Activation of human protein C by blood coagulation factor Xa in the presence of anionic phospholipids

Enhancement by sulphated polysaccharides

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The activation of protein C by thrombin is thought to occur at the endothelial cell surface in the presence of an essential membrane glycoprotein cofactor, thrombomodulin. In the present study it is demonstrated that, in the presence of hirudin, the most potent known inhibitor of thrombin, human protein C can be activated by human factor Xa (20 nM), but by a thrombomodulin-independent mechanism requiring only the presence of Ca^{2+} and phospholipid vesicles bearing a high proportion of negative charges (30–75 % phosphatidylserine, depending on the conditions). At an optimal concentration of phosphatidylserine/phosphatidylcholine (1:1, w/w) of 75 μM , the apparent K_m was 1 μM with a k_{cat} of 1 min^{-1} . At 25 μM -phospholipid the K_m was unchanged and the k_{cat} was 0.67 min^{-1} . At either lipid concentration, increasing the density of negative charges by the adjunction of sulphated polysaccharides, like pentosan polysulphate or standard heparin at optimal concentrations of 2–5 $\mu\text{g}/\text{ml}$ and 5–10 $\mu\text{g}/\text{ml}$ respectively, resulted in a 4-fold increase of the k_{cat} , without affecting the K_m . Sulphated polysaccharides alone were poor promoters of protein C activation by factor Xa. In any case the presence of Ca^{2+} was essential, the dependence being sigmoidal with Hill coefficients ranging from 1.4 to 2.0. No significant activation of 4-carboxyglutamic acid-domainless protein C, a chymotryptic derivative lacking the phospholipid-binding domain, could be detected in the presence of phospholipids and Ca^{2+} , with or without pentosan polysulphate. In a large molar excess, other phospholipid-binding entities like prothrombin fragments F1 or F_{1+2} could inhibit protein C activation by factor Xa, but pentosan polysulphate exerted a clear protective effect. Factor Xa irreversibly inhibited at its active centre, but not di-isopropyl phosphoro-thrombin, behaved as an inhibitor but in a more complex manner than simple Michaelis–Menten kinetics. Among several derivatives of pentosan polysulphate or of heparin which were tested, those having the higher degree of sulphation and/or molecular mass were the most efficient in enhancing the rate of activation of protein C by factor Xa in the presence of phospholipids. These results suggest that human factor Xa, at physiological concentrations, could activate human protein C in the presence of anionic phospholipids and that this activation could be potentiated by therapeutic concentrations of sulphated polysaccharides.

INTRODUCTION

Protein C is the central element of one of the major systems of control of blood coagulation. It circulates as the vitamin K-dependent precursor of a serine proteinase (Stenflo, 1988). Until now thrombin was the only known physiological activator of protein C in the presence of thrombomodulin, an essential membrane glycoprotein cofactor of endothelial origin (Esmon, 1987). Using purified systems, it has been observed that reconstitution of human placenta thrombomodulin (Freyssinet *et al.*, 1986) or of rabbit lung thrombomodulin (Galvin *et al.*, 1987) into phospholipid vesicles could enhance protein C activation. Activated protein C functions as a potent anticoagulant in the presence of its specific cofactor, protein S, another vitamin K-dependent protein, and

anionic phospholipids by degrading factors Va and VIIIa, thus limiting the (auto)amplification of thrombin generation (Walker, 1984; Mann, 1988). The physiological significance of the protein C pathway in the regulation of blood coagulation is clearly demonstrated by the increased risk of recurrent thromboembolism in patients presenting acquired or inherited protein C or S deficiency responsible for a decrease of biological activity below the pathological level of around 50 % of the normal (Bertina, 1988). The surprising absence of thrombomodulin from brain endothelium (Ishii *et al.*, 1986; Wen *et al.*, 1987) and its different concentrations due to different surface/volume ratios in the remainder of the vasculature raise the question of the existence of another pathway of protein C activation.

The ability of human factor Xa to activate human

Abbreviations used: $\text{iPr}_2\text{P-F}$, di-isopropyl phosphorofluoridate; FPR, D-phenylalanyl-L-prolyl-L-arginine aldehyde sulphate; FPRCK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; DNS-EGRCK, dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone; n-octyl glucoside, 1-O-n-octyl β -D-glucopyranoside; PC, phosphatidylcholine; PS, phosphatidylserine; GD-protein C, 4-carboxyglutamic acid-domainless protein C; F1, prothrombin fragment 1; F_{1+2} , prothrombin fragment F_{1+2} ; a.t.u., anti-thrombin unit.

