Phospholipase A_2 Activates Hemostasis

Thomas W. Stief

Department of Clinical Chemistry, University Hospital Giessen & Marburg, Germany.

Abstract

Background: Phospholipases A_2 (PLA_2) are aggressive enzymes that can destroy phospholipids of cell membranes. The resulting cell fragments trigger the kallikrein—mediated contact phase of coagulation. The aim of the present study was to expose citrated whole blood to PLA_2 and to quantify thrombin generation in recalcified plasma.

Methods: Normal citrated blood was exposed to bovine pancreatic or snake PLA_2, lipopolysaccharide (LPS), or zymosan A for 30–45 min (RT). After centrifugation the plasma samples were recalcified (10 + 1) with 250 mM $CaCl_2$ in the recalcified coagulation activity assay (RECA). After 0–45 min coagulation reaction time (CRT at 37°C) 1.6 M arginine (final test concentration) was added to stop hemostasis activation and to depolymerize non-crosslinked fibrin. The generated thrombin activity was chromogenically determined.

Results: 100 ng/ml bovine pancreatic or snake PLA_2 generates about 0.2–0.8 IU/ml thrombin after 15 min CRT. This thrombin generation is similar as that induced by 200 ng/ml LPS or 20 μg/ml zymosan A. Up to 60 ng/ml bovine pancreatic PLA_2 the generated thrombin activity is proportional to the PLA_2 activity used; 1 μg/ml PLA_2 induces much less thrombin, but PLA_2 at 10 μg/ml again results into thrombin generation of 0.1–3 IU/ml at 10–15 min CRT. As control, in pooled normal citrated plasma there is no significant change in thrombin generation when exposed to up to 10 μg/ml bovine pancreatic PLA_2.

Discussion: Elevated plasmatic PLA_2 activities (occurring e.g. in trauma, pancreatitis, or sepsis) activate the blood hemostasis system resulting in pathologic disseminated intravascular coagulation (PDIC). It is suggested to diagnose these life threatening states as early as possible, screening all patients for plasmatic thrombin activity.

Abbreviations: ΔA/t, absorbance increase per time; CRT, coagulation reaction time (37°C); CS-IIa, chromogenic substrate HD-cyclohexylglycyl-alanyl-arginyl-paranitroanilide for thrombin; F-well, flat bottom well Polysorp®; IU, international units; LPS, lipopolysaccharide; mA, milli absorbance units; PDIC, pathologic disseminated intravascular coagulation; PLA_2, phospholipase A_2; RECA, recalcified coagulation activity assay; RT, room temperature; $sPLA_2$, secretory PLA_2; U, units; ZyA, zymosan A.

Keywords: Phospholipase, PLA_2, thrombin, intrinsic hemostasis, contact phase, PDIC.

1. Introduction

Phospholipases A_2 (PLA_2) are aggressive enzymes and are found in high concentrations in blood of septic patients and in snake venoms [1–5]. After cell damage PLA_2 are released into the circulating blood. The recent specific and sensitive assay system for plasmatic thrombin, involving final supramolar concentrations of arginine (to inhibit hemostasis and to depolymerize non-crosslinked fibrin) and final chromogenic substrate concentrations below 0.6 mM (to detect only thrombin and not enzymes of the intrinsic phase of hemostasis) [6] was used to answer the question, if PLA_2 of different origin (bovine pancreas or snake) destroy blood cells, resulting in intrinsic thrombin generation by non-physiologic cell fragments (e.g. polynegative niches in DNA or in phospholipid microparticles).

2. Material and Methods

2.1. Snake PLA_2 compared with LPS and ZyA

800 μl freshly drawn citrated blood (1 part of 106 mM citrate + 9 parts of venous blood) of 4 healthy donors was incubated in 1 ml Eppendorf-cups with 20 μl 8200 ng/ml LPS (final conc. 200 ng/ml; LPS

Correspondence: T.W. Stief, M.D., Priv.-Doz., Department of Clinical Chemistry, University Hospital, D-35033, Marburg, Germany. Fax: +49-6421-286 5594; Email: thstief@med.uni-marburg.de

from E. coli 0111;B4 purified by gel filtration; Sigma, Deisenhofen, Germany; Article Nr. L 2637; 5 mg/ml stabilized in 0.5% human albumin (Kabi, Stockholm, Sweden) and freshly diluted for the experiment) in physiol. NaCl, 820 μg/ml (20 μg/ml final) zymosan A (ZyA; from *Saccharomyces cerevisiae*; Sigma; Article Nr. Z 4250) in physiol. NaCl, or 0–410 μg/ml (0–10 μg/ml final) snake PLA_2 (1 mg/ml PLA_2 from *Naja mossambica mossambica* (Sigma; Article Nr. P 7778) in 5% human albumin and freshly diluted with physiol. NaCl) for 30 min or 45 min (RT). All healthy donors gave informed consent for studying the 4.5 ml citrated blood drawn into monovettes containing 0.5 ml 106 mM sodium citrate (Sarstedt, Nümbrecht, Germany). The cups were centrifuged in an Eppendorf-centrifuge at 10000 rotations/min for 5 min at 23°C. 50 μl of plasma was pipetted in duplicate into a microtitre plate with flat bottom (F-well type C Polysorp®; NUNC, Wiesbaden, Germany; Article Nr. 446140). The hemolysis-control absorbance was measured at 405 nm with a microtitre plate reader with a 1 mA resolution (Milenia-DPC, Los Angeles, U.S.A.). 5 μl 250 mM $CaCl_2$ was added, using an Eppendorf-multipette with H_2O-rinsed and completely emptied new tips. After 0–45 min coagulation reaction time (CRT at 37°C) 100 μl 2.5 M arginine, pH 8.6 (Sigma) was added. After 20 min (23°C) 50 μl 1 mM thrombin substrate HD-CHG-Ala-Arg-pNA (CS-IIa; Pentapharm, Basel, Switzerland) in 1.25 M arginine, was added, the increase in absorbance per time (ΔA/t) at 405 nm (23°C) was determined, and the results were standardized against the 1 IU/ml bovine thrombin (DadeBehring, Marburg, Germany) in 6.7% human albumin standard. From the hemolysis control it resulted that 1–10 μg/ml PLA_2 destroyed about 0.2–0.5% of erythrocytes (increase of basal plasma absorbance at 405 nm by 400–1000 mA).

1 ml citrated blood of a healthy donor was incubated with 10 μl 0–100 μg/ml (0–1 μg/ml final conc.) snake PLA_2 in 0.5 % human albumin –0.9%. NaCl for 30 min (RT). After centrifugation 20 μl plasma was incubated in duplicate with 2 μl 250 mM $CaCl_2$ in U-well microtitre plates (NUNC) for 0–20 min CRT (37°C). 20 μl 2.5 M arginine was added. After 20 min (RT) 20 μl 0.77 mM CS-IIa in 2.3 M arginine, pH 8.6, was added and ΔA/t was measured. The experiment was repeated with a CRT of 40 min (37°C) for n = 4 different healthy donors.

2.2. Pancreas PLA_2 compared with LPS and ZyA

The experiment of 2.1. was repeated with blood of 4 healthy donors and with PLA_2 from bovine pancreas (Sigma; Article Nr. P 8913) in 2% human albumin and with final concentrations of 0–1000 ng/ml PLA_2, 200 ng/ml LPS, or 20 μg/ml ZyA.

2.3. Pancreas PLA_2 versus LPS, ZyA without or with hydrocortisone

400 μl citrated blood of n = 4 healthy donors (3 h RT old) was added in duplicate to 10 μl 0–12300 ng/ml (final blood conc. 0–300 ng/ml) bovine pancreas PLA_2 in 2% human albumin, 10 μl 410 ng/ml LPS (final blood conc. 10 ng/ml) or 4100 ng/ml LPS (final blood conc. 100 ng/ml) in 2% human albumin, 10 μl 410 μg/ml zymosan A (final blood conc.10 μg/ml) in 2% human albumin, or 10 μl 410 μg/ml zymosan A with 1 μl 100 mg/ml hydrocortisone (final blood conc. 243 μg/ml) (Pfizer, Karlsruhe, Germany). The samples were vortexed and after 30 min (RT) they were centrifuged for 5 min at 10000 rpm in an Eppendorf-centrifuge. 4 × 50 μl plasma were withdrawn and pipetted into a polystyrole flat bottom microtiter plate (F-well type F, NUNC). 5 μl 250 mM $CaCl_2$ was added with an Eppendorf-mutipette using H_2O rinsed new disposable tips. After 5 min, 10 min, 15 min, 20 min CRT (37°C) 100 μl 2.5 M arginine, pH 8.6, was added. After 20 min (RT) 50 μl 0.91 mM chromogenic thrombin substrate HD-CHG-Ala-Arg-pNA in 1.36 M arginine (10 μmoles + 5 ml H_2O + 6 ml 2.5 M arginine, pH 8.6) was added and ΔA at 405 nm (RT) was determined with a microtiterplate reader with a 1 mA resolution. The results were standardized against the 1 IU/ml bovine thrombin calibrator in 6.7% human albumin, replacing the plasma sample. 1 IU/ml thrombin had 14 mA/min RT, the maximal ΔA was 1400 mA, the linear range was up to 40% of 1400 mA = 560 mA.

