Mechanisms by Which Soluble Endothelial Cell Protein C Receptor Modulates Protein C and Activated Protein C Function*

(Received for publication, November 29, 1999, and in revised form, December 10, 1999)

Patricia C. Y. Liaw‡‡, Pierre F. Neuenschwander‡‡, Mikhail D. Smirnov‡‡, and Charles T. Esmon‡‡§§§§

From the ‡‡Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, the Departments of ‡‡Pathology and ‡‡Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, and the §§Howard Hughes Medical Institute, Oklahoma City, Oklahoma 73104

The endothelial cell protein C receptor (EPCR) functions as an important regulator of the protein C anticoagulant pathway by binding protein C and enhancing activation by the thrombin-thrombomodulin complex. EPCR binds to both protein C and activated protein C (APC) with high affinity. A soluble form of EPCR (sEPCR) circulates in plasma and inhibits APC anticoagulant activity. In this study, we investigate the mechanisms by which sEPCR modulates APC function. Soluble EPCR inhibited the inactivation of factor Va by APC only in the presence of phospholipid vesicles. By using flow cytometric analysis in the presence of 3 mM CaCl₂ and 0.6 mM MgCl₂, sEPCR inhibited the binding of protein C and APC to phospholipid vesicles (Kᵦ = 40 ± 7 and 33 ± 4 nM, respectively). Without MgCl₂, the Kᵦ values increased approximately 4-fold. Double label flow cytometric analysis using fluorescein-APC and Texas Red sEPCR indicated that the APC-sEPCR complex does not interact with phospholipid vesicles. By using surface plasmon resonance, we found that sEPCR also inhibited binding of protein C to phospholipid in a dose-dependent fashion (Kᵦ = 32 nM). To explore the possibility that sEPCR evokes structural changes in APC, fluorescence spectroscopy studies were performed to monitor sEPCR/FI-APC interactions. sEPCR binds saturably to FI-APC (Kᵦ = 27 ± 13 nM) with a maximum decrease in FI-APC fluorescence of 10.8 ± 0.6%. sEPCR also stimulated the amidolytic activity of APC toward synthetic substrates. We conclude that sEPCR binding to APC blocks phospholipid interaction and alters the active site of APC.

The protein C anticoagulant pathway is well established as a physiologically important mechanism for inhibiting the coagulation process (reviewed in Refs. 1 and 2). The pathway is initiated when thrombin binds to thrombomodulin (TM) on the endothelial cell surface. The thrombin-TM complex rapidly activates protein C to generate the anticoagulant enzyme activated protein C (APC). APC, in combination with its cofactor protein S, limits the amplification and progression of the coagulation cascade by inactivating factors Va and VIIIa, a process that occurs on the phospholipid surface.

Recently, an additional component of the protein C anticoagulant pathway has been identified (3). This protein, named endothelial protein C receptor (EPCR), is an endothelial cell-specific, type 1 transmembrane protein that binds both protein C and APC with high affinity (Kᵦ ≥ 30 nM) (4). The interaction between EPCR and protein C/APC is mediated primarily by the Gla domain of the latter (5). Binding of EPCR to protein C enhances the rate of protein C activation by the thrombin-TM complex on the endothelial cell surface (6, 7) and in phospholipid reconstituted systems (8) primarily by decreasing the Km for protein C. This is physiologically relevant since the Km for protein C activation by the thrombin-TM complex is significantly higher (~1 μM) than the circulating plasma concentration of protein C (65 nM) (9–11). Since reconstitution experiments revealed little affinity of EPCR for TM (8), the main determinants of protein C activation appear not to be direct protein-protein interactions between EPCR and TM but rather the lateral mobility of EPCR in the membrane and the ability of EPCR to bind and present protein C to the thrombin-TM activation complex on the cell surface. Unlike TM, which is expressed at comparable density on the surface of the endothelium in most large and small blood vessels, EPCR is expressed at the highest levels on the endothelium of large vessels (7, 12). We hypothesize that one of the physiological roles of EPCR is to concentrate protein C on the endothelium and regulate blood coagulation on the surface of large vessels.

A soluble form of EPCR has recently been detected in normal human plasma (13) and has been shown to bind protein C and APC with an affinity similar to that of intact membrane-bound EPCR (4, 13). In healthy individuals, sEPCR circulates at a concentration of ~2.5 nM, a level that can increase up to 5-fold in patients with sepsis or systemic lupus erythematosus (14). In contrast to membrane-bound EPCR, sEPCR inhibits protein C activation over large vessel endothelium in culture. This presumably reflects competition between the sEPCR and cell surface EPCR. sEPCR also inhibits APC antiocoagulant activity (15), but the mechanism responsible for this inhibition remains unclear. Since EPCR interacts with the membrane-binding Gla domain of protein C (15), it is possible that binding to sEPCR and phospholipid is mutually exclusive. Alternatively, sEPCR

* This research was supported in part by NHLBI Grant P01 HL54804 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ Recipient of a Research Fellowship from the Heart and Stroke Foundation of Canada.

