*A*) Time course and reversibility of specific ^{125}I-Factor X_a (×) or X_{ai} (●) binding to aortic segments. Aortic wells were pre-incubated with anti-antithrombin III IgG (600 μg/ml) for 30 min in 0.5 ml of buffer A; then ^{125}I-Factor X_a (1 nM) alone (total binding) or with 0.5 μM unlabeled Factor X_a (nonspecific binding), or ^{125}I-Factor X_{ai} (1 nM) alone or with unlabeled Factor X_{ai} (0.6 μM) was added to other wells. Dissociation studies were carried out as described in Methods starting at 3 and 18 min as indicated by the arrows. Solid lines show the time course of binding and broken lines show dissociation. (*B*) Saturability of specific ^{125}I-Factors X_a (●) and X_{ai} (×) binding to bovine aortic segments. Aortas (0.79 cm^2 wells) were pre-incubated with anti-antithrombin III IgG (600 μg/ml) for 40 min in 0.5 ml buffer A. Then the indicated amount of ^{125}I-Factor X_a or X_{ai} was added to one set of wells, and tracer together with a 100-fold molar excess of unlabeled protein was added to another set of wells. Incubation times were 4 and 20 min for ^{125}I-Factors X_a and X_{ai}, respectively. Each point is the mean of triplicates and in *B* the SEM is shown. The experiments were repeated five times. At an added ^{125}I-Factor X_a or X_{ai} concentration of 0.9 nM, binding was half-maximal in these experiments and total vessel binding was ~4% of the added tracer.

of specifically bound ^{125}I-Factors X_a and X_{ai} (Fig. 4 *B*) showed a class of saturable binding sites. These studies were performed in the presence of antibody to antithrombin III and after only a 4-min incubation of ^{125}I-Factor X_a with the vessel segments to minimize the contribution of the slower, irreversible phase of Factor X_a binding. Thus, at lower Factor X_a concentrations equilibrium may not have been achieved. ^{125}I-Factor X_{ai} was incubated with vessels for 20 min since there was no slow phase of Factor X_{ai} binding. The binding of ^{125}I-Factors X_a and X_{ai} under these conditions was saturable (29 and 27±3 fmol per 10^6 cells, respectively), and half-maximal binding occurred at an added concentration of 0.9 and 0.7±0.1 nM, respectively. At saturation this corresponded to ~1.7×10^4 molecules/cell of bound ^{125}I-Factor X_a or X_{ai}. Addition of thrombin (1 to 50 nM) in the presence of anti-antithrombin III IgG had no effect on the half-maximal or saturation point of this binding.

When ^{125}I-Factor X_a was incubated longer with the vessel segment the slower phase of binding became evident, although ^{125}I-Factor X_{ai} did not show a parallel increase in binding (Fig. 4 *A*). This suggests that the active site is required for the slow phase of binding. To study this, SDS-PAGE of bound ^{125}I-Factors X_a and X_{ai} after 2 and 30 min of incubation with the vessel was carried out (Fig. 5). At 2 min of incubation (Fig. 5 *B*), when the ^{125}I-Factor X_a was mainly bound reversibly, the radioactivity profile is similar to that of the initial tracer (Fig. 5 *A*). However, after longer incubation (Fig. 5 *C*), the radioactivity profile of bound ^{125}I-Factor X_a, but not of ^{125}I-Factor X_{ai} (Fig. 5 *D*), showed a new slowly migrating peak with a molecular weight of 76,000 on unreduced (V, Fig. 5 *C*) and 63,000 on reduced (V′) SDS-PAGE. The vessel-bound complex had a different molecular weight on unreduced SDS-PAGE (76,000) than did the Factor X_a-antithrombin III complex found in the supernatant (84,000) (Fig. 2). Also, formation of the vessel bound complex was not blocked by anti-antithrombin III IgG. ^{125}I-Factor X_a-vessel wall complexes, solubilized in Nonidet P-40 (1%) could not be immunoprecipitated with anti-antithrombin III IgG under conditions that immunoprecipitated Factor X_a-antithrombin III complexes.

