Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 913–917, February 1984
Medical Sciences

Activation of factor IX bound to cultured bovine aortic endothelial cells

(hemostasis/thrombosis/phorbol ester/tissue factor)

DAVID M. STERN*, MICHAEL DRILLINGS*, WALTER KISIEL†, PETER NAWROTH*, HYMIE L. NOSSEL*‡, AND KALLIOPE S. LAGAMMA*

*Department of Medicine, Columbia University College of Physicians and Surgeons, 630 West 168 Street, New York, NY 10032; and †Department of Biochemistry, University of Washington, Seattle, WA 98195

Communicated by Oscar D. Ratnoff, October 6, 1983

ABSTRACT **Previous studies have shown that factor IX and its activated form, factor IX_a, bind to cultured vascular endothelial cells and that cell-bound factor IX_a retains its procoagulant activity. The present studies provide evidence that factor IX bound to cultured bovine aortic endothelial cells can be activated. Factor IX activation was assessed by finding cleavage of the factor IX molecule on $NaDodSO_4$/polyacrylamide gel electrophoresis and by the generation of procoagulant activity as assessed by thrombin-treated factor VIII-dependent generation of factor X_a activity. Cell-bound factor IX (0.8 μg per 4×10^8 cells per ml) could be activated by factor XI_a (5 μg/ml) or by factor VII_a (0.1 μg/ml) without exogenous tissue factor when endothelial cells were treated with phorbol ester and acquired tissue factor-like procoagulant activity. Regardless of how factor IX was activated, the cell-bound factor IX_a required thrombin-treated factor VIII and calcium, but not exogenous phospholipid, to activate factor X. In further experiments, factor X bound to endothelial cells specifically and reversibly with a dependence on calcium and with a lower affinity (half-maximal at 480 nM) than factor IX. At saturation, 9.1×10^6 factor X molecules were bound per cell. After activation of factor X by factor IX_a, approximately 50% of the factor X_a formed could be eluted from the cells by 10 mM EDTA, suggesting that the factor X_a was cell associated. These observations indicate that endothelial cells can bind and promote the activation of factors IX and X in the absence of platelets or exogenous phospholipid.**

Cultured bovine aortic endothelial cells (BAEC) have high-affinity binding sites for factors IX/IX_a (1–3). The functional significance of this binding as a potential mechanism for localizing procoagulant activity depends on the ability of cell-bound factor IX to be activated and the activity of cell-bound factor IX_a in activation of factor X. This study reports the activation of cell-bound factor IX by the contact and tissue factor pathways. Having previously shown that cell-bound factor IX_a retains clotting activity (3), we now report that cell-bound factor IX_a can activate factor X in the absence of exogenous phospholipid but in the presence of added factor VIII. Interaction of factor X, the substrate for this reaction, with endothelial cells has been investigated in view of the possible importance of vessel wall in factor IX/X activation.

METHODS

Coagulation Factors. Bovine coagulation proteins were used throughout these studies except where otherwise specified. Factor IX was isolated by the method of Fujikawa *et al.* (4) or was generously provided by Charles Esmon (Oklahoma Medical Research Foundation). The factor IX was tritiated, activated, and characterized as described (3). Human factor XI_a was a gift of Paul Bajaj, University of California (San Diego). Factor VII was prepared as described (5) and was activated at 37°C in the presence of 10 mM $CaCl_2$ by elution from celite (6, 7) coupled to CNBr-activated Sepharose (Pharmacia). Activation of factor VII was complete by 90 min as monitored by coagulant assay (8) and $NaDodSO_4$/10% PAGE (9). Factor X_1 was isolated to homogeneity by the method of Fujikawa *et al.* (10) and tritiated as described by Silverberg *et al.* (11). Factor X_a was prepared using the purified factor X activator from Russell viper venom (12) immobilized on CNBr-activated Sepharose. Activation was complete by coagulant assay (13) and $NaDodSO_4$/PAGE (9) after 45 min. Human α-thrombin (2.5 NIH units/μg) was a gift from J. W. Fenton (New York State Department of Health) (14). Factor VIII was prepared from bovine plasma by a combination of fractional polyethylene glycol 6000 precipitation (15) and gel filtration (Sepharose CL-4B) and was generously provided by M. Chopek (University of Washington). Factor VIII (1.3 units/ml before activation) was activated with thrombin (0.01 NIH unit/ml) for 4 min at 37°C in 50 mM Tris·HCl, pH 8.0/175 mM NaCl/0.2% bovine serum albumin and the product was designated factor $VIII_t$ (16). Factor $VIII_t$ coagulant activity (16) was stable for up to 7 min. Protein concentrations were determined colorimetrically (17).

