The influence of phospholipid on thrombin-thrombomodulin-catalyzed activation of protein C has been studied by incorporating thrombomodulin into vesicles by dialysis from octyl glucoside-phospholipid mixtures. Thrombomodulin was incorporated into vesicles ranging from neutral (100% phosphatidylcholine) to highly charged (30% phosphatidylserine and 70% phosphatidycholine). Thrombomodulin is randomly oriented in vesicles of different phospholipid composition. Incorporation of thrombomodulin into phosphatidylcholine, with or without phosphatidylserine, alters the Ca²⁺ concentration dependence of protein C activation. Soluble thrombomodulin showed a half-maximal rate of activation at 580 μM Ca²⁺, whereas half-maximal rates of activation of liposome-reconstituted thrombomodulin were obtained between 500 μM Ca²⁺ and 2 mM Ca²⁺, depending on the composition (protein:phospholipid) of the liposomes. The Ca²⁺ dependence of protein C activation fits a simple hyperbola for the soluble activator, while the Ca²⁺ dependence of the membrane-associated complex is distinctly sigmoidal with a Hill coefficient greater than 2.4. In contrast, the Ca²⁺ dependence of γ-carboxyglutamic acid (Gla) domainless protein C activation is unchanged by membrane reconstitution (1/2max = 53 ± 10 μM) and fits a simple rectangular hyperbola.

Incorporation of thrombomodulin into pure phosphatidylcholine vesicles reduces the K_m for protein C from 7.6 ± 2 to 0.7 ± 0.2 μM. Increasing phosphatidylserine to 20% decreased the K_m for protein C further to 0.1 ± 0.02 μM. Membrane incorporation has no influence on the activation of protein C from which the Gla residues are removed proteolytically (K_m = 6.4 ± 0.5 μM).

The K_m for protein C observed on endothelial cells is more similar to the K_m observed when thrombomodulin (TM) is incorporated into pure phosphatidylcholine vesicles than into negatively charged vesicles, suggesting that the protein C-binding site on endothelial cells does not involve negatively charged phospholipids. In support of this concept, we observed that prothrombin and fragment 1, which bind to negatively charged phospholipids, do not inhibit protein C activation on endothelial cells or TM incorporated into phosphatidylcholine vesicles, but do inhibit when TM is incorporated into phosphatidylcholine:phosphatidylserine vesicles. These studies suggest that neutral phospholipids lead to exposure of a site, probably on thrombomodulin, capable of recognizing the Gla domain of protein C.

Thrombomodulin (TM) is a high affinity endothelial cell surface receptor for thrombin (1–4). The thrombin-thrombomodulin complex converts protein C, a vitamin K-dependent protein, to the anticoagulant enzyme activated protein C (5, 6).

TM has been purified to homogeneity from rabbit lung (7), bovine lung (8), and human placenta (9). TM is extracted from these tissues using detergent and cannot be extracted with high salt or low salt plus EDTA (7). This information, in conjunction with previous studies (1, 2) indicating that TM remains bound to the cell surface during protein C activation, suggests that TM is an integral membrane protein. TM, however, retains some biological activity after extraction with nonionic detergent (7–9). The properties of TM and the availability of significant quantities of purified protein suggested that this might prove a useful system for studying membrane involvement in coagulation.

In recent years, considerable interest has focused on the role of membranes in coagulation, largely because of the central role they play in assembly of the activation complexes (10). In the zymogen activations which lead ultimately to clot formation, a central theme is repeated. The enzyme, a pro tease, interacts with a regulatory protein on a surface, usually a membrane, to rapidly activate the substrate. In several of the complexes such as prothrombinase, the enzyme, regulatory protein, and substrate all bind reversibly to the membrane and bind effectively only to negatively charged membrane surfaces (10). Binding of two of the components, factor Xa and prothrombin, requires Ca²⁺ and the other component, factor Va, requires Ca²⁺ for stability. This makes the analysis of the role of Ca²⁺ or membrane surface charge on the function of any single component of the complex exceedingly difficult. A system more comparable to TM involves tissue factor. Tissue factor is an integral membrane protein which interacts with the vitamin K-dependent enzyme factor VII (VIIa) (11, 12) to activate either factor IX or factor X (13), both of which are also vitamin K-dependent factors. Thus, even in this system, it is difficult to directly determine the role of surface...
charge since it influences both the enzyme and substrate interaction with the surface. In addition, tissue factor is devoid of significant activity in solution, making comparative studies between the soluble and membrane-bound cofactor impossible.

