Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 723-731

BINDING OF COAGULATION FACTORS IX AND X TO THE ENDOTHELIAL CELL SURFACE

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Received February 8, 1983

Bovine coagulation factors IX and X bind to independent sites on bovine aortic endothelial cells. Binding studies with cells maintained serum-free showed that there are at least two classes of binding sites for factor IX and factor X with a dissociation constant of 4.9×10^{-9} M and 2.1×10^{-8} M for the respective high affinity sites. Ca^{+2} was required for specific binding and was reversed by addition of EDTA or EGTA. Competition experiments showed that factor IX and factor IX_a bind to the same sites, which are different from the factor X binding sites. Neither binding of factor IX or factor X is inhibited by addition of prothrombin or protein C. Indirect immunofluorescence of factor IX indicated that binding was diffuse on the cell surface.

In the vascular response to injury, thrombin is of particular interest because of its central role in hemostasis and thrombosis as well as evidence that it modulates endothelial cell prostaglandin synthesis (1) and plasminogen activator release (2). Recently, it has been shown that protein C, a vitamin K-dependent plasma protein activated by thrombin, binds to a cofactor on the endothelium which increases the rate of activation several fold (3). This suggests the possibility of other interactions of coagulation factors with one another and with receptors on the endothelial surface. The activation of factor X and prothrombin by factor IX_a and factor X_a, respectively, is thought to occur on phospholipid surfaces in complexes with the accessory proteins, factor VIII and factor V (see reference 4 for review). This report demonstrates the independent binding of factor IX and factor X to multiple sites on the surface of cultured endothelial cells.

Abbreviations used are: SDS, sodium dodecyl sulfate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N,N'-tetraacetic acid; NP-40, Nonidet P-40; BSA, bovine serum albumin.

0006-291X/83 $1.50

METHODS

Cultured Bovine Endothelial Cells. Endothelial cells isolated from bovine aorta were grown in culture as previously described (5). For binding studies cells between passages 7 and 12 were plated in 16 mm culture wells and allowed to grow to confluence in Waymouth's complete medium supplemented with 10% fetal bovine serum. Once at confluence cells were either fed twice weekly or put in serum-free medium and maintained for 4 days before using in experiments (6). Saturation density was approximately 1.4×10^5 cells per cm^2.

Bovine Coagulation Factors. Factor IX (7), factor IX_a (8), factor X_1 (9), protein C (10), and prothrombin (11) were prepared from bovine plasma and were homogeneous by SDS-polyacrylamide gel electrophoresis (data not shown). Protein concentrations were determined from the published extinction coefficients. Factor IX and factor X_1 were radiolabeled with tritium (12) and the resulting specific activities of [^{3}H]factor IX and [^{3}H]factor X were 82,400 dpm/µg and 22,500 dpm/µg, respectively. The purified and radiolabeled proteins were generous gifts from Drs. M. Chopek and W. Kisiel.

Binding Studies. The culture medium was removed by suction and the cell layer was washed three times with modified Hank's balanced salts containing 15 mM HEPES, pH 7.4, and 0.5% BSA. Cells were then incubated with [^{3}H]factor IX or [^{3}H]factor X in 0.25 ml for 5 minutes at 22°C. The incubation was terminated by removing the medium and washing three times with cold Dulbecco's phosphate-buffered saline (PBS) containing 0.1% BSA. The cells were solubilized for 30 minutes with 0.5% NP-40 in 20 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl and 2 mM EDTA washed and counted in Aquasol (NEN) in a Packard TriCarb liquid scintillation counter.

