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Continuous Flow and the Prothrombinase-Catalyzed Activation of Prothrombin

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Summary

The activation of prothrombin by prothrombinase was investigated in a continuous flow system at 25 °C. A glass capillary, containing a continuous phospholipid bilayer attached to the interior surface, was first perfused with factor Va. The factor Va bound to the phospholipid surface functioned as sites for the formation of prothrombinase, when subsequently a factor Xa and prothrombin containing solution was perfused. Under the conditions used, steady-state rates of prothrombin activation were attained after 4 to 15 min. The rates of prothrombinase formation increased with increasing factor Xa concentrations and flow rates, which is compatible with the assembly of prothrombinase being dependent on the flux of factor Xa to the phospholipid-bound factor Va. As long as factor Xa and prothrombin were present in the fluid phase the assembly of prothrombinase was apparently irreversible; during at least 20 min no loss of activity occurred. The steady-state rate of prothrombin activation was dependent on the surface concentration of prothrombinase, at 1.0 μM prothrombin and a shear rate of 82 s^{-1} the average rate was 870 mol thrombin/min per mol prothrombinase. In contrast to test tube experiments it was observed that in this flow system, the formation of α-thrombin is favoured above the formation of meizothrombin (des fragment 1).

Introduction

In vivo blood coagulation is a complex process, which requires actions of and interactions between the vascular wall, blood platelets and the coagulation system. Of the coagulation system many reactions are membrane bound processes, i.e. they take place on either the platelet or endothelial cell membrane. Consequently, during coagulation reactants must be transported from the blood stream to the membrane surface. These transport processes, and therefore the formation and activities of the membrane-bound enzymatic complexes, are dependent on the local flow conditions (1, 2).

Recently it has been pointed out by Gemmell et al. (3) that the progress of coagulation reactions during flow cannot be extrapolated from static, closed systems. They reported on the activation of factor X by membrane-bound tissue factor and factor VII(a), during flow, and found that the steady-state catalytic activity of the enzymatic complex is independent of the concentration of factor VII(a) in the fluid phase. In addition it was shown, that the factor VII(a) concentration in the perfused solution determined the time required to achieve the steady-state.

In the past, detailed studies have been published on the kinetics and mechanisms of prothrombin activation (see refs. 4 and 5, and references therein), however, they were all performed in systems where the dynamic aspects of flow could not be examined. Prothrombinase, a key enzymatic complex in blood coagulation, is a dissociable Ca^{2+}-dependent enzymatic complex, composed of the enzyme factor Xa, the non-enzymatic cofactor factor Va, and a suitable phospholipid surface. This complex is a highly efficient activator of prothrombin, which leads to the formation of thrombin. In this paper, we describe the activation of prothrombin by prothrombinase under well-defined flow conditions, obtained by perfusion of reactants through a phospholipid coated glass capillary.

Materials and Methods

Capillaries

Glass capillaries with an internal diameter of 0.58 mm and a length of 12.7 cm, were obtained from Brand, FRG. They were boiled for 30 min in 2 mg/ml Sparkleen (Fisher Scientific Co., Pittsburgh, USA). After extensive washing with deionized water the capillaries were placed overnight in a 30% chromic acid solution. The capillaries were then again extensively washed and kept in 50% ethanol. Capillaries were used the same day; prior to use they were dried.

Proteins and Phospholipid Vesicles

Bovine factor V and factor Va (6) and bovine prothrombin (7) were prepared and quantified as described. Bovine antithrombin III (ATIII) was isolated as reported previously (8). Human prothrombin was purified as described (9), and the molar concentration was determined, after complete activation with *Echis carinatus* venom (Sigma), by active site titration with p-NPGB (10). Human factor Xa was prepared by activation of purified factor X (11) with the factor X activating protein from Russell's Viper venom (KabiVitrum, Stockholm, Sweden) and isolated as described for the bovine protein (12). The molar concentration was determined by active site titration with p-NPGB (13).

