The effect of phospholipids on the activation of protein C by the human thrombin–thrombomodulin complex

Jean-Marie FREYSSINET, Josiane GAUCHY and Jean-Pierre CAZENAVE

Laboratoire de Biologie et de Pharmacologie des Interactions du Sang avec les Vaisseaux et les Biomatériaux, Centre Régional de Transfusion Sanguine, 10 rue Spielmann, 67085 Strasbourg Cédex, France

Human thrombomodulin, an endothelial-cell-membrane glycoprotein, has been purified from placenta by Triton X-100 extraction and by affinity chromatography on concanavalin A–Sephacrose and thrombin–Sephacrose. It has been characterized by its ability to promote the activation of human protein C by human α -thrombin in the presence of Ca^{2+} and fulfilled the requirements of a cofactor. Reconstitution of thrombomodulin into phospholipid vesicles containing anionic phospholipids resulted in an increased rate of activation of protein C. Cardiolipin and vesicles containing phosphatidylcholine/phosphatidylserine (1:1, w/w) were the most effective. The apparent K_m of the thrombin–thrombomodulin complex for protein C was $2\ \mu\text{M}$. It was not changed in the presence of phospholipid, whereas the V_{max} could be apparently increased up to 3.2-fold depending on the phospholipid and on its concentration, the catalytic-centre activity reaching 15.7 mol of activated protein C formed/min per mol of thrombin. Above their optimal concentrations, phospholipids inhibited the amidolytic activity of activated protein C. Phospholipids had no effect on the activation of 4-carboxyglutamic acid-domainless protein C, a proteolytic derivative of protein C lacking the 4-carboxyglutamic acid residues. These results show that the positive effect of anionic phospholipids in the activation of protein C by the thrombin–thrombomodulin complex involves a Ca^{2+} -dependent interaction between protein C and phospholipids. They suggest that the enhancement of thrombomodulin activity by such phospholipids may be of functional significance.

INTRODUCTION

Protein C is the vitamin K-dependent zymogen of a serine proteinase, activated protein C, which has potent anticoagulant capacity. Thrombin is the physiological activator of protein C in the presence of an essential endothelial-cell-membrane cofactor, thrombomodulin (for a recent review, see Esmon & Esmon, 1984). Activated protein C exerts its anticoagulant activity by proteolytically degrading factors Va and VIIIa in the presence of phospholipid and of a cofactor, protein S, another vitamin K-dependent protein (Walker, 1984). Phospholipids are also essential in the activation and in the action of the vitamin K-dependent blood coagulation factors. Procoagulant phospholipids are anionic phospholipids present in the plasma membrane of cells involved in haemostasis, in particular platelets, endothelial cells and erythrocytes (Zwoll, 1978). Vitamin K-dependent proteins bear modified glutamic acid residues at the N-terminal end of single-chain molecules or at the N-terminal end of the light chain of two-chain molecules. These residues (4-carboxyglutamic acid residues) are 4-carboxylated by a post-ribosomal vitamin K-dependent enzymic system (Stenflo & Suttie, 1977), and thus have two carboxylic functions which allow interaction with anionic phospholipids through calcium bridges.

This process is fundamental, since enzymes, cofactors and substrates can be concentrated at the site of damaged endothelium where procoagulant phospholipids

are liberated, thus providing a localized basis for efficient haemostasis (Esnouf, 1977; Jackson & Nemerson, 1980; Zwoll & Hemker, 1982; Mann, 1984). Although protein C is the precursor of a potent anticoagulant, its structure is close to that of the vitamin K-dependent zymogens of coagulant enzymes (Foster *et al.*, 1985). Hence the mechanisms of activation of the coagulant and the anticoagulant pathways should share some common features. Procoagulant phospholipids are also active in the expression of the anticoagulant activity of activated protein C (Walker, 1984).

In the present study, the influence of phospholipids on apparent thrombomodulin activity has been examined. This question was raised by Esmon *et al.* (1982) on purifying rabbit lung thrombomodulin. These investigators suggested that thrombomodulin activity might have been decreased upon loss of phospholipids during the purification process. By using a homogeneous human system we show that anionic phospholipids enhance the V_{max} of the activation of protein C by the thrombin–thrombomodulin complex, whereas the K_m is unchanged.

