Activation of Factor V during intrinsic and extrinsic coagulation

Inhibition by heparin, hirudin and D-Phe-Pro-Arg-CH₂Cl

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The validity of the hypothesis that Factor Xa activates Factor V in heparinized plasma was examined by establishing the temporal relationships between Factor V proteolysis and prothrombin consumption in plasma. Factor V was cleaved into Factor Va heavy chain (approx. 110 kDa) and an intermediate (approx. 230 kDa) 30 s after CaCl₂ was added to contact-activated plasma (CAP). The larger fragment was converted into Factor V activation peptide (approx. 150 kDa) and Factor Va light chain (approx. 80 kDa) 15 s later. Heparin (approx. 0.05 μM) delayed Factor V proteolysis in CAP by at least 30 s. On supplementing CAP with 1 nM-Factor Xa or 1 nM-thrombin, Factor V was activated 15 s later. Heparin prolonged by 15 s and 45 s the time required to demonstrate Factor V activation in CAP supplemented with Factor Xa and thrombin respectively. Factor V was activated 20 s after tissue factor and CaCl₂ were added to plasma, both in the absence and in the presence of approx. 0.05 μM-heparin. In contrast, hirudin and D-Phe-Pro-Arg-CH₂Cl (two thrombin inhibitors more effective than heparin) delayed Factor V activation in this plasma by at least 30 s. The fragments of Factor V obtained in heparinized CAP suggest thrombin escapes inhibition and contributes to Factor V activation in that plasma. Production of Factor Va heavy chain and the 230 kDa Factor V fragment invariably preceded efficient prothrombin activation. These observations suggest that heparin, hirudin and D-Phe-Pro-Arg-CH₂Cl delay Factor V activation by inhibiting thrombin. The availability of Factor Xa markedly moderates the ability of heparin to inhibit Factor V activation.

INTRODUCTION

Blood coagulation Factor V is a single-chain glycoprotein that is activated to provide the cofactor for efficient prothrombin activation [1–6]. Prothrombinase, which consists of Factor Xa (the enzyme) and Factor Va on a coagulant surface in a Ca²⁺-dependent reaction, activates prothrombin at a rate reported to be five orders of magnitude faster than Factor Xa [1,2,4]. Recent studies have proposed that heparin delays prothrombin activation in contact-activated plasma (CAP) by inhibiting thrombin-mediated activation of Factor V and Factor VIII [7–10]. Thrombin or Factor Xa can activate both Factor V and Factor VIII [1,5,6,11–14]. The roles of these two enzymes in catalysing the amplification reactions in plasma are not fully defined. Although heparin is unable to delay prothrombin activation in CAP supplemented with Factor Xa, it delays by 45 s prothrombin activation in CAP supplemented with thrombin [9], suggesting that Factor Xa catalyses the amplification reactions in heparinized CAP [8,9]. The experiments reported in the present paper demonstrate that heparin delays Factor V activation in CAP. Secondly, heparin inhibits Factor V activation for a shorter time in CAP supplemented with Factor Xa than in CAP supplemented with thrombin. We also demonstrate that Factor Xa can activate plasma Factor V when endogenous thrombin is inactivated by inhibitors more efficient than heparin.

MATERIALS AND METHODS

Materials

Rabbit anti-(human Factor V) serum was obtained from Wellmark Diagnostics (Guelph, Ontario, Canada). The rapid

inhibitors of thrombin and Factor Xa, D-Phe-Pro-Arg-CH₂Cl and dansyl-Glu-Gly-Arg-CH₂Cl respectively [15,16], were obtained from Terachem (Toronto, Ontario, Canada). DEAE-Affigel Blue, reagents and equipment for SDS/PAGE and equipment for transblotting proteins on to nitrocellulose membranes were obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Activated-partial-thromboplastin-time (APTT) reagent and rabbit brain coagulant phospholipids were obtained from Organon Technika (Toronto, Ontario, Canada). Rabbit brain tissue factor was a gift from Dr. Leon Poller (National Centre for Anticoagulant Reagents and Control, Manchester, U.K.). Recombinant hirudin, an efficient thrombin inhibitor [17], was a gift from Dr. R. B. Wallis (CIBA-GEIGY, Horsham, West Sussex, U.K.). Heparin was a gift from Organon (Oss, The Netherlands). Human α-thrombin (specific activity 2750 units/mg), prepared by the procedures of Fenton *et al.* [18], was a gift from Dr. J. W. Fenton, II (New York Department of Health, Albany, NY, U.S.A.). Factor V, isolated from human plasma and labelled with Na¹²⁵I by using Iodobeads (Pierce, Rockford, IL, U.S.A.) to a specific radioactivity of 1.0 mCi/mg, was a gift from Dr. M. E. Nesheim (Queen's University, Kingston, Ontario, Canada). Human Factor V was also obtained from Haematologic Technologies (Essex Junction, VT, U.S.A.).