Cell Culture and Suspension of BAEC. BAEC were isolated from calf aortas and cultured as described (3). Cells in these experiments were from passages 2–13 and were used within 24 hr of achieving confluence. Confluent BAEC in 10 × 35 mm culture dishes were suspended as follows: the monolayer was washed twice with 10 mM Hepes/137 mM NaCl/4 mM KCl/11 mM glucose, pH 7.45 (buffer A) and then incubated with Dulbecco's calcium/magnesium-free phosphate-buffered saline/25 mM $NaHCO_3$/10 mM sucrose/1 mM EDTA (1 ml per dish). After 10 min at 37°C, the cells were removed from the dishes, centrifuged at 135 × *g* for 5 min and suspended in buffer A/10 mM $CaCl_2$ containing fatty acid-free bovine serum albumin at 2 mg/ml (buffer B) to a final concentration of 4×10^8 cells per ml. Cell suspensions consisted of predominantly single cells and occasional small clumps of <5 cells. Throughout the activation experiments, cell viability was 85–95% as assessed by trypan blue exclusion.

Binding Studies. To achieve a higher cell density than could be obtained in monolayer, binding studies were done using suspended cells. Suspended cells in buffer B were pipetted into glass tubes, and factor IX (1.8–180 nM) in the same buffer was added. The cells were incubated for 30 min at 23°C with agitation every 5 min and were then washed with two 1-ml portions of buffer B and centrifuged (135 × *g*,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BAEC, bovine aortic endothelial cells; S2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; PMA, phorbol 12-myristate 13-acetate.

‡Deceased October 9, 1983.

5 min) between washes. In experiments using [^{3}H]factor IX, the radioactivity in the last wash was at the background level. Studies with [^{3}H]factor IX showed binding of similar affinity to that reported with monolayers (3) with 10^6 cells specifically binding a maximum of 34 fmol of factor IX (nonspecific binding, 21%) and half-maximal binding occurring at a total factor IX concentration of ≈2 nM. For activation studies, factor IX was bound to the cells and unbound ligand was washed away. Cells were then suspended in buffer B, and activating enzyme, factor XI_a, or VII_a was added for 10 min at 23°C. After cells were washed again, factors $VIII_t$ and X were added and the amount of cell-bound factor IX_a formed was assessed by monitoring factor X_a activity. The binding of [^{3}H]factor IX to phorbol 12-myristate 13-acetate (PMA)-treated BAEC and to control BAEC in monolayer was studied as described (3).

The binding of [^{3}H]factor X_1 to confluent BAEC was also studied as described for factor IX (3) except that binding assays were performed in 24-well cluster dishes with 16-mm wells at 23°C without agitation except for an initial mixing of components in the incubation mixture. Cells were washed three times with buffer A, and then 0.2 ml of buffer A/5 mM $CaCl_2$ containing fatty acid-free bovine serum albumin at 2 mg/ml (buffer C) was added. Monolayers were equilibrated for 10 min at 23°C in buffer C, then tracer and other components of the reaction mixture were added. After the incubation period, monolayers were washed four times (0.5 ml per wash) with ice-cold buffer C for a total of 6 sec then solubilized and radioactivity was determined.

Assessment of Coagulant Activity. Cell-associated factor IX_a coagulant activity was assessed in a two-stage assay. In the first stage, factor $VIII_t$ and factor X were added to the cells, and in the second stage, factor X_a was quantified by measuring the hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S2222). BAEC with bound factor IX_a were suspended in 50 μl of buffer B and 50 μl each of factor X and factor $VIII_t$ (16.5 μg/ml or 0.3 μM unless otherwise specified) were added. The mixture was incubated at 37°C and at intervals 25-μl aliquots were removed and diluted in 475 μl of 50 mM Tris·HCl, pH 7.9/175 mM NaCl/10 mM EDTA containing ovalbumin at 0.5 mg/ml; (buffer D); 200 μl of this sample was mixed with 300 μl of 3 mM S2222 and hydrolysis was followed at 405 nM (18). The amount of factor X_a formed was determined by comparison with the linear portion of a standard curve in which known amounts of factor X_a were incubated with S2222 under the same conditions.