TM is unique in the coagulation scheme of enzyme-effector activation systems. TM binds an enzyme, thrombin, which does not interact directly with the membrane. Thus, thrombin interaction is not dependent on the membrane surface charge. The thrombin-thrombomodulin complex activates protein C, which is a vitamin K-dependent protein with relatively lower affinity for bilayer membranes (12) than the other vitamin K-dependent proteins. TM, therefore, can be used to delineate the roles of membrane composition and Ca\(^{2+}\) on substrate activation without the added complication of altering the binding of the enzyme or the regulatory protein to the surface.

An additional question about expression of TM activity relates to the mechanism by which the endothelial cell surface promotes protein C activation. The endothelial cell surface is considered nonthrombogenic, yet TM on the endothelial cell surface saturates with respect to protein C at a 10-fold lower concentration (2) than soluble TM (9, 14). To determine whether this difference requires a specific membrane-binding site for protein C on the endothelial cell surface or whether the effect could be obtained with synthetic phospholipids, TM was incorporated into liposomes of varying phospholipid composition and the effect on protein C activation were evaluated.

**EXPERIMENTAL PROCEDURES**

**Materials**

1-Palmitoyl-2-oleoyl phosphatidylcholine (PC) and 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PS) were purchased from Avanti and used without further purification. Octyl glucoside was purchased from Behring Diagnostics. Aqueous, 2% scintillation mixture, formula 963, and L-1-palmitoyl-2-oleoyl-[oleoyl-1-\(^{14}C\)]phosphatidylcholine (51 mCi/mmol) were purchased from New England Nuclear. Frozen lungs from young rabbits were obtained from Sigma. Chymotrypsin was obtained from Worthington. All other chemicals were analytical reagent-grade.

**Methods**

**Protein Preparation**—Bovine protein C (15), thrombin (16), thrombomodulin (16), and antithrombin III (17) were purified by published methods. Glu-domainless protein C (14) and prothrombin fragment 1 (16) were made by previous methods; however, they were isolated on a fast protein liquid chromatography system equipped with a Mono Q HR5/5 column (Pharmacia).

**Purification of Thrombomodulin**—In order to purify sufficient TM for the phospholipid reconstitution studies, it was necessary to redesign the published TM preparative procedure (7) to increase the amount obtained and decrease the time required. The key step in the modified isolation procedure is the use of an immobilized monomeric monoclonal antibody to rabbit TM in place of the original thrombin column. The modified procedure is as follows: 80 pairs of frozen lungs from young rabbits were ground while frozen in a commercial, motor-driven meat grinder (International Edge Tool Co., Roseland, NJ). The lungs were ground three times before suspension. The ground lung was suspended in 8 liters of 0.25 M sucrose, 0.02 M Tris-HCl, 5 mM benzamidine-HCl, 0.02% NaN\(_3\), pH 7.5. All procedures throughout the preparation were performed at 4 °C. The tissue was collected by centrifugation at 4,800 \(\times g\) for 50 min. The pellets were resuspended by vigorous mixing with a stirring bar in sucrose buffer and collected by centrifugation as before. This process was repeated twice. The TM was extracted from the washed pellet with 2 liters of wash buffer made 2% in Triton X-100. The extraction was done with a 250-ml glass homogenizer with the pestle attached to a drill press. Insoluble material was removed by centrifugation at 40,000 \(\times g\) for 40 min. The extracts were quickly run through a 5 × 10-cm column of Sephadex G-50 to remove particulates, then adsorbed by adding 20 g of QAE-Sephadex G-50 pre-equilibrated in 2 liters of 0.4 M NaCl, 0.02 M Tris-HCl, 5 mM benzamidine-HCl, pH 7.5. The supernatant was decanted; 4) the Sephadex was packed back into the column and the effluent containing the TM activity was collected. The column was eluted with an additional 300 ml of 1 M NaCl in the above buffer. Unlike the extract, the QAE-Sepahedex eluate was a clear solution suitable for use with the monoclonal antibody column. The eluate (≈700 ml) was absorbed in a Teflon beaker with a monoclonal antibody to rabbit TM (70 ml coupled to Affi-Gel-10 (Bio-Rad) at a final concentration of 5 mg/ml antibody) for 2 h with gentle stirring; the gel was allowed to settle for 30 min, the supernatant siphoned off, and the beads packed into a 2.5 × 20-cm column. The column was washed sequentially with 4 liters of 1.0 M NaCl, 0.02 M Tris-HCl, 5 mM benzamidine-HCl, 0.5% Lubrol PX, 0.02% NaN\(_3\), pH 7.5, at a flow rate of 250 ml/h, 200 ml of this buffer without benzamidine-HCl, and 50 ml of 1 M NaCl, 0.5 mM guanidine-HCl, 0.02% M Tris-HCl, 0.5% Lubrol PX, pH 7.5. The TM activity was eluted with 1 M NaCl, 1.5 M guanidine-HCl, 0.5% Lubrol PX, 0.02% M Tris-HCl, pH 7.5. The flow rate on the last two steps was reduced to approximately 40 ml/h. TM was immediately desalted on a Sephadex G-25 column into 0.1 M NaCl, 0.02 M Tris-HCl, 0.1% Lubrol PX, pH 7.5.

