Effects of Calphobindin II (Annexin VI) on Procoagulant and Anticoagulant Activities of Cultured Endothelial Cells

Hideo YOSHIZAKI,*[,a] Sohei TANABE,[a] Koichi ARAI,[a] Akira MURAKAMI,[a] Yasushi WADA,[a] Masao OHKUCHI,[a] Yohichi HASHIMOTO,[b] and Masahiro MAKI[c]

Tokyo Research Laboratories, Kowa Co., Ltd.,[a] Noguchi-cho, Higashimurayama, Tokyo 189, Japan, Department of Biochemistry, Faculty of Science, Saitama University,[b] Urawa, Saitama 338, Japan, and Department of Obstetrics and Gynecology, School of Medicine, Akita University,[c] Akita, Akita 010, Japan. *Received November 21, 1991*

Effects of human placental calphobindin II (CPB-II) on the protein C activation and prothrombin activation on the cell surface of cultured calf pulmonary arterial endothelial cells have been investigated. CPB-II inhibited thrombin generation by factor Xa bound to the surface of the cultured endothelial cells in a dose-dependent manner. The amount (IC_{50}) of CPB-II causing the inhibition at 50% was estimated to be approximately 10 nM. CPB-II was found to be ineffective, however, in the protein C activation by thrombin–thrombomodulin (TM) complex on the cell surface. Assay using purified TM revealed that CPB-II was able to exhibit the inhibitory potency for the protein C activation exclusively in the reconstituted system with negatively charged phospholipids. These results suggest that the neutral phospholipids participate in the protein C activation through the thrombin–TM system on the endothelial cell surface. The ability of CPB-II to inhibit procoagulant activity without affecting anticoagulant activity on the cultured endothelial cells is probably related to its potential physiological function, while it is able to exert various degrees of influence upon these activities in blood coagulation by interacting with negatively charged phospholipids *in vitro*.

Keywords calphobindin; annexin; protein C activation; prothrombinase; endothelial cell

Introduction

In the previous works, we have reported the isolation and characterization of an anticoagulant protein, calphobindin II (CPB-II) from human placenta.[1] The primary structure was determined based on data of amino acid composition and sequence, and complementary deoxyribonucleic acid (cDNA) sequence analyses.[1-3] CPB-II was identified as annexin VI, a member of the Ca^{2+} dependent phospholipid-binding protein family. CPB-II did not affect the catalytic activity of factor Xa or thrombin, which convert prothrombin to thrombin or fibrinogen to fibrin, respectively, whereas it was able to act as inhibitor for the potentiation of prothrombinase activity by negatively charged phospholipid present as the constituent in prothrombinase complex *in vitro*. Competition assay using phospholipid vesicles revealed that CPB-II was able to compete with several coagulation factors for their binding to negatively charged phospholipids,[4] thus suggesting that anticoagulant activity of CPB-II might be attributable to its ability to block the binding of the coagulant factors to negatively charged phospholipids on the cell surface.

It has been established that thrombomodulin (TM), a thrombin receptor on the endothelial cell surface, is effective in enhancing thrombin-catalyzed protein C activation,[5] thereby providing activated protein C (APC) that acts as possible negative regulator for clotting process. The activity of thrombin–TM complex to active protein C was shown to be potentiated by phospholipids as assayed by a reconstituted system.[6,7] In this context, further evidence to solve the mechanism of anticoagulant activity of CPB-II is desirable since questions remain as to the incompatible ability of this protein which affects protein C activation by thrombin–TM complex and is active as a thrombotic factor on the endothelial cell surface.

In this paper, we report the influence of CPB-II upon prothrombin activation and protein C activation on the cultured endothelial cell surface. We also describe the effects of CPB-II on protein C activation by the thrombin–TM systems reconstituted in phospholipid vesicles and discuss the function of phospholipids on the surface of cultured endothelial cell.