Characterization of the Procoagulant Activity of PMA-Treated BAEC. Endothelial cells were incubated with PMA (Consolidated Midland, Brewster, NY) at a final concentration of 100 ng/ml [0.1% (vol/vol) acetone] in serum-containing culture medium. After 16 hr at 37°C, the cells were washed with buffer A and suspended as described above. PMA-treated endothelial cells were assayed for procoagulant activity after suspension to final concentration of 10^6 cells per ml in 50 mM Tris·HCl, pH 7.5/100 mM NaCl/0.1% bovine serum albumin. Cell suspensions were incubated with factor VII_a (2 nM) and factor X_1 (1.2 μM) in the presence of 10 mM $CaCl_2$ and then assayed for clot-promoting activity using factor VII-deficient plasma (19) and for S2222 hydrolytic activity as described above. Progressive factor X activation, dependent on the presence of factor VII_a, occurred with PMA-treated cells as shown by both assays.

RESULTS

Activation of Cell-Bound Factor IX by Factor XI_a. Activation of endothelial cell-bound factor IX by factor XI_a was assessed first by cleavage of the radiolabeled molecule as judged by $NaDodSO_4$/PAGE. The radioactivity profile of a $NaDodSO_4$ gel of the EDTA eluate of factor XI_a-treated cell-bound [^{3}H]factor IX is shown in Fig. 1*A*. Cleavage was limited but definite. The band labeled α at M_r = 38,000 presumably represents the heavy chain of factor IX with the activation peptide still attached (20). The band labeled β at M_r = 32,000 presumably represents the heavy chain alone (20). The band reflecting the activation peptide was quite small, suggesting that this peptide is not closely associated with cell-bound factor IX_a (3). In the control sample (Fig. 1*B*), factor XI_a was omitted and one major band of radioactivity (M_r = 56,000) was observed. A band at M_r = 95,000 was observed on both gels as previously reported (3). This band may reflect either binding of aggregated ligand or formation of a complex of ligand with a cell-associated protein that is dissociated from the cells by EDTA.

Activation of cell-bound factor IX was also monitored functionally as generated factor X_a activity after addition of factors $VIII_t$ and X. Cleavage of cell-bound [^{3}H]factor IX as judged by $NaDodSO_4$/PAGE was associated with formation of factor IX_a coagulant activity as shown in Table 1. Controls omitting factor XI_a, IX, or $VIII_t$ produced no factor X activation.

Experiments comparing the procoagulant activity of factor IX_a on the cells with that in solution (Fig. 2) showed that cell-bound factor IX_a activated factor X in the presence of added factor $VIII_t$ and calcium ions whereas ≈20-fold more factor IX_a in solution failed to do so. However, when phospholipid was added to factor IX_a in solution, factor X was activated (data not shown). Cell-bound factor IX_a activation of factor X in the presence of factor $VIII_t$ occurred with BAEC in suspension (line a) or in monolayer. The factor X_a generation curve shows a 1-min lag followed by linear factor X_a formation. The reason for this lag is unclear though it has been described by others (16) in cell-free systems.

Factor VII_a Dependent Activation of Cell-Bound Factor IX by PMA-Treated Endothelial Cells. Studies were then made with PMA-treated cells. Factor IX was found to bind similarly to both PMA-treated and control cells (Fig. 3). As shown