§ § Supported by NHLBI, National Institutes of Health Grant P50 54502 (to Naomi L. Eson).

‡‡§§ Investigator with the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute, Oklahoma Medical Research Foundation, 825 NE 13th St., Oklahoma City, OK 73104. Tel.: 405-271-7571; Fax: 405-271-3137; E-mail: Charles-Esmon@omrf.ouhsc.edu

The abbreviations used are: TM, thrombomodulin; APC, activated protein C; FI-APC, APC labelled at the active site with fluorescein; EPCR, endothelial cell protein C receptor; sEPCR, soluble EPCR; RU, resonance units; PC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidyl-choline; PS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine; PE, 1,2-dilinoleoyl-sn-glycerol-3-phosphatidylethanolamine; ATA-PPACK, N’-[acetylthiolo]acyetyl]-FPR-chloromethyl ketone.
could mask the factor Va binding site on APC or alter the macromolecular substrate specificity of the enzyme by altering the conformation of the extended substrate binding pocket. The goal of the current study is to elucidate which, if any, of the above mechanisms are responsible for the ability of sEPCR to modulate APC function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human protein C and APC (16), human protein S (17), and human EPCR (8) were prepared as described previously. Recombinant soluble EPCR (sEPCR) was isolated as described by Fukudome et al. (4). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (PS), and 1,2-dilauroyl-3-sn-glycero-3-phosphatidylcholine (PE) were from Avanti Polar Lipids, Inc. (Alabaster, AL). 1-Palmitoyl-2-[1-14C]oleoylPC was from NEN Life Science Products. Polystyrene latex beads were from Sigma. n-Octyl-p-glucopyranoside was from Calbiochem. Texas Red-x-succinimidyld ester and 5-iodoacetamidofluorescein were from Molecular Probes Inc. (Eugene, OR). H-57-carboxybenzoxazyl-L-prolyl-L-arginine-p-nitroanilide diactate (Sp-PCa) and H-5-hexahydrotryosyl-t-alanyl-L-arginine-p-nitroanilide diactate (Sp-Ipa) were from American Diagnostica (Greenwich, CT). H-Val-L-leucyl-L-arginine-p-nitroanilide dihydrochloride (S-2268) and H-isoleucyl-L-prolyl-L-arginine-p-nitroanilide-dihydrochloride (S-2288) were from Chromogenix (Molndal, Sweden). All other chemicals were of the highest grade commercially available.

**Preparation of Phospholipid Vesicles**—Sonicated vesicles were prepared as described previously (18). FC:PS:PE vesicles were 40% FC, 20% PS, and 40% PE.

**Influence of sEPCR on Factor Va Inactivation by APC—**Factor Va (0.2 nm), unilamellar, sonicated FC:PS:PE vesicles (10 µg/ml), and various concentrations of sEPCR (up to 1 µM) were incubated in the absence and presence of APC (5 or 20 pM) in HBS (20 mm Hepes, pH 7.4, 100 mM NaCl), containing 3 mM CaCl2, 0.6 mM MgCl2, and 0.1% gelatin. All reactions were run at room temperature in 96-well polystyrene plates (Costar, Cambridge, MA). After 30 min, the reaction was terminated by the addition of p-(amidinophenyl)methanesulfonyl fluoride (50 µM). Residual factor Va activity was monitored by its ability to enhance prothrombin activation in the presence of excess factor Xa (2 nM), prothrombin (1.4 µM), and 3 mM CaCl2. After activation for 5 min, the reactions were stopped with EDTA (8 mM). Thrombin formed during the activation was determined by amidolytic activity with Spectrozyme PCa as the substrate (0.2 nm).

**Fluorescent Labeling of HPC2 Monoclonal Antibody—**HPC2, a monoclonal antibody against human protein C, was labeled with fluorescein isothiocyanate as described by Goding (20).

**Fluorescent Labeling of sEPCR—**sEPCR was labeled with the amine-reactive probe Texas Red succinimidyld ester as described by the supplier (Molecular Probes, Eugene, OR). Briefly, 350 µl of 1.72 mg/ml sEPCR in 0.1 M sodium bicarbonate was incubated with 26 µl of 10 mg/ml Texas Red (in Me2SO) for 1 h at room temperature. The conjugate was then separated from unreacted probe on a PD-10 gel filtration column (Amersham Pharmacia Biotech), and the labeled EPCR was stored at −70 °C.

**Fluorescent Labeling of HPC2 Monoclonal Antibody—**HPC2, a monoclonal antibody against human protein C, was labeled with fluorescein isothiocyanate as described by Goding (20).