Indirect Immunofluorescence Microscopy. After binding factor IX (27 µg) to endothelial cells maintained serum-free in a 35 mm dish, the monolayer was washed and fixed for 30 minutes with 3% paraformaldehyde in PBS containing 1% sucrose at 4°C. The reactions were stopped by repeated washing with 0.05 M glycine in PBS. The monolayer was then exposed at room temperature for 1 hour to 1 ml rabbit anti-factor IX (0.2 mg/ml) in PBS containing 1% BSA. Rabbit anti-factor IX, provided by Dr. K. Fujikawa, was treated as described previously (7) with barium sulfate and fractionated with ammonium sulfate. After washing with PBS the cells were incubated for 1 hour with swine anti-rabbit immunoglobulins coupled to fluorescein isothiocyanate (Dako) in PBS containing 1% BSA and then washed. Photographs were taken on a Zeiss photomicroscope III with Kodak Tri-X film at an ASA of 800.

RESULTS

Binding of Factor IX and Factor X to Endothelial Cells. Factor IX and factor X were purified to homogeneity from bovine plasma and radiolabeled by incorporation of tritium according to the method of Van Lenten and Ashwell (12). Previous studies had suggested that this method retained biological activity while labeling at a high specific activity (11). When [^{3}H]factor IX and [^{3}H]factor X were incubated with confluent cultured bovine aortic endothelial cells, then washed and solubilized with 0.5% NP-40 containing EDTA, binding was observed. Maximum binding at 0.3 µg/ml of both coagulation

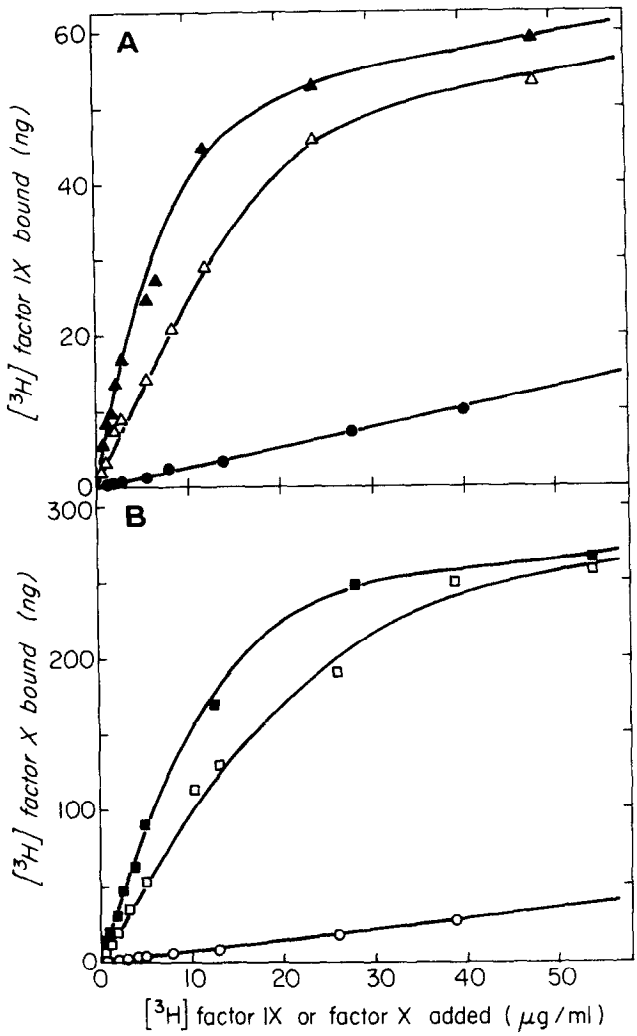

Figure 1. Binding of [^{3}H]factor IX and [^{3}H]factor X to confluent endothelial cells. Binding was measured as described in Methods to cells maintained in the presence of 10% fetal bovine serum or maintained for 4 days in the absence of serum. (A) Binding of [^{3}H]factor IX to cells maintained in the presence (△) or absence of serum (▲). (B) Binding of [^{3}H]factor X to cells maintained in the presence (□) or absence (■) of serum. Nonspecific binding was meausred in parallel reaction mixtures in the presence of a 100-fold excess of unlabeled factor IX (●) or factor X (○). Each point represents an average of three separate experiments.

factors to the cells was rapid and occurred within 5 minutes (data not shown). Additional incubation of up to 15 minutes showed no increase or decrease in bound radioactivity.

