measure plasma coagulation, namely turbidimetry and viscometry. The effect of addition of FII, both r-hFII and pd-hFII, on APTT in both normal platelet-poor plasma and FII immune-depleted plasma has been studied.

Materials and methods

Lyophilized pd-hFII was from Enzyme Research Laboratories, (South Bend, Indiana, USA) and correctly gamma-carboxylated r-hFII was produced by AstraZeneca R&D, Mölndal, Sweden. The commercial pd-hFII preparations did all contain low amounts of contaminating proteins, for example FXa (0.2 μg/mg, PC (0.1 mg/mg) and traces of FVIIa and FIXa and varying low amounts of FIIa. In the r-hFII preparations, no other proteins were present and less than 0.4 μmol FIIa/mol FII. Bovine serum albumin (BSA) 20% (w/v) solution in water and the phospholipid emulsion containing synthetic sources of phosphatidylserine, phosphatidylcholine and sphingomyelin (PL-TGT, 0.5 mmol\l) were from Rossix AB (Mölndal, Sweden). All solutions were prepared with deionized water that was further purified by reversed osmosis on an Elgastadt UHP (Elga Ltd., High Wycombe Bucks, England).

Platelet-poor plasma, from two in-house prepared normal human plasma pools, was used. These pools were prepared after approval of the local ethics committee (No ADS 180-01 T048-05) by collecting blood from 30 healthy volunteers employed by AstraZeneca R&D Mölndal, Sweden (nine volumes blood with one volume 129 mmol/l trisodium citrate). Normal plasma was prepared by centrifugation of the citrated blood at 2000*g* in a swing-out rotor for 20 min at 20°C. The plasma supernatant was put on ice, pooled, aliquoted and stored at −80°C.

The APTT assay was performed according to the instructions for the reagents but with 45 μl of citrated plasma incubated with 5-μl FII before the APTT reagents were added to a total of 150 μl. APTT was determined by turbidimetry, from the apparent change in optical absorbance at 405 nm (A405), by a Behring Coagulation System instrument from Siemens (Siemens Healthcare Diagnostics Inc., Marburg, Germany) applying an absorbance difference threshold. In addition, viscometry was used by a KC 10 A Micro from Heinrich Amelung GmbH (Lemgo, Germany).

Data analysis

For all nonlinear regression analyses, Grafit software version 5 (Erithacus Software Ltd., Horley, UK) was used. Data are expressed as mean values with the standard error.

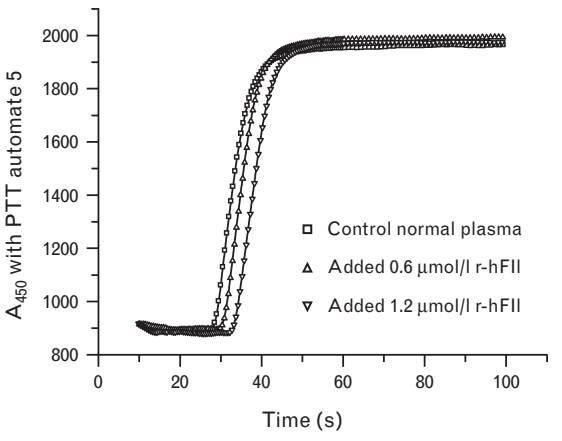
Thrombin is generated from the substrate, FII, as the result of the overall enzyme activity of the prothrombinase complex. The thrombin concentration is proportional with its activity as estimated from the fibrin formation rate, *id est* coagulation. The initial coagulation rate is thus in principle proportional to the inverse coagulation time, assuming that the time of coagulation, Δt, the same concentration of fibrin, the product ΔP is formed: $v = \Delta P/\Delta t$. The titration curves can therefore be analyzed applying Michaelis-Menten kinetics, by plotting $1/v =$ APTT versus the concentration of FII added to FII-depleted plasma. Thus, by using the reverse Michaelis-Menten equation, $1/v = 1 + ([FII]/K_M))/V_{max}$, the K_M for FII can be estimated by nonlinear regression analysis.

Results and discussion

Previously, in the comparison of different thrombin inhibitors on plasma coagulation, the PTT automate 5 reagent from Stago (Diagnostica Stago, Asnières, France), has been proven to be a sensitive assay [13,20]. However, in the present study we have found an unexpected increase in APTT with increasing concentrations of FII added to normal plasma employing the PTT automate 5 reagent, see Figs 1 and 2. An increase in APTT, as shown by the parallel shifts in the coagulation curves (Fig. 1), suggests a paradoxical inhibition of coagulation by FII. The plot of APTT versus the total FII concentration (Fig. 2a and b) showed both with pd-hFII and r-hFII a similar effect on APTT with the different reagents, indicating that the effect was not dependent on a contaminant in the plasma-derived FII. Moreover, neither adjustment of the final free Ca^{2+} concentration to 1.5 mmol/l nor addition of the PL-TGT phospholipids emulsion up to 16 μmol/l had an effect on the increase in APTT by added FII in the assay with PTT automate 5.

Different commercial reagents contain different initiators; Actin and Actin FS from Siemens contain ellagic

Fig. 1



Effect of FII on partial thromboplastin time (PTT) automate 5-induced activated partial thromboplastin time in normal plasma, incubated with added r-hFII and recorded by turbidimetry. Effect of addition of r-hFII on coagulation curves of plasma: from a total concentration of FII in plasma from 1.4 (control) to 2 and 2.6 μmol/l.