Materials and Methods

Human antithrombin III (AT-III) was obtained from Green Cross Co., Ltd., Osaka. Heparin was purchased from Shimizu Pharmaceutical Co., Ltd., Shizuoka. Boc–Val–Pro–Arg–MCA, Boc–Leu–Ser–Thr–Arg–MCA and 7-amino-4-methyl-coumarin (AMC) were obtained from Peptide Institute Inc., Osaka. Russell's viper venom, phosphatidylcholine dilinoleoyl (PC) and bovine brain phosphatidylserine (PS), and bovine serum albumin (BSA) were from Sigma Chemical Co., MO. (U.S.A.). Bovine thrombin was purified from a commercial preparation from Mochida Pharmaceutical Co., Ltd., Tokyo by the method of Lundblad.[8] Protein C, prothrombin and factor X were purified from bovine plasma as described by Hashimoto *et al.*[9] Factor Xa was obtained from factor X incubated with Russell's viper venom,[10] and its concentration was determined by active site titration.[11] Rabbit TM was purified according to the method of Esmon *et al.*[12] Activated protein C (APC) was prepared by full-activation method using thrombin–TM.[12] Concentrations of the following proteins were calculated based on the values of their A_{280} and molecular weights (M.W.): TM, A_{280} (8.8) and M.W. (74000)[12]; thrombin, A_{280} (21.4) and M.W. (37000)[13]; prothrombin, A_{280} (15.5) and M.W. (72000)[13]; protein C, A_{280} (13.7) and M.W. (62000).[14]

Cell Culture Calf pulmonary arterial endothelial cells (CPAEC) were purchased from American Type Culture Collection, MD. (U.S.A.). CPAEC were grown to confluence on 48 well culture plates with MEM medium (200 μl) containing 10% (v/v) fetal calf serum.

Preparation of Phospholipid Vesicles Containing TM TM was incorporated into phospholipid vesicles by the method of Mimms *et al.*[15] PC (2.3 mg) and PS (2.3 mg) were dissolved in $CHCl_3$ (230 μl), respectively, and evaporated to dryness by blowing nitrogen gas. The resulting lipid layers were dissolved in 2% (w/v) octyl glucoside solution (500 μl). The TM solution (100 μl, 40 μg/ml) was mixed with a suspension composed of 50 mM Tris–HCl buffer (100 μl, pH 7.4) containing 0.1 M NaCl, and a phospholipid solution (200 μl) containing either PC or a mixture of PC and PS in a molar ratio of 4:1 (PC/PS) and dialyzed against the buffer for 2 d at 25 °C. For the control, the buffer (200 μl) was added instead of the phospholipid solutions.

Prothrombin Activation on CPAEC Surface The twice washed CPAEC were incubated with factor Xa (1 nM) in *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer (200 μl/well, pH 7.4) containing 0.1% (w/v) BSA and 2.5 mM $CaCl_2$ for 15 min at 37 °C. After washing with HEPES buffer, the cells were incubated in HEPES buffer (200 μl, pH 7.4) containing 0.1% (w/v) BSA, 2.5 mM $CaCl_2$, bovine prothrombin (1 μM)

and various concentrations of CPB-II (0.003—0.3 μM) for 10 min at 37 °C. The amidolytic activity of the generated thrombin in each well was assayed in a reaction mixture (total volume, 400 μl) consisting of the supernatant (10 μl), 1 mM Boc–Val–Pro–Arg–MCA (100 μl) and HEPES buffer (190 μl, pH 7.4) containing 0.1% (w/v) BSA and 2.5 mM $CaCl_2$. After incubation at 37 °C for 5 min, 20% (v/v) acetic acid (300 μl) was added to terminate the reaction. The activity was calculated using a standard curve for purified thrombin.