MATERIALS AND METHODS

Materials

Human serum albumin (essentially fatty-acid-free), hirudin, PMSF, $\text{iPr}_2\text{P-F}$, methyl glucoside, Triton X-100,

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; $\text{iPr}_2\text{P-F}$, di-isopropyl phosphorofluoridate; methyl glucoside, 1-O-methyl α -D-glucopyranoside; DPG, diphosphatidylglycerol (cardiolipin); Con A, concanavalin A; QAE, quaternary aminoethyl; Phe-Pip-Arg-Nan $\cdot 2\text{HCl}$ ('S2238'), *H*-D-phenylalanyl-L-pipecolylarginine *p*-nitroanilide dihydrochloride; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; PG, phosphatidylglycerol; GD-protein C, 4-carboxyglutamic acid-domainless protein C; a.t.u., anti-thrombin unit; APC, activated protein C.

Taipan-snake (*Oxyuranus scutellatus scutellatus*) venom and dPG were products from Sigma, St. Louis, MO, U.S.A. Con A coupled to agarose (Con A-Sepharose), CNBr-activated-Sepharose 4B (CNBr-Sepharose 4B) and QAE-Sephadex were from Pharmacia, Uppsala, Sweden. Phospholipase C from *Bacillus cereus* was obtained from Boehringer, Mannheim, Germany. Phe-Pip-Arg-Nan·2HCl was from Kabi Vitrum, Stockholm, Sweden. Factor II concentrates (PPSB, about 250 prothrombin units/vial) were obtained from the Centre Régional de Transfusion Sanguine, Strasbourg, France, and used to isolate and purify human prothrombin by a previously published method (Freyssinet *et al.*, 1982). PC, PS, PA, PI and PG were purchased from Lipid Products, Redhill, Surrey, U.K. All these phospholipids were of grade 1, i.e. they contained less than 1% impurities as judged by t.l.c. α -Chymotrypsin from bovine pancreas (45–70 units/mg of protein) was from Worthington, Freehold, NJ, U.S.A. TSK DEAE 5PW ion-exchange column for h.p.l.c. was purchased from LKB, Bromma, Sweden. The kit for protein assay and Chelex resin were products from Bio-Rad, Richmond, CA, U.S.A.

Preparation of human protein C and thrombin

Protein C was purified to homogeneity (as judged by SDS/polyacrylamide-gel electrophoresis) from plasma cryosupernatant as previously described (Freyssinet *et al.*, 1985). Human α -thrombin [3000 National Institutes of Health (NIH) units/mg of protein] was obtained by activating purified prothrombin with Taipan-snake venom as described by Owen & Jackson (1973).

Preparation of thrombin-Sepharose

Thrombin-Sepharose was prepared by coupling 80 mg of human α -thrombin (1 mg/ml) in 0.75 M-NaCl to 40 ml of CNBr-activated Sepharose 4B as published by Salem *et al.* (1984b). By using such a procedure, the yield of coupling was close to 100%, with 2 mg of thrombin/ml of gel.

Preparation of human placenta thrombomodulin

Extraction of thrombomodulin and affinity chromatography on Con A-Sepharose were performed as previously described (Freyssinet *et al.*, 1986a). After elution from the Con A-Sepharose column by a methyl glucoside concentration gradient, fractions containing thrombomodulin activity were pooled and the material was dialysed for 24 h at 4 °C against 3 × 20 vol. of 50 mM-Tris buffer, pH 7.5, containing 0.05% Triton X-100, 0.1 M-NaCl, 1 mM-EDTA and 0.02% NaN₃. The dialysed sample (160 ml/placenta) was applied to a column (1.6 × 20 cm) of thrombin-Sepharose previously equilibrated in the above dialysis buffer at a flow rate of 30 ml/h. The column was washed with 800 ml of starting buffer and was eluted with the same buffer containing 1 M-NaCl at a flow rate of 15 ml/h. Fractions containing thrombomodulin activity were pooled and dialysed against 2 × 100 vol. of 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl and 0.02% NaN₃ for 24 h at 4 °C. The dialysed samples were either stored at –80 °C or at 4 °C for immediate use. No significant loss of activity could be detected on storage for several weeks at either –80 °C or 4 °C.