Meizothrombin (des fragment 1) was prepared as follows. Human prothrombin (16 μM) was activated by *Echis carinatus* venom (0.5 mg/ml) in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 10 mM $CaCl_2$, and 10 mM benzamidine-HCl. After 5 min the conversion of prothrombin was terminated by the addition of EDTA to a final concentration of 20 mM, and the mixture was applied to a column (1.5 × 30 cm) of QAE-Sepharose (Pharmacia) equilibrated in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM benzamidine-HCl at 4 °C. A linear salt gradient, from 0.10 to 0.60 M NaCl in 50 mM Tris-HCl (pH 7.5), 10 mM benzamidine-HCl (2 × 150 ml), was applied and meizothrombin (des fragment 1), assessed by its activity on S2238, eluted at about 0.25 M NaCl. The fractions with the highest activity were pooled. In these steps, the presence of 10 mM benzamidine-HCl was an absolute requirement; upon its omission the only product was α-thrombin. For further use, the benzamidine was rapidly removed by gel filtration. The preparation was applied to a column (1.6 × 60 cm) of Sephadex G50 (Pharmacia) in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl at 4 °C. Fractions were immediately pooled and stored at −70 °C. The final preparation was over 95% pure as judged by SDS polyacrylamide gelelectrophoresis (14), and the concentration of meizothrombin (des fragment 1) was assessed by active site titration with p-NPGB (10).

Abbreviations:

ATIII, antithrombin III; p-NPGB, p-nitrophenyl p′-guanidinobenzoate hydrochloride; HSA, human serum albumin; S2238, D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride.

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Small unilaminar vesicles, composed of 75% egg phosphatidylcholine and 25% brain phosphatidylserine were prepared by sonication in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl (7). To determine amounts of phospholipid covering the inner wall of the capillaries, also vesicles were prepared with tracer concentrations of dioleoylphosphatidyl-(^{14}C)serine (specific activity 2.0 GBq/mmol; Amersham, UK). The vesicle suspension had a specific activity of 97.8 MBq/mmol. Radioactivity measurements were performed with a Beckman LS3801 scintillation counter, utilizing a liquid scintillation cocktail (Formula-989, Biotechnology systems, Boston, USA).

The Activation of Prothrombin by Surface-Bound Prothrombinase

Capillaries were coated with phospholipid, by 20 min incubation with a 1 mM vesicle suspension, and then rinsed with Tris-buffer (50 mM Tris-HCl [pH 7.9] containing 175 mM NaCl, 3.0 mM $CaCl_2$, and 0.5 mg ovalbumin/ml) at a flow rate of 1.2 ml/min (wall shear rate 1,044 s^{-1}) for 2 min. The capillaries were then perfused with factor Va, diluted in the Tris-buffer, for 10 min at a flow rate of 30 µl/min (wall shear rate 26 s^{-1}). Immediately hereafter the capillaries were washed with Tris-buffer at a flow rate of 30 µl/min during 10 min, and then perfused with a solution of factor Xa and prothrombin in the same Tris-buffer. Drops were collected at the outlet and 20 µl aliquots were immediately diluted in 430 µl of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, containing 0.5 mg HSA/ml and assayed for thrombin by the addition of 50 µl chromogenic substrate (S2238, KabiVitrum, Stockholm, Sweden). The final concentration S2238 was 0.22 mM and the rate of absorbance increase was recorded at 405 nm on a dual wavelength spectrophotometer (reference wavelength: 500 nm) at 37 °C. The amounts of thrombin formed were calculated from a standard curve constructed with purified human α-thrombin (15) under identical conditions.

All perfusions were performed by the use of a syringe pump (Harvard Apparatus Co., Massachusetts, USA) resulting in a continuous laminar flow through the capillaries. The experiments were performed in a climated room at 25 ± 1 °C, and the conditions mentioned were routinely applied, unless otherwise indicated.

Determination of Factor Va Bound to the Phospholipid Coated Capillaries

At the end of each experiment the capillaries were emptied and subsequently washed with one capillary volume of 0.2% Triton X100 in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 3.0 mM $CaCl_2$. The solutions were mixed and a sample was diluted 10-fold with Tris-buffer, and assayed for factor Va.