Proteolysis of plasma Factor V

Plasma Factor V proteolysis was initiated by contact activation and CaCl₂ addition, or by adding tissue factor and CaCl₂ to defibrinated plasma. Pooled normal plasma was defibrinated with 0.15 unit of Arvin/ml of plasma [19]. The plasma (2 vol.)

Abbreviations used: CAP, contact-activated plasma; APTT reagent, activated-partial-thromboplastin-time reagent.

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was added to 1 vol. of APTT reagent. After a 5 min incubation at 37 °C, 1 vol. of 40 mM-CaCl₂ (pre-warmed to 37 °C) was added. Samples (10 µl) were withdrawn at 15 s intervals and added to 10 µl of an EDTA/inhibitor buffer to stop further proteolysis of Factor V. The EDTA/inhibitor buffer consisted of 10 mM-EDTA, 36 mM-sodium barbiturate, 36 mM-sodium acetate, 2 µM-D-Phe-Pro-Arg-CH₂Cl and 2 µM-dansyl-Glu-Gly-Arg-CH₂Cl, at pH 7.4. Electrophoretic sample buffer (80 µl) was then added, the resulting solution was boiled for 4 min, cooled to room temperature, and a 50 µl sample subjected to PAGE in 5–15 %-gradient slab gels containing 0.1 % SDS [20]. Assuming the plasma Factor V concentration to be approx. 5 µg/ml, approx. 12 ng of Factor V/Factor V fragments was subjected to electrophoresis in each channel. The procedures of Towbin *et al.* were used to transfer the proteins from polyacrylamide gels on to nitrocellulose membranes [21]. The nitrocellulose sheets were incubated with the anti-(human Factor V) IgG fraction labelled with Na¹²⁵I to a specific radioactivity of 20 mCi/mg by using the chloramine-T method [22]. Factor V and its proteolytic derivatives were then located by exposure to X-ray films for 7–14 days.

Factor V proteolysis was also initiated by adding 1 vol. of rabbit brain tissue factor to 2 vol. of defibrinated plasma and incubating the suspension at 37 °C for 3 min. Then 1 vol. of 40 mM-CaCl₂ (pre-warmed to 37 °C) was added to initiate the coagulation. Samples were withdrawn at 10 s intervals into equal volumes of the EDTA/inhibitor buffer and Factor V proteolysis was determined by using the procedures described above.

In some experiments ¹²⁵I-labelled human Factor V was added to the plasma simultaneously with the CaCl₂ used to initiate Factor V proteolysis. After electrophoresis the gels were dried under vacuum, and Factor V and its proteolytic derivatives were

located by autoradiography. The effects of unfractionated heparin, hirudin and D-Phe-Pro-Arg-CH₂Cl on plasma Factor V proteolysis were also determined by adding each inhibitor in turn to the plasma simultaneously with CaCl₂.

Prothrombin activation

Prothrombin activation in plasma, initiated by the addition of tissue factor or APTT reagent as described above for Factor V activation, was quantified by a specific e.l.i.s.a. [9]. The first antibody used to measure prothrombin activation was sheep anti-(human prothrombin) serum affinity-purified on thrombin-Sepharose 4B, yielding IgG that reacted with both thrombin and prothrombin but not with prothrombin fragment 1.2 [9]. The second antibody was a rabbit anti-(human prothrombin) serum that did not react with thrombin when diluted 1 in 10000 or greater [9].