2.4. Bovine pancreatic PLA_2 at 10 μg/ml

The experiment of 2.3. was performed with 20 ng/ml final conc. LPS, 2 μg/ml final conc. ZyA, 2% human albumin, 0.1 μg/ml, 1 μg/ml, 10 μg/ml PLA_2.

2.5. Plasma replacing whole blood

2.5.1. Pooled normal plasma

160 µl pooled normal plasma (standard human plasma®; DadeBehring) was incubated with 20 µl LPS (final conc. 100 ng/ml, ZyA (final conc. 10 µg/ml), or PLA2 (final conc. 0.1 µg/ml, 1 µg/ml, 10 µg/ml) in 1 ml Eppendorf-cups for 45 min (RT). 20 µl samples were withdrawn and pipetted into U-wells. The samples were recalcified (10+1) with 2 µl 250 mM CaCl2. 50 µl 2.5 M arginine, pH 8.6, stopped the CRT after 0–30 min (37°C). After 20 min (RT) 25 µl 0.91mM CS-IIa in 1.36 M arginine was added and ΔA/t at 405 nm (RT) was determined. The calibrator 1 IU/ml thrombin had 3.7 mA/min RT.

2.5.2. Fresh normal plasma

190 µl of freshly drawn citrated plasma (<2 h RT old) from one healthy donor with proper venipuncture and one healthy donor with improper venipuncture and 5 min venous stasis time was incubated with 10 µl 2% human albumin, or final conc. of 0.2 µg/ml PLA_2, 2 µg/ml PLA_2, 20 µg/ml PLA_2, 250 ng/ml LPS, 2.5 µg/ml LPS, 25 µg/ml LPS, 250 µg/ml LPS, 10 µg/ml ZyA, 100 µg/ml ZyA for 45 min (RT) in 1 ml Eppendorf-cups. After centrifugation, 20 µl samples were pipetted into U-well microtitre plates and analyzed in duplicate as described in 2.5.1.

2.6. Statistics

The intra-assay coefficients of variation (CV), as defined as standard deviation divided by mean value and multiplied by 100%, of the thrombin generation values measured were <8%. The data points were compared to the respective controls and tested for significance ($p < 0.05$), using the two-fold Yates-corrected chi-square comparison against the control-mean ($\bar{X}^2$0 Test) [7].

3. Results

Figures 1a-d are the assay results of n = 4 healthy donors, each graphic representing one individual donor. They demonstrate that 100 ng/ml snake PLA_2 activates hemostasis similarly as 200 ng/ml LPS or 20 µg/ml ZyA in 3 of 4 healthy donors. 1000 ng/ml PLA_2 might result into lower (Fig. 1a–e) or similarly high (Fig. 1f,g) thrombin activities comparable to 100 ng/ml PLA_2. Thrombin generation in the recalcified coagulation activity assay (RECA) also depends on the nature of the reaction well: flat bottom polystyrole wells (F-wells) possess a different surface to polystyrol U-wells. This might explain the different thrombin generation in Fig. 1a-d (F-wells) when compared to Fig. 1e (U-wells).

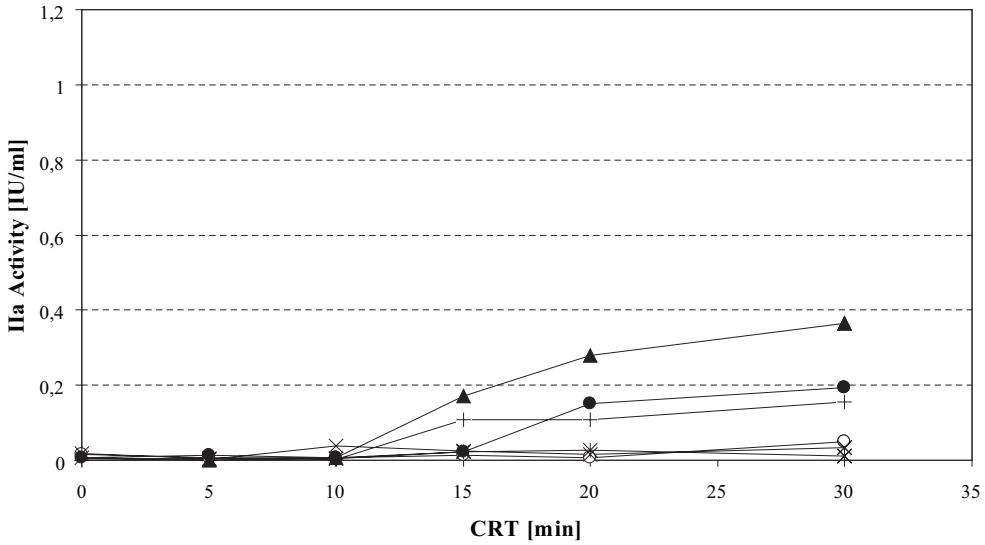
Also PLA_2 from bovine pancreas activates coagulation (Fig. 2). At 15 min coagulation reaction time 10–100 ng/ml PLA_2 generate about 0.2–0.8 IU/ml thrombin. 1000 ng/ml pancreas PLA_2 at 15 min CRT behaves like 0 ng/ml pancreas PLA_2. Coagulation reaction times >15 min (37°C) seem to result into activation of hemostasis, presumably by interaction of the plasma with the polystyrol of the microtiter plate. Fig. 2e shows the thrombin generation in the initial phase (CRT = 10 min) by pancreas PLA_2 as compared with that by snake PLA_2 (Fig. 1e).

Up to 60 ng bovine pancreatic PLA_2 added per ml blood the generated thrombin activity is proportional to the PLA_2 activity used (Fig. 3a–c): at 15 min CRT about 0.1–0.8 IU/ml thrombin is generated in 3 of 4 healthy donors; the blood hemostasis system of one donor (♦) behaved as resistant against PLA_2 attack. At PLA_2 concentrations ≤ 60 ng/ml the basal absorbance increase of plasma due to lysis of erythrocytes was <100 mA, i.e. < 0.05% of erythrocytes were lysed. Hydrocortisone at about 200 µg/ml blood concentration does not inhibit the hemostasis activation by 10 µg/ml zymosan A (Fig. 3d–f). This means that the anti-inflammatory agent hydrocortisone does not inhibit the hemostasis activation potency of extracellular PLA_2.

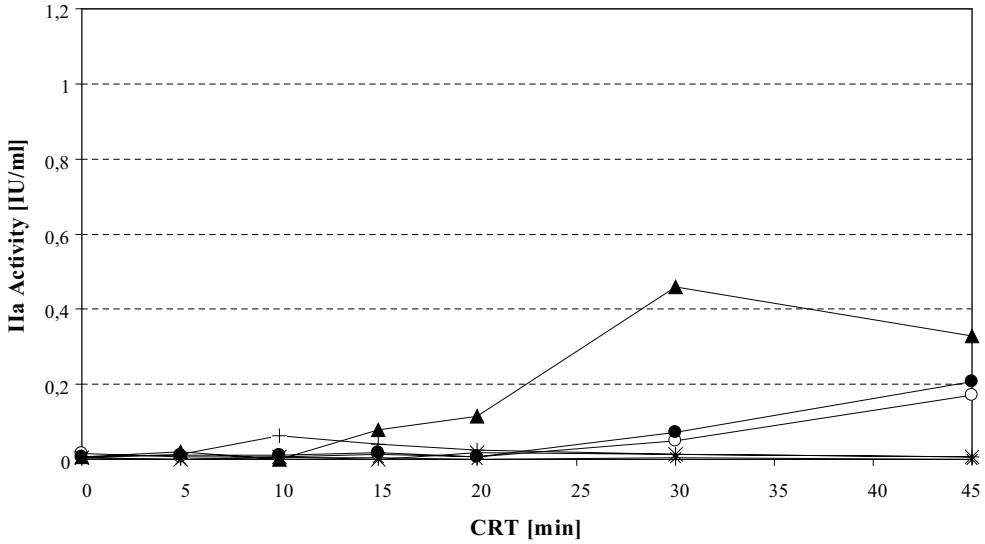
Figure 4 demonstrates that bovine pancreatic PLA_2 at a very high concentration (10 µg/ml final) again results into pronounced hemostasis activation with about 0.1–3 IU/ml at 10–15 min CRT. However, in one of four patients 0.1 µg/ml PLA_2 induced more thrombin than 10 µg/ml PLA_2 (17 min CRT: 0.29 IU/ml versus 0.12 IU/ml) (Fig. 4b).