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protein C was examined in the present study. Factor Xa could activate protein C to detectable extents only when calcium and phospholipid vesicles bearing a high proportion of negative charges (~ 50 – 75%) were present. This process was strictly thrombomodulin-independent. Increasing the density of negative charges by the adjunction of certain sulphated polysaccharides (of the pentosan polysulphate or heparin class) resulted in an enhanced rate of protein C activation due to a k_{cat} effect; the higher the degree of sulphation and/or size, the higher the efficiency. The presence of an efficient sulphated polysaccharide-like pentosan polysulphate allowed the reduction of the proportion of phospholipid negative charges down to $\sim 30\%$ for maximal effect. Furthermore this compound could exert a protective effect against possible interference of other vitamin K-dependent phospholipid-binding proteins or fragments.

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MATERIALS AND METHODS

Materials

Chondroitin sulphate (type B, from porcine skin), heparan sulphate, human serum albumin (essentially fatty-acid-free) and di-isopropyl phosphorofluoridate ($i\text{Pr}_2\text{P-F}$) were products from Sigma, St. Louis, MO, U.S.A. Dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone (DNS-EGRCK) and D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (FPRCK) were from Calbiochem, La Jolla, CA, U.S.A. D-phenylalanyl-L-propyl-L-arginine aldehyde sulphate (FPR) was a kind gift from Laboratoires Pierre Fabre, Castres, France. Phosphatidylcholine (PC) and phosphatidylserine (PS) from bovine spinal cord were purchased from Lipid Products, Redhill, Surrey, U.K. Chromogenic substrates S2366 (L-pyrogutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride) and S2222 (*N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride and its methyl ester) and low-molecular mass heparin 2165 were from Kabi Vitrum, Stockholm, Sweden. *n*-Octylglucoside was obtained from Boehringer, Mannheim, Germany. α -Chymotrypsin from bovine pancreas (45–70 units/mg of protein) was from Worthington, Freehold, NJ, U.S.A. The kit for protein assay and Chelex resin were products from Bio-Rad, Richmond, CA, U.S.A. Factor II concentrates (PPSB, about 250 prothrombin units/vial) and factor VIII concentrates (500 units/vial) were obtained from the Centre Régional de Transfusion Sanguine, Strasbourg, France. L- α -Di-[1- ^{14}C]palmitoyl phosphatidylcholine (110 mCi/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Russell's viper venom and highly purified hirudin [10000–12500 anti-thrombin units (a.t.u.) per mg] were from Diagnostica Stago, Asnières, France. Benzamidine-Sepharose and dextran sulphate ($M_r \sim 500000$) were products from Pharmacia, Uppsala, Sweden. Pentosan polysulphate (average M_r 4700, degree of sulphation = 1.9) and derivatives were gifts from Sanofi Recherche, Toulouse, France. Standard heparin, low-molecular-mass heparins CY 216 and CY 222 and pentasaccharide were generous gifts from Laboratoire Choay, Paris, France. Low-molecular-mass heparin PK 10169 was from Pharmuka, Gennevilliers, France.

Proteins

Human blood coagulation factors II and X were isolated by barium citrate adsorption followed by ion-exchange chromatography and affinity chromatography on insolubilized dextran sulphate (Freyssinet *et al.*, 1982). Human α -thrombin [3000 National Institutes of Health (NIH) units/mg of protein] was obtained as already described (Freyssinet *et al.*, 1986). Factor Xa was prepared by activating factor X with Russell's viper venom in the presence of 10 mM- CaCl_2 but in the absence of procoagulant phospholipids at an enzyme/substrate (w/w) ratio of 1:100 (Jesty & Nemerson, 1976). In order to minimize the formation of factor Xa_β , factor Xa was rapidly separated from factor X and Russell's viper venom by adsorption on benzamidine-Sepharose and elution with 0.5 M-benzamidine. Human protein C was purified to homogeneity (as judged by SDS/polyacrylamide-gel electrophoresis) from human plasma cryosupernatant as previously reported (Freyssinet *et al.*, 1985). 4-Carboxyglutamic acid-domainless protein C (GD-protein C) was prepared by limited proteolysis of protein C by α -chymotrypsin as already published (Freyssinet *et al.* 1986). Human factor V was obtained following the method of Nesheim *et al.* (1981). Human placenta thrombomodulin was extracted at pH 10.5 and was purified according to the method of Jakubowski *et al.* (1986). Protein concentrations were determined using the Bio-Rad protein assay, with bovine serum albumin as standard. $i\text{Pr}_2\text{P}$ -thrombin and DNS-EGR-Xa were prepared by incubating 9.3 μM -thrombin with 5 mM- $i\text{Pr}_2\text{P-F}$ and 9.8 μM -factor Xa with 1 mM-DNS-EGRCK for 30 min at 37 °C in 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl. Excess inhibitor was then removed by extensive dialysis against the same buffer.