RESULTS

Intrinsic-pathway activation of Factor V

Control plasma. Factor V (approx. 330 kDa) and several derivatives (approx. 230 kDa to approx. 280 kDa) were detected in CAP by Western blotting before the addition of CaCl₂ (Fig. 1a, lane 5). At 30 s after CAP was replenished with CaCl₂ the 230 kDa Factor V intermediate had become the predominant species (Fig. 1a, lane 1). After a further 15 s Factor V activation peptide (approx. 150 kDa) had also been generated (Fig. 1a, lane 2). Proteolysis of ¹²⁵I-Factor V in CAP was investigated. As shown in Fig. 1(b), Factor V and several derivatives (approx. 110 kDa to 280 kDa) were seen in control CAP before the addition of CaCl₂ (Fig. 1b, lane 5). Factor Va light chain

Fig. 1. Inhibition of intrinsic Factor V activation by heparin

(a) Effects of approx. 0.05 µM-heparin (0.66 µg/ml) on Factor V proteolysis in CAP. Control CAP was applied in lanes 1–3 and 5, with the following Ca²⁺ replenishment times: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s. CaCl₂ was not added to CAP in lane 5. Radioactive molecular-mass markers ranging from 200 kDa to 14 kDa were applied in lane 4. CAP applied in lanes 6–8 contained heparin with the following Ca²⁺ replenishment times: lane 6, 60 s; lane 7, 90 s; lane 8, 120 s. Native Factor V and fragments of Factor V were identified by using rabbit anti-(human Factor V) IgG labelled with Na¹²⁵I. Abbreviations: V, native Factor V; A, Factor V activation peptide (approx. 150 kDa). (b) Effects of approx. 0.05 µM-heparin (0.66 µg/ml) on the proteolysis of ¹²⁵I-Factor V after CaCl₂ was added to CAP. Control CAP was applied in lanes 1–3 and 5, with the following Ca²⁺ replenishment times: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s. CaCl₂ was not added to CAP in lane 5. Radioactive molecular-mass markers as in (a) were applied in lane 4. CAP in lanes 6–8 contained heparin with the following Ca²⁺ replenishment times: lane 6, 60 s; lane 7, 90 s and lane 8, 120 s. Abbreviations: V, native Factor V; A, Factor V activation peptide (approx. 150 kDa); L and H, light chain and heavy chain of Factor Va respectively.

Fig. 2. Scheme summarizing the cleavage products of plasma Factor V resulting from the limited proteolysis by endogenously generated thrombin (a) and by Factor Xa (c)

Only native Factor V and its fragment containing Factor V activation peptide (approx. 150 kDa and labelled A in the Figure) readily reacted with the anti-(Factor V) antibodies used. Formation of the light chains (L) and heavy chains (H) of Factor V (and which do not react with the antibodies) was observed by using ^{125}I -Factor V. The only apparent cleavage catalysed by Factor Xa in plasma results in the formation of Factor Va heavy chain and the 230 kDa intermediate (c). The cleavage catalysed by Factor Xa appears to be the preferred cleavage by thrombin (a). In addition, thrombin cleaves the 230 kDa intermediate into Factor V activation peptide (A) and Factor Va light chain. An alternative initial cleavage of plasma Factor V by thrombin into Factor Va light chain and 260 kDa intermediate was also seen (b). The larger fragment was subsequently cleaved by thrombin into Factor Va heavy chain and Factor V activation peptide.

(apparent molecular mass approx. 80 kDa) was first observed in the 30 s sample (Fig. 1b, lane 1). Subsequently the largest species of Factor V detectable was the 230 kDa intermediate (Fig. 1b, lanes 2 and 3).

The following points emerge when the results in Figs. 1(a) and 1(b) are considered together. Two cleavages of native Factor V are observed within 30 s after CaCl_2 is added to CAP. The preferred cleavage gives Factor Va heavy chain (approx. 110 kDa) and an intermediate fragment with molecular mass approx. 230 kDa. Cleavage of the larger fragment yields Factor Va light chain (approx. 80 kDa) and a Factor V activation peptide (approx. 150 kDa). An alternative cleavage of Factor V yields Factor Va light chain and a second intermediate with apparent molecular mass approx. 260 kDa. A subsequent cleavage of the 260 kDa intermediate yields Factor Va heavy chain and Factor V activation peptide. Under the conditions employed, therefore, three limit fragments (approx. 150 kDa, approx. 110 kDa and approx. 80 kDa) of Factor V, shown schematically in Figs. 2(a) and 2(b), are generated in human plasma. Factor V activation peptide (approx. 150 kDa) is the smallest limit fragment detected routinely with the ^{125}I -labelled antibodies to human Factor V used in this study. Thus the anti-(Factor V) antibodies used react primarily with native Factor V and Factor V fragments that also contain the 150 kDa activation peptide. Therefore detection of the 150 kDa Factor V activation peptide by Western blotting implies generation of both the heavy chain and the light chain of Factor Va.