Factor Va Assay

Factor Va samples (10 µl) were added to cuvettes, containing a solution of factor Xa, phospholipid vesicles and $CaCl_2$ in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, and 0.5 mg HSA/ml. After 2 min at 37 °C, 10 µl bovine prothrombin was added. The final concentrations were 0.10 nM factor Xa, 10 µM phospholipid, 10 mM $CaCl_2$, and 0.2 µM prothrombin in a final volume of 125 µl. After 5 min the conversion of prothrombin was terminated by the addition of 325 µl of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, and 0.5 mg HSA/ml. Hereafter, 50 µl 2.2 mM S2238 was added and the amount of thrombin formed was determined. The amounts of thrombin formed were linearly dependent on the amounts of factor Va present, up to at least 2.0 pM factor Va in the assay.

In samples derived from the capillaries, 0.01% Triton X100 was present. Because the assay was sensitive to such an amount of Triton X100, factor Va reference curves were constructed in the presence of the same detergent concentration. Each factor Va determination was performed in duplicate, and the differences between the individual values of each duplicate varied between zero and approximately 10% of the mean values.

Measurement of Meizothrombin (Des Fragment 1)

Samples (20 µl) from drops collected at the capillary outlet, were immediately diluted in cuvettes, containing 430 µl of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg HSA/ml, 4 nM ATIII, and 2 µg unfractionated heparin/ml (165 USP U/mg) at 37 °C. After 1 min 50 µl 2.2 mM S2238 was added and the residual amidolytic activity was determined. From previous work it follows that under these conditions α-thrombin activity will be completely neutralized, whereas this is not the case for meizothrombin (des fragment 1) activity (8). The amounts of meizothrombin (des fragment 1) present in the samples were calculated from the residual amidolytic activities utilizing a standard curve constructed with purified meizothrombin (des fragment 1).

Theory of Protein Adsorption to the Interior Surface of a Capillary

During laminar flow along the inner wall of a capillary, in which a fluid phase protein binds to the capillary wall, the transfer of the protein occurs in a thin diffusion layer between the bulk solution and the surface. It has been derived (16) that, provided the binding capacity of the surface is sufficiently large as compared to the protein content of the boundary layer, and if the binding sites are uniformly distributed, the rate of protein flow (v_S, mol cm^{-2} s^{-1}) towards the surface is given by:

$$v_S = 0.54\,(\gamma D^2/x)^{1/3}\,S_b \qquad (I)$$

where γ is the shear rate (s^{-1}), D is the diffusion coefficient of the protein (cm^2/s), x is the distance from the capillary entrance (cm), and S_b is the concentration of protein in the fluid phase (mol/ml). The shear rate is given by:

$$\gamma = 32\,Q/(\pi d^3) \qquad (II)$$

where Q is the flow rate (ml/s), and d is the internal diameter (cm) of the capillary.

Results

Determination of the Amounts of Phospholipid Bound to the Interior Surfaces of Capillaries

It has been demonstrated that, when glass coverslips are placed in a vesicle suspension, the coverslip surface becomes covered with a continuous, planar membrane (17). This technique has been used for the binding of phospholipids to glass capillaries (3). We used virtually the same technique, and in order to verify the formation of a phospholipid bilayer we determined the amounts of phospholipid bound, by use of vesicles containing ^{14}C-labelled phosphatidylserine. Clean and dry capillaries were filled with a suspension of the small unilaminar phospholipid vesicles, as outlined in "Materials and Methods". Non-bound phospholipid was removed by perfusion of Tris-buffer at a flow rate of 1.2 ml/min for 2 min. The capillaries were emptied, washed with approximately 0.5 ml of scintillant and broken into 3 segments. All was collected in a vial containing scintillant to a final volume of 5 ml and the radioactivity was measured.

Our results showed that 6.5×10^{-10} mol phospholipid was bound per cm^2 (n = 8; SD = 0.6×10^{-10} mol/cm^2). Such an amount of phospholipid agrees reasonably well with published data (ref. 3, and references therein). In addition, we demonstrated the bilayer to be stable. After the removing of non-bound phospholipid, capillaries were perfused for 10 min with 0.15 nM factor Va followed by a 30 min perfusion with Tris-buffer at a rate of 30 µl/min. The amount of radioactivity retained in the capillaries, assessed as described above, corresponded with 5.9×10^{-10} mol/cm^2 (n = 4; SD = 0.9×10^{-10} mol/cm^2) phospholipid bound to the capillary surfaces.