TM was further purified by chromatography on a diisopropylphosphorothio- phosphor-thrombin Affi-Gel 10 column (0.6 × 30 cm) equilibrated in 0.2 M NaCl, 0.02 M Tris-HCl, 0.1% Lubrol PX, 0.5 mM Ca\(^{2+}\). The TM was made 0.5 mM in Ca\(^{2+}\), applied to the column, and the column was washed with 15 ml of equilibration buffer, then with 15 ml of 0.4 M NaCl, 0.02 M Tris-HCl, 0.1% Lubrol PX, 0.1 mM EDTA EDTA before eluting the TM with 2 M NaCl, 0.02 M Tris-HCl, 0.1% Lubrol PX, 0.1 mM EDTA, pH 7.5. Traces of degradation products were removed and the Lubrol PX concentration reduced to 0.02% by chromatography on a Bio-Gel A-15m (0.6 × 200 cm) equilibrated in 0.2 M NaCl, 0.02% M Tris-HCl, 0.02% Lubrol PX, pH 7.5, at room temperature. A total of 15 mg (5 ml, 2.8 mg/ml) was applied with the TM activity eluting as a clear solution in the void volume of the column. The yield from a single preparation was 8–12 mg.

Bovine TM was purified by modification of the published procedure (8). After cold methanol precipitation, the supernatant was batch-adsorbed with 800 ml of DEAE-Sepharose previously equilibrated in 0.075 M NaCl, 0.02 M Tris-HCl, 0.5% Lubrol PX, 25% MeOH, pH 7.5, for 2 h at 4 °C. The gel was washed with 6 liters of equilibration in a Buchner funnel, packed into a 5 × 40-cm column and eluted with 2.5 liters of 0.75 M NaCl, 0.02% M Tris-HCl, 0.5% Lubrol PX, 25% MeOH, pH 7.5. Protein-containing fractions were pooled and dialyzed against 6.5 volumes of 0.02 M Tris-HCl, 0.5% Lubrol PX, pH 7.5, for 6 h. The sample was then batch-adsorbed with 150 ml of diisopropylphosphorothio-thrombin/agarose (BioGel A-15m, Bio-Rad), washed with 15 ml of 0.1 M NaCl, 0.02% M Tris-HCl, 0.05% Lubrol PX, pH 7.5, and step-eluted with 1 M NaCl, 0.02 M Tris-HCl, 0.02% Lubrol PX, pH 7.5. Fractions containing activity were pooled and dialyzed against 0.1 M NaCl, 0.02% M Tris-HCl, 0.02% Lubrol PX, pH 7.5. If concentration was necessary, this material was batch-adsorbed to a small amount (30–50 ml) of DEAE-Sepharose and step-eluted as before. The TM was then applied to a Mono Q HR5/5 column attached to a fast protein liquid chromatography system and developed with a 20-ml linear gradient from 0.1 to 1.4 M NaCl in 0.02 M Tris-HCl, 0.02% Lubrol PX, pH 7.5. The gradient was held at 0.4 M NaCl until 280 nm of adsorbed material fell to zero. Protein C, thrombin, and prothrombin fragment 1 Ekm 10.1, 24,000 (16). Extinction Coefficients and Molecular Weights—The protein concentrations were obtained by the following methods: 5) the bovine rabbit, E\(_{220}\) 8.8 and 74,000 (7); TM, bovine, E\(_{220}\) 10.0, 84,000 (8); thrombin E\(_{220}\) 21, 37,000 (16); protein C, E\(_{220}\) 13.7, 62,000 (19); Glu-domainless protein C, E\(_{220}\) 13.7, 58,000 (14); prothrombin, E\(_{220}\) 15.0, 72,000 (16); and prothrombin fragment 1 E\(_{220}\) 10.1, 24,000 (16).

**Thrombomodulin—Enzyme beads (Bio-Rad) and Na\(^{2+}\) (ICN), were used to radioactive TSs according to the packaging protocol for 15 min at room temperature. After labeling, the free iodide was removed by extensive dialysis at 4 °C against TBS plus 0.02% Lubrol PX. The specific activity of the TM was 0.7 μCi/μg.
The labeled TM was stored at 4 °C.  

Phospholipid Preparation—The purity of the phospholipids was confirmed by thin layer chromatography as described by Hauser (20). Aliquots of the PC and the PS in chloroform were transferred to borosilicate glass tubes and dried to a thin film on the wall of the tube under a stream of N₂. The phospholipid film was placed under vacuum to remove the last traces of solvent. The dried film was then flushed with N₂, sealed tightly with Teflon, and stored desiccated at 4 °C.  