Thrombomodulin was reconstituted into phospholipid vesicles as described by Galvin *et al.* (1987) and by Freyssinet *et al.* (1988). Phospholipid vesicles were formed by dialysis to eliminate non-ionic detergent used to solubilize the lipids. Organic solvent was carefully evaporated and phospholipids were dissolved in 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl and 2% (w/v) *n*-octyl glucoside at a final concentration of 2 mg/ml. The lipid mixtures were then dialysed against 3×100 vol. of the above buffer without *n*-octyl glucoside, in dialysis tubing of 12000 to 14000- M_r cut-off for 36 h at room temperature. The molar ratios of phospholipid-to-protein and phospholipid concentrations were estimated by counting the radioactivity of tracer [^{14}C]PC after dialysis and assuming $M_r = 780$ for the phospholipids (Freyssinet *et al.*, 1988).

Prothrombin fragments F1 and F_{1+2} were prepared according to Owen *et al.* (1974) and Esmon *et al.* (1974) respectively.

Methods

The activation of protein C by the thrombin-thrombomodulin complex (thrombomodulin being either pure or reconstituted in phospholipid vesicles) was performed in 50 mM-Tris, pH 7.5, 0.1 M-NaCl, 2 mM- CaCl_2 and 20 μM -human serum albumin in a final volume of 200 μl for 15 min or shorter periods at 37 °C. The reaction was terminated by the addition of 5 a.t.u. of hirudin in 5 μl . The incubation time and/or protein C concentration was adjusted so that less than 20% of the protein C was activated. Then 795 μl of 0.22 mM-S2366 was added and

the rate of hydrolysis was recorded at 405 nm for 3 min at 25 °C. The concentration of activated protein C formed was determined by reference to a standard curve constructed using known amounts of activated protein C.

The activation of protein C by factor Xa was carried out, at an enzyme concentration of 20 nM unless otherwise stated, in 50 mM-Tris, pH 7.5, 0.1 M-NaCl, 20 μ M-human serum albumin and 5 a.t.u. of hirudin in a final volume of 200 μ l for 30 min or shorter periods at 37 °C. The incubation mixture could contain phospholipids, sulphated polysaccharides and/or other proteins at various concentrations as given in the Results section. The reaction was terminated by the addition of 5 μ l of DNS-EGRCK, giving a final concentration of this specific inhibitor of factor Xa of 20 μ M or 1 μ M in case of verification of the activity of activated protein C towards factor VIIIa.

After generation by different activating systems, the anticoagulant effect of activated protein C on factor VIIIa activity was verified qualitatively according to Fay *et al.* (1982). The dilution of factor VIIIa was adjusted to give a clotting time of $\sim$ 100 s (i.e. corresponding to 1

unit of factor VIII/ml in our assay) and the dilution of the incubation mixture where activated protein C was generated was such that the inhibitor(s) had no significant effect on the prolongation of the clotting time, thus due only to the presence of activated protein C. Under these conditions, 10 ng of activated protein C gave a clotting time of $\sim$ 200 s, to be compared with a blank (i.e. with no factors VIII or VIIIa) of $\sim$ 250 s. A similar verification could be carried out with factor Va following a comparable procedure, but the sensitivity was lower, probably due to shorter clotting times; consequently, most of the assays were performed at the factor VIIIa level.

RESULTS

Apparent activation of protein C by factor Xa in the presence of thrombomodulin and phospholipids

In a human homogeneous system, factor Xa (20 nM) behaved as an apparent potent activator of protein C (0.6 μ M) in the presence of thrombomodulin (14 nM) reconstituted into PS/PC vesicles (1:1, w/w). The rate of protein C activation was comparable with that obtained

Table 1. Apparent activation of protein C by factor Xa

In these experiments protein C was 0.6 μ M, factor Xa was 20 nM, thrombomodulin (TM) was 14 nM, DNS-EGR-Xa was 20 nM and factor V was 20 nM. Where indicated, 5 a.t.u. of hirudin was present during activation. TM was reconstituted into PS/PC vesicles (1:1, w/w) at a protein-to-lipid molar ratio of 1:12500. The assay of generated activated protein C and other experimental details were as described in the Materials and methods section. Each value is the mean $\pm$ s.d. of four independent determinations carried out using two different factor Xa and protein C preparations. n.d., no detectable activation.

Activator	<i>v</i> (pmol/min per ml)
Factor Xa	n.d.
Factor Xa + TM	n.d.
Factor Xa + TM + PC vesicles	n.d.
Factor Xa + TM + PS/PC vesicles	42.4 $\pm$ 2.1
Factor Xa + TM + PS/PC vesicles + hirudin	10.5 $\pm$ 0.5
DNS-EGR-Xa + TM + PS/PC vesicles	n.d.
Factor Xa + TM + PS/PC vesicles + factor V	83.2 $\pm$ 3.5
Factor Xa + TM + PS/PC vesicles + factor V + hirudin	12.3 $\pm$ 0.7
Thrombin (2 nM) + TM + PS/PC vesicles	44.7 $\pm$ 2.2
Thrombin (10 nM) + TM + hirudin	n.d.
Thrombin (10 nM) + TM + PS/PC vesicles + hirudin	n.d.