We also established, with the use of the ^{14}C-labelled vesicles, that the procedure for the determination of the amounts of factor Va bound to the phospholipid surfaces, removed essentially all bound phospholipid. Again capillaries were filled with the vesicle suspension, and washed as described. Then each capillary was emptied in a vial containing 5 ml of scintillant and washed with one volume of the Tris-buffered Triton X100 solution. By this procedure we recovered an amount of radioactivity corresponding to 6.2×10^{-10} mol phospholipid/cm^2 (n = 4; SD = 0.1×10^{-10} mol/cm^2).

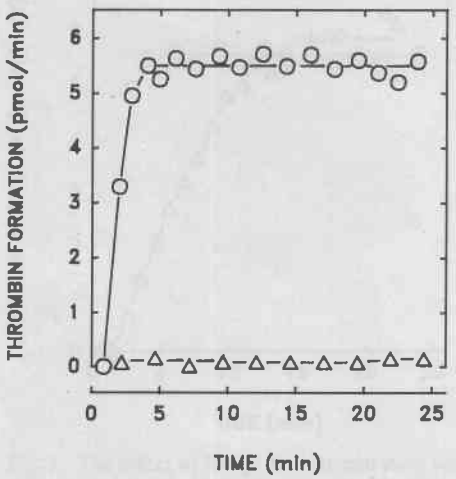

Fig. 1 The rate of thrombin formation as function of time. Prothrombin (1.0 μM) and factor Xa (0.50 nM) were perfused through a phospholipid coated capillary in the presence of 2.4 fmol factor Va/cm^2 (○), or a capillary which was not coated with phospholipid (△). See the text for further experimental details

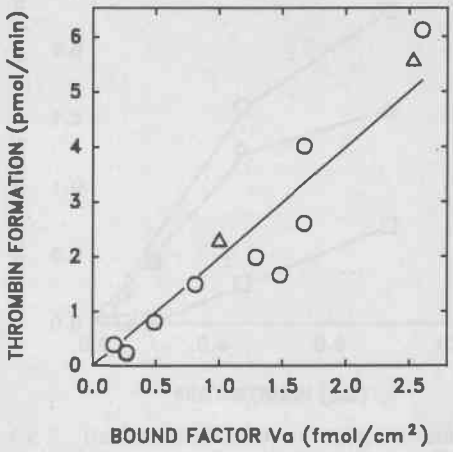

Fig. 2 The steady-state thrombin formation as function of the amount of bound factor Va. Phospholipid-coated capillaries were perfused with factor Va solutions of variable concentration. Steady-state rates of thrombin formation were determined at fluid phase concentrations of 1.0 μM prothrombin and either 0.05 nM (○) or 0.50 nM (△) factor Xa at a flow rate of 94 μl/min

Binding of Factor Va to Phospholipid-Coated Capillaries

In order to obtain surface-bound prothrombinase we first adsorbed factor Va to phospholipid coated capillaries. To this end, a phospholipid-coated capillary was perfused with 0.15 nM factor Va for 10 min (30 μl/min; wall shear rate 26 s^{-1}) and subsequently washed for 10 min with buffer (30 μl/min). The amount of factor Va bound to the phospholipid-coated capillary, determined as outlined in "Materials and Methods", was 2.4 fmol/cm^2. Given the interior surface of the capillary (2.31 cm^2) and the factor Va influx (300 μl of 0.15 nM) it can be calculated that 12% of the amount of factor Va perfused became bound. We investigated the distribution of the factor Va bound to the phospholipid surface, by breaking a capillary, perfused with factor Va and washed with buffer, into two pieces of approximately equal length. An amount of 2.6 fmol factor Va was bound in the inlet part (l = 6.4 cm) and 2.1 fmol in the outlet part of the capillary (l = 6.3 cm); hence the ratio of the amounts is 1:0.81.