Protein C Activation on CPAEC Surface CPAEC were rinsed twice with HEPES buffer (consisting of 11 mM HEPES, 137 mM NaCl, 4 mM KCl and 11 mM D-glucose, pH 7.4) containing 0.1% (w/v) BSA and 2.5 mM $CaCl_2$, incubated with thrombin (45 nM) in HEPES buffer (200 μl/well, pH 7.4) containing 0.1% (w/v) BSA and 2.5 mM $CaCl_2$ for 30 min at 37 °C, and then washed with the buffer. To the cells were added HEPES buffer (200 μl/well) containing protein C (0.2 μM) and CPB-II (0.01—5 μM) and the cell suspension was incubated at 37 °C for 30 min. The reaction was terminated by the addition of a mixture (10 μl) composed of AT-III (10 μg/ml) and heparin (1 U/ml), and the cells thus treated were sedimented by centrifugation at 3000 × *g* for 5 min. The amidolytic activity of the generated APC was fluorimetrically measured at 37 °C using the initial rate method in a reaction mixture (total volume, 300 μl) consisting of 10 mM Boc–Leu–Ser–Thr–Arg–MCA (30 μl), 50 mM Tris–HCl buffer (180 μl, pH 7.4) and the protein C-activating system (90 μl) treated as above. The amount of AMC liberated was determined in a Hitachi F-4010 fluorescence spectrophotometer using 380 nm (excitation) and 460 nm (emission). Purified APC was used to prepare a standard curve for the activity.

Effect of CPB-II on Protein C Activation in Phospholipid–TM Complex Soluble TM or reconstituted TM with PC or PC/PS (100 μl) was mixed with 50 mM Tris–HCl buffer (100 μl, pH 7.4) containing 0.1 M NaCl and 20 mM $CaCl_2$, and thrombin (100 μl, final concentration 0.25 nM). After incubation for 5 min at 37 °C, aliquots (7.5 μl) of the mixture were withdrawn and mixed with various concentrations of protein C (5 μl, final concentrations of 0.29—2.4 μM) and CPB-II (7.5 μl, final concentrations of 0.3—10 μM). The reaction mixtures were incubated at 37 °C for 10 min. The reaction was terminated by the addition of a mixture (80 μl) containing AT-III (10 μg/ml) and heparin (1 U/ml). The activity of the APC generated was measured as above.

Results

Prothrombin Activation on CPAEC Surface Figure 1 depicts the curve showing the effect of varying concentrations of CPB-II on the conversion of prothrombin to thrombin by the action of factor Xa bound to the surface of CPAEC, on which factor Va was also located.[16,17] Estimation of the amidolytic activity due to the generated thrombin revealed that the activation of prothrombin occurred on CPAEC that had been pretreated with factor Xa and Ca^{2+}. The thrombin generated without CPB-II in the system was estimated to be about 0.4 nM. As can be seen from the curve, the amounts of the generated thrombin decreased nearly proportionally with increasing concentrations (0.003—0.3 μM) of CPB-II. The concentration (IC_{50}) of CPB-II capable of inhibiting the thrombin generation at 50% was approximately 10 nM in the prothrombinase system.

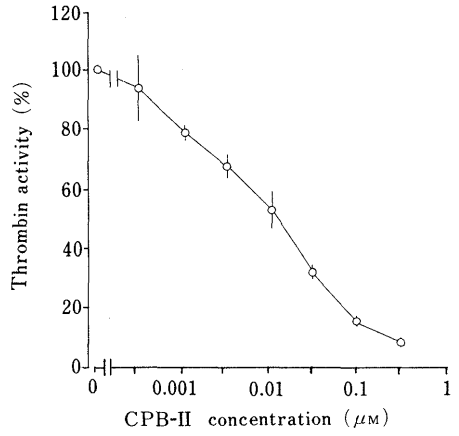

Fig. 1. Effect of CPB-II on Factor Xa-Catalyzed Prothrombin Activation on the Surface of CPAEC

CPACE (5 × 10^4 cells/1.0 cm^2 well) were incubated with factor Xa (1 nM) for 20 min at 37 °C. The cells were twice washed, then incubated with prothrombin (1 μM) and various amounts of CPB-II for 10 min at 37 °C. The amidolytic activity of the thrombin generated was measured fluorimetrically as described in Materials and Methods, and expressed as the activity relative to that of control. Mean ± S.D. (n = 3).