Binding of factor IX and factor X to endothelial monolayers was measured as a function of coagulation factor concentration. Initially the amount of cell-associated factor IX (Fig. 1A) or factor X (Fig. 1B) increased rapidly,

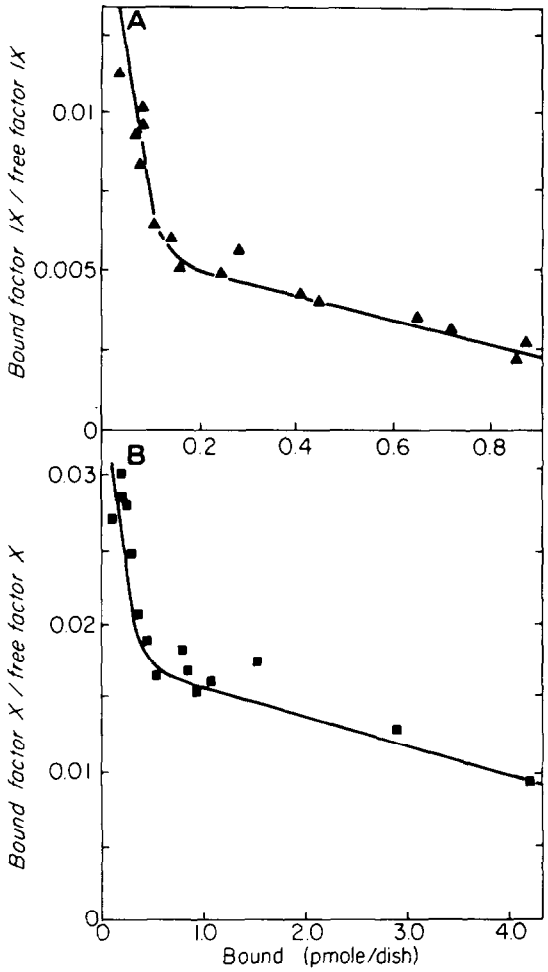

Figure 2. Scatchard analysis of specific binding of [^{3}H]factor IX (A) and [^{3}H]factor X (B) to confluent endothelial cells. Nonspecific binding was determined at each point and subtracted from the data. Least squares analysis was used to determine limiting slopes of the curves.

but at levels of 10 to 20 μg/ml the slope of the binding curve decreased and became constant. For cells maintained in the presence of 10% fetal bovine serum up to the time of the experiment, the binding of factor IX and factor X was decreased and required a higher concentration of added factor to reach saturation than cells maintained in the absence of serum. The corrected specific binding was saturable at 49 ng and 230 ng per 2.8 x 10^5 cells for factor IX and factor X, respectively. The half-maximal binding for [^{3}H]factor IX to serum-free cells was at 4.8 μg/ml and for [^{3}H]factor X was at 7.8 μg/ml.

The data for specific binding were analyzed in the form of a Scatchard plot (13) in Figure 2. When the binding data with cells maintained serum-free

TABLE 1

Additions	ng bound/well ± S.D.
[^{3}H]factor IX	24.0 ± 1.3
+ factor IX	1.6 ± 0.1
+ factor X	23.6 ± 1.8
+ prothrombin	21.3 ± 1.7
+ protein C	22.5 ± 1.6
+ factor IX_a	6.1 ± 0.3
+ EDTA	3.2 ± 0.1
[^{3}H]factor X	88.5 ± 2.6
+ factor IX	87.2 ± 2.9
+ factor X	3.3 ± 0.2
+ prothrombin	81.5 ± 7.6
+ protein C	75.5 ± 6.3
+ EDTA	8.2 ± 0.3