Table 2. Effect of antibodies to human placenta thrombomodulin and of the absence of thrombomodulin on the apparent activation of protein C by factor Xa and on the activation by thrombin

In these experiments protein C was 0.6 μ M, factor Xa was 20 nM, thrombomodulin (TM) was 14 nM, thrombin was 4 nM and IgG fractions were present at a final concentration of 1.2 mg/ml. Thrombomodulin was reconstituted into PS/PC vesicles (1:1, w/w) at a protein-to-lipid molar ratio of 1:12500. When PS/PC vesicles were used without TM, the phospholipid concentration was 75 μ M. Hirudin (5 a.t.u.) was added where indicated. The assay of generated activated protein C and other experimental details were as described in the Materials and methods section. Each value is the mean $\pm$ s.d. of four independent determinations carried out using two different factor Xa and protein C preparations. n.d., no detectable activation.

Activator	<i>v</i> (pmol/min per ml)
Factor Xa + TM + PS/PC vesicles + hirudin	10.5 $\pm$ 0.5
Factor Xa + TM + PS/PC vesicles + hirudin + control rabbit IgG	12.2 $\pm$ 0.6
Factor Xa + TM + PS/PC vesicles + hirudin + anti-TM IgG	11.1 $\pm$ 0.7
Thrombin + TM + PS/PC vesicles + control rabbit IgG	78.3 $\pm$ 2.9
Thrombin + TM + PS/PC vesicles + anti-TM IgG	0.1 $\pm$ 0.1
Factor Xa + PS/PC vesicles $\pm$ hirudin	9.2 $\pm$ 0.3
Thrombin + PS/PC vesicles	n.d.

Fig. 1. Substrate concentration dependence of the activation of human protein C by factor Xa

Protein C, at various concentrations, was activated by factor Xa (20 nM) in the presence of 75 μ M-phospholipid (PS/PC vesicles, 1:1, w/w) and Ca^{2+} (2 mM), and in the absence (●) or in the presence (▲) of pentosan polysulphate at a final concentration of 5 μ g/ml. The buffer was 50 mM-Tris, pH 7.5, containing 0.1 M-NaCl, 20 μ M-human serum albumin and 0.02 % NaN_3 . Hirudin (5 a.t.u.) was present during activation. The assay of generated activated protein C and other experimental details were as described in the Materials and methods section. The insert represents the double-reciprocal plot of the data constructed using linear regression analysis (all points were taken into account for calculation but the last one was not plotted in order to respect proportions). In the absence of pentosan polysulphate the apparent K_m was 1 μ M with a corresponding V_{max} of ~ 20 pmol/min per ml; in the presence of 5 μ g of pentosan polysulphate/ml, the K_m remained unchanged but the V_{max} was enhanced 4-fold. Each value is the mean of three independent determinations with s.d. never exceeding 6%.

in the presence of 2 nM-thrombin-thrombomodulin complex (Table 1). When thrombomodulin was incorporated into PC vesicles or used without lipids, no significant activation occurred. In the presence of hirudin during the activation, the rate of protein C activation by factor Xa and thrombomodulin incorporated into negatively charged phospholipid vesicles was reduced by approx. 75 % (Table 1). When hirudin was added at the end of the activation period only, the reduction of amidolytic activity was of the order of 10 % when compared with that measured in the absence of hirudin, thus testifying to the presence of generated thrombin. In this system with 2 nM-thrombin (i.e. only 0.21 NIH units) as a substitute for factor Xa, protein C was activated at the same rate. When factor Xa was pretreated with its specific irreversible inhibitor DNS-EGRCCK (20 μ M final concentration), no significant activation of protein C could be detected either in the presence or absence of hirudin. In the presence of 20 nM-factor V, the apparent activation of protein C by factor Xa and thrombomodulin reconstituted into PS/PC vesicles was more efficient. Once again hirudin, when added at the same time as factor Xa, was responsible for an 85 % decrease of protein C activation (Table 1).