Reconstitution of Thrombomodulin into Phospholipid Vesicles—The conditions used to incorporate purified TM into phospholipid vesicles were derived from Mimms et al. (21). All procedures were carried out at room temperature, well above the transition temperature of the phospholipids. Octyl glucoside dissolved in TBS was added to the thin film of phospholipids at a 15-fold molar excess. The detergent phospholipid mixture was mixed and allowed to completely solubilize before mixing the PC and PS. TM in TBS containing 0.02% Lubrol PX was added, producing a final concentration of Lubrol PX of <0.001%. The initial molar ratio of phospholipid to protein was calculated to be 75,600:1 (assuming Mᵣ = 787 for the phospholipids) unless stated otherwise. In some experiments, tracer amounts of ¹²⁵I-TM and [³⁵S]phosphatidylcholine were added to the protein/phospholipid/membrane detergent mixture to quantify the protein and phospholipid in the final sample. The samples (0.2 ml of each mixture) were dialyzed against 1-3 changes, 1 liter each, of TBS at room temperature for 72 h. The phospholipid/protein mixture became turbid as the detergent was removed.  

Bovine TM was incorporated into liposomes by the same procedures. However, to allow direct comparison with kinetic parameters obtained using endothelial cells on tissue culture, the liposomes were formed and centrifuged in HEPES-buffered Hanks' salt solution (HyClone).  

Sucrose Density Centrifugation—The reconstituted TM (0.2 ml) was layered on top of a 30-ml 5-30% discontinuous sucrose gradient prepared in TBS and centrifuged in an SW 28 rotor (Beckman) for 12-16 h at 131,000 x g. In some preparations, the samples were incorporated into the 25% sucrose layer of the gradient before centrifugation. The sucrose gradient was formed and centrifuged in HEPES-buffered Hanks' salt solution (HyClone). Unless otherwise specified, all activations were performed in HEPES-buffered Hanks' salt solution (HyClone).  

As shown), the sucrose gradient was fractionated into 1-ml aliquots and [³⁵S]PC counted. The [¹²⁵I]-TM was counted on a Nuclear Enterprises NE 1600 instrument (Edinburgh, Scotland, UK). The [³⁵S]phosphatidylcholine was counted on a Beckman LS-100C counter. Samples were prepared for scintillation counting by the addition of 150 μl of 0.1% Triton X-100 to eliminate the quenching effect of the sucrose before the addition of 2 ml of Aquasol-2. After at least 2 h in the dark, the carbon-14 counts were measured and corrected for ¹²⁵I count spillover. The phospholipid content of each sample was calculated based on the specific activity of the starting protein/phospholipid mixture.  

Assay of Thrombomodulin Activity—The assay for activation of protein C or Gla-domainless protein C by purified soluble or reconstituted TM was performed as previously described (14). Unless otherwise specified, all activations were performed in TBS containing 0.1% gelatin and 5 mM Ca²⁺ at 37 °C. All proteins were dialyzed against TBS before use. To determine the relationship between the concentration of TM added and the incorporation, TM concentration was varied (5 pg/ml–1.0 mg/ml) at a constant concentration of phospholipid (20 μg/ml).  

RESULTS  

Reconstitution of Thrombomodulin into Phospholipid Vesicles—TM was reconstituted into phospholipid vesicles ranging in composition from 100% PC to 70% PC, 30% PS as described under “Experimental Procedures.” To determine the relationship between the concentration of TM added and the incorporation, TM concentration was varied (5 pg/ml–1.0 mg/ml) at a constant concentration of phospholipid (20 μg/ml). To assess incorporation, [¹²⁵I]-TM was added as a tracer. Sucrose density gradients were utilized (see “Experimental Procedures,” Methods) to separate the incorporated from the free TM. TM, determined by both [¹²⁵I]-TM incorporation and functional activity, was separated into two regions. Functional TM activity was measured using Gla-domainless protein C as the substrate to avoid overestimation of the TM concentration in the liposomes (see later sections). The top 5 ml of the gradient were rich in phospholipid and TM, while the TM that sedimented was devoid of phospholipid. As a control, TM was sedimented in the absence of phospholipid and resulted in <1% of the added TM being found in the top 5 ml. All of the detectable activity sedimented near the bottom of the gradient. The TM activity present in the top 5 ml of the gradient (16% of the total volume) was considered bound and plotted as a function of TM added (Fig. 1). TM incorporation increased as a nearly linear function of added TM up to 1 mg/ml TM. The total TM activity incorporated and the [¹²⁵I]-TM incorporated were in good agreement only if the TM activity was assayed after solubilization of the liposomes with Lubrol PX. Without detergent, the [¹²⁵I]-TM incorporated 2-2.5 times as high as the functional activity in the case of the PC vesicles and 3-3.5 times higher in the case of the PC/PS vesicles (Fig. 1). These studies imply that at least half of the TM is oriented toward the inside of the liposome. It is clear that the extent of incorporation or expression was not influenced to a substantial extent by the surface charge of the phospholipids employed. At the highest TM concentrations employed, 1 TM molecule was incorporated per 9,000 phospholipid molecules based on functional data and 1 per 10,000 based on [¹²⁵I]-TM incorporation.  