Preparation of GD-protein C

Human GD-protein C was prepared as described by Esmon *et al.* (1983) for the preparation of bovine GD-protein C, by incubating human protein C and α -chymotrypsin (400:1, w/w) with the following modifications. The incubation was carried out for 6 min at 37 °C in 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl and 0.02% NaN₃ and the action of α -chymotrypsin was stopped by the addition of 5 mM-iPr₂P-F (final concn.). The incubation mixture was immediately applied to the TSK DEAE 5PW column previously equilibrated in the incubation buffer. GD-protein C was not retained in the column and was recovered as the breakthrough peak. The column was eluted with a 70 ml linear NaCl gradient from 0.1 to 1 M in the equilibration buffer. Residual protein C (less than 10%) was eluted at 0.4 M-NaCl, whereas the cleaved peptide containing the 4-carboxyglutamic acid residues (Esmon *et al.*, 1983) was eluted at 0.55 M-NaCl. The purity of the different products was assessed by SDS/polyacrylamide-gel electrophoresis. Only the light chain of protein C was altered by the chymotryptic digestion. An incubation time of human protein C with α -chymotrypsin greater than 6 min resulted in a loss of the activatability of GD-protein C or of the activity of activated GD-protein C.

Preparation of phospholipid vesicles

Phospholipid vesicles containing a single phospholipid or a mixture, (1:1, w/w) of two phospholipids were prepared as previously described (Freyssinet *et al.*, 1982) in the absence of Ca²⁺ by the method of Huang & Thompson (1974), except that the phospholipid suspension was sonicated three times for 1 min instead of 2 h in order to obtain larger vesicles, the phospholipid concentration being 10 mg/ml.

Protein concentrations

Protein concentrations were determined by using the Bio-Rad protein assay, with bovine serum albumin as standard. Protein C and thrombin concentrations were also determined from the measurement of A₂₈₀; absorption coefficients of 14.5 (Kisiel & Davie, 1981) and 18.3 (Fenton *et al.*, 1977) respectively were assumed.

Thrombomodulin assay

Thrombomodulin activity was detected by its ability to enhance the activation of protein C by thrombin. In the routine assay, 0.75 μ M-protein C was incubated at 37 °C for 20 min with 20 nM-thrombin (0.5 NIH units), and samples of thrombomodulin in a total volume of 200 μ l containing 50 mM-Tris, pH 7.5, 0.1 M-NaCl, 5 mM-CaCl₂ and 20 μ M-human serum albumin. In other assays, the concentration of thrombin was varied. The activation of protein C was terminated by neutralization of thrombin with 5 a.t.u. of hirudin in 5 μ l. Then 795 μ l of 0.22 mM-Phe-Pip-Arg-Nan·2HCl in the above buffer was added and the amount of activated protein C was assayed for amidolytic activity by measuring, at 405 nm, the rate of hydrolysis of Phe-Pip-Arg-Nan·2HCl at 25 °C. The concentration of APC formed was determined by reference to a standard curve constructed by using known amounts of APC (Salem *et al.*, 1984b; Freyssinet *et al.*, 1986a). In routine assays, one unit of thrombomodulin was arbitrarily defined as the amount of

cofactor that resulted in the formation of 20 pmol of APC/min per ml. As recommended by Salem *et al.* (1984b), less-than-saturating concentrations of protein C were used in order to conserve material, and thrombomodulin concentrations were adjusted so that less than 15% of the protein C was activated. Most routine assays were performed under conditions where anionic-phospholipid concentration was kept below that at which inhibition of the amidolytic activity of activated protein C occurred (see the Results section).

Apparent effect of phospholipids on thrombomodulin activity

The effect of phospholipids on thrombomodulin activity was assessed by their ability to enhance the activation of protein C by thrombin. Thrombomodulin and phospholipid vesicles (or buffer in control experiments) were preincubated for 15 min at 25 °C in 150 μ l of 50 mM-Tris (pH 7.5)/0.1 M-NaCl/6.7 mM-CaCl₂/0.02% NaN₃. In all experiments the dilution of thrombomodulin was such that the concentration of Triton X-100 was decreased below its critical micelle concentration, i.e. below 0.02% (Hjelmeland & Chrambach, 1984). After this preincubation at 25 °C, thrombin, human serum albumin and protein C or GD-protein C were added. The final volume was 200 μ l, containing 5 nM-thrombomodulin–thrombin complex, 5 mM-CaCl₂, 20 μ M-human serum albumin and various concentrations of protein C or GD-protein C and phospholipids. The activation of protein C was stopped by the addition of 5 a.t.u. (5 μ l) of hirudin and the amount of APC was assayed as described above.

Fig. 1. Ca²⁺-dependence of human protein C activation by the human thrombin–thrombomodulin complex

Measurements were performed with 0.75 μ M-protein C, 5 nM-thrombomodulin and 20 nM-thrombin in 50 mM-Tris, pH 7.5, containing 0.1 M-NaCl, 20 μ M-human serum albumin, 0.02% NaN₃, and the indicated concentrations of Ca²⁺. The generated APC was assayed as described in the Materials and methods section. Buffers were treated with Chelex (Bio-Rad) before use. The inset shows a double-reciprocal plot for the same data. Each value is the mean of two determinations. Similar results were obtained with different thrombomodulin preparations.