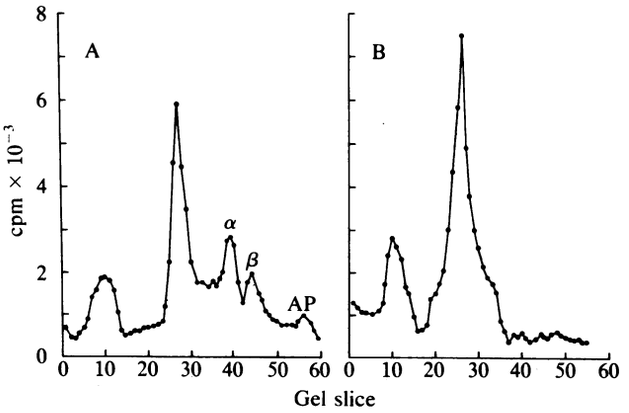

FIG. 1. $NaDodSO_4$/PAGE of cell-bound [^{3}H]factor IX with and without factor XI_a treatment. (*A*) Cleavage of bound [^{3}H]factor IX by factor XI_a: 1.5×10^7 BAEC (P_6) in buffer B (50 μl) were incubated with 5 μl of [^{3}H]factor IX (17 nM) for 20 min at 23°C, washed, and suspended in 50 μl of buffer B; then 5 μl of factor XI_a (final concentration, 31 nM) in 50 μl buffer B was added. After 17 min at 23°C, the cells were washed once with buffer B without added albumin and then suspended in 20 μl of 10 mM $NaPO_4$ buffer, pH 7.0/10 mM EDTA for 5 min at 23°C. Cells were removed by centrifugation and $NaDodSO_4$ (5 μl) and 2-mercaptoethanol (3 μl) at final concentrations of 1% and 10%, respectively, were added to the supernatant. The samples were boiled for 3 min and analyzed by $NaDodSO_4$/PAGE (8.5% acrylamide). Gels were electrophoresed and sliced, and radioactivity was determined as described (4) except the electrode buffer was 0.1 M $NaPO_4$, pH 7.0/0.1% $NaDodSO_4$. The bands labeled α and β are as described in the text; that labeled AP is due to an activation peptide. (*B*) [^{3}H]Factor IX bound to BAEC: Conditions were identical to *A*, except that factor XI_a was omitted.

Table 1. Activation of cell-bound factor IX by factor XI_a

Factor IX added, μg/ml*†	Factor XI_a added, μg/ml	Factor $VIII_t$ added	Factor X_a formed in 5 min, ng/ml
0	5	0	<50
2.5	0	+	<50
2.5	5	0	<50
2.5	5	+	1,760

Endothelial cells (P_6) (2×10^7) in 50 μl of buffer B and 5 μl of factor IX were added to each tube and the tubes were incubated for 30 min at 23°C. The cells were washed twice and then factor XI_a (5 μl) was added in buffer B to a final volume of 50 μl. After 10 min at 23°C, the cells were washed and resuspended in 50 μl of buffer B, and 50 μl of factor X (final concentration, 0.3 μM = 16.5 μg/ml) and 50 μl of factor $VIII_t$ or 50 μl of buffer B containing thrombin (0.01 unit/ml) were added. After 5 min incubation at 23°, S2222 hydrolysis was measured. Each result is the mean of duplicate determinations and each experiment was repeated four times.

*Concentrations refer to the reaction mixture at each stage.

†Experiments using [^{3}H]factor IX showed 2.1 ng (38 fmol) of cell-bound factor IX per 10^6 cells after initial incubation and washing. After incubation with activators and washing, there was 1 ng (18.2 fmol) of cell-bound factor IX per 10^6 cells.

in Table 2, PMA-treated endothelial cells acquired potent factor VII_a-dependent ability to activate cell-bound factor IX. Factor IX_a required thrombin-treated factor VIII to activate factor X. The failure of the cells to directly activate X in these experiments may have resulted from the removal of factor VII_a by the washing procedure. Since the tissue factor-like activity of PMA-treated cells remains associated with the cell pellet after these reactions, factor VII_a endothelial cell-derived tissue factor is the presumed activator of factor IX. When PMA-treated endothelial cells are incubated

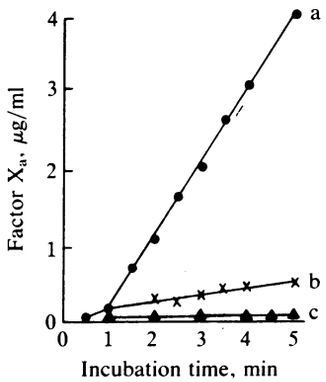

FIG. 2. Activation of factor X by cell-bound factor IX_a and not by factor IX_a in solution. Line a. Factor IX_a bound to suspended BAEC; 2×10^7 BAEC in 50 μl of buffer B were incubated with [^{3}H]factor IX_a (180 nM) for 30 min at 23°C. The cells were washed twice, centrifuged, and resuspended in buffer B. The radioactivity in an aliquot of the cell suspension was determined and the concentration of cell-associated factor IX_a was 11 nM or 28 fmol per 10^6 cells. Factor $VIII_t$ (1.3 units/ml prior to activation) and factor X (final concentration, 0.9 μM = 50 μg/ml) were added and the mixture was incubated at 37°C. At the times indicated, an aliquot was removed, diluted, and assayed for factor X_a activity. Line b. Factor IX_a bound to monolayer; confluent BAEC in 10 × 35 mm dishes (1.4×10^6 cells per dish) were incubated with factor IX_a as above, then unbound material was removed by washing six times. There was 34 fmol per dish of bound IX_a (24.3 fmol per 10^6 cells). Factors $VIII_t$ and X were added in buffer B at the concentrations given above in a final volume of 300 ml, and the mixture was incubated at 37°C and assayed for factor X_a activity. Line c. Factor IX_a in solution; factor IX_a (93 nM, 5.1 μg/ml) in 50 μl of buffer B was added to factors $VIII_t$ and X. The mixture was incubated at 37°C and assayed for factor X_a activity. Each point is the mean of duplicate determinations and the experiment was repeated three times.