Effects of heparin. Heparin delayed Factor V proteolysis in CAP, and a minimum incubation of 60 s was required before

Factor V proteolysis could be detected by Western blotting when CAP contained approx. $0.05 \mu\text{M}$ -heparin (Fig. 1a, lane 6). Factor V activation peptide (approx. 150 kDa) was generated in the 90 s and 120 s samples (Fig. 1a, lanes 7 and 8). Heparin similarly delayed ^{125}I -Factor V proteolysis in CAP; incubations of 60 s or longer were necessary before Factor V activation peptide was generated (Fig. 1b, lanes 6–8). Improved inhibition of both endogenous Factor Xa and thrombin by heparin thus delays Factor V activation. Additionally, the same Factor V limit fragments are generated in control and heparinized CAP.

Effects of D-Phe-Pro-Arg- CH_2Cl and hirudin. D-Phe-Pro-Arg- CH_2Cl or recombinant hirudin was added to CAP to inhibit thrombin in order to assess directly the role of Factor Xa in Factor V proteolysis. D-Phe-Pro-Arg- CH_2Cl inhibited Factor V proteolysis in CAP (Fig. 3, lanes 1–3). Hirudin inhibited Factor V activation in CAP also. Thus Factor V had not been cleaved in CAP containing $1.0 \mu\text{M}$ -hirudin up to 90 s after CaCl_2 addition (Table 1). One-tenth this concentration of hirudin inhibited Factor V activation less effectively (Table 1). Inhibition of Factor V activation by these two thrombin inhibitors was confirmed with ^{125}I -Factor V (results not shown). On the basis of the results summarized in Figs. 1 and 3 and Table 1, thrombin is the major enzyme to activate Factor V in CAP. Hirudin and D-Phe-Pro-Arg- CH_2Cl effectively inhibit thrombin, and thereby delay Factor V activation.

Proteolysis of purified Factor V by thrombin and Factor Xa

Purified Factor V (150 nM) was incubated with an equal volume of a suspension consisting of 20 nM-thrombin, $12 \mu\text{g}$ of

Fig. 3. Inhibition of plasma Factor V proteolysis by 1 μ M-D-Phe-Pro-Arg-CH₂Cl

The chloromethyl ketone and CaCl₂ were simultaneously added to CAP in lanes 1–3, or to plasma with tissue factor (in lanes 5–8). Ca²⁺ replenishment times were as follows: lane 1, 30 s; lane 2, 45 s; lane 3, 60 s; lane 6, 10 s; lane 7, 20 s; lane 8, 30 s. CaCl₂ was not added in lane 5. Lane 4 contained radioactive molecular-mass markers. Abbreviation: V, native Factor V.

rabbit brain coagulant phospholipids/ml and 10 mM-CaCl₂ in Michaelis buffer, pH 7.4. After incubations of 15 s to 600 s, thrombin was inactivated by adding 4 vol. of a buffer consisting of 1 μ M-hirudin and 5 mM-EDTA, and the Factor V proteolysis achieved was assessed. Partial conversion of Factor V into Factor Va (detected by Western blots as Factor Va heavy chain and Factor V activation peptide) had occurred 15 s (result not shown) and 60 s after Factor V was incubated with thrombin (Fig. 4, lane 1). Fig. 4 also shows that 1 μ M-hirudin (lane 2) or 1 μ M-D-Phe-Pro-Arg-CH₂Cl (lane 3) abrogated thrombin-mediated proteolysis of purified Factor V.

The 230 kDa intermediate and a 150 kDa fragment were the major Factor V products obtained with Factor Xa after a 600 s incubation (Fig. 4, lanes 4–6). The latter fragment was probably not Factor V activation peptide but the N-terminal fragment of

Fig. 4. Effects of D-Phe-Pro-Arg-CH₂Cl and hirudin on the proteolysis of purified Factor V by thrombin and Factor Xa

Each enzyme (20 nM) was added in a suspension with 20 μ g of rabbit brain coagulant phospholipids and 20 mM-CaCl₂ to an equal volume of Factor V (150 nM) in Michaelis buffer, pH 7.4. After various incubation times the proteolysis of Factor V was assessed by Western blotting. Factor V in lanes 1–3 was incubated with thrombin for 60 s, and Factor V in lanes 4–6 was incubated with Factor Xa for 600 s. Lane 1, Factor V+thrombin; lane 2, Factor V+thrombin+20 nM-hirudin; lane 3, Factor V+thrombin+20 nM-D-Phe-Pro-Arg-CH₂Cl; lane 4, Factor V+Factor Xa; lane 5, Factor V+Factor Xa+1 μ M-hirudin; lane 6, Factor V+Factor Xa+1 μ M-D-Phe-Pro-Arg-CH₂Cl; lane 7, molecular-mass markers; lane 8, Factor V. Abbreviations: V, Factor V; A, Factor V activation peptide (approx. 150 kDa); H, Factor V heavy chain (approx. 110 kDa).

