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The activation *in vitro* of porcine pancreatic prophospholipase A_2 by lysosomal enzymes

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Pancreatic phospholipase A_2 (PLA_2*) (EC 3.1.1.4) is produced and secreted as an inactive zymogen. Hydrolysis of the arg-7-ala-8 linkage by trypsin (EC 3.4.21.4) in the small intestine results in a heptapeptide and the active enzyme [1] which cleaves phospholipids producing 1-acyllysophosphoglycerides and fatty acids [2].

Premature liberation of the digestive enzymes within the pancreas may be responsible for the tissue damage in acute pancreatitis [3]. Especially the lysocompounds of lecithin and cephalin, produced by PLA_2, are supposed to cause extensive vascular and parenchymatous necrosis (for review see Ref. 4).

Investigations of experimental acute pancreatitis have indicated that lysosomal enzymes participate in the premature activation of pancreatic digestive enzymes [5–7]. The liberation of trypsin by cathepsin B (EC 3.4.22.1) [8–10], was discussed as one possible mechanism leading to the hydrolysis of other zymogens by the released trypsin [11]. Therefore, trypsin inhibitors like camostate mesilate were studied as therapeutic agents for pancreatitis [12]. We were interested in whether different liberation pathways, without the involvement of trypsin, were possible.

Materials and Methods

Materials. Methyl-α-D-mannoside, phosphatidylcholine, Z-Phe-Arg-NMec and trypsinogen were purchased from Sigma (Taufkirchen, F.R.G.). NEFA C Kit was obtained from Wako Chemicals GmbH (Neuss, F.R.G.) and camostate mesilate† from Schwarz Pharma AG (Monheim, F.R.G.). Concanavalin A-Sepharose and Sephadex G-75 sf were products of Pharmacia (Freiburg, F.R.G.). Arg-NNap and bovine serum albumin were purchased from Serva (Heidelberg, F.R.G.). Trypsin was obtained from Boehringer (Mannheim, F.R.G.). Bz-Arg-$NPhNO_2$ and all other chemicals were products of Merck (Darmstadt, F.R.G.). E-64 was kindly provided by Dr Hanada, Ohmiya, Japan.

Isolation of pancreatic $PPLA_2$. $PPLA_2$ was isolated from porcine pancreas according to Nieuwenhuizen *et al.* [13]. To prevent activation of the zymogen during preparation, 1 μM camostate mesilate was added in the first steps. The isolated zymogen showed one single protein band after SDS–PAGE.

Isolation of lysosomal proteases. These were extracted from minced porcine liver with 4% NaCl solution containing 0.2% Triton X-100 and 1.5 mM EDTA at pH 4.0. After centrifugation, the proteins of the supernatant were precipitated by ammonium sulfate (35–65% saturation), dissolved in water to a protein concentration of 24 mg/mL ("crude extract"), and separated by gel chromatography on Sephadex G-75 sf [14]. Fractions containing proteins with a molecular weight between 30,000 and 22,000 were concentrated and applied to a column of concanavalin A-Sepharose equilibrated with buffer, pH 5.3 (0.1 M sodium acetate and 0.5 M NaCl). Elution was done with 0.3 M methyl-α-D-mannoside.

A preparation of lysosomes from porcine liver was made as described for rat liver by Bohley *et al.* [15].

Activation of $PPLA_2$ and trypsinogen by lysosomal proteases. For maximal activity the lysosomal enzymes were preincubated at 38° for 20 min in sodium acetate buffer (0.05 M, pH 5.0) with 1.25 mM DTE and 1.25 mM EDTA. This solution was mixed with 6.8 μg of isolated $PPLA_2$ or 120 μg trypsinogen to a final volume of 600 μL. Aliquots of 50 μL were withdrawn periodically and diluted with the same volume of 0.3 M Tris buffer (pH 7.9), before measuring the PLA_2 activity or mixed with 50 μL of E-64 (0.1 mM) before estimating the trypsin activity.

Enzyme assays. The lysosomal proteinases were assayed with Arg-NNap and Z-Phe-Arg-NMec [16] and with Bz-Arg-$NPhNO_2$ essentially as published [17].

For estimating the $PPLA_2$ activity an enzymatic colorimetric assay was used [18].

Trypsin was assayed in samples of 100 μL with Bz-Arg-$NPhNO_2$ [19]. The reaction was stopped by adding camostate mesilate at a final concentration of 1.7 μM.

Results and Discussion

When incubating the $PPLA_2$ with the crude extract from porcine liver a progressive increase of PLA_2 activity was noticed (Fig. 1). Addition of 24 ng trypsin to the zymogen led to a similar activation rate as using 150 μL of the crude extract. In both cases, the PLA_2 activity reached a maximum after 60 min.

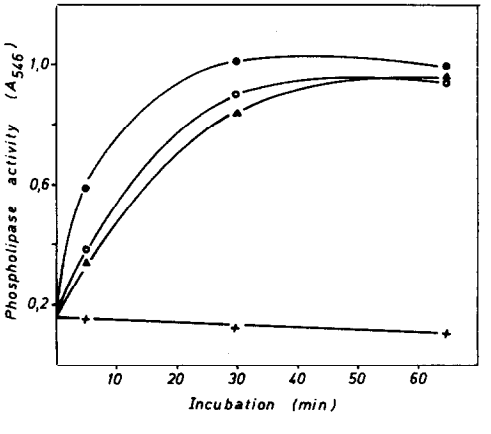

Fig. 1. Liberation of PLA_2 from its zymogen by 24 ng trypsin (▲), crude porcine liver extract (○, 150 μL; ●, 300 μL) and without addition of proteases (+).