Estimation of Surface Expression of TM on the Phospholipid Vesicles—TM incorporation into membranes could alter the activity by at least three different mechanisms: 1) altering the
K_m), 2) altering K_m for protein C, and 3) altering the K_a for thrombin. To estimate the influence of TM incorporation on K_m, we attempted to measure the TM concentration on the surface of the liposomes. From previous studies, it is clear that TM binds thrombin with very high affinity (≤1 nM) (2, 7, 23, 24). Since TM concentrations could be obtained that were significantly higher than the K_m, we mixed TM-containing vesicles with excess thrombin to saturate surface TM, and the TM containing vesicles were then separated from free thrombin by flotation through a sucrose density gradient as described under “Experimental Procedures.” Three types of assays were performed: 1) thrombin determination by chromogenic substrate assay; 2) Gla-domainless protein C activation rate; and 3) protein C activation rate (Table I). The TM concentration based on thrombin binding and activity on Gla-domainless protein C correlated well for both the PC vesicles and the PC/PS vesicles. In contrast, the rate of protein C activation was substantially greater than either of the other values. These results suggest the Gla-domainless protein C can be used to assess surface expression of TM and that the protein C activation rate overestimates TM concentration.

Influence of Membrane Insertion and Surface Charge on Protein C Activation—To examine the mechanism by which membrane incorporation enhances the activation of protein C, we incorporated TM into vesicles ranging in composition from 100% PC to (70:30) PC/PS and studied the influence of protein C concentration on the rate of activation (Fig. 2A). Surprisingly, incorporation into 100% PC vesicles enhanced protein C activation by decreasing the K_m. Increasing the phosphatidylserine content decreased the K_m somewhat further. PS content did not influence the concentration dependence of Gla-domainless protein C activation (Fig. 2A, inset). The influence of phospholipid composition on protein C activation is shown in Table II. These results reflect pooled data from three separate experiments and were obtained by linear least squares analysis of the transformed data. One of the features we observed in attempts to measure the K_m for protein C in the systems that included phospholipid vesicles (especially negatively charged vesicles) was that above 1 μM protein C, significant deviation from simple Michaelis-Menten kinetics occurred. This appeared to correspond to the presence of a much lower affinity component (K_m ≈ 5 μM). This effect was not observed if Gla-domainless protein C was used as substrate. Because the concentrations are far above physiological and because this could be caused by many factors (i.e. substrate dimerization, vesicle fusion, etc.), these values were not included in the above data analysis and no attempt was made to treat the data as from a two-site model.

As can be seen from Table II, incorporation of TM into PC vesicles decreases the K_m for protein C at least 10-fold, while incorporation into PC/PS (80:20) vesicles decreases the K_m an additional 3- to 7-fold. Incorporation into vesicles had

![Figure 1](image1.png)

**Fig. 1.** Incorporation of thrombomodulin into phospholipid vesicles. TM, at the concentrations indicated, was incorporated into phospholipid and the liposomes isolated as described under “Experimental Procedures.” Concentration of total TM present in the liposomes was determined by incorporation of [³H]TM (•) and biological activity (●) assessed using Gla-domainless protein C as substrate in the presence of 0.1% Lubrol PX. Phospholipid concentration was determined based on [³H]PC incorporated. Surface-expressed TM was determined by biological activity towards Gla-domainless protein C in the absence of detergent (△). A, incorporation into 100% PC vesicles. B, incorporation into 80% PC, 20% PS vesicles.

![Figure 2](image2.png)

**Fig. 2.** Substrate concentration dependence of protein C activation by soluble and liposome-incorporated TM. A, TM liposome concentration was adjusted such that 0.07–0.1 μM expressed TM was present in the assay in the presence of 5 nM thrombin, 5 mM Ca²⁺, and the concentrations of protein C indicated on the horizontal axis. For illustration purposes, values were normalized to those obtained with 8 μM protein C. Soluble TM (●); TM incorporated into liposomes (100 μg of TM, 23 ng of phospholipid) of the following compositions: 100% PC (■), PC/PS, 80:20 (△), PC/PS, 80:20, (○); PC/PS, 70:30, (□). Inset, Gla-domainless protein C was used as substrate and values normalized to the rate obtained with 5 μM substrate, the highest concentration used. Bars indicate the range of values obtained for all TM preparations. The different preparations showed random variation within the values indicated. B, equivalent to A, except that bovine TM was used, and the buffer for lipidome preparation and kinetic assays was HEPES-buffered Hanks' salt solution. Soluble TM (●); TM reconstituted into 100% PC vesicles (■); 80%:20% PS/PC vesicles (△); bovine aortic endothelial cells, (□).