Factor V cleaved by Factor Xa and slowly converted into Factor Va heavy chain and a 71 kDa activation peptide [14]. Hirudin and D-Phe-Pro-Arg-CH₂Cl did not inhibit Factor V proteolysis by Factor Xa (Fig. 4, lanes 5 and 6 respectively). These results (observable with 100 ng of Factor V/Factor V degradation products channel) confirmed that the antiserum used for Western blots reacted primarily with Factor V and its fragments 150 kDa or larger (including the 150 kDa Factor V activation peptide). With this rather high concentration of Factor V products, the antibody reacted marginally with Factor Va heavy chain but not

Table 1. Inhibition of thrombin-mediated activation of Factor V in CAP by heparin (approx. 0.05 μ M, or 0.1 unit/ml) and by hirudin (0.1 μ M and 1.0 μ M)

CaCl₂ (final concentration 10 mM) was added to CAP for up to 90 s to initiate Factor V proteolysis. In heparinized plasma the CaCl₂ was added to CAP, both with and without 1 nM-thrombin (final concentration) and the effect on Factor V proteolysis was assessed. The times at which the 230 kDa Factor V intermediate and Factor V activation peptide (approx. 150 kDa) were first noted are reported in the Table. Generation of either fragment coincides with Factor Va cofactor activity. Abbreviation: N.D., not detected up to 90 s after CaCl₂ had been added to CAP.

Coagulant stimulus	Inhibitor	Time for the appearance of 230 kDa intermediate (s)	Time for the appearance of Factor V activation peptide (~ 150 kDa) (s)
CAP	None	30	45
CAP	~ 0.05 μ M-Heparin	60	75
CAP+1 nM-thrombin	None	15	15
CAP+1 nM-thrombin	~ 0.05 μ M-Heparin	30	60
CAP	0.1 μ M-Hirudin	60–75	90
CAP	1.0 μ M-Hirudin	N.D.	N.D.

Fig. 5. Effects of addition of Factor Xa (1 nM) (lanes 1–3 and 5) and of addition of Factor Xa + heparin (approx. 0.05 μ M) (lanes 4, 7 and 8) on Factor V proteolysis in CAP

Ca²⁺ replenishment times were: lane 1, 15 s; lane 2, 30 s; lane 3, 60 s; lane 4, 15 s; lane 7, 30 s; lane 8, 60 s. Control CAP to which no heparin was added was applied in lane 5. Lane 6 contained radioactive molecular-mass markers. Abbreviations: V, native Factor V; A, Factor V activation peptide (approx. 150 kDa).

with Factor Va light chain. As plasma contains approx. 80 μ g of protein/ μ l, the amount of plasma Factor V-derived proteins that could be applied per channel was about 10% that of purified Factor V used in Fig. 4. The results with purified Factor V are

consistent with thrombin being the major enzyme that catalyses the production of Factor Va heavy and light chains in CAP.

Effects of exogenous Factor Xa and of thrombin on Factor V proteolysis in CAP

Exogenous Factor Xa accelerates prothrombin activation in CAP [9]. Consistent with this observation, 1 nM-Factor Xa accelerated Factor V activation. Factor V activation peptide was generated in the 15 s sample (Fig. 5, lane 1) compared with the 45 s sample in control CAP (Fig. 1a, lane 2). Heparin (approx. 0.05 μ M) prolonged by 15 s production of this fragment in CAP supplemented with Factor Xa (Fig. 5, lanes 7 and 8). Note, however, that cleavage of Factor V into the 230 kDa intermediate had occurred in the 15 s sample (Fig. 5, lane 4). Thrombin (1 nM) similarly accelerated Factor V activation in CAP (Table 1). However, heparin (approx. 0.05 μ M) could effectively delay Factor V proteolysis in CAP supplemented with thrombin, and Factor V activation peptide was first seen in the 60 s sample (Table 1).