The effect of various additions on the binding of [^{3}H]factor IX and [^{3}H]factor X to endothelial cells maintained serum free. Either 1.1 µg of [^{3}]factor IX or 1 µg of [^{3}H]factor X and with the following additions: factor IX (109 µg), factor X (108 µg), prothrombin (96 µg), protein C (82 µg), factor IX_a (90 µg), and EDTA (5 mM) were added to the binding reaction as described in the Methods section.

were plotted, the graphs for factor IX and factor X were curvilinear indicating at least two apparent classes of sites of quite different affinity and number. Least squares analysis of the limiting slope of the Scatchard plots gave values for the high affinity dissociation constant for [^{3}H]factor IX of 4.9×10^{-9} M and for [^{3}H]factor X of 2.1×10^{-8} M. The number of high affinity sites per cell was 2.8×10^5 and 18.2×10^5 for factor IX and factor X. The apparent dissociation constants (K_d) for the low affinity sites were approximately the same for cells maintained in the presence or absence of serum. In addition, only the low affinity sites were seen with cells maintained in the presence of serum for factor IX $K_d = 2.4 \times 10^{-7}$ M and for factor X $K_d = 3.2 \times 10^{-7}$ M.

Specificity of Binding. The specificity of [^{3}H]factor IX and [^{3}H]factor X binding to endothelial monolayers was further shown by the ability of excess unlabeled factor IX and factor X to compete for binding of the respective labeled factor (Table 1). Addition of excess unlabeled factor IX_a also inhibited the binding of radiolabeled factor IX, apparently binding to the

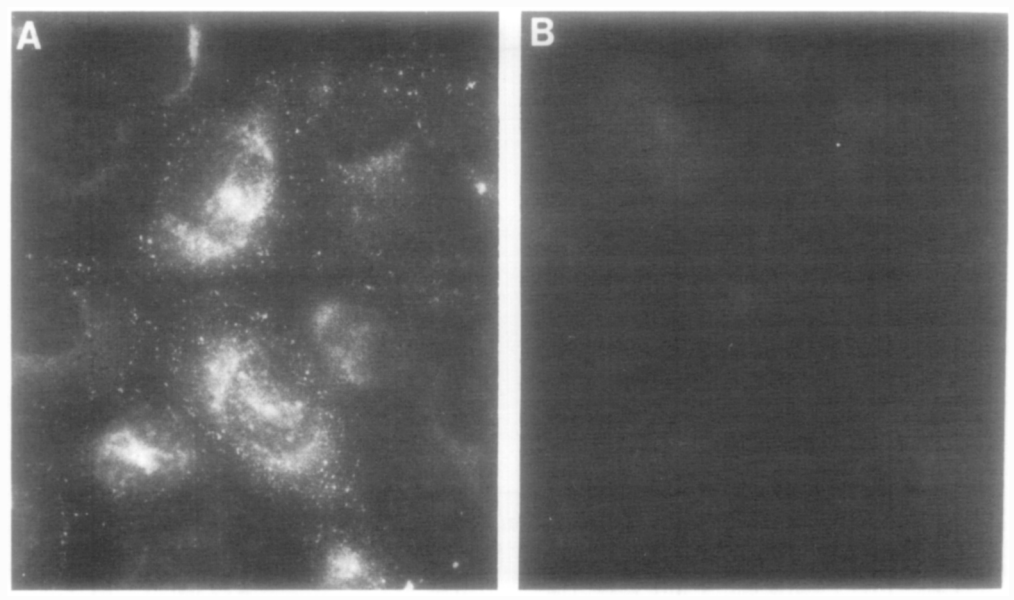

Figure 3. Indirect immunofluorescence with anti-factor IX. Monolayers of endothelial cells were incubated in the presence (A) or absence (B) of factor IX, washed, and then fixed in paraformaldehyde and processed for indirect immunofluorescent localization of binding.

same sites. Excess cold factor IX, however, did not inhibit the binding of [^{3}H]factor X and excess factor X did not inhibit the binding of [^{3}H]factor IX. Prothrombin or protein C do not compete with binding of either [^{3}H]factor IX or [^{3}H]factor X. The specific binding for both coagulation factors to endothelial cells required calcium in the incubation medium and was nearly completely inhibited by addition of either 5 mM EDTA or EGTA.