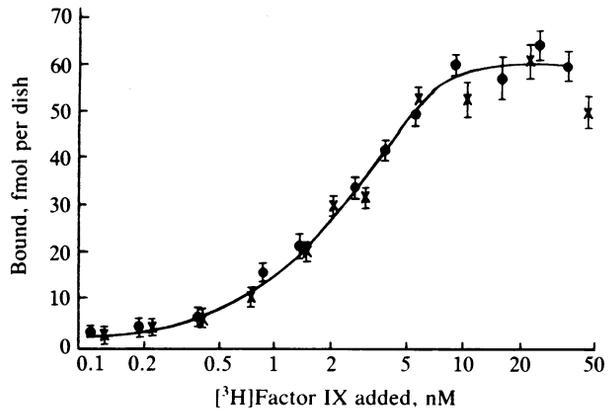

FIG. 3. Saturability of [^{3}H]factor IX binding to TPA-treated (x) and control (●) BAEC. Results are plotted semilogarithmically as specifically bound [^{3}H]factor IX against concentration of added tracer. Binding was studied by using confluent BAEC (1.35×10^6 cells per dish) after a 2-hr incubation at 4°C. Each observation was made in duplicate dishes and the experiment was repeated twice. Results represent mean ± SEM.

simultaneously with factors VII_a and X, factor X activation also occurs. Control experiments (Table 2) showed minimal activation of factors IX and X in the absence of factor VII_a with either PMA-treated or control cells.

Interaction of Factor X with Endothelial Cells. ^{3}H-labeled bovine factor X_1 binds in a time-dependent manner to BAEC (Fig. 4*A*). Studies of the calcium dependence of the binding (Fig. 4*B*) showed that specific binding increased with increasing amounts of added calcium in the incubation buffer to a maximum at 5 mM. Binding is partially reversible (Fig. 4*C*), as shown by the elution of cell-bound [^{3}H]factor X. This elution does not appear to be a first-order process and is thus similar to, though faster than, the complex dissociation kinetics of factor IX from BAEC (3). Cell-bound [^{3}H]factor X from the cells was virtually instantaneously eluted by EDTA (Fig. 4*C*). The lesser degree of displacement of bound factor X by unlabeled protein as compared with EDTA suggests that binding may be occurring to more than one site with different properties. Incubation of BAEC with increasing amounts of [^{3}H]factor X showed that binding was saturable with half-maximal binding at an added factor X concentration of 0.48 μM (Fig. 4*D*), which is ≈2.5 times the plasma concentration of factor X in humans. At saturation, 9.1×10^6 molecules were bound per cell. Competition studies in the presence of unlabeled factors X (40 μM), IX (35 μM), and

Table 2. Factor VII_a activation of factor IX bound to PMA-treated and untreated endothelial cells

Factor IX added, μg/ml	Factor VII_a added, μg/ml	Factor $VIII_t$ added	Factor X_a formed in 5 min, ng/ml: PMA-treated BAEC	Factor X_a formed in 5 min, ng/ml: Untreated BAEC
0	0	+	<50	51
0	1	0	60	<50
7	0	+	61	53
7	1	0	70	52
7	1	+	870	<50

Factor IX was incubated with PMA-treated or untreated endothelial cells at room temperature, then unbound ligand was washed away. Cells were incubated with factor VII_a, then washed, and formation of cell-bound activated factor IX was assessed by subsequent factor X_a formation after the addition of factors $VIII_t$ and X. Specific experimental conditions were the same as those described in Table 1 except that BAEC were P_4. Each value is the mean of duplicate determinations and the experiment was repeated twice.