**Adsortion of Liposomes onto Latex Beads—**A 5% suspension of polystyrene latex beads (Sigma) (500 ml) was pelleted in Eppendorf tubes and washed 3 times with HBS by centrifugation at 6,000 × g for 1 min. Liposomes (200 µl at 2 mg/ml total phospholipid in HBS) were added to the beads and incubated overnight at room temperature with shaking. 30 µl of 140 mg/ml bovine serum albumin was added to the mixture and mixed for an additional 2 h. After three washes with HBS containing 1 mg/ml bovine serum albumin, the beads were resuspended in HBS containing 0.02% NaN3. Total phospholipid concentration was determined by the method of VanBlerkom (23). The mixing and washing were performed using a Beckman model LS 6000 SE scintillation counter. The final concentration of phospholipid was 685 µg/ml latex suspension.

**Flow Cytometric Analysis of Protein-Phospholipid Binding—**The affinities of Fl-APC and protein C for FC:PS:PE phospholipids were determined as follows. Briefly, liposome-adsorbed latex beads (0.1 mg of total phospholipid/ml) were suspended in HBS containing 1 mM CaCl2, 1% bovine serum albumin, 0.02% NaN3, in the absence or presence of 0.6 mM MgCl2. The solution was incubated with increasing concentrations of Fl-APC at 25 °C for 10 min in the dark. To monitor protein C binding to the liposome-adsorbed beads, the solution was first incubated with various concentrations of protein C for 15 min at room temperature, then with 8 Fl-HPC2, a fluorescently labeled monoclonal antibody to protein C for 10 min at room temperature. Binding was analyzed on a FACSCalibur (Becton Dickinson). Values of Kd were determined by fitting binding isotherms with a hyperbolic equation using the TableCurveTM program (Jandel Scientific, San Rafael, CA). To calculate Kd values, 22 nM Fl-APC or 22 nM protein C (+Fl-HPC2) was incubated with 4.5 µM FC:PS:PE in the presence of increasing amounts of Fl-APC. 4.5 µM sEPCR-coated FC:PS:PE (40:20:40) vesicles, at a concentration of 76 nM, protein C/APC (21, 22). The Kd values were determined using the following quadratic Equation 1,

\[
F_\text{C} - 1 - \frac{[\text{E}] + [\text{Kd}]}{\sqrt{[\text{E}] + [\text{Kd}] + (\frac{[\text{E}] - [\text{Kd}])^2}}} = \frac{1}{2[E]}
\]

\[(\text{Eq. 1})\]

where [E] is the initial fractional concentration of Fl-APC or protein C and [I] is the concentration of sEPCR. The Kd value was then calculated from Kd, using the following relationship (shown in Equation 2),

\[
K_{\text{diss}} = K_d \left(1 + \frac{U}{K_d}\right)^2
\]

where \([I]\) is the lipid-binding site concentration, and \(K_d\) is the experimentally determined dissociation constant for the protein C–or APC-lipid interaction. All data were fitted to the indicated equations using TableCurveTM.

**Protein-Phospholipid Binding Studies Using Surface Plasmon Resonance—**Real-time biomolecular interactions between sEPCR, protein C, and lipid were studied using a BIAcoreTM 1000 biosensor instrument (Biacore Inc.). Unless stated otherwise, all experiments were performed at 25 °C at a flow rate of 10 µl/min in 50 mM Heps, pH 7.5, 150 mM NaCl containing 3 mM CaCl2, and 0.6 mM MgCl2. All buffer solutions were filtered through a 0.2-µm membrane and degassed thoroughly prior to use.

Phospholipid vesicles were captured onto the surface of a Pioneer L1 sensor chip (Biacore Inc.), which contains dextran modified with lipo-philic compounds. Unilamellar, sonicated FC (100%), FC:PS:PE (80:20), or FC:PS:PE (40:20:40) vesicles, at a concentration of 76 µM, were introduced onto the surface of the sensor chip for 30 s, which produced a signal of approximately 2000 resonance units (RU). Binding of protein C or protein C-sEPCR complexes to the phospholipid membrane was then monitored in real time by measuring changes in RU (1000 RU corresponds to ~1 ng of bound protein/mm²). After each protein injection, the sensor chips were regenerated by the injection of 10 µl of 40 mM octyl glucoside, followed by washing for 2 min with buffer before reinjecting phospholipid vesicles for the next cycle. For each set of experiments, the proteins were introduced onto the surface of a sensor chip that lacked phospholipids (control sensor chip). The sensorsgrams of the control sensor chip were subtracted from the sensorsgrams of the lipid-containing flow cells to remove the effects of nonspecific binding to the dextran surface.