**TABLE I**

| Concentration of thrombomodulin in reconstituted liposomes | Surface expressed TM | Thrombin binding | Gla-domainless protein C biological activity | Protein C biological activity |
|-------------------------------------------------------------|----------------------|------------------|---------------------------------------------|-----------------------------|
| 100% PC  | 1.0 mg/mL | 0.5 mg/mL | 0.5 mg/mL | 0.5 mg/mL |
| 80:20, PC/PS | 1.0 mg/mL | 0.5 mg/mL | 0.5 mg/mL | 0.5 mg/mL |

See “Experimental Procedures” for experimental conditions.

*The concentration of TM listed corresponds to the apparent concentration based on a standard curve with soluble TM.

1. TM was incorporated into liposomes as described under “Experimental Procedures” at a ratio of 1 μg of TM, 23 mg of phospholipid.

2. Assays were performed in the presence of 0.1% Lubrol to disrupt the liposomes.
## TABLE II

**Kinetic constants of soluble thrombomodulin and liposome reconstituted thrombomodulin**

| Protein C* | Gla-domainless protein C* |
|------------|----------------------------|
|            | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ |
| Soluble TM | 7.6   | 370       | 7.2   | 260       |
| 100% PC    | 0.7   | 335       | 5.7   | 210       |
| 80:20, PC/PS | 0.1  | 214       | 6.2   | 230       |

* Protein C was varied from 0.08 to 8 μM.
* Gla-domainless protein C was varied from 0.5 to 8 μM.
* All reactions contained 0.1 nM expressed TM, 10 nM thrombin, 5 mM Ca$^{2+}$. Data were pooled from three experiments and the values shown were determined by linear regression of the transformed data. All r values were ≥0.99.

### Little or no effect on the $K_{cat}$ of activation

To eliminate the possibility that these results were unique to rabbit TM, or due to the use of proteins of heterologous species, the experiments were repeated with bovine TM (Fig. 2B) and virtually identical results were obtained. As indicated by the dashed line, values obtained with bovine endothelial cells in culture more closely approximate those of the TM in 100% PC vesicles than PS containing vesicles. The influence of the incorporation of TM into PC vesicles was surprising since vitamin K-dependent proteins do not bind significantly to these surfaces. Based on thin layer chromatography, it is unlikely the response results from contaminating negatively charged phospholipids.

### Inhibition of Protein C Activation by Prothrombin and Prothrombin Fragment 1

Under physiological conditions, protein C circulates at <5% of the concentration of prothrombin. Hence, unless protein C has a specialized site for interaction with membrane surfaces, prothrombin would effectively inhibit activation. To determine if the nature of the functional sites were different on the PC vesicles versus the PC/PS vesicles, the influence of prothrombin and prothrombin fragment 1 was investigated. Fragment 1 inhibited protein C activation when TM was incorporated into PC/PS vesicles with 50% inhibition occurring at 2.9 μM fragment 1 (Fig. 3A). With either soluble TM or TM incorporated into 100% PC vesicles, fragment 1 inhibition was not detectable.

Prothrombin was also studied as an inhibitor and gave results similar to those obtained with fragment 1 (Fig. 3B). Prothrombin demonstrated slight but reproducible enhancement of protein C activation with soluble TM. As with fragment 1, there was very little inhibition of protein C activation when TM was incorporated into phosphatidylcholine vesicles. When TM was incorporated into PC/PS (70:30) vesicles, prothrombin inhibited even more effectively than prothrombin fragment 1, with 50% inhibition occurring at 0.7 μM prothrombin.

This allowed examination of the nature of the protein C activation site on endothelial cells. Endothelial cell surface catalysis of protein C activation is very similar to that observed with 100% PC vesicles ($K_m$ = 0.7 μM) for the cell surface). To determine if the cell surface catalytic site is more similar to the PC vesicles than the PC/PS vesicles, protein C activation was studied over bovine endothelial cells in culture in the presence and absence of prothrombin or fragment 1.