Reversibility studies (Fig. 4 *A*) showed that the same amount of ^{125}I-Factor X_a dissociated at 3 min of incubation, when no covalent Factor X_a-vessel wall complex (M_r 76,000) had formed, and at 18 min of incubation, when the latter complex had formed. The amount of nondissociable bound ^{125}I-Factor X_a increased from 3 to 18 min of incubation in parallel with formation of the M_r 76,000 complex. Unreduced SDS-PAGE showed that the radioactivity profile of the dissociated ^{125}I-Factor X_a, in the presence of anti-antithrombin III IgG (500 μg/ml), was identical to that of the initial tracer. Factor X_a in the 76,000 mol wt complex remained vessel associated (data not shown). The ^{125}I-Factor X_a-vessel wall complex (M_r 76,000) thus accounts for the steadily increasing phase of irreversible binding.

Competitive binding studies carried out in the presence of antibody to antithrombin III (Fig. 6) showed no significant inhibition of ^{125}I-Factor X_a binding by Factors IX, IX_a, X, prothrombin, thrombin, or protein C. The experiment in Fig. 6 was carried out with a 20-min incubation of coagulation proteins with the vessel, and ~50% of the bound ^{125}I-Factor X_a was in the M_r 76,000 complex. Competitive binding studies

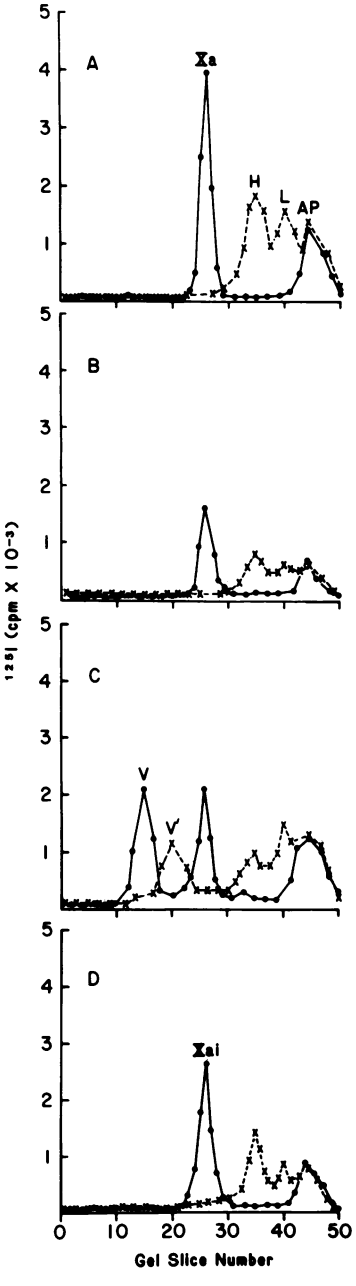

Figure 5. SDS-PAGE (7.5%) of ^{125}I-Factors X_a and X_{ai} bound to bovine aortic segments at different times. Aortic wells were pre-incubated with anti-antithrombin III IgG (600 μg/ml) and then ^{125}I-Factor X_a (5 nM) or X_{ai} was added. After the indicated incubation time wells were washed with buffer A and bound ^{125}I-Factor X_a or X_{ai} was solubilized with 1% Nonidet P-40. Labeled peaks are designed as follows: Xa, ^{125}I-Factor X_a; Xai, ^{125}I-Factor X_{ai}; L and H, ^{125}I-Factor X_a light and heavy chain, respectively; AP, activation peptide; V, complex of bound ^{125}I-Factor X_a and vessel wall binding site (M_r 76,000 unreduced); V', same complex as V but reduced (M_r 63,000); •, unreduced; ×, reduced. (*A*) ^{125}I-Factor X_a, no aorta; (*B*) ^{125}I-Factor X_a after a 2-min incubation with aorta; (*C*) ^{125}I-Factor X_a after a 30-min incubation with aorta; (*D*) ^{125}I-Factor X_{ai} after a 30-min incubation with aorta.

were also carried out with a 4-min incubation, when >90% of the Factor X_a was reversibly bound, and at 90 min, when ~80% of the Factor X_a was in the M_r 76,000 complex. In each case, the other cogulation proteins did not inhibit ^{125}I-Factor X_a-vessel wall binding. Binding of ^{125}I-Factor X_a was calcium dependent, maximal at 3 mM Ca^{++}, and decreased by 20–30% from 3 to 10 mM Ca^{++}.