Tissue-factor-dependent activation of plasma Factor V

Effects of heparin. Tissue-factor-dependent generation of Factor V activation peptide was first observed in the 20 s sample (result not shown). The minimum concentration of heparin to delay Factor V proteolysis was approx. 0.25 μ M, as Factor V activation peptide (approx. 150 kDa) was first observed in the 30 s sample (Fig. 6a, lane 8). Tissue-factor-dependent proteolysis of ¹²⁵I-Factor V in control plasma was also examined. The intensity of the band corresponding to Factor Va heavy chain (approx. 110 kDa) had increased in the 10 s sample (Fig. 6b, lane 1). Factor Va light chain (approx. 80 kDa) and Factor V activation peptide (approx. 150 kDa) were generated 10 s later (Fig. 6b, lane 2). Both ¹²⁵I-Factor Va heavy and light chains had

Fig. 6. Effect of heparin on tissue-factor-dependent Factor V activation

(a) Effect of heparin on plasma Factor V proteolysis initiated by adding tissue factor and CaCl₂. The concentration of heparin in lanes 1–3 was approx. 0.05 μ M (0.66 μ g/ml), that in lanes 5–8 was approx. 0.25 μ M (3.3 μ g/ml). The following incubation times were used: lanes 1 and 6, 10 s; lanes 2 and 7, 20 s; lanes 3 and 8, 30 s. Lane 4 contained plasma to which no CaCl₂ was added. Lane 5 contained radioactive molecular-mass markers. Abbreviations: V, native Factor V; A, Factor V activation peptide (approx. 150 kDa). (b) Tissue-factor-dependent proteolysis of ¹²⁵I-Factor V in control plasma (lanes 1–3) or in plasma containing approx. 0.25 μ M-heparin (lanes 4, 5, 7 and 8). The proteolysis of ¹²⁵I-Factor V was initiated by adding tissue factor and CaCl₂ for the following incubation times: lane 1, 10 s; lanes 2 and 4, 20 s; lane 3, 30 s; lane 7, 40 s; lane 8, 60 s. Lane 5 contained control plasma. Lane 6 contained radioactive molecular-mass markers. Abbreviations: V, native Factor V; A, Factor V activation peptide; H and L, Factor Va heavy chain and light chain respectively.

Fig. 7. Inhibition of tissue-factor-dependent Factor V activation by hirudin

The concentrations of hirudin were as follows: lanes 1–3, 0.1 μM ; lanes 5–8, 1.0 μM . The durations of the incubations were as follows: lanes 1 and 6, 10 s; lanes 2 and 7, 20 s; lanes 3 and 8, 30 s. Lane 5 contained control plasma. Lane 4 contained molecular-mass markers. Abbreviations: V, native Factor V; A, Factor V activation peptide (approx. 150 kDa).

been generated in the 20 s sample in plasma containing approx. 0.25 μM -heparin (Fig. 6*b*, lane 4). The earlier appearance of Factor Va heavy and light chains with ^{125}I -Factor V reflects a higher proportion of the labelled Factor V having been proteolysed before CaCl_2 was added to the plasma (Fig. 6*b*, lane 5).

Effects of D-Phe-Pro-Arg-CH₂Cl and of hirudin. D-Phe-Pro-Arg-CH₂Cl delayed tissue-factor-dependent Factor V proteolysis by 20 s (Fig. 3, lanes 5–8). The predominant Factor V species generated in the 30 s sample were the 230 kDa fragment and (by inference) Factor Va heavy chain. Hirudin similarly inhibited tissue-factor-dependent proteolysis of Factor V. Factor V ac-

tivation peptide (approx. 150 kDa) was first demonstrated in the 30 s sample in plasma with 0.1 μM -hirudin (Fig. 7, lane 3). Only the 230 kDa fragment had been generated in the 60 s sample when the concentration of hirudin was increased to 1 μM (result not shown).

Results summarized in Figs. 3, 6 and 7 suggest that during extrinsic coagulation the same three major Factor V limit peptides generated in CAP are also generated. Efficient inhibition of endogenous thrombin by 1 μM -D-Phe-Pro-Arg-CH₂Cl (Fig. 3) or 1 μM -hirudin (Fig. 7) limits production of Factor V activation peptide. When thrombin action is thus suppressed, Factor Xa converts plasma Factor V into the 230 kDa fragment and Factor Va heavy chain, the latter detected by using ^{125}I -Factor V. A scheme summarizing the two Factor V fragments generated by Factor Xa is shown in Fig. 2(*c*). A less efficient inhibition of endogenously generated thrombin by heparin plus antithrombin III (Figs. 6*a* and 6*b*, lanes 4, 7 and 8) or 0.1 μM -hirudin (Fig. 7) results in thrombin-mediated cleavage of the 230 kDa intermediate into Factor V activation peptide and Factor Va light chain.