In commercially available lyophilized pooled normal plasma there is no significant increase in thrombin activity when exposed to up to 10 µg/ml bovine pancreatic PLA_2 (Fig. 5a). Fresh plasma of the donor with proper venipuncture shows no thrombin generation by PLA_2 or LPS, only by extreme concentrations of ZyA (100 µg/ml)

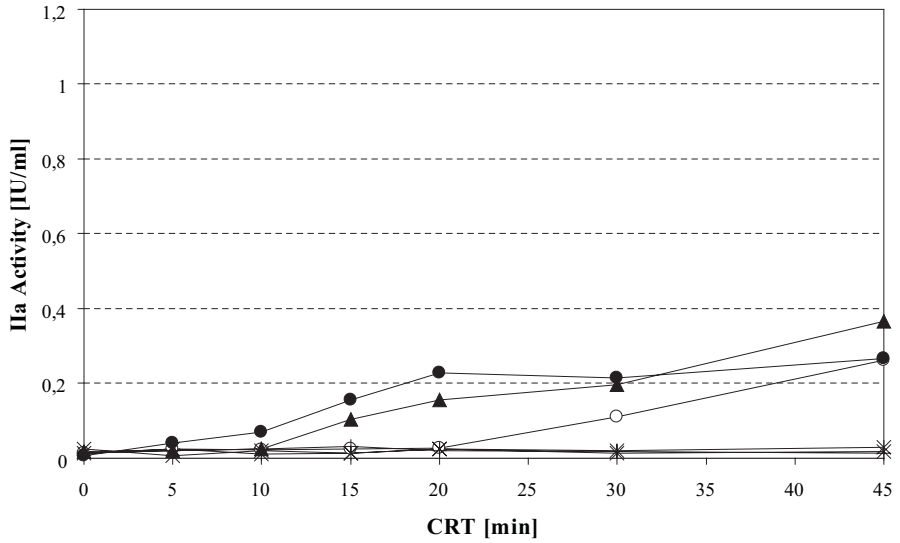
1a


IIa Activity [IU/ml]
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
25
30
35
CRT [min]

1b


IIa Activity [IU/ml]
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
25
30
35
40
45
CRT [min]

1c


IIa Activity [IU/ml]
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
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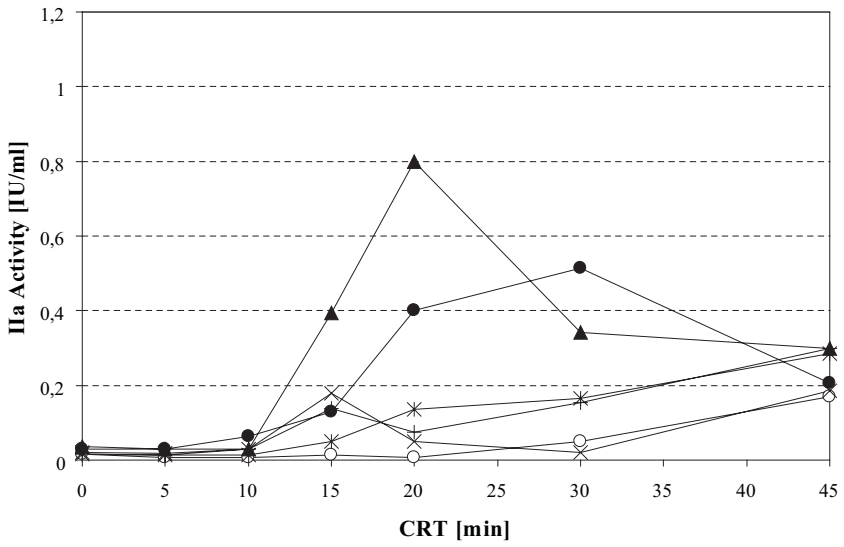
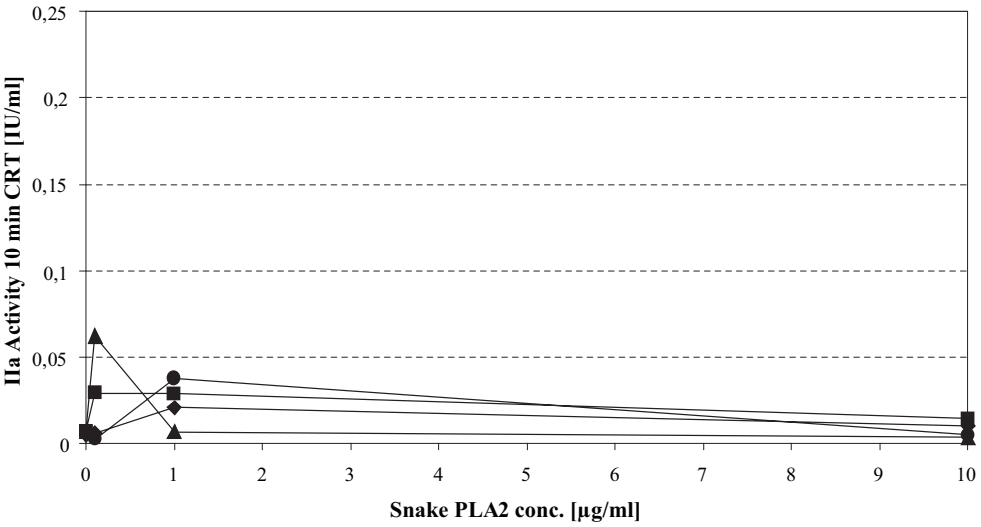
30
35
40
45
CRT [min]

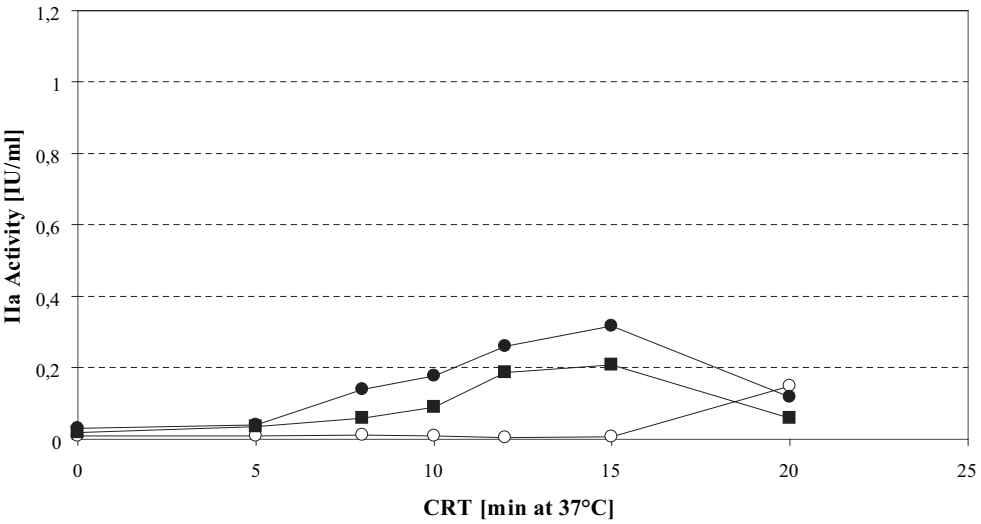
1d


1,2
1
0,8
0,6
0,4
0,2
0
IIa Activity [IU/ml]
0
5
10
15
20
25
30
35
40
45
CRT [min]

1e


0,25
0,2
0,15
0,1
0,05
0
IIa Activity 10 min CRT [IU/ml]
0
1
2
3
4
5
6
7
8
9
10
Snake PLA2 conc. [µg/ml]

1f


1,2
1
0,8
0,6
0,4
0,2
0
IIa Activity [IU/ml]
0
5
10
15
20
25
CRT [min at 37°C]