The Course of Thrombin Generation by Surface-Bound Prothrombinase

Fig. 1 shows thrombin generation obtained by the perfusion of 0.50 nM factor Xa and 1.0 μM prothrombin at a flow rate of 94 μl/min (wall shear rate 82 s^{-1}), after a phospholipid-coated capillary had been perfused with a solution of 0.15 nM factor Va for 10 min. It is seen that under these conditions thrombin is measurable at the outlet after about 1 min. Then the rate of thrombin formation rapidly increases until a steady-state rate of approximately 5.5 pmol/min is obtained, which takes about 4 min. The steady-state thrombin formation is stable for at least 20 min, which could suggest that during the course of the perfusion with factor Xa and prothrombin no significant amount of factor Va is eluted from the phospholipid coated capillary. In order to verify this we determined the amount of bound factor Va, at the end of the perfusion with factor Xa and prothrombin, and found 2.5 fmol/cm^2. An amount which is identical to the amount of factor Va bound at the onset of the perfusion with factor Xa and prothrombin, as determined in the previous section.

The observed thrombin formation is truly the consequence of prothrombinase-catalyzed activation of prothrombin because in the absence of phospholipids thrombin generation was not detectable (Fig. 1). Also when the factor Va perfusion step was omitted thrombin activity was not measurable. Thus, factor Xa alone did not produce thrombin at detectable rates whether phospholipids were present or not.

The Steady-State Rate of Thrombin Formation as Function of the Amount of Phospholipid-Bound Factor Va

We were intrigued by the stable steady-state rate of thrombin formation, and we were curious to find out to what extent the steady-state was a measure for the amount of surface-bound prothrombinase. Thus we varied the amount of factor Va bound, which was achieved by varying the factor Va concentration of the perfused solution. Subsequently we determined the steady-state rates of thrombin formation by perfusion of factor Xa and prothrombin.

The results, shown in Fig. 2, demonstrate that the steady-state rate of thrombin formation increases linearly with the amount of bound factor Va up to 2.5 fmol factor Va/cm^2. It is also seen that the steady-state levels are independent of the fluid phase factor Xa concentration when it varies from 0.05 to 0.50 nM. In this range of factor Xa concentrations the phospholipid-bound factor Va can thus be saturated with factor Xa irrespective of the concentration of the latter. Linear regression analysis of the data revealed that 1 fmol factor Va/cm^2 results in a steady-state rate of thrombin formation of 2.0 ± 0.1 (SE) pmol/min. With an interior capillary surface of 2.31 cm^2, thrombin is thus generated with a rate of 870 ± 40 mol/min per mol prothrombinase.

The Influence of the Fluid Phase Factor Xa Concentration and the Flow Rate on the Rate of Generation of Prothrombinase

Although, the steady-state level of prothrombinase activity is not influenced by the fluid phase factor Xa concentration, the formation rate of prothrombinase decreases drastically with decreasing factor Xa concentrations, which is shown in Fig. 3.

In the presence of 0.50 nM factor Xa thrombin generation starts at about 1 min and reaches the steady-state at approximately 4 min. In the presence of 0.05 nM factor Xa thrombin generation starts at roughly the same time, however, it takes

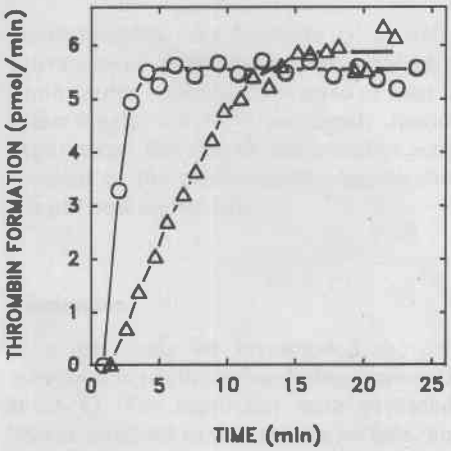

Fig. 3 The effect of factor Xa concentration on the course of the rate of thrombin formation. Capillaries containing phospholipid-bound factor Va were perfused with 1.0 μM prothrombin and factor Xa at 94 μl/min. The experiments were conducted with 0.50 nM factor Xa and 2.5 fmol factor Va/cm^2 (○) or 0.05 nM factor Xa and 2.6 fmol factor Va/cm^2 (△)