Fig. 2. Phospholipid-dependence of human protein C activation by the human thrombin–thrombomodulin complex

Protein C (0.75 μ M) was activated by the thrombin–thrombomodulin complex (5 nM) after reconstitution of thrombomodulin into purified phospholipid vesicles. Reconstitution, assay of the generated APC and other experimental details are as described in the Materials and methods section. Buffer was 50 mM-Tris, pH 7.5, containing 0.1 M-NaCl, 20 μ M-human serum albumin, 5 mM-CaCl₂ and 0.02% NaN₃. Above their optimal concentrations for the activation of protein C, PC/PS (1:1, w/w) vesicles ($\blacktriangle$) and dPG ($\bullet$) were responsible for an apparent inhibition of APC (broken lines) (see Table 1). Other experimental details are as described in the Materials and methods section.

Effect of phospholipids on the amidolytic activity of activated protein C

The effect of phospholipids on the amidolytic activity of APC was assessed by mixing APC [0.75 μ M (final concn.) prepared as previously described (Freysinet *et al.*, 1986a)] with various phospholipids at different concentrations, in the presence of 5 mM-Ca²⁺, in a final volume of 200 μ l. APC and phospholipids were incubated for 10 min at room temperature before APC was assayed by measuring the rate of hydrolysis of Phe-Pip-Arg-Nan·2HCl as described above.

RESULTS

Purification of human placenta thrombomodulin

Thrombomodulin was purified to homogeneity, a three-step procedure being adopted: detergent extraction, affinity chromatography on Con A–Sephacryl and thrombin–Sephacryl affinity chromatography. The overall yield was 1.5% when thrombomodulin was assayed after relipidation (see below). When the material eluted from the thrombin–Sephacryl column was analysed by SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969), a single faint band corresponding to an apparent M_r of 69 000 and 73 000, respectively before and after reduction, could be detected by silver staining.

Functional characterization of thrombomodulin activity

With purified thrombomodulin, the rate of activation of protein C was saturable with respect to either

Table 1. Influence of various phospholipids on the rate of activation of human protein C (0.75 μ M) by the human thrombin-thrombomodulin complex (5 nM) and the effect of these phospholipids on the amidolytic activity of 0.75 μ M APC

See the Materials and methods section for further experimental details

Phospholipid	Concn. (mg/ml)	Rate of activation of 0.75 μ M-protein C (pmol/min per ml)	Inhibition of amidolytic activity of 0.75 μ M-APC (%)
PC	0.04	9.6	< 3
	0.2	9.5	< 3
	1	9.3	< 3
PS	0.04	17.8	< 3
	0.2	20.5	22
	1	17.6	41
PG	0.04	16.9	< 3
	0.2	21.0	16
	1	10.2	79
PI	0.04	19.3	< 3
	0.2	19.6	22
	1	16.4	51
dPG	0.02	20.2	< 3
	0.04	24.4	< 3
	0.2	18.7	30
PC/PS	1	1.1	96
	0.04	19.1	< 3
	0.2	24.4	9
PC/PG	1	19.1	37
	2	10.9	79
	0.04	14.6	< 3
PC/PI	0.2	18.4	18
	1	13.9	56
	0.04	17.5	< 3
PC/PA	0.2	20.1	13
	1	14.5	44
	0.04	12.5	< 3
PC/dPG	0.2	15.3	< 3
	1	17.7	20
	0.04	13.4	< 3
Control with buffer	0.2	15.7	< 3
	1	16.8	26
		7.5	-

thrombin or thrombomodulin concentration. In the presence of 5 nM-thrombomodulin, the rate of protein C activation was at saturation at a thrombin concentration of 5–8 nM and, conversely, saturation was attained at 5–8 nM thrombomodulin in the presence of 5 nM-thrombin (results not shown). These results demonstrate that thrombomodulin behaves as a cofactor and suggest a stoichiometric interaction between thrombin and thrombomodulin (Esmon *et al.*, 1982; Salem *et al.*, 1984b).