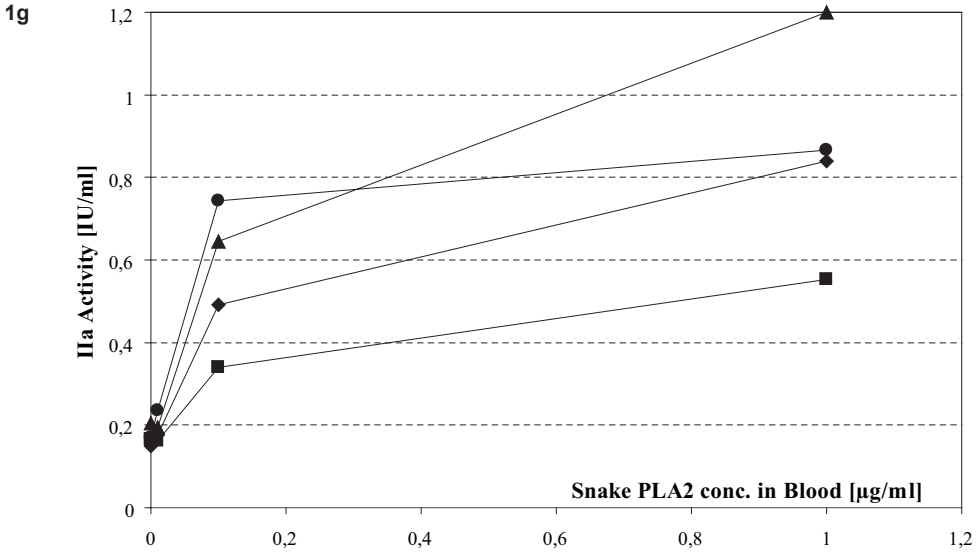
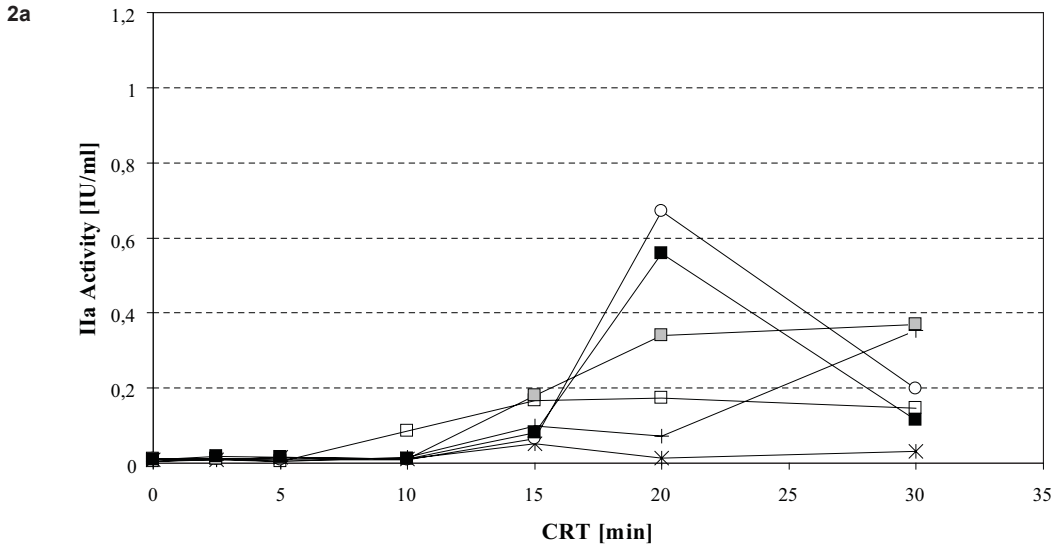
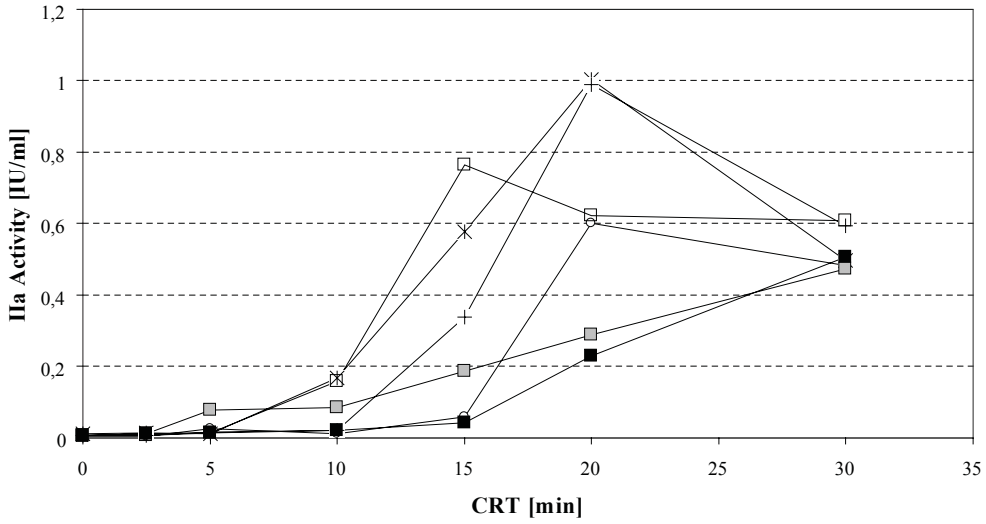


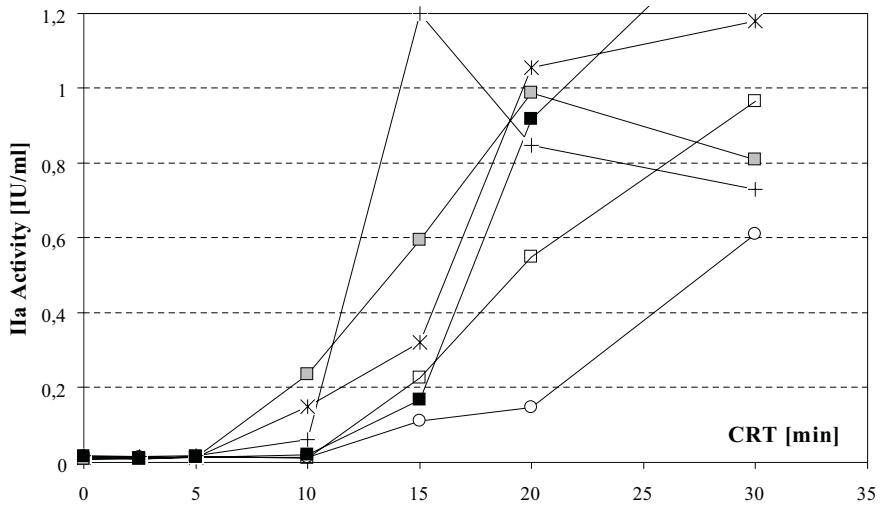
Figure 1. Snake PLA2 compared to LPS and ZyA.
800 µl freshly drawn citrated blood of 3 healthy donors (Fig. 1a-c) or a donor with acute bacterial tonsillitis (CRP = 21 mg/l) (Fig. 1d) was incubated in 1 ml Eppendorf-cups with final concentrations of 200 ng/ml LPS (●), 20 µg/ml ZyA (▲), or 10 µg/ml (*),1 µg/ml (x), 0.1 µg/ml (+), 0 µg/ml (O) snake PLA_2 for 30 min (RT). After centrifugation 50 µl of plasma was pipetted into microtitre plates with flat bottom. 5 µl 250 mM $CaCl_2$ was added. After 0–45 min coagulation reaction time (CRT) 100 µl 2.5 M arginine, pH 8.6, was added. After 20 min (RT) 50 µl 1 mM CS-IIa in 1.25 M arginine, was added, ΔA at 405 nm was determined, and the results were standardized against the 1 IU/ml thrombin in 6.7 % human albumin calibrator. Fig. 1e: 10 min CRT values of Figs. 1a-d; each donor has an individual symbol (●,▲,♦,■). 1 ml citrated blood of a healthy donor was incubated at final conc. of 0 µg/ml (O), 0.1 µg/ml (■), 1 µg/ml (●) snake PLA_2 for 30 min (RT) (Fig. 1f). After centrifugation 20 µl plasma was incubated with 2 µl 250 mM $CaCl_2$ in U-well microtitre plates for 0–20 min CRT (37°C). 20 µl 2.5 M arginine was added. After 20 min (RT) 20 µl 0.77 mM CS-IIa in 2.3 M arginine, pH 8.6, was added and ΔA/t was measured. The experiment was repeated with a PLA_2 preincubation time of 45 min and a CRT of 40 min for n = 4 healthy donors; each donor has an individual symbol (●,▲,♦,■) (Fig. 1g).