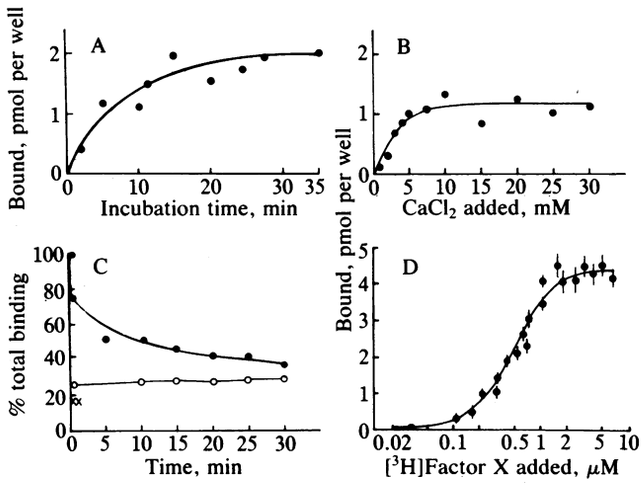

FIG. 4. Factor X binding to BAEC. (*A*) Time course of specific [^{3}H]factor X binding to BAEC at 23°C. Confluent wells of BAEC (2.6×10^5 cells per well) were incubated with [^{3}H]factor X (0.45 μM) alone or with tracer along with unlabeled factor X (44 μM). Cells were washed four times, then solubilized, and radioactivity was determined. Nonspecific binding (not shown) was 27% of total binding. Specific binding is the difference between total and nonspecific binding. For each experiment (*A–C*), the mean of duplicates is plotted and experiments were repeated three times. (*B*) Calcium dependence of [^{3}H]factor X-specific binding to BAEC. BAEC (2.7×10^5 cells per well) were incubated with [^{3}H]factor X (0.25 μM) alone or together with unlabeled factor X (30 μM) in buffer A containing bovine serum albumin at 2 mg/ml and the indicated concentration of $CaCl_2$ for 20 min at 23°C. (*C*) Elution of [^{3}H]factor X from BAEC in the presence of unlabeled factor X and EDTA. BAEC (2.5×10^5 cells per well) and [^{3}H]factor X (0.45 μM) were incubated at 23°C. To one series of wells, unlabeled factor X (44 μM) was added at the beginning of the experiment (○). To a second set of wells, the same amount of unlabeled factor X was added at 20 min (●). To a third set of wells, EDTA (final concentration, 10 mM) was added at 20 min (×). 0 time is the time after addition of unlabeled factor X (●) or EDTA (×) or 20 min after the start of incubation (○). (*D*) Saturability of [^{3}H]factor X binding to BAEC. Results are plotted semilogarithmically as specifically bound [^{3}H]factor X against concentration of added tracer. Binding was studied by using confluent BAEC (2.9×10^5 cells per well) after a 40-min incubation at 23°C. (*A–C*) Results represent means of duplicate determinations and each experiment was repeated three times. (*D*) Results represent mean ± SEM of duplicate determinations and the experiment was repeated four times.

prothrombin (50 μM) showed that only factor X inhibited the binding of [^{3}H]factor X (0.5 μM). No specific binding of [^{3}H]factor X to the wells occurred in the absence of cells. $NaDodSO_4$/PAGE of cell-bound [^{3}H]factor X eluted with EDTA (Fig. 5) is identical to that of the original factor X tracer. Without reduction, both cell-associated and native tracer have a M_r of 56,000 (Fig. 5*A*) and with reduction of 40,000 (Fig. 5*B*), indicating that the radiolabel is on the heavy chain. There is no evidence of covalent complex formation with a cell-associated protein. Analysis of [^{3}H]factor X that still remained bound after EDTA elution was carried out by dissolving the cells in 5 mM Tris·HCl, pH 7.5/140 mM NaCl/0.5% Nonidet P40 and electrophoresing (data not shown) under the same conditions as in Fig. 5. The cell-associated factor X was also similar to the native tracer. During the incubation of [^{3}H]factor X with BAEC at 23°C for 50 min, there was no increase in the baseline solubility of the surface-bound tracer or tracer in the supernatant in 5% trichloroacetic acid (<5% of the total radioactive material is soluble in trichloroacetic acid).

Association of Factor X_a with Endothelial Cells. Binding of factor X_a to BAEC was not studied directly; however, when factor IX_a bound to BAEC was incubated with factors $VIII_t$ and X in the presence of calcium, the factor X_a formed was ≈50% cell associated (Table 3).

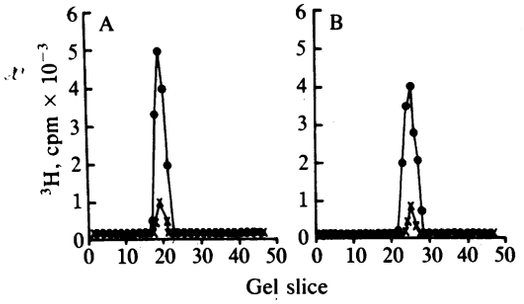

FIG. 5. $NaDodSO_4$/polyacrylamide (7.5%) gel electrophoresis of unreduced (*A*) and reduced (*B*) [^{3}H]factor X in solution (●) and after elution from BAEC (×). The latter sample was obtained by binding [^{3}H]factor X (0.9 μM) to BAEC at 23°C for 20 min and washing away unbound tracer. Bound factor X was eluted with 10 mM EDTA. For reduction, samples were made 10% in 2-mercaptoethanol. Samples were further processed and gels were run and sliced as described in Fig. 1.