Prothrombin activation

Prothrombin activation was initiated after contact activation or by addition of tissue factor to plasma. Maximum prothrombinase activity, and the times that this was observed in each plasma, are summarized in Table 2. A comparison of the results summarized in Fig. 1, Figs. 3, 5, 6 and 7 and Table 2 suggest that highest prothrombinase activity was observed only when both Factor Va heavy and light chains were generated as exemplified by: control plasmas, CAP with approx. 0.05 μM -heparin, and plasma to which both tissue factor and 0.1 μM -hirudin were added. Note that heparin and hirudin delayed the availability of maximum prothrombinase activity in the last two plasmas. High prothrombinase activity was also observed when the 230 kDa Factor V intermediate was generated, suggesting that significant levels of Factor Va cofactor activity coincide with formation of this fragment in plasma, as reported recently by Monkovic & Tracy [14]. Generation of the 230 kDa intermediate and the associated prothrombin consumption are exemplified in the following plasmas: CAP containing 0.1 μM -hirudin, and plasma containing 1.0 μM -hirudin or 1.0 μM -D-Phe-Pro-Arg-CH₂Cl with tissue factor as the coagulant stimulus.

Table 2. Ability of heparin, hirudin and D-Phe-Pro-Arg-CH₂Cl to delay and inhibit prothrombinase formation

Prothrombin activation was initiated by adding CaCl_2 to CAP. The contact activator used was micronized silica that also contained rabbit brain coagulant phospholipids (APTT reagent). Samples were withdrawn at 15 s intervals and the concentration of prothrombin consumed was measured by e.l.i.s.a. Prothrombin activation was also initiated by adding a suspension of tissue factor and CaCl_2 to plasma. Samples were withdrawn at 10 s intervals and prothrombin consumption was measured to estimate the concentration of prothrombinase activity generated in each sample. Maximum prothrombinase activity is expressed as the nmol of prothrombin consumed/min per l during the time interval studied. In no case did the Ca^{2+} replenishment time exceed 90 s.

Coagulant stimulus	Inhibitor	Maximum prothrombinase activity (nmol/min per l)	Time interval (s)
APTT reagent	None	3480	30–45
APTT reagent	~ 0.05 μM -Heparin	3200	60–75
APTT reagent	0.1 μM -Hirudin	480	60–75
APTT reagent	1.0 μM -Hirudin	< 100	75–90
APTT reagent	1.0 μM -D-Phe-Pro-Arg-CH ₂ Cl	< 100	75–90
Tissue factor	None	6570	20–30
Tissue factor	0.1 μM -Hirudin	5400	30–40
Tissue factor	1.0 μM -Hirudin	2250	40–50
Tissue factor	1.0 μM -D-Phe-Pro-Arg-CH ₂ Cl	2520	30–40

DISCUSSION

The aims of the present studies were twofold: (1) to determine the temporal relationships between Factor V proteolysis and prothrombin consumption in plasma; (2) to relate the inhibition of Factor V activation to that of prothrombin consumption. Initial proteolysis of Factor V in CAP produced two fragments of molecular masses approx. 230 kDa and approx. 110 kDa, the latter being the heavy chain of Factor Va. The 230 kDa fragment was subsequently cleaved into Factor V activation peptide (approx. 150 kDa) and Factor Va light chain (approx. 80 kDa) 15 s later. Thus Factor Va was generated approx. 45 s after CaCl_2 had been added to CAP. Consistent with the requirement of Factor Va for prothrombinase formation [1,2,4,14], maximum prothrombin consumption in CAP was observed approx. 15 s after the initial cleavage of Factor V into the 230 kDa intermediate and Factor Va heavy chain (Table 2). This pattern of Factor V proteolysis was entirely consistent with thrombin action on human Factor V [14].