The determination of kinetic parameters for the binding reactions was performed using the BiaEvaluation 3.0 software. The dissociation rate constant (1/kd) and association rate constant (k+a) were determined separately from individual dissociation and association kinetics data, respectively, using a simple 1:1 binding model. The value of the equilibrium dissociation constant, Kd, was calculated from the ratio k/d/k+a.
the sEPCR-dependent fluorescence intensity change of Fl-APC. Fluorescence studies were performed on 900 μl of 12.5 nM Fl-APC in a 1 × 1-cm quartz cuvette using an SLM 8000 photon-counting spectrofluorometer. The sample was stirred continuously with a magnetic stirrer. Excitation and emission wavelengths were set to 490 and 520 nm, respectively, with excitation and emission slit widths of 4 nm. After readings were taken of Fl-APC alone (Io), known quantities of sEPCR were then added to the cuvette, and after 10 min of mixing, the change in fluorescence was monitored (I).

\[ I = \frac{\alpha}{2} \times \left( 1 + \frac{K_d + [sEPCR]}{[Fl-APC]} \right) - \frac{4x[sEPCR]}{[Fl-APC]} + 1 \quad (Eq. 3) \]

where \( \alpha \) is the maximum fluorescence change and assumes a stoichiometry of 1:1 (23).

**Influence of sEPCR on APC-mediated Hydrolysis of Chromogenic Substrates**—The chromogenic activity of 5 nM APC in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 3 mM CaCl2, 0.6 mM MgCl2, 0.1% (w/v) gelatin was determined with 0–250 mM various chromogenic substrates in the absence or presence of 200 mM sEPCR. A 50-μl aliquot of reaction solution was mixed with 50 μl of chromogenic substrate on a microtiter plate, and the change in absorbance with time at 405 nm was determined on a microplate reader (Molecular Devices). All samples were assayed in duplicate. For each substrate, the \( K_d \) (nM) and \( k_{cat} \) (s⁻¹) values were calculated by nonlinear regression analysis using the Michaelis-Menten equation in TableCurve (Jandel Scientific, San Rafael, CA).

**TABLE I**

| System          | \( K_d \) (nM) | \( K_{cat} \) (s⁻¹) |
|-----------------|---------------|---------------------|
| \(+CaCl_2\) + MgCl₂ | 186 ± 8       | 40 ± 7              |
| \(+CaCl_2\) no MgCl₂ | 137 ± 58     | 33 ± 4              |

**FIG. 1. Effect of sEPCR on factor Va inactivation by APC in the presence (A) or absence (B) of PC:PS:PE vesicles.** A, PC:PS:PE vesicles (10 μg/ml) were incubated with various concentrations of sEPCR (0 nM to 1 μM) in the presence of factor Va (0.2 nM) and 0 nM APC (○), 5 nM APC (●), or 20 nM APC (ivre). B, various concentrations of sEPCR (0 to 1 μM) were incubated with factor Va (20 nM) in the presence of 1 nM APC (●) or 5 nM APC (ivre). The amount of remaining intact factor Va was assayed in a prothrombinase assay as described under “Experimental Procedures.”

**RESULTS**

**Influence of sEPCR on Factor Va Inactivation by APC**—Previous studies demonstrated that sEPCR inhibits APC anticoagulant activity in plasma in a dose-dependent fashion (15). In this study, we used a purified factor Va inactivation assay to investigate the membrane dependence of this phenomenon. As expected, in the presence of PC:PS:PE liposomes, sEPCR inhibited APC-mediated factor Va inactivation as measured by residual prothrombinase activity (Fig. 1A). However, in the absence of phospholipids, sEPCR had no effect on the ability of APC to inactivate factor Va (Fig. 1B). Thus, sEPCR prevented inactivation of factor Va by APC in a lipid-dependent manner.

**Lipid-Protein Interactions Measured by Flow Cytometry**—One possible explanation for the lipid dependence of the inhibitory effect of sEPCR on APC activity is that sEPCR inhibits the binding of APC to phospholipids. To explore this possibility, flow cytometry was used employing PC:PS:PE vesicles immobilized on polystyrene beads. Our studies were performed in the presence of 3 mM CaCl2 and 0.6 mM MgCl2, conditions that have previously been shown to be optimal for the binding of membrane-bound EPCR to APC (4). The data are summarized in Table I. Protein C and Fl-APC bind to PC:PS:PE vesicles with similar affinities both in the presence and absence of MgCl2. Holding the protein C and Fl-APC concentrations constant (22 nM), binding of these proteins to the liposomes decreased as a function of increasing sEPCR concentration, until virtually all of the binding was inhibited (not shown). The equilibrium inhibition constant (\( K_i \)) for sEPCR bound to protein C and Fl-APC at the liposome surface was 40 ± 7 and 33 ± 4 nM, respectively (Table I). In the absence of MgCl2, the \( K_i \) values increased approximately 4-fold, suggesting that the interaction between sEPCR and protein C and APC is magnesium–as well as calcium-dependent.