In order to eliminate the contribution of traces (less than 0.5 NIH units) in the activation of protein C by factor Xa, all further experiments involving factor Xa as

Fig. 2. Influence of the proportion of anionic phospholipids on the factor Xa-dependent activation of protein C

Protein C (0.75 μ M) was activated by factor Xa (20 nM) in the presence of Ca^{2+} (2 mM) and of phospholipid vesicles (50 μ M-phospholipid) containing various proportions of negative charges (i.e. PS) and in the absence (■) or in the presence (▲) of pentosan polysulphate at a final concentration of 5 μ g/ml. Hirudin (5 a.t.u.) was present during activation. The assay of generated activated protein C and other experimental details were as described in the Materials and methods section. Each value is the mean of three independent determinations with s.d. never exceeding 6%.

the activating enzyme were performed in the presence of a large excess of hirudin (5 a.t.u.), which would block any protein C activation by the 10 nM-thrombin-thrombomodulin complex (see Table 1). Antibodies to thrombomodulin had no influence on the activation of protein C by factor Xa in the presence of thrombomodulin and negatively-charged phospholipids. Such antibodies were able to block protein C activation by thrombin (Table 2). Furthermore, a 350-fold molar excess of iPr_2P -thrombin had no significant influence on the activation of protein C by factor Xa in the presence of phospholipid-reconstituted thrombomodulin. Finally, Table 2 shows that factor Xa could activate protein C only in the presence of negatively charged phospholipids either in the presence or absence of hirudin. In the absence of hirudin, but still in the presence of Ca^{2+} , contaminant thrombin could not activate protein C without thrombomodulin (Freyssinet *et al.*, 1986).

In any case, with the appropriate dilutions and controls, it could be deduced that activated protein C was the enzyme generated by the above systems by assaying qualitatively its ability to destroy factor VIIIa procoagulant activity, 10 ng of activated protein C being responsible for a prolongation of the clotting time of ~ 100 s (see the Materials and methods section). Furthermore, protein C activated by factor Xa gave an electrophoretic pattern indistinguishable from that obtained after activation by thrombin (results not shown).

The above results suggest that factor Xa alone could activate protein C in a reaction depending only on the presence of anionic phospholipids and not on thrombomodulin. Replacing hirudin by other thrombin inhibitors like FPR or FPRCK (used at a final concentration of 1 μ M) led to the same conclusions but with apparently lower rates of activation because of their

Fig. 3. Calcium dependence of the activation of protein C by factor Xa

Protein C ($0.75 \mu\text{M}$) was activated by factor Xa (20 nM) in the presence of $75 \mu\text{M}$ -phospholipid (PS/PC vesicles, 1:1, w/w) and in the absence (▲) or in the presence (●) of pentosan polysulphate at a final concentration of $5 \mu\text{g/ml}$, at various Ca^{2+} concentrations. Buffers were treated with Chelex (Bio-Rad) before use. Hirudin (5 a.t.u.) was present during activation. Other experimental conditions are detailed in the Materials and methods section. Each value is the mean of two independent determinations with s.d. never exceeding 8%.

Fig. 4. Pentosan polysulphate concentration and phospholipid concentration dependence of the activation of protein C by factor Xa

Protein C ($0.75 \mu\text{M}$) was activated by factor Xa (20 nM) in the presence of Ca^{2+} (2 mM) and of phospholipids (PS/PC vesicles, 1:1, w/w) at various final concentrations (◆, $5 \mu\text{M}$; ●, $15 \mu\text{M}$; ■, $25 \mu\text{M}$; ▲, $75 \mu\text{M}$) and of pentosan polysulphate (PPS) also at various final concentrations. Hirudin (5 a.t.u.) was present during activation. See the Materials and methods section for further experimental details. Each value is the mean of two independent determinations with s.d. never exceeding 7%.

interference in the expression of the activity of either factor Xa or activated protein C, or both. This was probably due to the close specificities of these enzymes; consequently, hirudin was the only thrombin inhibitor to be used in further studies.

Activation of protein C by factor Xa in the presence of phospholipids and of pentosan polysulphate

Protein C at various concentrations could be activated by factor Xa (20 nM) in the presence of 2 mM - CaCl_2 and of PS/PC vesicles (1:1, w/w). At $75 \mu\text{M}$ -phospholipid, a V_{max} of the order of $20 \text{ pmol/min per ml}$ was measured, corresponding to a k_{cat} of 1 min^{-1} ; the apparent K_m was $1 \mu\text{M}$ (Fig. 1). At $25 \mu\text{M}$ -phospholipid, the k_{cat} was 0.67 min^{-1} but the K_m remained unchanged at $1 \mu\text{M}$ (results not shown). Vesicles containing a high density of negative charges were the most efficient in promoting this activation (Fig. 2). Therefore, an interesting possibility was that of increasing the concentration of negatively-charged groups by incorporating a sulphated polysaccharide in the activation medium. In the presence of pentosan polysulphate ($5 \mu\text{g/ml}$) in this factor-Xa-dependent system, the V_{max} and consequently the k_{cat} values were enhanced 4-fold at either phospholipid concentration without any significant change in the K_m (Fig. 1). Factor V (20 nM) had no noticeable effect either in the presence or in the absence of pentosan polysulphate. In the absence of pentosan polysulphate, vesicles containing up to 75% PS had the strongest ability to promote protein C activation by factor Xa, while this proportion could be lowered to 33% in the presence of this sulphated polysaccharide (Fig. 2). In any case, we noticed that phospholipid vesicles had to be freshly prepared by the dialysis method for optimal effect, whereas vesicles prepared by sonication were less efficient.