The activation of protein C by the human thrombin-thrombomodulin complex was dependent on Ca^{2+} , the rate being saturable with respect to Ca^{2+} (Fig. 1). Half-maximal rate was attained at 0.35 mM- Ca^{2+} . Mg^{2+} was not able to replace Ca^{2+} , whereas Mn^{2+} was found to be almost as effective as Ca^{2+} in the activation of protein C. At 5 mM- Mg^{2+} , the rate of activation was only about 5% of that observed at 5 mM- Ca^{2+} , whereas at 5 mM- Mn^{2+} the rate was 80% of that obtained with 5 mM- Ca^{2+} . In the presence of either 0.5 or 5 mM- CdCl_2 , no significant activation of protein C could be detected. In the presence of 1 mM-EDTA, 5 nM-thrombin could activate 0.75 μ M-protein C at a rate of 54 fmol/min per

ml, i.e. 1/130 of the rate observed with the 5 nM-thrombin-thrombomodulin complex. In the presence of 5 mM- Ca^{2+} , thrombin on its own, at a concentration of 5 nM, activated 0.75–2.5 μ M-protein C at a rate that was 1/450 of that measured with the 5 nM-thrombin-thrombomodulin complex.

The above results characterize thrombomodulin extracted and purified from human placenta and confirm the specificity of the activating system of human protein C. In all cases, the anticoagulant activity of APC could be qualitatively verified through the prolongation of the clotting time of normal human plasma (activated partial thromboplastin time; Freyssinet *et al.*, 1986a).

Apparent effect of phospholipids on thrombomodulin activity

Although purified human placenta thrombomodulin preparations exhibited specific biological activity after elimination of the bound lipids during the extensive washing of the two successive affinity-chromatography columns, relipidation resulted in a substantially increased activation of protein C. In the absence of phospholipid, treatment of thrombomodulin by phospholipase C

Table 2. Comparison of the rate of activation of human protein C and of human GD-protein C in the presence and in the absence of phospholipids

PC, phosphatidylcholine; PS, phosphatidylserine. See the Materials and methods section for further experimental details.

Substrate	Rate of activation (pmol/min per ml)				
	Cofactor . . . Crude Triton X-100 extract*	Purified thrombomodulin (5 nM)	Purified thrombomodulin (5 nM) + PC/PS (0.2 mg/ml)	Purified thrombomodulin (5 nM) + dPG (0.04 mg/ml)	PC/PS (0.2 mg/ml) or dPG (0.04 mg/ml) (control)
Protein C (0.75 μM)	22.5	7.5	23.5	24.3	N.A.†
GD-protein C (0.75 μM)	7.5	7.9	6.9	7.0	N.A.

* The amount of crude Triton X-100 extract was adjusted so that it gave a rate of protein C activation comparable with that obtained with purified thrombomodulin and phospholipids.

† Abbreviation used: N.A., no detectable activation.

(10 units/ml of thrombomodulin solution) had no influence on its biological activity. When thrombomodulin was reconstituted with phospholipids, the rate of activation of protein C was enhanced in the presence of vesicles bearing a net negative charge (Fig. 2, Table 1). The most effective phospholipids were PC/PS vesicles and cardiolipin. Fig. 2 shows the concentration-dependence of the enhancing effect of PC/PS vesicles and dPG on the rate of activation of protein C by the thrombin–thrombomodulin complex. Optimal concentration was reached at 0.2 mg/ml with PC/PS vesicles and 0.04 mg/ml with dPG, which represents a phospholipid thrombomodulin ratio (w/w) of 540 with PC/PS vesicles and of 108 with dPG. At the optimal concentration of either phospholipid, the apparent enhancement of

protein C activation was about 3.2-fold, from 7.5 pmol of APC generated/min per ml in the absence of phospholipids to 24.4 pmol/min per ml in their presence. Above 0.4 mg of PC/PS vesicles/ml and above 0.08 mg of dPG/ml, the observed enhancement decreased with increasing phospholipid concentrations. In order to determine whether this diminished capacity of high phospholipid concentrations to enhance the rate of protein C activation was due to a decreased activation of protein C or to a reduced amidolytic activity of APC, the influence of phospholipid concentration on the amidolytic activity of APC was examined. It was found that most of this diminished capacity was attributable to an apparent inhibition of the amidolytic activity of APC by high phospholipid concentrations (Table 1). It was

Fig. 3. Substrate-concentration-dependence of human protein C activation by the human thrombin–thrombomodulin complex in the presence and in the absence of phospholipid

(a) Protein C, at various concentrations, was activated by the thrombin–thrombomodulin complex (5 nM), either without phospholipid or after reconstitution of thrombomodulin into PC/PS (1:1, w/w) vesicles as described in the Materials and methods section. The buffer was 50 mM-Tris, pH 7.5, containing 0.1 M-NaCl, 20 μM -human serum albumin, 5 mM- CaCl_2 and 0.02% NaN_3 . The assay of generated APC and other experimental details are as described in the Materials and methods section.