We next used double label flow cytometric analysis to see if Texas Red-sEPCR (sEPCR labeled with the amine-reactive probe Texas Red) could bind to the lipid surface in an APCDependent fashion. The average degree of labeling of Texas Red-sEPCR was 3.2 indicating that all three of the primary amino

\[ I_o = \frac{\alpha}{2} \times \left( 1 + \frac{K_d + [sEPCR]}{[Fl-APC]} \right) - \frac{4x[sEPCR]}{[Fl-APC]} + 1 \quad (Eq. 3) \]
concentration-dependent decrease in fluorescence emission in- a function of sEPCR concentration (Fig. 4). sEPCR elicited a ability to bind phospholipid vesicles. A plot of the maximum that sEPCR evokes structural changes in APC upon bind- (310 nM) was incubated with increasing concentrations of Fl-APC (0 to 275 nM) in the presence of PC:PS:PE-adsorbed latex beads (0.34 mg/ml) in HBS buffer containing 3 mM CaCl2 and 0.6 mM MgCl2. After washing, bound Fl-APC (●) or bound Texas Red-sEPCR (▲) was analyzed by flow cytometry. As a control experiment, binding of Fl-APC to lipids in the absence of Texas Red-sEPCR was monitored (○). Mean channel fluorescence is plotted for each Fl-APC concentration.

Surface Plasmon Resonance Studies—We next used surface plasmon resonance to see if the binding characteristics of the sEPCR-protein C complex to lipids are similar to those of the sEPCR-Fl-APC complex to lipids. Surface plasmon resonance is an optical, label-free measuring technique that monitors the binding interactions between molecular partners. Binding parameters were determined for the interaction between protein C or the protein C-sEPCR complex with immobilized PC:PS:PE vesicles formed on a lipophilic surface.

The interaction between protein C and immobilized PC:PS:PE vesicles was monitored by flowing various concentrations of protein C (from 0 to 7.5 μM) over the lipid-coated chip surface. From the overlaid dose-response binding curves, the Kd values of protein C binding to PC:PS:PE were calculated as 203 nM as described under “Experimental Procedures” (not shown).

To determine the binding characteristics of the protein C-sEPCR complex to PC:PS:PE vesicles, a fixed amount of protein C (0.5 μM) was preincubated with sEPCR at molar ratios of 1:0, 1:0.2, 1:1, 1:2, and 1:5 and injected over the surface of the lipid-coated chip. As shown in Fig. 3A, at a protein C:sEPCR molar ratio of 1:5, no binding of protein to the lipid surface was observed. The arrows indicate the start and end of the association phase of the interaction. These studies suggest that protein C, when in complex with sEPCR, loses the ability to bind phospholipid vesicles. A plot of the maximum RU values of the binding isotherms shown in A were plotted versus sEPCR concentration. The IC50 value (224 nM) was determined by nonlinear regression analysis of the curve.

Fluorescence Spectroscopy Studies—To explore the possibility that sEPCR evokes structural changes in APC upon binding, fluorescence intensity changes of Fl-APC were analyzed as a function of sEPCR concentration (Fig. 4) sEPCR elicited a concentration-dependent decrease in fluorescence emission intensity both in the presence and absence of MgCl2. The curve in the absence of MgCl2 was right-shifted, however, indicating decreased affinity between the proteins. To calculate the affinity of the Fl-APC-sEPCR interaction, the I/IO values were plotted versus sEPCR concentration. The data was fit to Equation 3 using nonlinear regression. In the presence of 3 mM CaCl2 and 0.6 mM MgCl2 (Fig. 4A), the calculated Kd value was 27 ± 13 nM and a maximum decrease in Fl-APC fluorescence of 10.8 ± 0.6%. In the absence of MgCl2, the Kd value increased to 71 ± 39 nM, again suggesting that the interaction between sEPCR and Fl-APC is magnesium- as well as calcium-dependent. The observed sEPCR-dependent changes in Fl-APC fluorescence intensity were specific because (a) there was no change in fluorescence intensity of Fl-APC when chicken egg albumin was used in place of sEPCR, (b) titration of the formed Fl-APC-sEPCR complex with unlabeled APC returned the I to the initial value of unbound Fl-APC, and (c) addition of 10 mM EDTA to the formed Fl-APC-sEPCR complex returned the I to the initial value of the unbound Fl-APC (data not shown).

We also examined the effect of sEPCR on the rates of APC-mediated hydrolysis of chromogenic substrates. These chromogenic substrates interact on the unprimed side of the primary specificity pocket of APC. The kinetic parameters of APC toward synthetic substrates in the absence and presence of sat-
urating concentrations of sEPCR are shown in Table II. The enhanced catalytic activities observed are consistent with the active site environment being altered upon binding of sEPCR to APC.