Activation. Prothrombin activation studies were performed on aortic segments to assess the coagulant activity of bound ^{125}I-Factor X_a. Vessel-bound ^{125}I-Factor X_a activated added prothrombin as measured by increasing levels of amidolytic activity in the S2238 assay (Fig. 7). Thrombin formation depended upon the presence of Factors X_a and prothrombin, as well as on the binding of ^{125}I-Factor X_a to the endothelium, since no activation of prothrombin by Factor X_a was observed on aortic segments previously treated with collagenase. Furthermore, when aliquots of the reaction mixture supernatant were withdrawn from contact with the endothelium at intervals and incubated in a test tube, no further thrombin formation occurred. The amount of thrombin formed on aortic segments was correlated with the amount of bound ^{125}I-Factor X_a (Fig. 7) and depended upon the interaction of Factor X_a with an endothelial cell Factor V-like molecule, as shown by 75–90% inhibition of prothrombin activation in the presence of anti-Factor V IgG (Fig. 7), though this concentration of antibody blocked ^{125}I-Factor X_a binding by only 10–30% (data not shown). The effect of anti-antithrombin III IgG on the activation of prothrombin by vessel-bound ^{125}I-Factor X_a was studied by comparing the amount of thrombin generation in the supernatant of wells incubated with anti-antithrombin III IgG or nonimmune IgG (Fig. 8). In the presence of anti-antithrombin III IgG (600 μg/ml), formation of Factor X_a-antithrombin III complex was inhibited by 90–95% and formation of thrombin-antithrombin III complex was inhibited by ~70% as judged by SDS-PAGE of reaction mixtures. Wells pre-ihcubated with anti-antithrombin III IgG (Fig. 8 *A*) showed 30–50-fold more thrombin activity in the supernatant. The apparent increase in thrombin activity correlated well with the dose of anti-antithrombin III IgG and reached a plateau at 500–800 μg/ml of added antibody (Fig. 8 *B*). In this range of antibody concentration, complex formation between antithrombin III and the two proteases was not blocked further by addition of more IgG (data not shown).

Discussion

These studies were initiated to examine the interaction of bovine Factors IX, IX_a, and X_a with a continuous layer of native endothelium. Previously it has been reported that Factors IX, IX_a, and X_a bind to cultured bovine aortic endothelial cells (8–13). Bound Factor IX_a can activate Factor X in the presence of VWF/Factor VIII (10) and Factor X_a can activate prothrombin when the cultured cells are subconfluent (14). Factor V synthesis by cultured endothelial cells has also been reported (44). Since endothelial cells are generally considered to inhibit hemostasis and thrombosis, we have investigated whether these clot-promoting phenomena observed with cultured cells can also be found with native endothelial cells. In an in vivo system, Factor X_a binding to mouse aortas has been observed (45). In our studies the source of native endothelium was calf aortas because of the relative ease of obtaining and handling aortic tissue.

The presence of Factor IX antigen in the EDTA-eluates from vessels harvested immediately after slaughter (Table I) strongly suggests that there is Factor IX binding in vivo and supports previous observations of Factor IX binding to endo-