Indirect Immunofluorescence of Factor IX Binding. Monolayers of serum-free endothelial cells were incubated with factor IX, washed and fixed with paraformaldehyde. Then they were treated with rabbit antisera directed against purified factor IX and finally with fluorescein coupled swine anti-rabbit immunoglobulins. The fluorescence appeared as discrete randomly arranged dots on the entire surface of the cell (Fig. 3A). The fluorescence pattern was observed without permeabilization of the cells with membrane disrupting agents. Only faint nonspecific staining was observed in cells in the absence of factor IX (Fig. 3B) or rabbit anti-factor IX.

DISCUSSION

We have described the interaction of factor IX and factor X at independent high affinity sites on endothelial cells maintained in culture serum-free. The half-maximal binding of factor IX and factor X was similar to their plasma concentration which is approximately 5 and 10 μg/ml, respectively (7, 9). The high affinity binding sites have a K_d of 4.9×10^{-9} M for factor IX and of 2.1×10^{-8} M for factor X. These findings for factor IX are consistent with a recent abstract published while this work was in progress (14). In preliminary studies binding was also observed to bovine aortic smooth muscle cells for [^{3}H]factor IX and [^{3}H]factor X (data not shown).

Our data also show low affinity binding sites of factors IX and X to the endothelium. The dissociation constants for the low affinity binding sites for factor IX and factor X to endothelial cells are similar to the calcium-mediated binding of fragment 1 of prothrombin to phospholipid vesicles (15), although for factor X some differences have been reported (16, 17). The amino acid sequence in this part of the molecule shows extensive homology to the amino-terminal γ-carboxyglutamic acid containing part of the light chain of factor IX and factor X. Thrombin, which has lost fragment 1, also interacts with at least two independent classes of sites on endothelial cells with a K_d of 3×10^{-9} M for the high affinity sites with 4.4×10^4 sites/cell (18, 19).

Indirect immunofluorescence employing rabbit anti-factor IX shows that the coagulation factor binds to the endothelial surface. The presence of high affinity binding sites on the surface of the endothelium suggests an explanation for the early phase of rapid clearance seen in radiolabeled tracer studies in factor IX-deficient patients (20, 21). This may not represent a real clearance but binding to the surface of endothelium.

Platelets can shorten the *in vitro* clotting time of plasma, however, 20 times more platelet phospholipid is required than the activity present in intact membranes (22). This suggests that platelets provide more than a phospholipid surface. Thrombin but not prothrombin binds to platelets stimulating secretion and aggregation (23). Recently, high affinity binding

of factor X_a was shown to platelets which have undergone the release reaction induced by thrombin and further accelerates the activation of prothrombin (24, 25).

Our results suggest that zymogens of coagulation factors exist complexed with receptors on the endothelial surface ready to respond to the appropriate stimuli. Esmon and Owen (3) have already proposed that the complex of thrombin with a receptor enhances the activation of protein C, a potent anti-coagulant (10). Since the endothelium normally functions not only as a barrier but is active in maintaining compatibility between blood and the vessel wall, binding may inhibit activation. In contrast, we also need to consider the possible role of the endothelium in localizing coagulation in response to injury.

ACKNOWLEDGMENTS

We thank Drs. M. Chopek, W. Kisiel, and K. Fujikawa from the University of Washington, Seattle, for the generous gifts and helpful discussions. This work is supported in part by funds from the Washington Affiliate of the American Heart Association and from the National Heart, Lung, and Blood Institute from a Cardiovascular Pathology Training Grant (HL-07312). SMS is an Established Investigator of the American Heart Association.

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