DISCUSSION

Previous studies demonstrated that sEPCR blocks the anticoagulant activity of APC in a plasma clotting system and factor Va inactivation in a purified system (24), but the molecular mechanisms responsible for these effects were not elucidated. Since our findings indicate that sEPCR blocks the ability of protein C and APC to interact with negatively charged phospholipid surfaces, an interaction that is necessary for efficient inactivation of factors Va and VIIIa by APC (2), this mechanism alone would be sufficient to explain the inhibition of APC anticoagulant functions. That inhibition of phospholipid binding is the primary mechanism responsible for the loss of APC anticoagulant activity is supported by the observation that sEPCR does not influence APC inactivation of factor Va in solution. The simplest explanation for the ability of EPCR to inhibit phospholipid binding by APC is that since EPCR interacts with the Gla domain of APC, this interaction sterically blocks access of EPCR to the membrane surface. This explanation would be consistent with the observations that the $K_d$ value determined in solution is essentially identical to the $K_i$ value for sEPCR inhibition of phospholipid interaction as determined by both flow cytometry and surface plasmon resonance. Further supporting this model, sEPCR did not interact with phospholipids even in the presence of APC suggesting that binding of APC/protein C to EPCR or phospholipid is mutually exclusive.

Whereas the concept of a simple blockage by sEPCR of the phospholipid interaction is attractive due to its simplicity and the known interaction of EPCR with the membrane binding Gla domain of protein C, it is not the only possible explanation for the inhibition of the phospholipid binding interactions. In this regard, the observation that sEPCR can alter the environment near the catalytic center of APC (indicated by the change in fluorescence yield of fluorescein near the active site in Fl-APC and by the increased cleavage rates of peptide chromogenic substrates) is indicative of rather global changes in APC conformation. Given these long distance effects in APC conformation, the possibility that the loss of lipid binding following complex formation with sEPCR is due to conformational changes in the Gla domain of APC cannot be excluded.

The observation that sEPCR alters the amidolytic activity of APC and the fluorescence intensity of Fl-APC suggests that sEPCR induces structural changes at the active site of the protease. Protein C is a vitamin K-dependent glycoprotein composed of a vitamin K-dependent Gla-rich membrane binding domain, a helical aromatic segment, two epidermal growth factor (EGF)-like domains, and a trypsin-like serine protease domain. From the crystal structure of Gla domainless protein C (25) and from fluorescence resonance energy transfer studies (26), the Gla domain, the major site of EPCR binding (5), and the protease domain are located far apart, approximately 60 Å. There are several possible explanations for the ability of sEPCR to modulate the active site of APC. Binding of sEPCR to the Gla domain of APC could induce a long range reorganization of the active site. To our knowledge, this would be the first report of direct allosteric linkage between a Gla domain and an active site, at least for a protein ligand. Related to linkage between the protease domain and the Gla domain, Persson and Petersen (27) have shown that metal ion (Ca$^{2+}$) binding to the Gla domain of factor VIIa stimulates the amidolytic activity of the protease, and Sugo et al. (28) have shown that all of the disulfide bonds in protein C are much more stable in the presence of Ca$^{2+}$ when the Gla domain is present again supporting the possibility of altering protease domain conformation by interactions with the Gla domain. An alternative possibility is that sEPCR binds not only to the Gla domain of APC but also to the anion-binding exosite, located on the prime side of the

![Fig. 4. Binding of sEPCR to Fl-APC. Increasing amounts of sEPCR were mixed with 12.5 nM Fl-APC in HBS buffer containing 3 mM CaCl$_2$ in the presence (A) or absence (B) of 0.6 mM MgCl$_2$. Changes in fluorescent intensity were monitored with constant mixing of the samples. I/I$_0$ is plotted versus peptide concentration, where I is the fluorescent intensity at a given concentration of sEPCR, and I$_0$ is the initial fluorescence intensity of Fl-APC. The $K_i$ values were determined by nonlinear regression analyses of the data (line).](https://www.jbc.org/)

### Table II

**Effect of sEPCR on the rates of APC-mediated hydrolysis of chromogenic substrates**

The chromogenic activity of 5 nM APC was determined with 0 to 2.5 mM of various chromogenic substrates in the absence or presence of 200 nM sEPCR. For each substrate, the $K_m$ and $k_{cat}$ values were calculated by non-linear regression analysis using the Michaelis-Menten equation. The values correspond to the mean and S.E. of at least two determinations.

| Substrate | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | $K_m$ + APC (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) |
|-----------|------------|---------------------|-----------------------------|------------------|---------------------|-----------------------------|
| Sp-PCa    | 183.5 ± 2.5 | 107.0 ± 3.4         | 0.58                        | 180.5 ± 0.5      | 140.6 ± 1.5         | 0.78                        |
| S-2266    | 135.5 ± 12.5| 23.3 ± 0.5          | 0.17                        | 167.3 ± 20.7     | 32.9 ± 0.25         | 0.20                        |
| S-2288    | 183.5 ± 15.5| 91.6 ± 1.7          | 0.50                        | 173.5 ± 8.5      | 114.5 ± 0.6         | 0.66                        |
| Sp-IIa    | 154.8 ± 8.4 | 126.9 ± 0.4         | 0.82                        | 153.5 ± 7.1      | 143.1 ± 0.25        | 0.93                        |
active site of APC (25), and/or to the 148 loop at the rim of the active site. Although previous studies indicate that the Glu domain is required for protein-protein contacts with EPCR, the data do not rule out the contribution of other regions of protein C to the interaction (5). The 148 loop has been shown to be conformationally linked to the substrate binding pocket of APC (29). A third possibility is that sEPCR also binds to the flexible linking peptide between EGF-1 and EGF-2 of APC, a region of high flexibility (25), and repositions the Glu domain so that it is no longer able to bind to phospholipids.