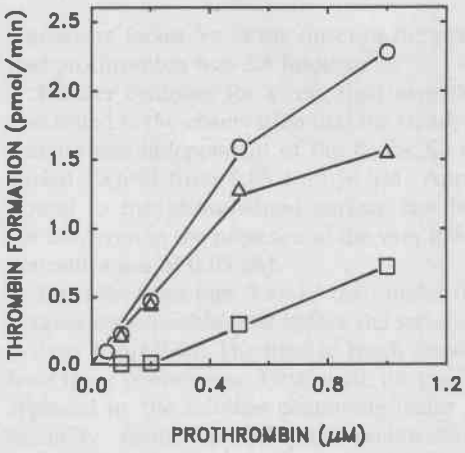

Fig. 5 The steady-state rate of thrombin formation as function of the prothrombin concentration. Prothrombin was activated by 0.05 nM factor Xa in the presence of 1.9 fmol factor Va/cm^2 at a flow rate of 30 μl/min. The amounts of total amidolytic thrombin activity (○), as well as the amounts of α-thrombin (△) and meizothrombin (des fragment 1) (□) formed were determined

about 15 min before the steady-state is reached. At the steady-state thrombin was generated at an average rate of 5.8 pmol/min by approximately 6.0 fmol prothrombinase. The first derivatives of the curves reveal that at the onset of thrombin, its rate of formation increases with 5.3 pmol/min per min and 0.60 pmol/min per min at 0.50 and 0.05 nM factor Xa, respectively. Thus, after the lag-phase prothrombinase activity is generated at a rate of 5.5 fmol/min (0.50 nM factor Xa) and 0.62 fmol/min (0.05 nM factor Xa).

The rate of formation of prothrombinase is also influenced by the flow rate. This is seen in Fig. 4, where thrombin formation was studied at a flow rate of 48.4 μl/min (wall shear rate 42 s^{-1}). Thrombin generation now starts at approximately 2 min and reaches a steady-state end-level at 7 min, and the prothrombinase activity is generated at a rate of 2.6 fmol/min.

Steady-State Rates of Generation of α-Thrombin and Meizothrombin (Des Fragment 1) at Varying Prothrombin Concentrations

When prothrombin activation by prothrombinase is monitored via amidolytic activity measurements we cannot discriminate α-thrombin and meizothrombin (des fragment 1) as the products formed. It has been shown in static, closed systems that, depending on the prothrombin concentration, large amounts of meizothrombin (des fragment 1) are formed during prothrombin activation by complete prothrombinase (4, 8). Therefore, we investigated whether the formation of meizothrombin (des fragment 1) also occurs in our continuous flow system.

We assayed the amidolytic thrombin activity determined at the capillary outlet for the presence of meizothrombin (des fragment 1). This was achieved by taking advantage of the fact that α-thrombin is rapidly scavenged by ATIII in the presence of heparin, whereas the inhibition of meizothrombin (des fragment 1) by ATIII is slow and is not enhanced by heparin (8). The steady-state rates of α-thrombin and meizothrombin (des fragment 1) formation as function of the fluid phase prothrombin concentration are depicted in Fig. 5. Our data show, that up to 0.20 μM prothrombin no significant amounts of meizothrombin (des fragment 1) are formed; when the prothrombin concentration increases further up to 1.0 μM the relative amount of meizothrombin (des fragment 1) formed increases up to 32%. In addition, the steady-state rates of formation of overall thrombin activity (α-thrombin plus meizothrombin [des fragment 1]) are linearly dependent on the prothrombin concentration up to 0.50 μM of prothrombin. A deviation from linearity is seen when the prothrombin concentration increases to 1.0 μM. Thus during continuous flow the relative rates of formation of α-thrombin and