### Ca$^{2+}$ Dependence of Protein C Activation

The previous sections indicate that incorporation of TM into either neutral or negatively charged liposomes enhances protein C activation by decreasing the $K_m$ for protein C, but has no influence on TM activation of Gla-domainless protein C. Ca$^{2+}$ is required for activation of either protein C or Gla-domainless protein C by the thrombin-TM complex. In addition, optimal binding of protein C to phospholipid vesicles requires Ca$^{2+}$. If the interaction between protein C and the site exposed upon reconstitution into PC vesicles also required Ca$^{2+}$, the Ca$^{2+}$ concentration dependence of the activation with soluble TM and membrane-incorporated TM might differ. Comparison of the Ca$^{2+}$ dependence of protein C activation with the soluble versus the membrane-incorporated form of TM did reveal substantial differences in the Ca$^{2+}$ dependence (Fig. 4). With purified TM, the dependence was characterized by a simple hyperbolic relationship between Ca$^{2+}$ concentration and initial rate of protein C activation. With TM incorporated into PC vesicles, the Ca$^{2+}$ dependence became distinctly sigmoidal. When these data are plotted using the Hill equation (25), a value of 2.4 for the Hill coefficient is obtained. When TM is incorporated into negatively charged vesicles, a very similar sigmoidal change is observed (Hill coefficient = 3.3). Half-maximal rates of activation are obtained at approximately 580 μM Ca$^{2+}$ when the TM is soluble. The Ca$^{2+}$ concentration required for half-maximal rates of activation for liposome-incorporated TM varied between 0.5 and 2 mM (Table III), depending on the TM/phospholipid ratio used to form the

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**Fig. 3. Effect of prothrombin and prothrombin fragment 1 on protein C activation.** *A,* protein C activation was measured in reactions containing 0.2 nM expressed TM, 4 nM thrombin, 1 μM protein C, and the indicated concentrations of fragment 1 for 6 min at 37°C. *B,* as in A, replacing prothrombin for fragment 1 at the concentrations indicated.

**Ca$^{2+}$ Dependence of Protein C Activation**—The previous sections indicate that incorporation of TM into either neutral or negatively charged liposomes enhances protein C activation by decreasing the $K_m$ for protein C, but has no influence on TM activation of Gla-domainless protein C. Ca$^{2+}$ is required for activation of either protein C or Gla-domainless protein C by the thrombin-TM complex. In addition, optimal binding of protein C to phospholipid vesicles requires Ca$^{2+}$. If the interaction between protein C and the site exposed upon reconstitution into PC vesicles also required Ca$^{2+}$, the Ca$^{2+}$ concentration dependence of the activation with soluble TM and membrane-incorporated TM might differ. Comparison of the Ca$^{2+}$ dependence of protein C activation with the soluble versus the membrane-incorporated form of TM did reveal substantial differences in the Ca$^{2+}$ dependence (Fig. 4). With purified TM, the dependence was characterized by a simple hyperbolic relationship between Ca$^{2+}$ concentration and initial rate of protein C activation. With TM incorporated into PC vesicles, the Ca$^{2+}$ dependence became distinctly sigmoidal. When these data are plotted using the Hill equation (25), a value of 2.4 for the Hill coefficient is obtained. When TM is incorporated into negatively charged vesicles, a very similar sigmoidal change is observed (Hill coefficient = 3.3). Half-maximal rates of activation are obtained at approximately 580 μM Ca$^{2+}$ when the TM is soluble. The Ca$^{2+}$ concentration required for half-maximal rates of activation for liposome-incorporated TM varied between 0.5 and 2 mM (Table III), depending on the TM/phospholipid ratio used to form the

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**Fig. 4. Effect of Ca$^{2+}$ on protein C activation by prothrombin and prothrombin fragment 1.** *A,* protein C activation was measured in reactions containing 0.2 nM expressed TM, 4 nM thrombin, 1 μM protein C, and the indicated concentrations of fragment 1 for 6 min at 37°C. *B,* as in A, replacing prothrombin for fragment 1 at the concentrations indicated.
endothelial cell surface. Thus, the site responsible for protein C recognition that forms upon incorporation into PC vesicles is specific for protein C and shares many properties in common with the endothelial cell surface. This phospholipid-dependent effect requires the presence of the Gla domain in protein C since no influence on the activation of Gla-domainless protein C is observed. These general characteristics of TM incorporated into PC vesicles are very similar for either rabbit or bovine TM. Since both the species and the preparative methods are very different, it is unlikely that the observed effects are due to a species specificity or to a loosely bound contaminant in the preparation. It should be noted that the rabbit TM was washed very extensively with 1 M NaCl containing buffer in the presence of high levels of detergent, and briefly with low levels of guanidine before elution with 1.5 M guanidine-HCl, further supporting the concept that the observed effect of phospholipid incorporation is dependent on a structural component of TM.