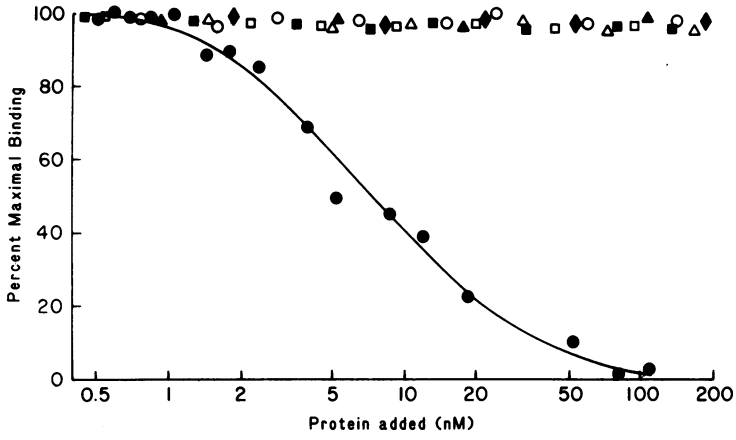

Figure 6. Competitive binding studies. Aortic segments (0.79 cm^2 wells) were pre-incubated with anti-antithrombin III IgG (600 μg/ml) for 40 min in buffer A (0.5 ml). Then ^{125}I-Factor X_a (1 nM) alone or the same amount of ^{125}I-Factor X_a in the presence of increasing concentrations of unlabeled Factor X_a (●), Factor X (○), Factor IX (□), Factor IX_a (■), prothrombin (▲), thrombin (△), or protein C (♦) was added for 20 min. Wells were then washed and solubilized as described. Maximal binding (23 fmol/10^6 cells) was defined as the amount of ^{125}I-Factor X_a bound when ^{125}I-Factor X_a was incubated with aortic segments alone minus the amount of ^{125}I-Factor X_a bound in the presence of a 100-fold molar excess of unlabeled Factor X_a. The mean of duplicates is plotted and the experiment was repeated three times.

thelial cells in culture. Animals slaughtered according to kosher dietary laws were selected for these studies because in this procedure the blood drains rapidly from the aorta at the time of slaughter. Although the radioimmunoassay for Factor IX could not distinguish the zymogen from its activated form, clotting assays indicated that <3% of the eluted Factor IX antigen was Factor IX_a. This suggests that Factor IX binding in vivo was responsible for Factor IX antigen in the EDTA eluate, rather than selective Factor IX_a binding accompanying activation of the coagulation system at the time of slaughter. However, killing of animals by any technique certainly could be associated with perturbation of the endothelium, and infusion studies may provide further insight into the binding of Factor IX to the vessel wall in vivo. Factor VII, a coagulation factor reported not to bind specifically to bovine aortic endothelial cells in culture (46), was selected as a control for the vessel wall elution studies of Factor IX (Table I).

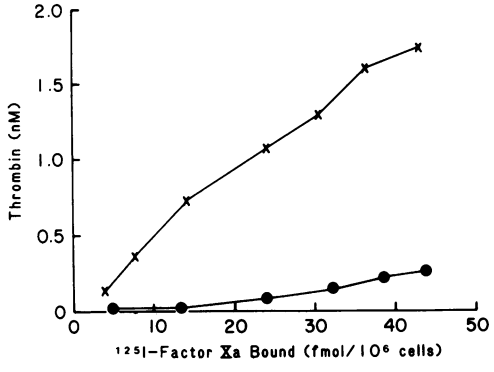

Figure 7. Activation of prothrombin by Factor X_a on aortic segments. Wells (0.79 cm^2) were preincubated with either 5 ml (200 μg/ml) of anti-Factor V IgG (●) or control burro IgG (×) for 40 min. ^{125}I-Factor X_a (5 μl; 3–30 nM) was then added to the wells and incubated for 5 min. Unbound ^{125}I-Factor X_a was removed by washing with buffer A, and 0.5 ml of buffer A containing either anti-Factor V IgG or control IgG was added to each well. The same IgG as for the initial incubation followed by 7.5 μl of prothrombin (1.4 μM) was then added. Reaction mixtures were incubated for 10 min and terminated by the addition of 0.5 ml buffer C, and 0.2-ml aliquots were assayed for thrombin activity as described in Methods. Specific ^{125}I-Factor X_a binding was determined as described in Fig. 3. Thrombin concentration at 10 min is plotted against the amount of specifically bound ^{125}I-Factor X_a. The mean of duplicate measurements is shown, and the experiment was repeated three times.

Factor IX_a bound to vessel segments activated Factor X in the presence of exogenous VWF/Factor VIII and in the absence of exogenous phospholipid (Fig. 1, *A* and *B*). In the experiments shown in Fig. 1, all of the specifically bound ^{3}H-Factor IX_a was considered to be active in Factor X cleavage. Specifically bound Factor IX_a was responsible for the observed Factor X activation since activation was prevented in the presence of a 100-fold molar excess of Factor IX, which prevents specific Factor IX_a binding, or by elution of bound Factor IX_a by calcium-free buffer. Further studies will be required to determine if all or only a portion of the specifically bound Factor IX_a has procoagulant activity.

To study Factor X_a generation, vessels were incubated with Factor IX_a and VWF/Factor VIII, and washed, and then catalytic amounts of thrombin were added and Factor X activation was assessed. Since the vessel was essential for Factor X_a formation, a complex of bound Factor IX_a and activated VWF/Factor VIII may have formed on the vessel wall and functioned in a fashion analogous to the Factor X_a-V_a complex in the activation of prothrombin (47–49). These results raise a number of questions concerning VWF/Factor VIII-vessel wall interactions.