(b) Double-reciprocal plot for the same data. The K_m for protein C was 2 μM . ●, No phospholipid; ▲, 0.2 mg/ml PC/PS; ■, 1 mg/ml PC/PS.

verified, by using purified α -thrombin, that high phospholipid concentrations had no influence on the chromogenic substrate, Phe-Pip-Arg-Nan-2HCl, itself.

When thrombomodulin was reconstituted with phospholipid having a net neutral charge, i.e. with PC, the enhancement of protein C activation was weak, with about 9.5 pmol of APC generated/min per ml in the presence of PC vesicles as compared with 7.5 pmol/min per ml in the absence of phospholipid (Table 1). The effect of PC was not concentration-dependent.

In the presence of 1 mM-EDTA, the activation of protein C by 5 nM-thrombin in the presence of 5 nM-thrombomodulin was not significantly affected by phospholipids. For the reconstitution of thrombomodulin with phospholipids, a preincubation of 15 min at 25 °C in the presence of Ca^{2+} was sufficient to allow the optimal development of thrombomodulin activity. In the absence of thrombomodulin, phospholipids on their own were not able to promote the activation of protein C by thrombin. Treatment of PS or PC/PS vesicles with phospholipase C (10 units/mg of phospholipid) abolished their capacity to enhance the rate of protein C activation by the thrombin-thrombomodulin complex.

In the absence of phospholipid, the rate of activation of GD-protein C by the thrombin-thrombomodulin complex was very close to that of protein C, whereas in the presence of phospholipids the rate of activation of GD-protein C remained of the order of 7 pmol/min per ml and that of protein C was more than three times higher (Table 2). The same rate was also observed with crude Triton X-100 as a source of thrombomodulin and of phospholipids.

The rate of activation of protein C by the 5 nM-thrombin-thrombomodulin complex increased with respect to protein C, in the absence and in the presence of phospholipid (Fig. 3a), with an apparent K_m of 2 μM calculated from the double-reciprocal plot (Fig. 3b) in both cases. In the absence of phospholipid the calculated V_{max} was 25.5 pmol/min per ml, whereas it was 78.8 pmol/min per ml in the presence of 0.2 mg of PC/PS vesicles/ml. The catalytic-centre activity was 5.1 mol of APC formed/min per mol of thrombin in the absence of phospholipid, whereas it was 15.7 mol of APC formed/min per mol of thrombin in the presence of 0.2 mg of PC/PS vesicles/ml, which represents an enhancement of about 3.1-fold. The presence of 1 mg of PC/PS vesicles/ml did not change the apparent K_m , but the V_{max} was decreased to about 55 pmol/min per ml. Thus, taking the V_{max} at 0.2 mg of PC/PS vesicles/ml as a reference, phospholipids at high concentrations, i.e. above 0.2 mg/ml, behaved as non-competitive inhibitors in the activation of protein C by the thrombin-thrombomodulin complex.

The above results clearly demonstrate the role of anionic phospholipids in the activation of protein C by the thrombin-thrombomodulin complex in a purified system.

DISCUSSION

In vivo, the activation of protein C by thrombin is under cellular control, requiring an endothelial-cell-membrane cofactor, thrombomodulin (Esmon & Esmon, 1984). In the present study *in vitro*, involving purified components, it is demonstrated that the activation of

protein C by the thrombin-thrombomodulin complex is enhanced by anionic phospholipids, including dPG.

When compared with the purification scheme for thrombomodulin from human placenta reported by Salem *et al.* (1984b), our overall yield of 1.5% is about five times lower, our specific activity being four times lower. Thus it seems that these discrepancies could be attributed to the general difficulties encountered in the isolation of membrane proteins and also to the possible selection of a less-active species of thrombomodulin molecules by Con A-Sepharose affinity chromatography. However, thrombomodulin, purified by the above procedure, fulfils the requirements of a cofactor in the activation of protein C by thrombin. The expression of cofactor activity is dependent on Ca^{2+} , as is that of rabbit lung thrombomodulin, with saturation at 2.5 mM- Ca^{2+} (Esmon *et al.*, 1982) but is different from that reported by Salem *et al.* (1984b) for human placenta thrombomodulin. In that study, Ca^{2+} concentrations above 1 mM was found to have an inhibitory effect. Again, this difference might be due to the use of different thrombomodulin preparations. The slight increase of cofactor activity after the reconstitution of thrombomodulin into PC vesicles may just reflect the preference of thrombomodulin for a phospholipid environment rather than a non-ionic-detergent environment.