Protein C Activation on CPAEC Surface Figure 2 shows the effect of CPB-II on the protein C activation on CPAEC surface. The amount of APC generated without CPB-II was estimated to be about 0.8 nM under the reaction conditions. The curve of the enzyme activity indicates that the addition of CPB-II (0.01 to 5 μM) to this system does not exert any appreciable influence upon the protein C activation on CPAEC surface. On the other hand, a dose-dependent inhibition of APC generation was observed by the addition of AT-III to the reaction mixture instead of CPB-II (data not shown).

Protein C Activation in Reconstituted TM To determine

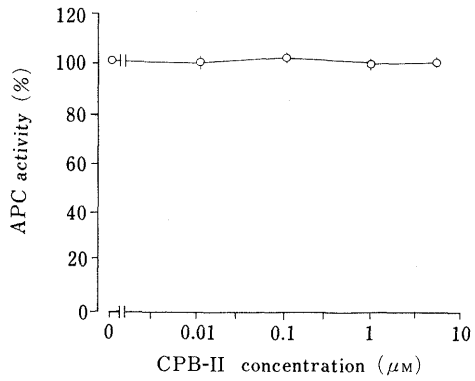

Fig. 2. Effect of CPB-II on Protein C Activation on the Surface of CPAEC

CPAEC (5 × 10^4 cells/1.0 cm^2 well) were incubated with thrombin (1 U) for 30 min at 37 °C. The cells were washed, then incubated with protein C (0.2 μM) and various amounts of CPB-II for 30 min at 37 °C. The reaction was terminated by the addition of AT-III and heparin. The amidolytic activity of the APC generated was measured fluorimetrically and expressed as the activity relative to that of control. Mean ± S.D. (n = 3).

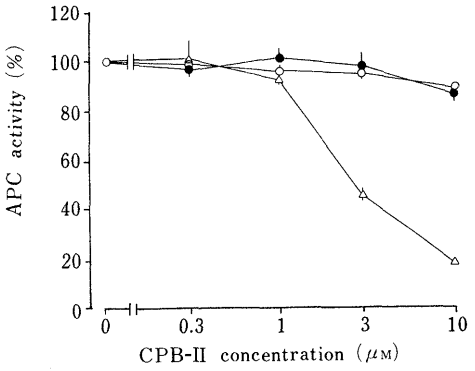

Fig. 3. Effect of CPB-II on the Protein C Activation by Thrombin–TM Systems Involving Soluble and Phospholipids Reconstituted TM

TM in soluble TM or that reconstituted with phospholipid vesicles and thrombin (0.25 nM) were mixed and incubated for 5 min at 37 °C. To the mixture (7.5 μl), various amounts of protein C (0.29—2.4 μM; 5 μl), and CPB-II (7.5 μl) were added, mixed and incubated for 10 min at 37 °C. The reaction was terminated by the addition of AT-III and heparin. The amidolytic activity of the APC generated was measured fluorimetrically. Soluble-TM, —○—; PC-TM, —●—; PC/PS (4 : 1) TM, —△—. Mean ± S.D. (n = 3).