2b


IIa Activity [IU/ml]
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
25
30
35
CRT [min]

2c


IIa Activity [IU/ml]
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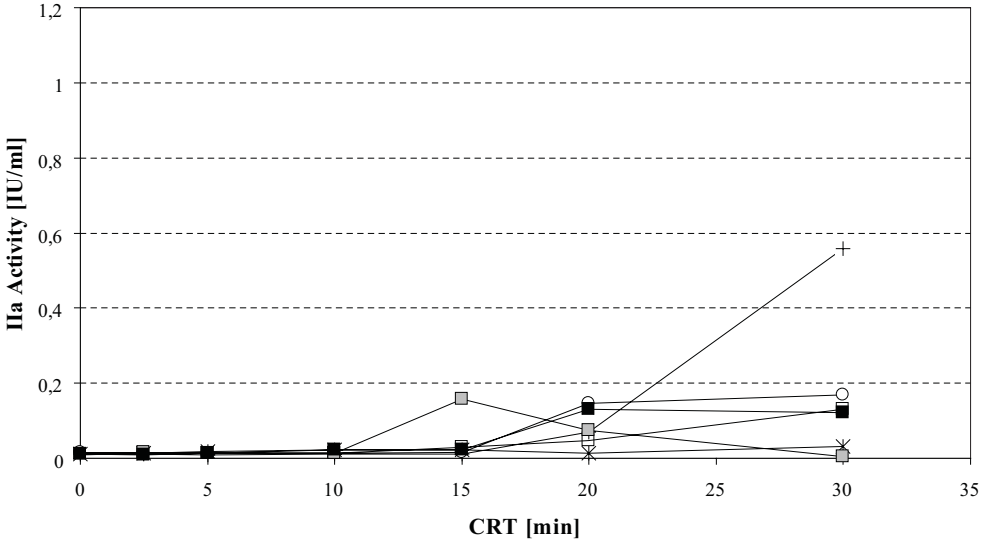
0,4
0,2
0
0
5
10
15
20
25
30
35
CRT [min]

2d


IIa Activity [IU/ml]
1,2
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5
10
15
20
25
30
35
CRT [min]

2e

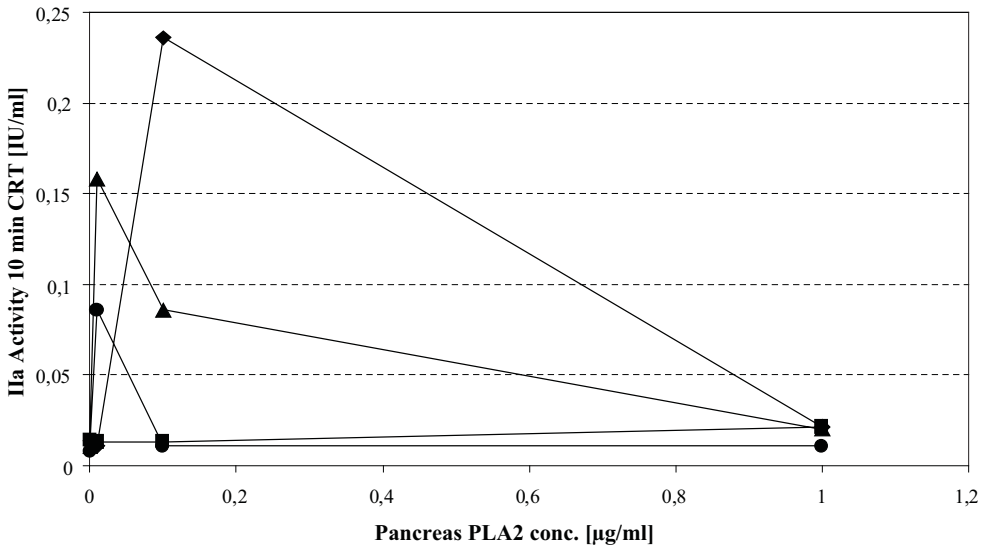
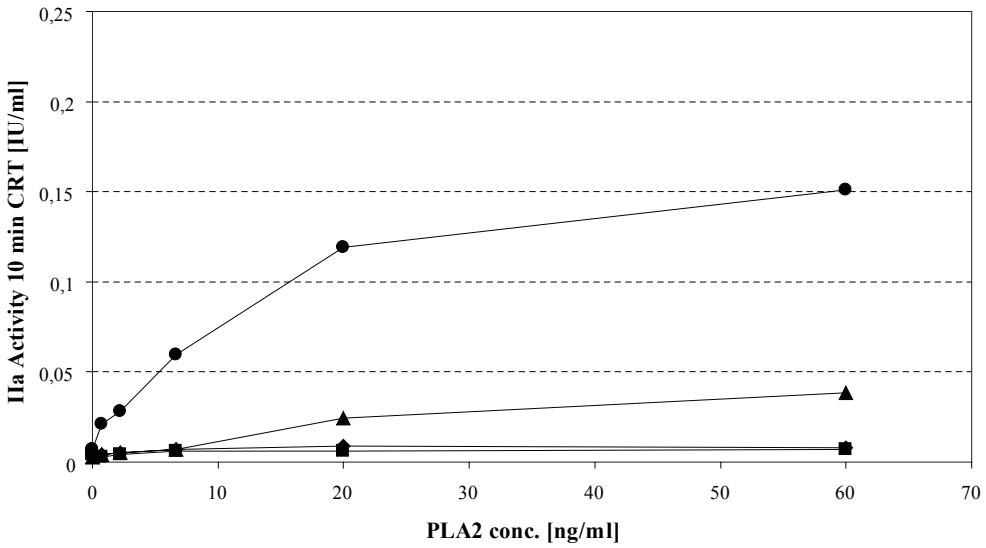


Figure 2. Pancreas PLA_2 compared with LPS and ZyA.
The experiment of figure 1 was repeated with blood of 4 healthy donors (Fig. 2a-d) and with PLA2 from bovine pancreas at final concentrations of: 0 ng/ml (O), 10 ng/ml (□), 100 ng/ml (grey□), 1000 ng/ml PLA_2 (■), or 200 ng/ml LPS (*), 20 µg/ml ZyA (+). Fig. 2e: 10 min CRT values of Figs. 2a-d; each donor has an individual symbol (●,▲,♦,■).