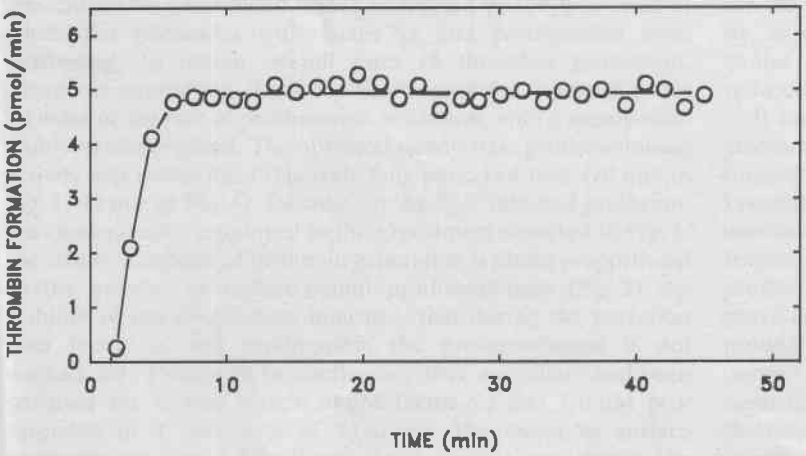

Fig. 4 Time course of the thrombin generation rate. Prothrombin (1.0 μM) and factor Xa (0.50 nM) were perfused at a flow rate of 48.4 μl/min through a factor Va containing phospholipid coated capillary. The amount of factor Va was 3.3 fmol/cm^2

meizothrombin (des fragment 1), as well as the overall rates of activation of prothrombin are dependent on the fluid phase prothrombin concentration up to at least 1.0 μM of prothrombin. Interestingly, we have previously found that, in vesicle type experiments the rate of prothrombin activation is virtually independent of the prothrombin concentration at concentrations of 0.6 μM and higher (8).

Discussion

In this study we investigated the prothrombinase-catalyzed activation of prothrombin during laminar flow through capillaries, at 25 °C. The capillaries were provided with a phospholipid bilayer attached to the interior surface, and then perfused with a factor Va containing solution. The amount of bound factor Va was determined after washing, and we found that significant amounts of factor Va were retained. A factor Va influx of 0.15 nM at a flow rate of 30 μl/min for 10 min resulted in a 12% factor Va binding of 2.4 fmol/cm^2. It has been reported that factor Va-binding to phospholipid vesicles, composed a 20–25% phosphatidylserine and 75–80% phosphatidylcholine, is satured at a phospholipid to protein ratio (mol/mol) varying between 57 and 100 (18, 19). Phospholipid vesicles are highly curved, however, relative to the molecular dimensions of proteins, the phospholipid bilayer attached to a capillary can be regarded as a plane. Since maximum surface concentration (factor V, factor X, prothrombin) seems to be independent of the curvature of the surface (20), it can be estimated, from the factor Va-binding data (18, 19) and the amount of phospholipid bound to the capillaries (6.5×10^{-10} mol/cm^2), that under the conditions used, less than 0.04% of the maximum factor Va binding capacity was used.

At such a low surface concentration the adsorption of factor Va is limited by the diffusion-dependent flux of factor Va towards the surface (20). In which case, according to eq. I, the adsorption of factor Va in a given time should decrease for increasing distances from the tube entrance. Thus, the inlet part of a capillary should contain a significant higher amount of factor Va as compared to the outlet part. We investigated the distribution of the bound factor Va by breaking a capillary into two pieces of equal length. The observed distribution was almost homogeneous (1:0.81). A full explanation for the apparent deviation from theory is not yet at hand, however, it can be speculated that the phospholipid-bound factor Va can diffuse laterally across the surface, as has been described for lipid-bound antibodies in phospholipid monolayers (21).