DISCUSSION

Previous studies demonstrated specific, saturable, high-affinity, calcium-dependent binding of factors of IX and IX_a to cultured endothelial cells (1–3). As an initial step in determining the functional significance of cell-bound factor IX, we determined whether it could be activated by the contact and tissue factor pathways and found activation via both factor XI_a from the contact system and tissue factor–factor VII_a. We found, as did Lyberg and colleagues (21) using cultured human umbilical vein endothelial cells, that BAEC treated with PMA acquired tissue factor-like procoagulant activity whereas untreated cells were inactive, as reported previously (22, 23). Factor IX bound to PMA-treated endothelial cells was activated by factor VII_a, as indicated directly by cleavage of the molecule and indirectly by factor $VIII_t$-dependent activation of factor X. These data suggest physical contiguity of factors IX, $VIII_t$, X, and VII_a on the endothelial cell surface. Although cell-bound factor IX can be activated by both the intrinsic and extrinsic pathways, the relative efficiency of factor XI_a and factor VII_a–tissue factor activation on the cell surface and the comparative efficiency of activation on the cell surface and in solution remain to be determined.

Factor IX_a bound to BAEC has been shown to retain its specific procoagulant activity as assessed by ability to accelerate the clotting time of factor IX-deficient plasma (3). In the present study, purified proteins and direct assay of factor X_a formation were used to assess the activity of cell-bound factor IX_a. Factor IX_a bound to BAEC activates factor X as assessed by factor X_a cleavage of S2222. Factor $VIII_t$ and calcium are required but not exogenous phospholipid, indi-

Table 3. Association of factor X_a activity with endothelial cells

Sample	Factor X_a formed in 6 min, μg/ml
Whole reaction mixture	4.54
Supernatant	1.61
EDTA eluate of cell pellet	2.23

The protocol for whole reaction mixture activity was the same as in Fig. 2 for cells in suspension except that BAEC were P_4. For supernatant activity, the cell suspension was centrifuged at 135 × *g* for 5 min and then a 25-μl aliquot was taken for factor X_a determination. The cell pellet from the centrifugation was washed with 1 ml of buffer B, centrifuged, and then incubated with 0.1 ml buffer A/10 mM EDTA for 5 min at 23°C for the EDTA eluate sample. Each value is the mean of duplicate determinations and the experiment was repeated twice.

cating that endothelial cells can provide the needed phospholipid. How much factor X is cell associated under these conditions is not known. Relationships between these endothelial cell localized reactions and those which occur on the platelet surface is an unexplored area.

Activation of factor IX bound to PMA-treated endothelial cells by factor VII_a indicates that the sequence of activation on the cell surface could be factor VII_a cleavage of factor IX with subsequent factor VIII-dependent factor X activation. Alternatively, if factor X bound to BAEC, direct factor X activation on the cell surface could also occur. The data presented here show that factor X binds to BAEC and that, after activation, at least part of the factor X_a remains associated with the cells. Heimark and Schwartz (2) have also observed factor X binding to BAEC. Comparison of the binding of factors IX and X shows that both bind specifically, reversibly, and in a calcium-dependent manner. Activation does not occur with binding in either case. However, the cells bind factor IX with a much higher affinity than they bind factor X, though there are a greater number of factor X binding sites (2, 3). Since the cultured bovine aortic endothelial cell can bind factors IX and X, and under appropriate stimulation can acquire potent tissue factor-like procoagulant activity, further studies in this system are likely to provide information about the comparative efficiency and the interactions between the activation of factors IX and X on the cell surface. However, the significantly lower affinity of factor X binding suggests that on the cell surface factor VII_a activation of factor IX may occur preferentially. Further studies are required to define the association of factor X_a with the cell surface.

The concentrations of factor XI_a (37 nM), VII_a (4.4 nM), and X (300 nM) used in this study are similar to the concentrations of these proteins in human plasma whereas the concentration of cell bound factor IX_a (5.5 nM) is about 10% the concentration of factor IX in normal plasma. Although *in vivo* binding remains to be demonstrated, the activity of cell-bound factor IX_a *in vitro* suggests its potential physiologic importance. To determine the true physiologic significance of this cellular surface, studies with cultured cells are only the first step and must be followed up with experiments using perfused vascular beds in *in vitro* and finally *in vivo* studies.