3a



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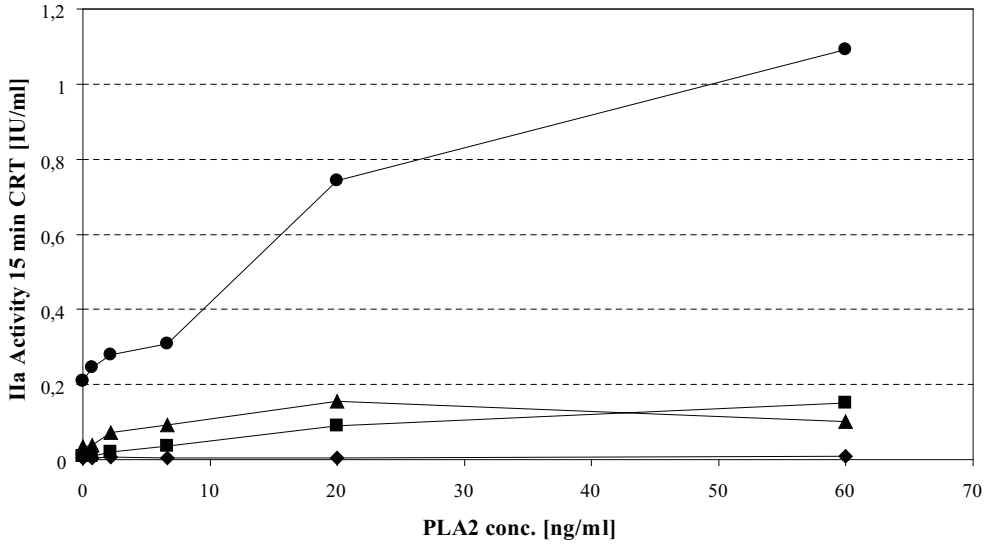
IIa Activity 15 min CRT [IU/ml]
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1
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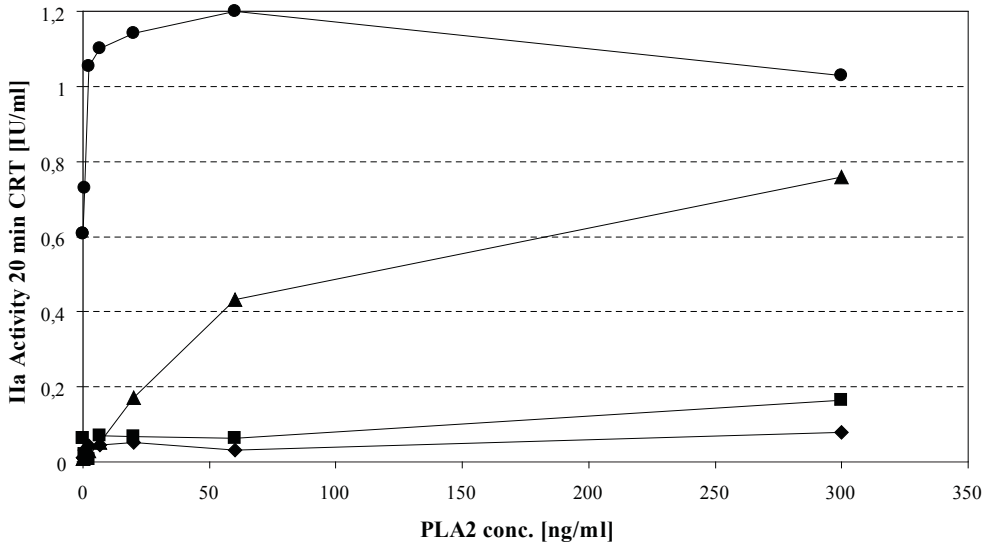
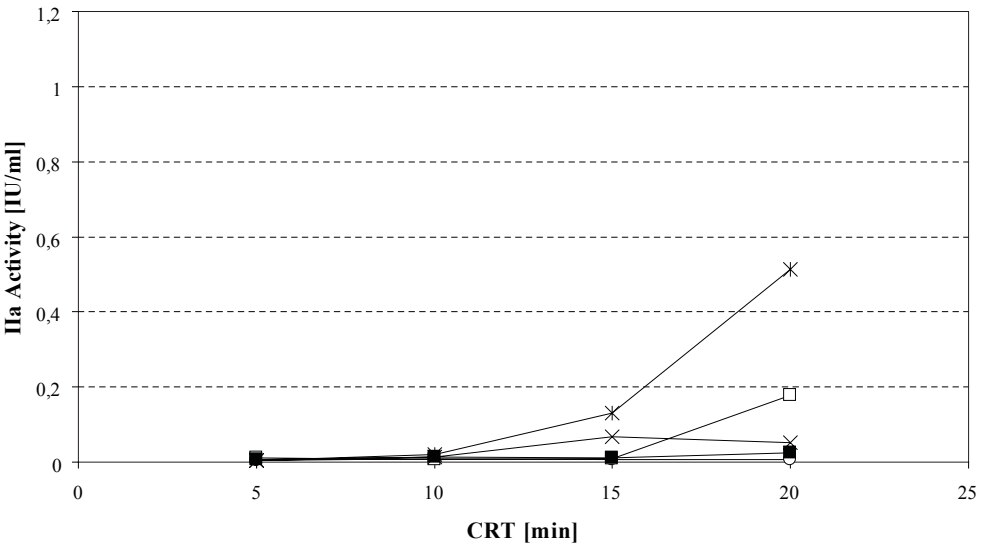
0,6
0,4
0,2
0
0
10
20
30
40
50
60
70
PLA2 conc. [ng/ml]

3c


IIa Activity 20 min CRT [IU/ml]
1,2
1
0,8
0,6
0,4
0,2
0
0
50
100
150
200
250
300
350
PLA2 conc. [ng/ml]

3d


IIa Activity [IU/ml]
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
25
CRT [min]

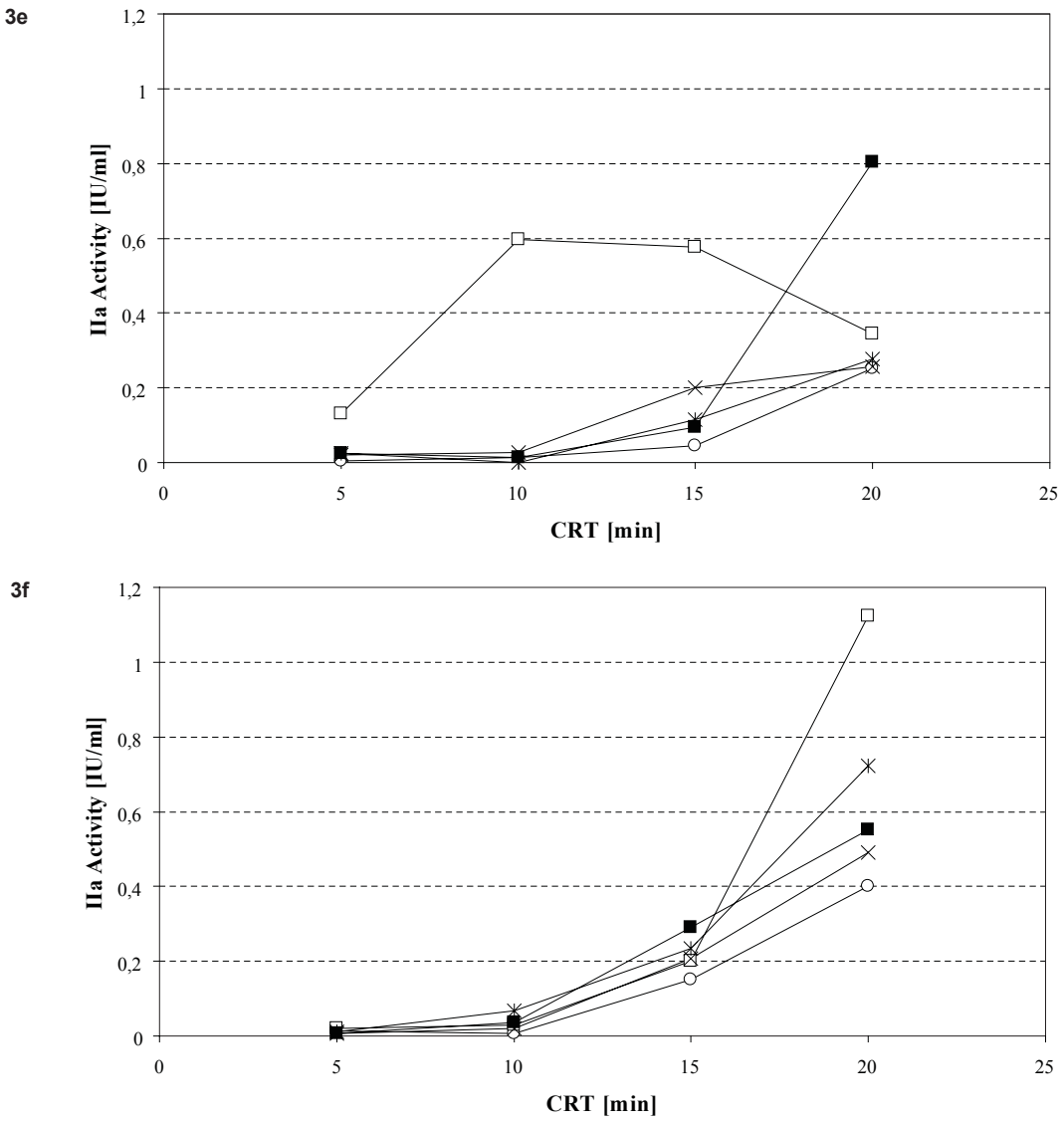
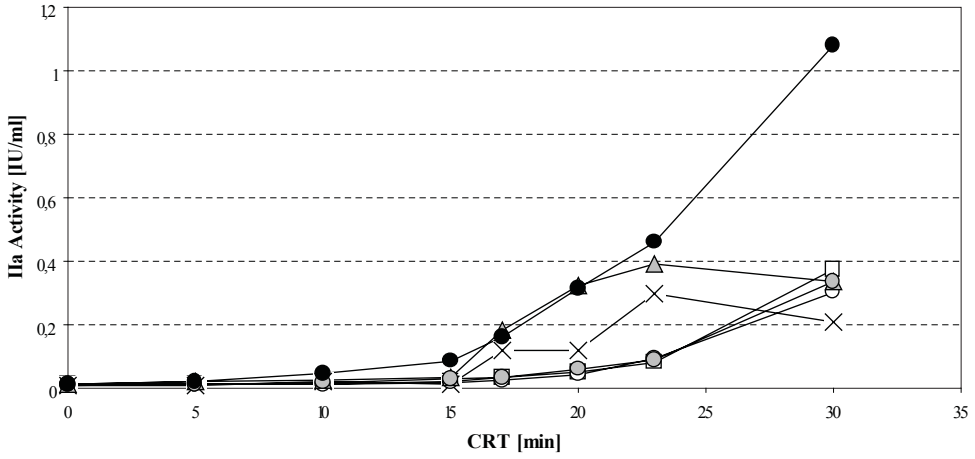
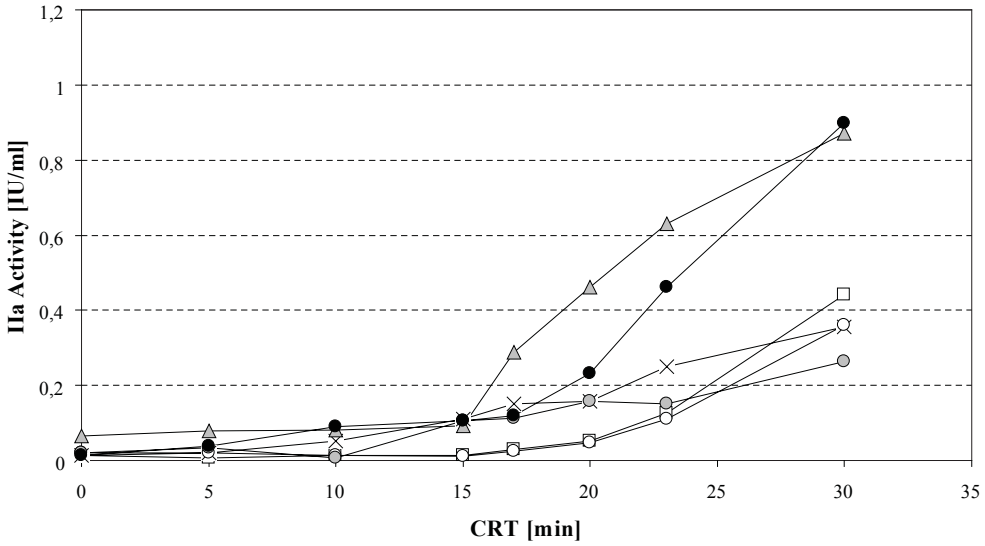