The present study shows that optimal activation of protein C by thrombin in the presence of thrombomodulin and Ca^{2+} occurs if anionic phospholipids are present. The maximum apparent enhancement of the rate of activation is about 3-fold, but it must be emphasized that, as for other membrane proteins, the reconstitution of thrombomodulin in purified phospholipid vesicles could lead to a random distribution of thrombomodulin molecules between the outer and the inner sides of the vesicles (Nicholls, 1981). This could be particularly true when reconstitution is accompanied by Ca^{2+} -induced fusion of these negatively charged vesicles. Therefore, this enhancement factor might well be underestimated, since thrombomodulin molecules located at the inner side would not participate in the activation because of their lack of accessibility. In the activation of protein C by the thrombin-thrombomodulin complex, the apparent K_m for protein C is 2 μM . This value is about four to five times smaller than that determined by other investigators with rabbit lung thrombomodulin (Esmon *et al.*, 1983) or with human placenta thrombomodulin (Salem *et al.*, 1984b), but is identical with that recently redetermined by Ishii & Majerus (1985) with human thrombomodulin. However, it is still much higher than the normal physiological concentration of protein C in plasma, which is about 65 nM (Griffin, 1984). The present study shows that phospholipids do not provoke a change in the apparent K_m and that the activation of GD-protein C is not potentialized by phospholipids. GD-protein C has also been found to be poorly activated by thrombin, as compared with protein C, in the presence of endothelial cells in culture (Esmon *et al.*, 1983). Thus it might be possible that phospholipids are responsible for a conformational change of protein C occurring through a Ca^{2+} -dependent interaction between substrate and membrane, resulting in an increased rate of activation by the thrombin-thrombomodulin complex. The overall apparent enhancement due to human thrombomodulin and phospholipids in the activation of human protein C by human α -thrombin is about 1350. Such a value is rather

modest when compared with 300 000-fold, which is the enhancement factor for the activation of prothrombin by factor Xa in the presence of factor Va and of phospholipids (Mann, 1984). Furthermore, human thrombomodulin does not seem to be as efficient as rabbit lung thrombomodulin in promoting the activation of protein C (Esmon & Owen, 1981). The above results tend to prove that the mechanism of activation of protein C is different from that of other vitamin K-dependent proteins. The optimal ratio (w/w) of 540 between PC/PS vesicles and thrombomodulin is comparable with that of procoagulant phospholipids and tissue-factor apoprotein from bovine brain (Bach *et al.*, 1981) or from human brain (Broze *et al.*, 1985) and, as for tissue factor, it is not known why a molar ratio of phospholipid to protein of approx. 50 000 is necessary for the development of full activity after reconstitution. Beyond their respective optimal concentrations, PC/PS vesicles, dPG and other acidic phospholipids inhibit the amidolytic activity of activated protein C.

It has recently been shown that factor Va binds to endothelial cells and that its light chain can act in concert with thrombomodulin to promote the activation of protein C (Maruyama *et al.*, 1984; Salem *et al.*, 1984a). Hence procoagulant phospholipids might well co-operate with thrombomodulin and factor Va in enhancing the activation of protein C, since they have been shown to interact with factor Va through its light chain (Mann, 1984). Although dPG is as potent as PC/PS vesicles at a 5-fold lower concentration, the basis of its effect is more difficult to interpret. dPG represents more than 10% of the total phospholipid of the inner membrane of the mitochondria (Alberts *et al.*, 1983). It is not known whether this complex phospholipid might be present in the endothelial-cell plasma membrane, but its presence in plasma is more likely due to tissue damage. It has been demonstrated that patients with circulating lupus anticoagulants of IgG or IgM classes and with high levels of anti-dPG antibodies are at higher risk of thrombosis (Harris *et al.*, 1983), and we have shown that an IgM lupus anticoagulant was able to neutralize the enhancing effect of PC/PS vesicles or of cardiolipin on purified thrombomodulin activity (Freyssinet *et al.*, 1986b). If the positive effect of phospholipid is of physiological importance, its neutralization by such anti-phospholipid antibodies might be responsible for thrombosis, since such patients would have only one-third of their protein C activated, i.e. below the pathological level of 50% of the normal (Griffin, 1984).

The present results suggest that anionic phospholipids, including dPG, should be seriously considered as potentiators of thrombomodulin activity in the activation of protein C by thrombin.