At both phospholipid concentrations and in the pres-

ence or absence of pentosan polysulphate, the Ca^{2+} -dependence of protein C activation by factor Xa was clearly sigmoidal, with Hill coefficients ranging from 1.4 to 2.0 (Fig. 3, data shown only for $75 \mu\text{M}$ -phospholipid). No activation occurred in the absence of Ca^{2+} , i.e. in the presence of 1 mM -EDTA. This dependence could be explained by a tentative multistep saturation scheme, with the saturation of the intrinsic high-affinity Ca^{2+} -binding sites of factor Xa and protein C followed by that of the Ca^{2+} -dependent interaction of these proteins with phospholipids and/or that of negatively-charged phospholipids.

The dependence of protein C activation by factor Xa on the concentration of pentosan polysulphate was examined at several concentrations of phospholipid vesicles containing equal amounts of PS and PC (Fig. 4). Optimal conditions were achieved at 25 – $75 \mu\text{M}$ -phospholipid and at 2 – $10 \mu\text{g}$ of pentosan polysulphate/ml. Above $10 \mu\text{g/ml}$, the sulphated polysaccharide interfered in the amidolytic activity of activated protein C, causing an apparent decrease. When phospholipids were omitted in these assays, pentosan polysulphate at concentrations ranging from 5 to $20 \mu\text{g/ml}$ was a poor promoter of protein C activation by factor Xa, with rates of activation less than 5% of those obtained in the presence of vesicles.

In all of the above experiments involving factor Xa as the activating enzyme in co-operation with phospholipids, and with or without sulphated polysaccharides, no significant activation of GD-protein C ($0.5 \mu\text{M}$) could be detected. At equivalent concentration, the rate of activation of GD-protein C by the thrombin–thrombomodulin complex was similar to that of protein C (Freyssinet *et al.*, 1986; Galvin *et al.*, 1987).

When present in a large molar excess during protein C activation by factor Xa and phospholipids, prothrombin fragments F1 and F_{1+2} behaved as inhibitors, but

Table 3. Protective effect of pentosan polysulphate on the inhibition of factor Xa-dependent activation of protein C by prothrombin fragments F1 and F₁₊₂

In these experiments protein C was 0.6 μM , factor Xa was 20 nM, phospholipids (PS/PC, 1:1, w/w) were 50 μM , Ca^{2+} was 2 mM and 5 a.t.u. of hirudin was present during activation. See the Materials and methods section for further experimental details. PPS, pentosan polysulphate. Each value is the mean $\pm$ S.D. of three independent determinations. n.d., no detectable activation.

Activator	Phospholipid antagonist...	<i>v</i> (pmol/min per ml)		
		None	F1 (3 μM)	F ₁₊₂ (1.5 μM)
Factor Xa + PS/PC vesicles		10.4 $\pm$ 0.5	n.d.	1.7 $\pm$ 0.5
Factor Xa + PS/PC vesicles + 5 μg of PPS/ml		41.2 $\pm$ 2.3	13.9 $\pm$ 0.7	18.6 $\pm$ 0.9

Table 4. Effect of various sulphated polysaccharides on the activation of protein C by factor Xa in the presence of PS/PC vesicles

In these comparative experiments protein C was 0.6 μM , factor Xa was 20 nM, phospholipids (PS/PC, 1:1, w/w) were 50 μM , Ca^{2+} was 2 mM and 5 a.t.u. of hirudin was present during activation. Other experimental conditions are detailed in the Materials and methods section. All sulphated polysaccharides were present at 5 $\mu\text{g}/\text{ml}$. Each value is the mean of two independent determinations with S.D. never exceeding 7%. Control protein C activation (factor Xa + PS/PC vesicles only) was 9.2 pmol/min per ml.

Sulphated polysaccharide present	<i>v</i> (pmol/min per ml)
PPS	38.7
SR 25493*	32.9
SR 25185†	44.5
SR 25839‡	18.0
MSB 3A 65§	9.1
Standard heparin	35.4
CY 216	28.1
CY 222	17.8
PK 10169	27.7
Kabi 2165	31.5
Pentasaccharide	8.8
Chondroitin sulphate type B¶	9.3
Heparan sulphate	9.0
Dextran sulphate	45.7

* Low M_r pentosan polysulphate (PPS), M_r = 3160, degree of sulphation = 1.85.

† High M_r PPS, M_r = 9600, degree of sulphation = 1.9.

‡ Partially desulphated PPS, M_r = 3740, degree of sulphation = 1.04.

§ Almost fully desulphated PPS, M_r = 2100, degree of sulphation = 0.4.

|| Minimum structure able to bind to anti-thrombin III.