These findings link a major portion of the prothrombin activation mechanism to the endothelial cell, starting with initiation by tissue factor and extending via factor IX to factor X activation. Factor V is an important cofactor for factor Xa activation of prothrombin (24), and the recent report of factor V synthesis by the endothelial cell (25) provides the basis for prothrombin activation on the cell surface. Perturbation of the cells, leading to synthesis and expression of tissue factor-like activity, which could then function as a cofactor in the activation of cell-bound factors IX and X, generating a potent cell-associated prothrombin activator, is a potential mechanism for initiating and localizing thrombosis on the endothelial cell surface.

D.M.S., M.D., W.K., P.N., and K.S.L. acknowledge our debt to Hymie L. Nossel for advice and support. Ronald Bach provided reagents and advice for the tissue factor assays. We wish to thank Karen Kaplan and Jeffrey Weitz for reading the manuscript. D.M.S. is supported by a Clinician–Scientist award from the American Heart Association. P.N. was supported by a grant from the Deutsche Forschungsgemeinschaft. This work was supported by grants from the National Institutes of Health (HL-15486, HL-21006, and HL-16919) and by Training Grant HL-07461.

1. Stern, D., Hurlet-Jensen, A. & Nossel, H. L. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41,** 1087 (abstr.).
2. Heimark, R. L. & Schwartz, S. (1983) *Biochem. Biophys. Res. Commun.* **111,** 723–731.
3. Stern, D. M., Drillings, M., Nossel, H. L., Hurlet-Jensen, A., LaGamma, K. S. & Owen, J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4119–4123.
4. Fujikawa, K., Thompson, A. R., Legaz, M. E., Meyer, R. G. & Davie, E. W. (1973) *Biochemistry* **12,** 4938–4945.
5. Kisiel, W. & Davie, E. W. (1975) *Biochemistry* **14,** 4928–4934.
6. Nossel, H. L. (1964) *The Contact Phase of Blood Coagulation* (Blackwell, Oxford), pp. 124–125.
7. Kisiel, W., Fujikawa, K. & Davie, E. W. (1977) *Biochemistry* **16,** 4189–4194.
8. Nemerson, Y. & Clyne, L. P. (1974) *J. Lab. Clin. Med.* **83,** 301–303.
9. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412.
10. Fujikawa, K., Legaz, M. E. & Davie, E. W. (1972) *Biochemistry* **11,** 4882–4891.
11. Silverberg, S. A., Nemerson, Y. & Zur, M. (1977) *J. Biol. Chem.* **252,** 8481–8488.
12. Kisiel, W., Hermodson, M. A. & Davie, E. W. (1976) *Biochemistry* **15,** 4901–4905.
13. Fujikawa, K., Legaz, M. E. & Davie, E. W. (1972) *Biochemistry* **11,** 4892–4899.
14. Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M. & Finlayson, J. S. (1977) *J. Biol. Chem.* **252,** 3587–3598.
15. Newman, J., Johnson, A. J., Karpatkin, M. H. & Puszkin, S. (1971) *Brit. J. Haematol.* **21,** 1–20.
16. van Dieijen, G., Tans, G., Rosing, J. & Hemker, H. C. (1981) *J. Biol. Chem.* **256,** 3433–3442.
17. Lowry, O. H., Rosebrough, N. M., Farr, L. S. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
18. Aurell, L., Friberger, P., Karlsson, G. & Claeson, G. (1977) *Thrombosis Res.* **11,** 595–609.
19. Bach, R., Nemerson, Y. & Konigsberg, M. (1981) *J. Biol. Chem.* **256,** 8324–8331.
20. Lindquist, P. A., Fujikawa, K. & Davie, E. W. (1978) *J. Biol. Chem.* **253,** 1902–1909.
21. Lyberg, T., Galdal, K. S., Evensen, S. A. & Prydz, H. (1983) *Br. J. Haematol.* **53,** 85–95.
22. Maynard, J. R., Dreyer, B. E., Stemerman, M. B. & Pitlick, F. A. (1977) *Blood* **50,** 387–396.
23. Rodgers, G. M., Broze, G. J. & Shuman, M. A. (1982) *Circulation* **66,** 810 (abstr.).
24. Davie, E. W. & Fujikawa, K. (1975) *Annu. Rev. Biochem.* **44,** 799–829.
25. Cerveny, T. J., Fass, D. N. & Mann, K. G. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42,** 4339 (abstr.).