We thank Dr. J. N. Mulvihill for reviewing the English of this paper and Mrs. C. Helbourg and Mrs. M. Voyat for typing the manuscript. J.-M.F and J.-P.C are research investigators supported by INSERM, respectively Chargé de Recherche and

Directeur de Recherche. This work was supported by a research grant (no 85-3-21-5-E) from C.N.A.M.T.S.

REFERENCES

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. (1983) *Molecular Biology of the Cell*, Garland Publishing, New York
- Bach, R., Nemerson, Y. & Konigsberg, W. (1981) *J. Biol. Chem.* **256**, 8324–8331
- Broze, G. J., Leykam, J. E., Schwartz, B. D. & Miletich, J. P. (1985) *J. Biol. Chem.* **260**, 10917–10920
- Esmon, C. T. & Esmon, N. L. (1984) *Semin. Thromb. Hemostasis* **10**, 122–130
- Esmon, C. T. & Owen, W. G. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2249–2252
- Esmon, N. L., Owen, W. G. & Esmon, C. T. (1982) *J. Biol. Chem.* **257**, 859–864
- Esmon, N. L., DeBault, L. E. & Esmon, C. T. (1983) *J. Biol. Chem.* **258**, 5548–5553
- Esnouf, M. P. (1977) *Br. Med. Bull.* **33**, 213–218
- Fenton, J. W. II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M. & Finlayson, J. S. (1977) *J. Biol. Chem.* **252**, 3587–3598
- Foster, D. C., Yoshitake, S. & Davie, E. W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4673–4677
- Freyssinet, J.-M., Thevenon, D., Souque, A. & Suscillon, M. (1982) *Thromb. Haemostasis* **48**, 120–124
- Freyssinet, J.-M., Grunebaum, L., Wiesel, M.-L., Cazenave, J.-P., Fritsché, R., Greber, S., Lampart, A. & Spaethe, R. (1985) in *Protein C: Biochemical and Medical Aspects* (Witt, L., ed.), pp. 107–116, Walter de Gruyter, Berlin
- Freyssinet, J.-M., Brami, B., Gauchy, J. & Cazenave, J.-P. (1986a) *Thromb. Haemostasis* **55**, 112–118
- Freyssinet, J.-M., Wiesel, M.-L., Gauchy, J., Boneu, B. & Cazenave, J.-P. (1986b) *Thromb. Haemostasis* **55**, in the press
- Griffin, J. H. (1984) *Semin. Thromb. Hemostasis* **10**, 162–166
- Harris, E. N., Gharavi, E. A., Boey, M. L., Patel, B. M., Mackworth-Young, C. G., Loizou, S. & Hughes, G. V. R. (1983) *Lancet* **ii**, 1211–1214
- Hjelmeland, L. M. & Chrambach, A. (1984) *Methods Enzymol.* **104**, 305–318
- Huang, C. & Thompson, T. E. (1974) *Methods Enzymol.* **22**, 485–489
- Ishii, H. & Majerus, P. W. (1985) *J. Clin. Invest.* **76**, 2178–2181
- Jackson, C. M. & Nemerson, Y. (1980) *Annu. Rev. Biochem.* **49**, 765–811
- Kisiel, W. & Davie, E. W. (1981) *Methods Enzymol.* **80**, 320–332
- Mann, K. G. (1984) *Prog. Hemostasis Thromb.* **7**, 1–23
- Maruyama, I., Salem, H. H. & Majerus, P. W. (1984) *J. Clin. Invest.* **74**, 224–230
- Nicholls, P. (1981) *Int. Rev. Cytol.* **12**, 327–388
- Owen, W. G. & Jackson, C. M. (1973) *Thromb. Res.* **3**, 705–714
- Salem, H. H., Esmon, N. L., Esmon, C. T. & Majerus, P. W. (1984a) *J. Clin. Invest.* **73**, 968–972
- Salem, H. H., Maruyama, I., Ishii, I. & Majerus, P. W. (1984b) *J. Biol. Chem.* **259**, 12246–12251
- Stenflo, J. & Suttie, J. W. (1977) *Annu. Rev. Biochem.* **46**, 157–172
- Walker, F. J. (1984) *Semin. Thromb. Hemostasis* **10**, 131–138
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Zwall, R. F. A. (1978) *Biochim. Biophys. Acta* **515**, 163–205
- Zwall, R. F. A. & Hemker, H. C. (1982) *Haemostasis* **11**, 12–39