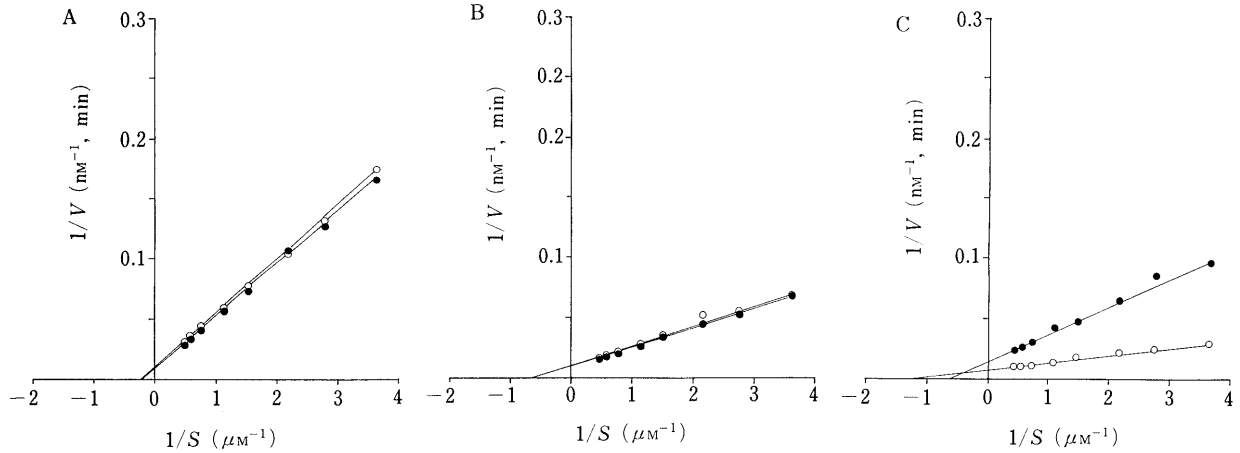

Fig. 4. Lineweaver-Burk Plots of the Amidolytic Activity Due to the APC in Thrombin–TM Systems Involving Soluble and Phospholipid Reconstituted TM

A, soluble-TM; B, PC-TM; C, PC/PS (4:1)-TM, with CPB-II (10 μM), —●—; without CPB-II, —○—.

Table I. Kinetic Constants for Protein C Activation by Thrombin–TM Systems Involving Soluble and Phospholipid Reconstituted TM

TM system	CPB-II (μM)	K_m (μM)	V_{max} (nM/min)
Soluble	None	4.3±0.4	8.2±1.1
	10	3.9±0.3	7.2±1.7
Phosphatidylcholine (100%)	None	1.4±0.2	7.6±1.4
	10	1.4±0.2	7.6±2.0
Phosphatidylcholine (80%) + phosphatidylserine (20%)	None	0.83±0.13	10.7±2.1
	10	1.7±0.4	6.4±0.6

Mean±S.D. ($n=3$).

the participation of phospholipid on the catalytic capacity of TM on CPAEC, the effects of CPB-II on the protein C activation by soluble TM (soluble-TM), TM reconstituted in PC vesicles (PC-TM) or TM reconstituted in PC/PS vesicles (PC/PS-TM) with thrombin were investigated. Approximately 9, 25 and 60 nM of APC were estimated to be generated respectively in soluble-TM, PC-TM and PC/PS-TM under standard reaction conditions. Figure 3 shows the curves of the relative enzyme activities resulting from APC generated in the different TM systems, to which various concentrations (0.3—10 μM) of CPB-II were added. The amounts of the APC generated in PC/PS-TM decreased remarkably with increasing concentrations of CPB-II. Only 20% of protein C was converted to the activated form at 10 μM of CPB-II, while more than 85% of protein C was activated in both soluble TM and PC-TM systems even when 10 μM of CPB-II was added. Obviously, thrombin-catalyzed protein C activation was practically insensitive to CPB-II in these systems as well as on the CPAEC surface. Figure 4 A—C shows Lineweaver-Burk plots of the enzyme activities due to the APC generated in the reaction systems. The influence of CPB-II upon the kinetic parameters for protein C activating systems in PC/PS-TM is easily seen from the plots in Fig. 4C. Table I summarizes the kinetic parameters (K_m and V_{max} values) based on the assay for the enzyme activity of the APC generated by the different TM systems. The K_m value in PC-TM decreased to approximately 1/3 for that obtained in soluble-TM while the V_{max} values in the two systems remained unchanged. No significant effect of CPB-II was observed on the kinetic parameters. With PC/PS-TM, a decrease in the K_m and a slight increase in the V_{max} indicated a similar accelerating effect on the protein C activation. However, the kinetic data with PC/PS-TM revealed that CPB-II not only caused about a 2-fold increase in the K_m value but also lowered the V_{max} values by about 1/2 for the protein C activation, as compared with those obtained in the absence of CPB-II. Moreover, a K_m value of approximately 1.2 μM for protein C was obtained upon the assay for APC generated on CPAEC. This value is virtually the same as those obtained with PC-TM or PC/PS-TM irrespective of the presence of CPB-II.