Recent studies suggest that heparin delays prothrombin activation in CAP by delaying thrombin-mediated activation of Factor V and Factor VIII [7–10]. Heparin increased the lag phase associated with Factor V proteolysis in CAP by approx. 30 s. Thrombin accelerated Factor V activation in CAP, an acceleration readily inhibitable by heparin. In partial support of the concept that Factor Xa activates Factor V in heparinized plasma [8,9], supplementation of CAP with Factor Xa accelerated Factor V activation, both in the presence and in the absence of heparin. Together with observations that heparin does not delay prothrombin activation in CAP supplemented with Factor Xa [9], Factor Xa would appear to be a more efficient Factor V activator in heparinized CAP than is thrombin. As discussed presently, however, thrombin probably participates in Factor V activation in heparinized plasma. D-Phe-Pro-Arg- CH_2Cl and hirudin inhibited Factor V activation more effectively than did heparin. This relative inability of heparin to sustain the inhibition of plasma Factor V activation probably reflects the lower affinity of thrombin for heparin/antithrombin III compared with its affinity for hirudin or D-Phe-Pro-Arg- CH_2Cl [9,15,23]. The weaker affinity of thrombin for heparin/antithrombin III probably permits transient escape of thrombin to participate in Factor V activation in heparinized CAP. The pattern of Factor V proteolysis observed in heparinized CAP and in plasma containing hirudin and D-Phe-Pro-Arg- CH_2Cl suggest that both thrombin and Factor Xa contribute to Factor V activation in heparinized CAP.

The Factor VII–Factor VIIa–tissue factor complex activates Factor X [24], thus making it feasible that Factor Xa generated *in situ* can initiate tissue-factor-dependent Factor V activation. The fragments of Factor V observed in the presence of micro-molar hirudin or D-Phe-Pro-Arg- CH_2Cl suggest that Factor Xa can activate Factor V during tissue-factor-dependent coagulation. Inhibition of tissue-factor-dependent formation of Factor V activation peptide by hirudin and D-Phe-Pro-Arg- CH_2Cl suggests that thrombin catalyses formation of Factor V activation peptide (approx. 150 kDa) and Factor Va light chain in plasma (Fig. 2). Using burro anti-(human Factor V) antibodies reactive with all Factor V fragments, Monkovic & Tracy concluded that the 150 kDa activation peptide and Factor Va light chain were produced by thrombin action [14].

A recent study reported that, when coagulation is initiated with dilute tissue factor, Factor X activation precedes Factor V and prothrombin activation [25]. Maximum prothrombinase activity was observed approx. 60 s after initiating coagulation [25], compared with 20–30 s in this study. Heparin (approx. $0.2 \mu\text{M}$) delayed Factor V and prothrombin activation by about

5 min and 6 min respectively. Inhibition of thrombin generation by heparin was reversed by the addition of Factor Va, suggesting that inhibition of Factor V activation, and not inhibition of Factor X activation, was the major action of heparin. The authors also suggested that thrombin was the sole agent to activate plasma Factor V [25]. We could demonstrate extrinsic Factor V activation by Factor Xa probably because we used undiluted tissue factor, an approach that probably reduced the need for both Factor IX and Factor VIII activation for efficient tissue-factor-dependent Factor X activation [26]. The present study also established the two species of plasma Factor Va species produced by Factor Xa and thrombin.

We have demonstrated in this study that approx. $0.05 \mu\text{M}$ -heparin delays Factor V proteolysis in CAP. However, 5 times this concentration of heparin is required to delay Factor V activation initiated with undiluted tissue factor. Factor V activation initiated with thrombin is inhibited more readily by heparin than is that initiated with Factor Xa. This observation probably accounts for the inability of heparin to delay prothrombin activation when CAP is supplemented with Factor Xa [9]. The ability of Factor Xa to initiate Factor V proteolysis was confirmed by adding undiluted tissue factor to plasma to which hirudin or D-Phe-Pro-Arg- CH_2Cl was also added. The Factor V fragments obtained with Factor Xa in the presence of each thrombin inhibitor provided Factor Va activity. Monkovic & Tracy reported that Factor Xa initially cleaved purified Factor V into two fragments (150 kDa and 220 kDa) that provide Factor Va activity, and that subsequently the 150 kDa fragment is slowly cleaved into Factor Va heavy chain (105 kDa) and a 71 kDa fragment [14]. Although we could detect the 150 kDa fragment reported by Monkovic & Tracy when using purified Factor V (Fig. 4), we were unable to demonstrate this intermediate as being generated by Factor Xa in plasma, either with ^{125}I -Factor V or with the rabbit anti-(Factor V) antibody used in this study. We could, however, detect generation of ^{125}I -Factor Va heavy chain when tissue factor was used to initiate coagulation in plasma containing hirudin or D-Phe-Pro-Arg- CH_2Cl . Additional studies will be necessary to determine the reasons for the differences observed between plasma and purified Factor V. Both Factor Xa and thrombin nonetheless contribute to Factor V activation during coagulation.

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