* Abbreviations: PLA_2, phospholipase A_2; $PPLA_2$, prophospholipase A_2; E-64, 1-(*trans*-epoxysuccinyl-L-leucylamino)-4-guanidinobutane; Arg-NNap, L-arginine-naphthylamide; Bz-Arg-*N*-$PhNO_2$, *N*-α-benzoyl-L-arginine-*p*-nitroanilide; Z-Phe-Arg-NMec, *N*-α-benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-(4-methyl)coumarylamide; DTE, dithioerythritol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

† Trivial name: Camostate mesilate, *N,N*-dimethylcarbamoylmethyl - 4 - (4-guanidinobenzoyloxy)phenylacetate methansulfonate.

Table 1. The liberation of PLA_2 from its zymogen by crude porcine liver extract in the presence of inhibitors

Inhibitor	Final concentration	PLA_2 activity (%)
None		100
E-64	10 μM	0
Leupeptin	10 μM	0
Pepstatin	10 μM	104
EDTA	1 mM	105
Camostate mesilate	10 μM	100

Contrary to the activation by trypsin the pH optimum of the hydrolysing activity of the liver extract was about 5.0 (data not shown). The activation could be inhibited by E-64 or leupeptin, both being inhibitors for cysteine proteases [20, 21]. Pepstatin, an inhibitor for aspartic proteases [21], and camostate mesilate, an inhibitor for trypsin and similar serine proteases [22], as well as EDTA, inhibiting Ca-dependent enzymes, did not influence the enzymatic cleavage of $PPLA_2$ (Table 1). These results pointed to lysosomal cysteine proteases as being responsible for the activation.

To confirm this assumption, a preparation of lysosomes from porcine liver was incubated with $PPLA_2$. An increase of PLA_2 activity was noticed here, too, and could be inhibited by E-64 or leupeptin (data not shown).

To identify the $PPLA_2$ activating protease, a purification of the crude liver extract was carried out with gel chromatography on Sephadex G-75 sf. The protease was eluted between 30,000 and 22,000 and could be further purified by adsorption on concanavalin A-Sepharose.

The lysosomal cysteine proteinases cathepsin B, H (EC 3.4.22.16) and L (EC 3.4.22.15) with molecular weights of 21,000 to 27,000 are considered to be the most active in the body [16]. Cathepsin B has been reported to activate trypsinogen at acid pH [8–10].

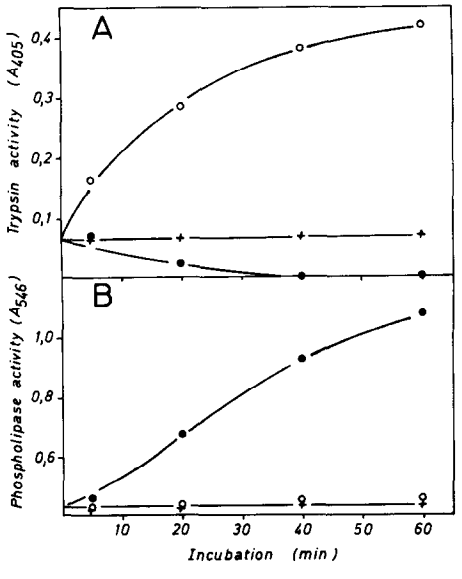

Fig. 2. Activation of digestive enzymes with porcine liver extract after concanavalin A-Sepharose chromatography (A, activation of trypsinogen; B, activation of $PPLA_2$). (○) Unadsorbed protein; (●) eluted protein; (+) no addition of liver extract.

Our investigations have shown that a lysosomal cysteine protease is capable of activating $PPLA_2$. This protease resembles cathepsin B: a molecular weight between 30,000 and 22,000, an activating optimum at acid pH and inhibition by E-64 or leupeptin. But the chromatography on concanavalin A-Sepharose separated cathepsin B and the $PPLA_2$ activating enzyme. An activation of $PPLA_2$ could be measured only with eluted protein (Fig. 2B), and contrary to this, trypsinogen was activated only with unadsorbed enzyme (Fig. 2A). Elution fractions did not activate trypsinogen but inactivated the small amount of pre-existing trypsin activity.

Both cathepsins H and L have affinity to concanavalin A-Sepharose [16]. However, a participation of cathepsin H in the activation of $PPLA_2$ can be excluded because this aminopeptidase is unaffected by leupeptin [23] which was confirmed by our own investigations. Therefore, it is assumed that cathepsin L is responsible for the activation of pancreatic $PPLA_2$.

Based on these results, at least two different activation procedures by lysosomal proteases on zymogens of pancreatic digestive enzymes are conceivable: (1) the cysteine proteinase cathepsin B is able to liberate trypsin from trypsinogen; and (2) the conversion of $PPLA_2$ to the active PLA_2 is catalysed by a different cysteine protease, possibly cathepsin L.

The premature liberation of the pancreatic digestive enzymes by lysosomal enzymes has been discussed as an early step leading to pancreatic autodigestion [24]. Here, especially cathepsin B is considered to play a key role because of its ability to activate trypsinogen. Active trypsin, responsible for the activation of all other zymogens, can be inhibited with camostate mesilate.

Our investigations have shown that an activation of pancreatic $PPLA_2$ is possible without the participation of trypsin, i.e. by the direct attack of a lysosomal cysteine protease different from cathepsin B. This reaction cannot be inhibited by camostate mesilate and should be taken into account by the development of new concepts in the therapy of inflammatory pancreatitis.

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Schwarz Pharma AG
Alfred-Nobel-Str. 10
4019 Monheim; and
†*Dept. of Physiological Chemistry*
University of Bonn
Nußallee 11
D-5300 Bonn
Federal Republic of Germany

CORNELIA LIPPERHEIDE
ROBERT MÜLLER*
KLAUS OTTO†

* To whom correspondence should be addressed.

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