Figure 3. Pancreas PLA_2 versus LPS, ZyA without or with hydrocortisone.
400 μl citrated blood of n = 4 healthy donors (3 h RT old; ●,▲,♦,■) was added to final blood conc. of 0–300 ng/ml bovine pancreas PLA_2. After 30 min (RT) the samples were centrifuged for 5 min at 10000 rpm, 50 μl plasma was recalcified by 5 μl 250 mM $CaCl_2$. After 10 min (Fig. 3a), 15 min (Fig. 3b), 20 min (Fig. 3c) coagulation reaction time (CRT at 37°C) 100 μl 2.5 M arginine, pH 8.6, was added. After 20 min (RT) 50 μl 0.91 mM CS-IIa in 1.36 M arginine was added and ΔA at 405 nm (RT) was determined. The results were standardized against the 1 IU/ml bovine thrombin calibrator in 6.7 % human albumin, replacing the plasma sample. Fig. 3d-f: 400 μl citrated blood of 4 healthy donors was incubated for 30 min (RT) with human albumin (O), 10 ng/ml LPS (□), 100 ng/ml LPS (■), 10 μg/ml ZyA (x), 10 μg/ml ZyA + 243 μg/ml hydrocortisone (*). Then the samples were zentrifuged, recalcified, and analyzed as described in Fig. 3a-c; Fig. 3e: haemolytic blood.

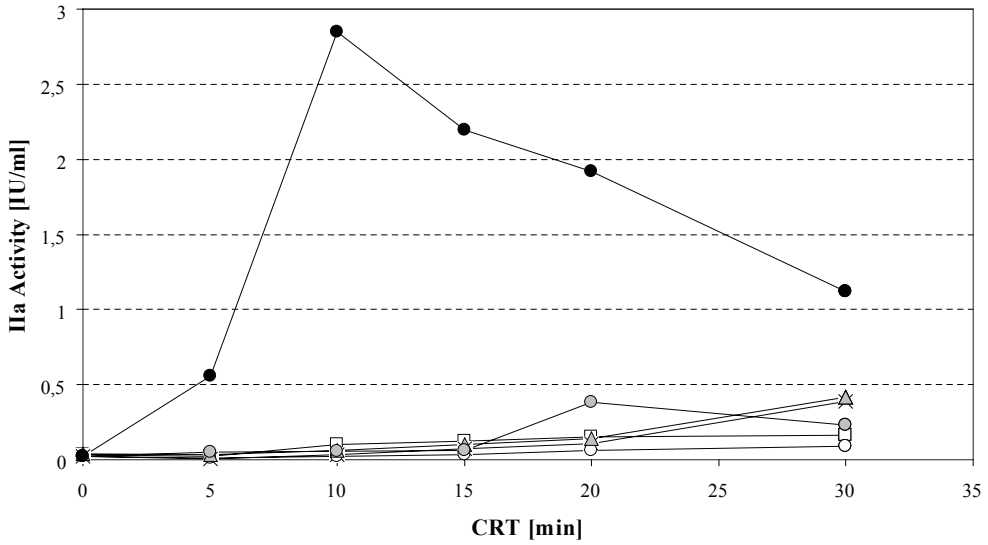
4a


IIa Activity [IU/ml]
CRT [min]

4b


IIa Activity [IU/ml]
CRT [min]

4c


IIa Activity [IU/ml]
CRT [min]

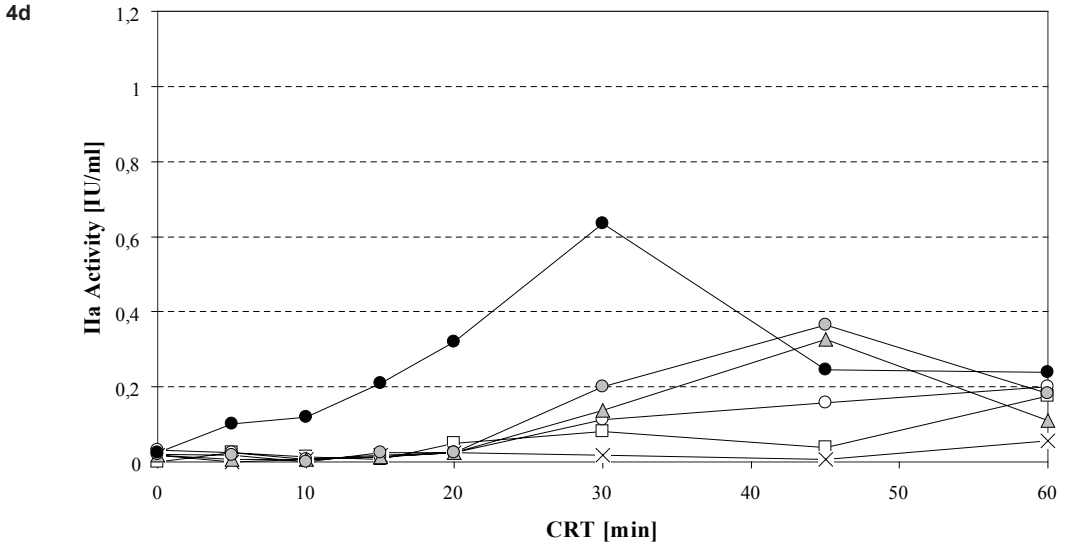
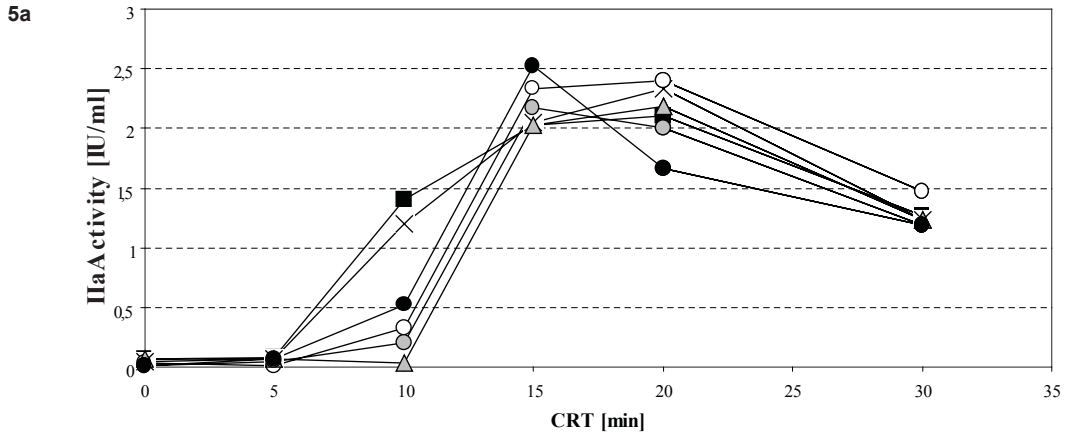
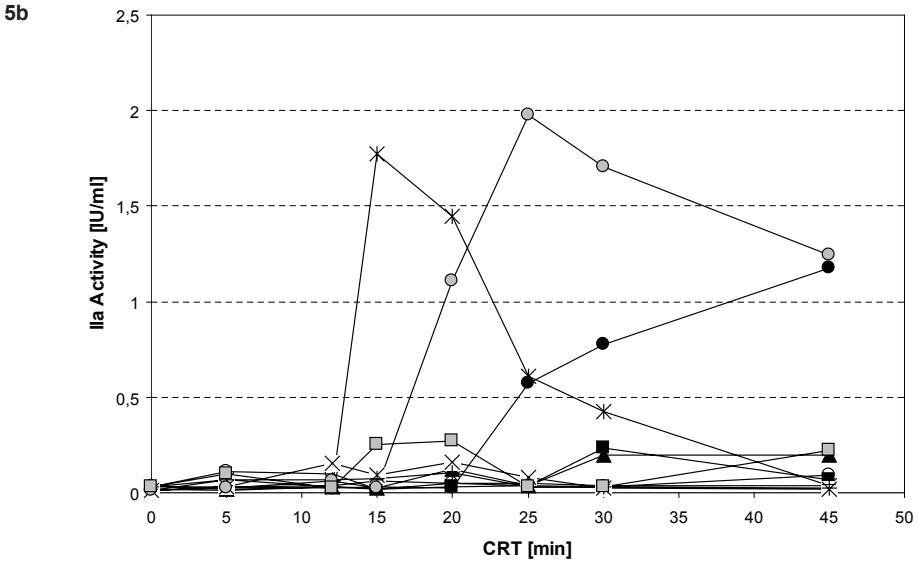