The present study provides the first direct evidence that Mg$^{2+}$ plays a direct role in protein-protein interactions between EPCR and APC. In the absence of MgCl$_2$, the $K_d$ for the sEPCR-APC interaction increases to 71 ± 39 nM (Fig. 4), indicating that, like the intact membrane-bound EPCR (4), the soluble form of EPCR also requires Mg$^{2+}$ for optimal interaction with APC. The influence of Mg$^{2+}$ on the EPCR-APC interaction seems quite specific to this complex since Mg$^{2+}$ had no discernible effect on the affinity of APC or protein C for phospholipid when the experiments were performed at saturating calcium ion concentrations (Table I).

The observation that sEPCR blocks factor Va inactivation only on membrane surfaces and appears to do so by blocking APC-phospholipid interaction might be taken as evidence that EPCR does not change APC substrate specificity. The ability of sEPCR to alter the fluorescence yield of FI-APC and to increase chromogenic substrate hydrolysis argues against this contention. In the case of the four chromogenic substrates tested, the enhanced catalytic activity against the substrates was due mainly to an increase in the $k_{cat}$ of the reaction (Table II). The increase in $k_{cat}/K_m$ values for substrate hydrolysis ranged from 1.13- to 1.34-fold (Table II). Although the magnitude of these changes is modest, they are comparable to those observed in the TM-mediated modulation of chromogenic substrate hydrolysis by thrombin (30). The interaction between TM and thrombin results in small changes in the rates of cleavage of peptide chromogenic substrates, yet interaction with TM switches the macromolecular specificity of thrombin from fibrinogen to protein C (31).

In normal individuals, the concentration of soluble EPCR is approximately 2.5 nM, but this level can rise to at least 13 nM in some disease states (14). This is still considerably less than the combined protein C activated protein C concentration of approximately 65 nM and hence would be expected to have a relatively small effect on the anticoagulant function of the system. There are two situations in which soluble EPCR levels might be sufficiently high to inhibit the protein C pathway. One is severe sepsis where the levels of protein C can be reduced to less than 10% of normal (32). Since EPCR levels are elevated in this disease and protein C levels are reduced, a significant percentage of the protein C/APC is likely to be in complex and therefore inactive as an anticoagulant and resistant to activation. A second situation where sEPCR levels might be sufficient to inhibit the protein C pathway is in the developing thrombus. Thrombin is known to be a major mediator of EPCR shedding in vivo (33). In the thrombus, where flow is restricted and thrombin concentrations relatively high, the thrombin-mediated shedding of the receptor could cause the local EPCR concentration to rise to levels where most of the protein C/APC is in complex, thus impairing protein C pathway functions.

The present results indicate that the ability of sEPCR to block APC binding to a phospholipid surface would be sufficient to explain the loss of anticoagulant function that occurs upon complex formation between these proteins. However, the finding that the active site conformation and activity toward synthetic substrates is altered upon complex formation suggests that the complex may have alternative substrates in solution. Consistent with this possibility, inhibition of protein C/APC interaction with EPCR exacerbates the host response to Escherichia coli, eliciting a capillary leak syndrome (34). Since EPCR concentrations are very low in the capillaries, these data suggest that the soluble EPCR-APC complex might play an important role in preventing vascular damage. The changes seen here in the catalytic activity of APC provide the first experimental data supporting this possibility.

Acknowledgment—We thank Dr. Naomi L. Esmon for critical reading of the manuscript and for many helpful discussions.