Factor IX_a bound to aortic segments in the presence of adequate amounts of VWF/Factor VIII can initiate a series of vessel-dependent reactions leading to thrombin formation (Fig. 1 *C*). Since the presumed activator of prothrombin was vessel-bound Factor X_a interaction with an endothelial cell Factor V-like molecule (14, 44), we next examined the interaction of Factor X_a with vessel segments. When Factor X_a was incubated with vessel segments, rapid inactivation occurred (Fig. 2). This is principally due to inactivation of the Factor X_a by antithrombin III initially associated with the vessel. The details of this inactivation are uncertain (i.e., whether inactivation of

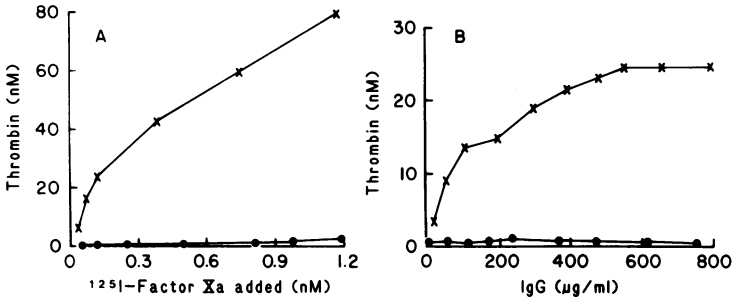

Figure 8. Effect of anti-antithrombin III IgG on the activation of prothrombin by Factor X_a on aortic segments. (*A*) Aortic wells were pre-incubated for 30 min with 600 μg/ml of either anti-antithrombin III IgG (×) or control rabbit IgG (•). ^{125}I-Factor X_a (0.05–1.2 nM) was then added to the wells for 5 min, and the wells were washed. Buffer A (0.5 ml), antibody, and prothrombin (1.4 μM) were added. Reaction mixtures were incubated for 10 min and the supernatants were assayed for thrombin formation as described in Methods. (*B*) Wells were incubated with varying amounts of either anti-antithrombin III IgG (×) (2–800 μg/ml) or control rabbit IgG (•) (2–800 μg/ml) in 0.5 ml buffer A for 30 min. ^{125}I-Factor X_a (0.13 nM) was added to the wells and incubated for an additional 20 min. Wells were then washed, and filled with fresh buffer A (0.5 ml), antibody (same as initially used for each well), and prothrombin (1.4 μM). Reaction mixture supernatants were assayed for thrombin after 10 min of incubation. The mean of duplicate measurements is shown, and the experiment was repeated three times.

Factor X_a results from Factor X_a binding to antithrombin III localized on the vessel surface with subsequent dissociation of Factor X_a-antithrombin III complex or whether antithrombin III dissociates first and then Factor Xa-antithrombin III complex formation occurs in the supernatant), but the antithrombin III is clearly associated with the vessel at the start of the experiment (Table I). The mechanism responsible for this localization of antithrombin III to the vessel is currently under study. One possibility is that plasma antithrombin III binds to the vessel in vivo (50). Alternatively, constitutive synthesis of antithrombin III by endothelium, which has been reported for cultured human umbilical vein endothelial cells (51), may be involved.