¶ Also termed dermatan sulphate.

pentosan polysulphate at 5 $\mu\text{g}/\text{ml}$ exerted a clear protective effect (Table 3).

DNS-EGR-Xa, i.e. Factor Xa irreversibly inhibited at its active centre by DNS-EGRCK, inhibited protein C activation by factor Xa in the presence of PS/PC vesicles, either in the absence or presence of pentosan polysulphate, 50% inhibition being observed in the range 1.6–2.2 μM -DNS-EGR-Xa. However, in both cases, significant deviation from simple Michaelis–Menten kinetics

occurred. Inhibition became significant at only $\sim 0.8 \mu\text{M}$ -DNS-EGR-Xa in the absence of pentosan polysulphate and at $\sim 1 \mu\text{M}$ in its presence, total inhibition being achieved at $\sim 5 \mu\text{M}$ - and 7.5 μM -DNS-EGR-Xa respectively. This behaviour might be explained by a complex competition between factor Xa and DNS-EGR-Xa for binding to phospholipids and/or protein C.

Effect of various sulphated polysaccharides on the activation of protein C by factor Xa in the presence of phospholipids

Several sulphated polysaccharides including high- and low-molecular-mass fractions of pentosan polysulphate with different degrees of sulphation, standard and low-molecular-mass heparins as well as chondroitin sulphate (type B), heparan sulphate and dextran sulphate were assayed for their ability to accelerate the activation of protein C by factor Xa in the presence of PS/PC vesicles (Table 4). The compounds having a high molecular mass and a high degree of sulphation were found to be the most efficient.

DISCUSSION

The activation of protein C by thrombin is under cellular control, requiring an endothelial-cell-membrane cofactor, thrombomodulin (Esmon, 1987). There is experimental evidence demonstrating that this is also true *in vivo* (Comp *et al.*, 1982). The interference of thrombomodulin in the inhibition of factor-Xa-mediated activation of prothrombin has been reported, and was attributed to factor Xa–thrombomodulin interaction (Thompson & Salem, 1986). In another study, Esmon & Esmon (1986) found that factor Xa could activate protein C in the presence of detergent-solubilized thrombomodulin. The reaction rate was considerably enhanced upon reconstitution of thrombomodulin into negatively charged phospholipid vesicles with a concomitant enhancement of the affinity constant of the enzyme for the cofactor; a further increase was noticed in the presence of factor Va. In contrast, Thompson & Salem (1988) could not detect activation of protein C by factors Xa, IXa or XIa, or by activated protein C itself in the presence of either detergent-solubilized thrombomodulin or thrombomodulin reconstituted into phospholipid vesicles. In the present article we report different results suggesting that human protein C could be activated by human factor Xa by a mechanism independent of thrombomodulin but necessitating the presence of negatively charged phospholipids. Such an activation was

enhanced in the presence of sulphated polysaccharides of the pentosan polysulphate class or of the heparin class. Furthermore, these sulphated compounds were found to act as protective agents against inhibition of protein C activation by phospholipid-binding entities like prothrombin fragments F1 and F₁₊₂.

In a homogeneous human system, factor Xa at physiological concentrations could apparently activate protein C in the presence of thrombomodulin, but only when the latter was reconstituted into negatively charged phospholipid vesicles. However, when the same experiments were repeated in the presence of hirudin, the most potent and specific known thrombin inhibitor ($K_i \sim 20 \times 10^{-15}$ M; Braun *et al.*, 1988), the level of protein C activation was reduced by approx. 80 %. This observation strongly suggests that traces of thrombin could have been generated from contaminant traces of prothrombin present in protein C preparations which appeared very difficult to eliminate even by affinity chromatography on immobilized anti-prothrombin IgG (and even after three consecutive passages on the affinity column). This conclusion was further supported by an even more efficient apparent activation of protein C in the presence of factor V, but in the absence of hirudin, probably due to the assembly of the complete prothrombinase complex which is able to generate thrombin more rapidly.

When the contribution from traces of thrombin was totally eliminated in the presence of a large molar excess of hirudin, the apparent activation of protein C by factor Xa did not depend any longer on thrombomodulin, since it could occur in the presence of antibodies neutralizing the cofactor activity. However, the reaction remained strictly dependent on the presence of negatively charged phospholipids and Ca²⁺. Generated activated protein C displayed a strong ability to destroy factor VIIIa procoagulant activity. Hence these results demonstrate that human factor Xa can activate human protein C in the presence of anionic phospholipids by a thrombomodulin-independent mechanism.