REFERENCES
1. Esmon, C. T. (1992) Arterioscler. Thromb. 12, 135–145
2. Castellino, F. J. (1995) Trends Cardiovasc. Med. 5, 55–62
3. Fukudome, K., and Esmon, C. T. (1994) J. Biol. Chem. 269, 26486–26491
4. Fukudome, K., Kurosawa, S., Stearns-Kurosawa, D. J., He, X., Rezaie, A. R., and Esmon, C. T. (1996) J. Biol. Chem. 271, 17491–17498
5. Regan, L. M., Mollica, J. S., Rezaie, A. R., and Esmon, C. T. (1997) J. Biol. Chem. 272, 26279–26284
6. Stearns-Kurosawa, D. J., Kurosawa, S., Mollica, J. S., Ferrell, G. L., and Esmon, C. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10212–10216
7. Fukudome, K., Ye, X., Tsuneyoshi, N., Tokunaga, O., Sugawara, K., Mizokami, H., and Kimoto, M. (1998) J. Exp. Med. 187, 1029–1035
8. Xu, J., Esmon, N. L., and Esmon, C. T. (1999) J. Biol. Chem. 274, 6704–6710
9. Owen, W. G., and Esmon, C. T. (1981) J. Biol. Chem. 256, 5532–5535
10. Galvin, J. B., Kurosawa, S., Moore, K., Esmon, C. T., and Esmon, N. L. (1987) J. Biol. Chem. 262, 2196–2205
11. Nishioka, J., Ido, M., Hayashi, T., and Suzuki, K. (1996) Thromb. Haemostasis 75, 275–282
12. Laszik, Z., Milro, A., Taylor, F. B., Jr., Ferrell, G., and Esmon, C. T. (1997) Circulation 96, 3633–3640
13. Kurosawa, S., Stearns-Kurosawa, D. J., Hidari, N., and Esmon, C. T. (1997) J. Clin. Invest. 100, 411–418
14. Kurosawa, S., Stearns-Kurosawa, D. J., Carson, C. W., D'Angelo, A., Della Valle, P., and Esmon, C. T. (1998) Blood 91, 725–727
15. Regan, L. M., Stearns-Kurosawa, D. J., Fukudome, K., Mollica, J., Fukudome, K., and Esmon, C. T. (1996) J. Biol. Chem. 271, 17499–17503
16. Esmon, C. T., Esmon, N. L., Le Bonne, B. F., and Johnson, A. E. (1993) Methods Enzymol. 222, 359–385
17. Taylor, P., Chang, A., Ferrell, G., Mather, T., Callett, R., Bicket, K., and Esmon, C. T. (1991) Blood 78, 357–363
18. Smirnov, M. D., Safa, O., Regan, L., Mather, T., Stearns-Kurosawa, D. J., Kurosawa, S., Rezaie, A. R., Esmon, N. L., and Esmon, C. T. (1998) J. Biol. Chem. 273, 9031–9040
19. Bock, P. E. (1988) Biochemistry 27, 6633–6639
20. Goding, J. W. (1976) J. Immuno. Methods 13, 215–226
21. Malthet, O., Gouescu, G. L., and Broderius, M. (1977) Biochemistry 16, 4172–4177
22. Prigent-Dachary, J., Faucon, J.-F., Boisseau, M.-R., and Dufourcq, J. (1986) EMBO J. 5, 6822–6831
23. Persson, E., and Petersen L C. (1995) Eur. J. Biochem. 234, 293–300
24. Sugio, T., Bjork, I., Holmgren, A., and Stenflo, J. (1984) J. Biol. Chem. 259, 4172–4177
25. Bode, W. (1996) Biochemistry 35, 9102–9108
26. Shen, L., Villoutreix, B. O., and Dahlback, B. (1999) Thromb. Haemostasis 82, 1078–1087
27. Ye, J., Liu, L., Esmon, C. T., and Johnson, A. E. (1992) J. Biol. Chem. 267, 11023–11028
28. Castellino, F. J., Stafford, A. R., and Weitz, J. I. (1997) J. Biol. Chem. 272, 25493–25499
29. Regan, L. M., Stearns-Kurosawa, D. J., Kurosawa, S., Mollica, J., Fukudome, K., and Esmon, C. T. (1996) J. Biol. Chem. 271, 17499–17503
30. Mather, T., Oganesyan, V., Hed, P., Huber, R., Foundling, S., Esmon, C., and Bode, W. (1996) EMBO J. 15, 6822–6831
31. Yegneswaran, S., Wood, G. M., Esmon, C. T., and Johnson, A. E. (1997) J. Biol. Chem. 272, 25013–25021
32. Peresson, E., and Petersen L C. (in press) Eur. J. Biochem.
33. Taylor, P. B., Jr., Kurosawa, S., Ferrell, G., Chang, A., Laszik, Z., Kusanne, P., Peer, G., and Esmon, C. T. (2000) Blood, in press
34. Taylor, P. B., Jr., Stearns-Kurosawa, D. J., Kurosawa, S., Ferrell, G., Chang, A., Laszik, Z., Kusanne, P., Peer, G., and Esmon, C. T. (2000) Blood, in press
Mechanisms by Which Soluble Endothelial Cell Protein C Receptor Modulates Protein C and Activated Protein C Function
Patricia C. Y. Liaw, Pierre F. Neuenschwander, Mikhail D. Smirnov and Charles T. Esmon

J. Biol. Chem. 2000, 275:5447-5452.
doi: 10.1074/jbc.275.8.5447

Access the most updated version of this article at http://www.jbc.org/content/275/8/5447

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 19 of which can be accessed free at
http://www.jbc.org/content/275/8/5447.full.html#ref-list-1