Incubation of Factor X_a with calf aortic segments showed the presence of time-dependent, specific binding (Fig. 3). The presence of antithrombin III decreased Factor X_a-vessel wall binding, whereas addition of anti-antithrombin III IgG, which blocked formation of ^{125}I-Factor X_a-antithrombin III complex (Fig. 2, *B* and *C*), promoted binding. This suggested that ^{125}I-Factor X_a, complexed with antithrombin III, no longer binds to the vessel segments. This suggestion was confirmed by experiments in which ^{125}I-Factor X_a, pre-incubated with a twofold molar excess of antithrombin for 60 min at 37°C, did not subsequently bind specifically to the vessels (unpublished observation). Also, SDS-PAGE of vessel-bound ^{125}I-Factor X_a did not reveal the presence of ^{125}I-Factor X_a-antithrombin III complex (Fig. 5, *B* and *C*). Binding of ^{125}I-Factor X_a to aortas in the presence of the plasma concentration of antithrombin III suggested that Factor X_a binding to the vessel wall may occur physiologically. This observation led us to investigate further Factor X_a-vessel wall interaction. Time-dependent binding of Factor X_a to aortic segments (Fig. 4) was biphasic, with an initial rapid, largely reversible phase and a subsequent irreversible phase. Reversible binding was similar for ^{125}I-Factor X_a and ^{125}I-Factor X_{ai} (Fig. 4), suggesting that integrity of the active site was not necessary for this vessel wall interaction. In contrast, the slower irreversible phase of binding required the active site and was due to formation of a covalent complex between Factor X_a and a vessel-associated protein (Fig. 5). Factor X_a bound to this site remained on the surface of the vessel since brief trypsin treatment (2 mg/ml trypsin for 4 min at 21°C) did not remove the endothelium but did result in rapid dissociation of bound radioactivity. The nature of this ^{125}I-Factor X_a vessel wall complex is unclear, though it appears to differ in its molecular weight and antigenic characteristics from Factor X_a-antithrombin III complex. Competitive binding studies (Fig. 6) demonstrated no inhibition of ^{125}I-Factor X_a binding by thrombin, suggesting that this was not the irreversible thrombin binding site observed by Lollar et al. (40) on cultured human umbilical vein endothelial cells. Further studies will be required to determine whether this binding site is an endogenous vessel wall nexin-like molecule (52) or an absorbed plasma protease inhibitor. Irreversible Factor X_a binding due to covalent complex formation between Factor X_a and an endothelial cell surface protein as well as binding of active site-blocked Factor X_a were not seen by Rodgers and Shuman (14). These differences might be due to the variety of methods used to inactivate Factor X_a or to different sources of endothelium, i.e., cultured vs. native endothelium.

Factor X_a bound to aortic segments activated prothrombin (Fig. 7), though only small amounts of thrombin were detectable in the supernatant. Studies with anti-antithrombin III IgG (Fig. 8) suggested that most of the Factor X_a added and thrombin formed were inactivated by antithrombin III initially associated with the surface of the vessel. Thus, in the presence of anti-antithrombin III IgG, which prevented antithrombin III protease complex formation, the amount of bound ^{125}I-Factor X_a (Fig. 3) and the amount of detectable thrombin in the amidolytic S2238 assay increased. The actual amount of thrombin formed per mole of Factor X_a bound may have been unchanged. Under conditions of compromised blood flow in which exposure of the vessel wall to plasma antithrombin III might be reduced and/or antithrombin III associated with the vessel might dissociate, it is possible that more of the Factor X_a and thrombin formed may remain active, potentially leading to fibrinogen cleavage and clot formation.

The presence of antithrombin III on the vessel surface is one mechanism by which the vessel wall is protected from activation of the coagulation system. Another potential protective mechanism is competition by Factors IX and IX_a for the same cellular binding site. When small amounts of Factor IX_a

are formed in the supernatant, no Factor IX_a binding will occur as long as there is continuous blood flow to ensure a large excess of the zymogen, Factor IX. However, when the amount of Factor IX_a in the supernatant increases, Factor IX_a binding could occur, as Factor IX already bound to the endothelium dissociates. (50% of bound Factors IX/IX_a dissociates in 30 min at 4°C (9) and in 10 min at 23°C.) Alternatively, activation of cell-bound Factor IX would lead to Factor IX_a localized to the vessel wall. This cell-bound Factor IX_a could then participate in Factor X activation since the dissociation of Factor IX_a from its cellular binding site is slow. We have previously demonstrated activation of Factor IX bound to quiescent endothelial cells by Factor XI_a and activation of Factor IX bound to endotoxin-treated endothelial cells, which express tissue factor, by Factor VII_a (10). The cell-bound Factor IX_a formed did activate Factor X in the presence of VWF/Factor VIII. Therefore, an endothelial cell-dependent pathway of coagulation, initiated by perturbed endothelial cells expressing tissue factor and culminating in thrombin formation, may be an important mechanism for activation of the coagulation system in localized areas of vessel wall pathology. In these areas Factor IX_a could be formed on the surface of endothelial cells, and Factor X and prothrombin activation could proceed rapidly when the amount of antithrombin III on the vessel wall is decreased.

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