In many respects the observed influence of TM incorporation into phospholipids suggests the formation of a substrate-binding site. Support for this concept also can be derived from studies of the Ca\textsuperscript{2+} dependence. When protein C activation is studied with solubilized TM, the reaction is Ca\textsuperscript{2+}-dependent but not dependent on the presence of the Gla domain (14). The Ca\textsuperscript{2+} dependence of the activation rate is represented by a simple hyperbolic function. In contrast, when TM is incorporated into vesicles, the Ca\textsuperscript{2+} dependence is sigmoidal, suggesting positive cooperativity at least two sites. This characteristic is dependent on the Gla domain since both the Ca\textsuperscript{2+} dependence and the activation kinetics of the substrate were unaffected if this region was removed from the substrate proteolytically. In our view it is probable, but certainly not demonstrated unambiguously, that TM incorporation into vesicles results in expression of a site(s) on the TM molecule which interacts directly with the Gla domain of protein C in a Ca\textsuperscript{2+}-dependent process. Further support for this concept is presented in the accompanying paper.

Although this study does not directly address the question of the mechanism of TM-phospholipid interaction, it is likely that this interaction involves hydrophobic interactions with the phospholipid. This is supported by the observation that TM incorporated equally well into phospholipids that had no net charge (PC) or a high negative surface charge (PC/PS, 70:30). These findings are compatible with our earlier observations than nonionic detergents are required to extract TM from cell cultures or tissues. The recent demonstration of circulating soluble TM (26) is probably explained by limited proteolysis of the surface-bound molecule (26, 27), and not a reversible equilibrium between the bound and free state.

In a recent report by Freysinet et al. (28), human TM in Triton X-100 was added to sonicated liposomes and the influence on protein C activation studied. They observed a 3.2-fold increase in activity which they attributed to a V\textsubscript{max} effect with no observed change in K\textsubscript{m}. In their study this effect was dependent on negatively charged phospholipids. Both their quantitative and qualitative results are very different from those reported here. Several differences may explain these discrepancies. No direct interaction of TM with the vesicles was demonstrated, the method of incorporation was very different, and human TM was utilized. In our experience, it is difficult to prevent generation of altered forms of human TM during isolation. The kinetics of altered forms of TM may be affected differently by the presence of a membrane surface. It is interesting that Freysinet et al. (28) also observed that the Gla domain was essential for activation, suggesting that membrane interaction with the substrate is

### Table III

| Protein C [Ca\textsuperscript{2+}] | Hill coefficient | Gla-domainless PC [Ca\textsuperscript{2+}] |
|-----------------------------------|------------------|-------------------------------------------|
| Soluble TM                       | 0.58             | 0.042                                     |
| 100% phosphatidyl-choline         | 1.9              | 0.064                                     |
| 80:20, PC/PS                     | 1.9              | 0.059                                     |

\* Concentration of Ca\textsuperscript{2+} leading to half-maximal rate of activation determined by computer fit.
\* Activation reactions contained 0.1 nM thrombin, 1 nM protein C, or Gla-domainless protein C.
\* Soluble TM was present at 2 nM.
\* Liposomes were formed at a ratio of 1 mg of TM, 23 mg of phospholipid and their concentrations in the reactions adjusted to 2 nM expressed TM.

![FIG. 4. The role of calcium ions in protein C activation by reconstituted TM](image)

The activation mixtures contained protein C (1 μM), 0.1 nM thrombin, 2 nM expressed TM, and the concentrations of calcium indicated on the horizontal axis. Liposomes were made using 1 mg/ml TM, 23 mg/ml phospholipid. Soluble TM (●); TM in 100% PC vesicles (■); TM in 80% PC, 20% PS vesicles (○).

**DISCUSSION**

TM incorporation into neutral phospholipid vesicles results in substantially enhanced protein C activation due primarily to an approximate 10- to 20-fold decrease in K\textsubscript{m}. When negatively charged phospholipid is employed, the K\textsubscript{m} decreases further. Several observations suggest that it is unlikely that these negatively charged phospholipids play an important role in protein C activation by the thrombin-TM complex. First, the additional decrease in K\textsubscript{m} observed with the negatively charged phospholipids is almost totally reversed by the presence of physiological concentrations of prothrombin (≈2 μM). Second, whereas protein C activation by the vesicle system containing negatively charged phospholipids is inhibited by prothrombin, endothelial cell surface activation is not. Third, the K\textsubscript{m} for protein C obtained with TM incorporated into PC vesicles is very similar to that obtained on the
an essential component of the activation process.

The nature of the substrate recognition site exposed upon membrane reconstitution is uncertain. Recently, Bourin et al. (29) proposed that TM has an acidic heparin-like domain tightly associated (perhaps covalently) with the TM molecule. They felt that the ability of TM to inhibit thrombin clotting was mediated through this domain. Alternatively, such an acidic region could constitute the substrate-binding site. Nevertheless, the presence of membrane properties capable of supporting membrane reconstitution could play a role in properly orienting this site for interaction with the substrate. Regardless of the nature of the site, it is apparent that membrane properties capable of supporting coagulation (10).

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