Figure 4. Pancreatic PLA_2 at highest concentrations.
The experiment of figure 3 was performed with 20 ng/ml LPS (□), 2 μg/ml ZyA (x), human albumin (O), 0.1 μg/ml (greyΔ), 1 μg/ml (greyO), 10 μg/ml PLA_2 (●) in 4 normal donors (Fig.4a-d).





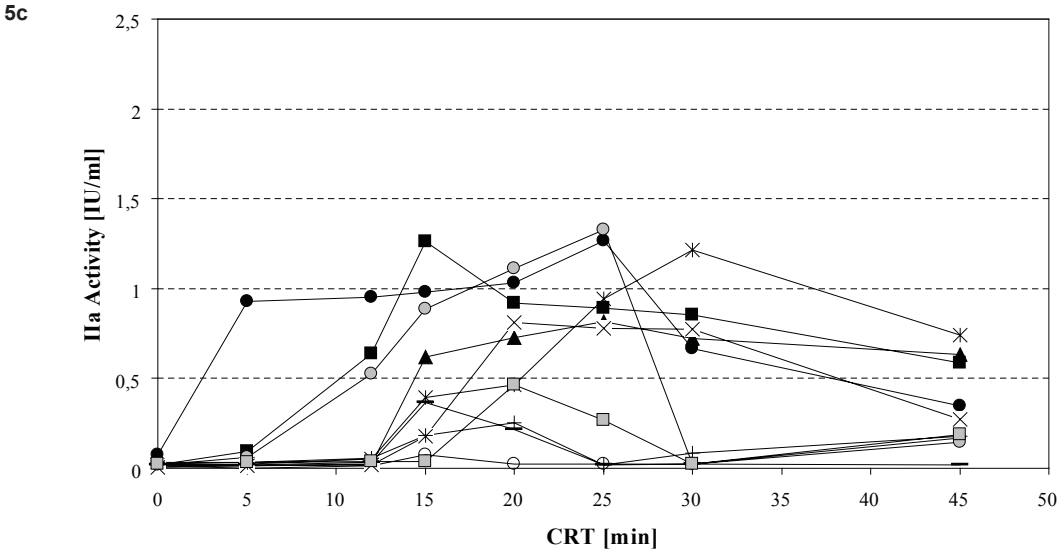


Figure. 5. Plasma replacing whole blood
Figure. 5a. Standard human plasma®
160 μl standard human plasma® was incubated with 20 μl LPS (final conc. 100 ng/ml (■)), ZyA (final conc. 10 μg/ml (x)), or PLA_2 (final conc. 0 μg/ml (0), 0.1 μg/ml (greyO), 10 μg/ml (●)) in 1 ml Eppendorf-cups for 45 min (RT). 20 μl samples were withdrawn and pipetted into U-wells. The plasmas were recalcified with 2 μl 250 mM $CaCl_2$. 50 μl 2.5 M arginine, pH 8.6, stopped the CRT after 0–30 min (37°C). After 20 min (RT) 25 μl 0.91 mM CS-IIa in 1.36 M arginine was added and ΔA/t at 405 nm (RT) was determined.

Figure. 5b,c. Fresh normal plasma
190 μl of freshly drawn citrated plasma (<2 h RT old) from one healthy donor with proper venipuncture (Fig. 5b) and one healthy donor with improper venipuncture and 5 min venous stasis time (Fig. 5c) was incubated with 10 μl 2 % human albumin (O), or final conc. of 0.2 μg/ml PLA_2 (▲), 2 μg/ml PLA_2 (■), 20 μg/ml PLA_2 (●), 250 ng/ml LPS (-), 2.5 μg/ml LPS (+), 25 μg/ml LPS (×), 250 μg/ml LPS (*), 10 μg/ml ZyA (greyΔ), 100 μg/ml ZyA (grey O) for 45 min (RT) in 1 ml Eppendorf-cups. After centrifugation, 20 μl samples were pipetted into U-well microtitre plates and analyzed in duplicate as described in Fig. 5a.

additional thrombin is generated (Fig. 5b). In fresh plasma of the donor with poor venipuncture and 5 min stasis time hemostasis is activated by PLA_2, LPS, and ZyA, when compared to the zero control (Fig. 5c).

4. Discussion

Phospholipase A_2 activates hemostasis in whole blood. This pathophysiology of PLA_2 can already be observed at PLA_2 concentrations in the range of 10–100 ng/ml. Higher concentrations might increase or decrease hemostasis activation. This might be due to different effects of PLA_2 on whole blood: on the one hand PLA_2 destroys cells, and the cell fragments activate the contact phase of hemostasis, on the other hand PLA_2 inhibits certain phospholipid-dependent pathways of coagulation [8–12], the intrinsic pathway is more susceptible than the extrinsic one [13], and the activation of the prothrombinase by factor Xa is inhibited by PLA_2 [14]. PLA_2 interacts with hemostasis by its enzymatic acitivity or by its binding to critical hemostasis components [8]. Blood hemostasis seems to be especially susceptible to very low blood flow, such as when drawing blood after prolonged venous stasis (Fig. 5c): the contact system of hemostasis might be activated thereby, this activation is enhanced by PLA_2, LPS, or ZyA. Among healthy individuals there appears some variability of resistance against extracellular PLA_2 or LPS [6]: some human beings seem to be more susceptible against these triggers of intrinsic thrombin generation than others. It would be of particular interest characterize physiologic PLA_2 or LPS inhibitors or inhibitors of non-physiologic polynegative niches.

Not all of the PLA_2 molecules found in patient plasmas may have the same cell killing power, some PLA_2 enzymes might act preferentially as signal enzymes (e.g. the platelet PLA_2), others as membrane destructors (e.g. the pancreas PLA_2). Furthermore, some PLA_2 molecules in plasma might be present in a cryptic (inactive) form, another folding by matrix change (e.g. dilution) or enzyme action on them might activate them.

The present data support the pathophysiologic importance of PLA_2 in disseminated intravascular coagulation (DIC): some pathogenetic aspects of DIC seem to be similar to a snake bite: pathologic PLA_2 activities in blood can occur by a wide range of diseases [1–5], many of them can give rise to PDIC. In PDIC an about 10–100 fold increase of the normal plasmatic PLA_2 activity can be found [15,16]. It is suggested to screen patients for plasmatic thrombin generation to stop the pathogenesis of PDIC in time. Of particular importance could be physiologic inhibitors of PLA_2 [17] or of intrinsic thrombin generation [18].

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