Discussion

Tanabe *et al.*[17] reported that factor Xa bound to the cell surface and endogenous factor Va are sufficient for activating prothrombin on a cultured endothelial cell system in the presence of Ca^{2+}. Our data on the effect of CPB-II on prothrombin activation revealed it to be inhibitory for prothrombinase activity on the CPAEC in a dose-dependent manner (Fig. 1). Likewise, CPB-II has been found to be inhibitory on the thrombin generation by the prothrombinase system reconstituted in negatively charged phospholipid vesicles that provide surface onto which all the protein components could be adsorbed.[1] Hence, it is most likely that the conversion of prothrombin to thrombin proceeds in the prothrombinase complex associated with negatively charged phospholipids on the cell surface of CPAEC.

It is currently accepted that TM is an essential receptor for thrombin on the endothelial cell surface, and that its binding to thrombin accelerates the limited proteolysis converting protein C to the active form (APC). Using the activating system involving the purified proteins, protein C activation by thrombin has been shown to be enhanced by phospholipid vesicles into which TM was incorporated; this is revealed by the lowered K_m values in PC-TM and PC/PS-TM (Table I). A comparable K_m value (1.2 μM) for protein C with that (1.4 μM) in PC-TM was obtained on the cell surface of CPAEC. No appreciable inhibitory effect of CPB-II could be detected on the protein C activation by CPAEC as true of PC-TM; however, the former abolished the stimulatory effect of negatively charged phospholipid on protein C activation by PC/PS-TM. It is therefore feasible

that the neutral phospholipid might participate in the protein C activation by the thrombin–TM system on the endothelial cell surface.

Galvin *et al.*[6] reported that the K_m value of 7.6 μM for protein C activation in a soluble thrombin–TM system reduced markedly to 0.7 or 0.1 μM, respectively, in PC or PC/PS vesicles into which TM is incorporated, while the K_{cat} values remain unaltered regardless of the reaction systems. Contrasting results have been presented by Freyssinet *et al.*[7] whose data indicate that the addition of phospholipids to a human thrombin–TM complex has no influence upon the K_m value of 2 μM for the protein C activation, whereas the V_{max} increases 3.2-fold, depending upon phospholipids added. Our results with bovine thrombin and rabbit TM were similar to the data of Galvin *et al.*,[6] which indicated a marked reduction of the K_m values and a slight change in V_{max} values for the protein C activation in the system involving PC-TM and PC/PS-TM. Lupus anticoagulant (LA), a spontaneously acquired inhibitor of blood coagulation in the plasma of patients with auto-immune disorders has been characterized as a phospholipid-binding protein.[18] Inconsistent with its anticoagulant activity, a high frequency of thrombotic events has been reported among patients carrying LA.[19] Thrombosis in such patients was presumed to be due to the suppression of protein C activation caused by the abolition by LA of the accelerating effect of phospholipids on the thrombin–TM system.[20] Indeed, Comp *et al.*[21] and Cariou and his colleagues[22,23] have presented supportive data for such an inhibitory potency of LA for protein C activation on cultured endothelial cells. On the contrary, prothrombin and fragment-1, Ca^{2+} dependent phospholipid-binding coagulation proteins, have been reported to exhibit the inhibitory potency for the protein C activation exclusively depending upon negatively charged phospholipids, but they are inactive as an inhibitor of thrombin–TM complex on PC vesicles and endothelial cell surface.[6] In the present work, we have demonstrated that CPB-II exerts influence similar to that of blood coagulation factors on protein C activation. Hence, CPB-II differs from LA primarily in its mechanism of action of this activation. These findings imply that the *in vivo* effects of CPB-II are different from those of LA and that CPB-II can be an effective anticoagulant.

The prothrombinase system on the CPAEC surface was susceptible to inhibition by CPB-II that did not exhibit any appreciable effect on protein C activation. Neutral phospholipids on the endothelial cell surface thus appear to be essential for the expression of the thrombin–TM system while the coagulant system requires negatively charged phospholipids for the activity.

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