Factor Va containing phospholipid-coated capillaries were perfused with factor Xa and prothrombin, drops were collected at the capillary outlet and assayed for thrombin. The thrombin concentrations determined where multiplied by the flow rates at which the perfusions with factor Xa and prothrombin were performed, to obtain overall rates of thrombin generation. Thrombin generation showed a short lag-phase followed by an increase of the rate of prothrombin activation, until a steady-state end-level was reached. The obtained steady-state prothrombinase activity was stable for a relatively long period of time (20 min in Fig. 1, 40 min in Fig. 4). Because, at the flow rate and prothrombin concentration employed in the experiment depicted in Fig. 1, the steady-state rate of thrombin generation is direct proportional to the amount of surface-bound prothrombinase (Fig. 2) the stability of the steady-state indicates, that during the perfusion with factor Xa and prothrombin the prothrombinase is not washed out. This could be confirmed; after a capillary had been perfused for 25 min with 0.50 nM factor Xa and 1.0 μM prothrombin at a flow rate of 94 μl/min, the factor Va surface concentration was 2.5 fmol/cm^2. In a control experiment the amount of factor Va at the onset of the perfusion with factor Xa and prothrombin was 2.4 fmol/cm^2.

Further evidence for a very tight assembly of prothrombinase was found in the observation that the steady-state prothrombinase activity was independent of the factor Xa concentration when it varied 10-fold from 0.05 to 0.50 nM. Apparently the factor Va bound to the phospholipid surface can be saturated with factor Xa, even in the presence of the very low fluid phase factor Xa concentration of 0.05 nM.

It is seen from Figs. 3 and 4 that, under the specific conditions, it takes considerable time before the steady-state prothrombinase activity is reached. The time to reach steady-state depends on at least three phenomena. First of all, the capillary content has to be replaced by the solution containing factor Xa and prothrombin, secondly, factor Xa and prothrombin have to be transferred through the boundary layer to the phospholipid surface, and thirdly, the thrombin formed has to move from the catalytic sites into the bulk solution in order to leave the capillary.

The rate of formation of prothrombinase depended on the fluid phase factor Xa concentration and the flow rate. At a flow rate of 94 μl/min and in the presence of 0.05 M factor Xa the rate of formation of prothrombinase was 0.62 fmol/min, with 0.50 nM factor Xa the rate was 5.5 fmol/min (Fig. 3). At a flow rate of 48.4 μl/min, in the presence of 0.50 nM factor Xa the rate of formation of prothrombinase was 2.6 fmol/min (Fig. 4). Because the prothrombin concentrations were at least 2,000-times in excess over the factor Xa concentrations, the diffusion limited rate of transport of prothrombin to the phospholipid surface is at least 3 orders of magnitude higher than the diffusion limited rate of transport of factor Xa to the surface. Therefore it is well feasible that under these conditions, the time delay required to reach the steady-state prothrombinase activity is dependent on the flux of factor Xa to the phospholipid-bound factor Va. According to eqs. I and II (Materials and Methods) the diffusion controlled transfer of factor Xa to the surface indeed increases with increasing fluid phase factor Xa concentrations and flow rates.

We have previously demonstrated, that if factor Xa is present as part of the fully assembled prothrombinase, a significant amount of meizothrombin (des fragment 1) is formed (8). The first step in the formation of this product is the generation of meizothrombin. Meizothrombin is formed when prothrombin is specifically cleaved at only one of the factor Xa-cleavage sites present in prothrombin. This cleavage has as consequence that the thrombin active site becomes exposed. Meizothrombin is very rapidly processed further, and gives rise to the formation of meizothrombin (des fragment 1). Meizothrombin (des fragment 1) is a "dead-end" product, because the protein, lacking the phospholipid- and Ca^{2+}-binding regions, is not as good a substrate for the prothrombinase as prothrombin is. Moreover its reactivity, as compared with thrombin, towards macromolecular substrates, e.g. fibrinogen, antithrombin III, and factor V, is greatly reduced.

It has been shown in static, closed systems, that the relative amounts of α-thrombin and meizothrombin (des fragment 1) formed depend on the prothrombin concentration (4, 8). In keeping with these findings we observed, that also during continuous flow, the relative contribution of meizothrombin (des fragment 1) to the total amidolytic activity increases when the prothrombin concentration increases. However, in contrast to previous work (8), where at 1.0 μM prothrombin 84% of the product was meizothrombin (des fragment 1), we observed that during flow the relative amount of meizothrombin (des fragment 1) formed is diminished to 32%. Apparently, during flow the major prothrombin activation product is α-thrombin instead of meizothrombin (des fragment 1).

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