An interesting observation is that of the enhancing effect of sulphated polysaccharides on the activation of protein C by factor Xa in the presence of negatively charged phospholipids and Ca²⁺. This suggests that, in addition to their well-documented direct anticoagulant effects (Fischer *et al.*, 1982; Rosenberg, 1987), these compounds could be involved in the control of thrombin generation due to their capacity to up-regulate thrombomodulin-independent activation of protein C. On the one hand, they would compete with vitamin K-dependent procoagulant factors for binding to phospholipids, thus reinforcing their anticoagulant action (Walker & Esmon, 1979; Ofosu *et al.*, 1980), and on the other hand they would co-operate with the same phospholipids to promote the activation of protein C by factor Xa. Furthermore, they could exert a protective effect against other vitamin K-dependent phospholipid-binding proteins or fragments which could interfere in the factor Xa-dependent activation of the anticoagulant protein C pathway. These results would also provide a supplementary explanation for the anticoagulant properties of pentosan polysulphate (Fischer *et al.*, 1982), since it could favour the inactivation of factors VIIIa and/or Va.

It is important to emphasize that, in this study, the concentrations of the various components involved in the activation of protein C by factor Xa are compatible

with their respective physiological or therapeutic circulating concentration. The circulating concentration of factor X is ~ 140 nM, while that of factor V is ~ 20 nM, and since factors Xa and Va assemble in a tight 1:1 complex to form prothrombinase, the degree of activation of factor V is the rate-limiting step in the expression of the coagulant activity of factor Xa (Nemerson, 1988). Thus it is conceivable that the excess of factor Xa might be involved in the control of the extension of coagulation, for instance through its participation in the activation of protein C. A phospholipid concentration of $40 \mu\text{M}$ is close to that contributed by about 3×10^8 platelets per ml (Bevers *et al.*, 1982). The inner half of platelet plasma membrane bears 34 % (mol/mol) PS (Zwaal *et al.*, 1977), but the lateral distribution of these negatively charged groups is not known. However, upon platelet or other cell-type activation, it might well be possible that clusters of anionic phospholipids become exposed, thus increasing the local density of negative charges to provide an optimal catalytic surface for the activation of protein C. The existence of a more complex activator patch remains an open question. It might involve endogenous heparin, but in this case its composition should vary since this sulphated polysaccharide is not equally distributed among the organs and the vasculature of different species (Nader *et al.*, 1980; Hovingh *et al.*, 1986). For preventive therapeutic treatment, the current unit dose of pentosan polysulphate is 50 mg, which would give an instantaneous plasma concentration of $\sim 20 \mu\text{g/ml}$. For standard heparin figures are comparable, higher doses being used under acute circumstances.

The integrity of protein C must be preserved to allow its activation in these factor Xa-dependent systems since no activation of GD-protein C, a chymotryptic derivative lacking the phospholipid-binding domain, could be detected. In the light of the requirements for a relatively high proportion of anionic phospholipids and/or a high degree of sulphation of the polysaccharide, it seems reasonable to hypothesize that these negatively charged entities could act as a template, accommodating both enzyme and substrate, to catalyse protein C activation. The catalytic efficiency of factor Xa and phospholipids, i.e. the second-order rate constant $k_{\text{cat.}}/K_m$, is only one order of magnitude smaller than that reported for protein C activation by thrombin in the presence of cultured human vascular endothelial cells as a natural source of thrombomodulin (for a review see Freyssinet & Cazenave, 1988). This difference can be reduced to a factor of two in the presence of sulphated polysaccharides.

Of course, the present results, obtained *in vitro*, raise the crucial question of their physiological significance. Thrombomodulin, the essential cofactor of thrombin for protein C activation, is absent from brain endothelium (Ishii *et al.*, 1986; Wen *et al.*, 1987). Yet subjects with heterozygous protein C deficiency do not seem to exhibit an increased frequency of cerebral thrombotic events when compared with the general population (Miletich *et al.*, 1987), whereas newborns with homozygous protein C deficiency frequently present massive thrombosis of the central nervous system (Marlar *et al.*, 1988). This pathophysiological evidence militates in favour of the existence of a thrombin-thrombomodulin-independent system of activation of protein C. Factor Xa in association with tissue phospholipids and possibly with

endogenous heparin could constitute such an alternative activator. Although apparently less efficient, from a catalytic point of view *in vitro*, than the thrombin-thrombomodulin complex, its contribution would mainly depend on the local concentration of insolubilized factor Xa on to exposed active phospholipids. Since the concentration of thrombomodulin is elevated in the microcirculation due to favourable surface/volume ratios, it is expected that thrombin-dependent activation of protein C is the dominant step for the control of blood coagulation in this type of vasculature. However, in larger vessels like veins where stasis is the determinant condition for the development of thrombosis, factor Xa could efficiently participate in the activation of protein C even to a low extent, since activated protein C seems to exert its anticoagulant action *in vivo* on factors Va and VIIIa at concentrations considerably lower than 1 nM (Bauer *et al.*, 1984). Hence, in this context, the up-regulation of the factor Xa-dependent activation of protein C by sulphated polysaccharides would appear to be an interesting pharmacological possibility.

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