

# Annexin II inhibits calcium-dependent phospholipase A<sub>1</sub> and lysophospholipase but not triacyl glycerol lipase activities of rat liver hepatic lipase

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Received 11 November 1991

A member of the annexin family (the heterotetrameric annexin II<sub>2</sub>pl 1<sub>2</sub> complex purified from porcine intestinal epithelium) was tested for its ability to affect different calcium-dependent intrinsic lipolytic activities of rat liver hepatic lipase (HL). Whereas annexin II in the presence of calcium failed to interfere with HL triacyl glycerol lipase (EC 3.1.1.3) activity, it inhibited HL phospholipase A<sub>1</sub> (EC 3.1.1.32) and lysophospholipase (EC 3.1.1.5) activities. Inhibition could be overcome by increasing the substrate concentration. Under phospholipase A<sub>1</sub> assay conditions, annexin II did not bind to the purified HL enzyme. These results therefore suggest that only inhibitor/substrate interactions lead to inhibition of HL phospholipase A<sub>1</sub> and lysophospholipase activities, an obviously general mechanism of phospholipase inhibition by annexins. Possible implications of HL inhibition in vivo by annexins are discussed.

Hepatic lipase; Annexin; Phospholipase A<sub>1</sub>; Lysophospholipase; Triacyl glycerol lipase

## 1. INTRODUCTION

Annexins represent a family of calcium- and phospholipid-binding proteins [1,2]. Among them the glucocorticoid-inducible lipocortins have been considered regulators of phospholipase A<sub>2</sub> [3,4], C [5] and D [6] activities. It has been suggested that phospholipases are non-specifically inhibited by annexins through 'substrate depletion' [7,8]. This process involves preferentially acidic phospholipids [7], and requires calcium in the micromolar range [9]. HL is a lipolytic enzyme with broad substrate specificity and requires calcium for optimal activity with triacyl glycerol as well as phospholipid substrates [10–15]. In the present study, we have addressed the question as to whether annexins affect phospholipase A<sub>1</sub> and other intrinsic lipolytic activities of hepatic lipase (HL) as well.

**Abbreviations:** HL, hepatic lipase; [<sup>14</sup>C]PC, 1,2-di[1-<sup>14</sup>C]palmitoyl-*sn*-glycero-3-phosphorylcholine; [<sup>14</sup>C]LPC, 1-[1-<sup>14</sup>C]palmitoyl-*sn*-glycero-3-phosphorylcholine; [<sup>14</sup>C]PS, 1,2-dioleoyl-*sn*-glycero-3-phosphoryl-L-[3-<sup>14</sup>C]serine; [<sup>14</sup>C]LPS, 1-oleoyl-*sn*-glycero-3-phosphoryl-L-[3-<sup>14</sup>C]serine; [<sup>3</sup>H]TO, glycerol tri[9,10(*n*)-<sup>3</sup>H]oleate.

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## 2. MATERIALS AND METHODS

### 2.1. Chemicals and biological material

All reagents and solvents used were of analytical grade and, with the exception of those named below, were obtained from Sigma (Deisenhofen). Organic solvents and precoated silica gel 60 TLC plates (20 × 5 × 0.025 cm<sup>3</sup>) were from E. Merck (Darmstadt). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoryl[*N*-methyl-<sup>14</sup>C]choline (52 mCi/mmol), 1-[1-<sup>14</sup>C]palmitoyl-*sn*-glycero-3-phosphorylcholine (56 mCi/mmol), 1,2-dioleoyl-*sn*-glycero-3-phosphoryl-L-[3-<sup>14</sup>C]serine (53 mCi/mmol) and glycerol tri[9,10(*n*)-<sup>3</sup>H]oleate (500 mCi/mmol) were purchased from Amersham Buchler (Braunschweig). 1-Oleoyl-*sn*-glycero-3-phosphoryl-L-[3-<sup>14</sup>C]serine (53 mCi/mmol) was prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphoryl-L-[3-<sup>14</sup>C]serine, by treatment with *Crotalus adamanteus* phospholipase A<sub>2</sub> (EC 3.1.1.4) at 10 mM CaCl<sub>2</sub> and pH 7.2 (50 mM Tris-HCl) and purifying the resulting lysophosphatidylserine by thin-layer chromatography (system as described below). Protein A Sepharose 4B was from Pharmacia LKB (Freiburg). The radioactive phospholipids were adjusted to the specific radioactivity required (so that 20,000 dpm were present per assay sample), by adding the corresponding unlabeled phospholipid and subsequently analysing radioactivity and phosphorus. The heterotetrameric annexin II<sub>2</sub>pl 1<sub>2</sub> complex was purified from porcine intestinal epithelium as described previously [16]. Hepatic lipase was purified from rat liver homogenate by a modification [17] of the method described by Twu et al. [18]. The electrophoretically homogeneous preparation displayed a specific catalytic activity of 383 μmol fatty acid released per min at 37°C, when assayed with 2.5 mM trioleoyl glycerol suspensions in arabic gum in the presence of CaCl<sub>2</sub>, BSA and Triton N-101 [17]. Anti-HL polyclonal antiserum against rat liver HL was raised in rabbits as described recently [17]. The antiserum obtained was capable of inhibiting approximately 13.6 mU HL/μl serum, when the activity was assayed with the trioleoyl glycerol substrate.

### 2.2. Phospholipase A<sub>1</sub> and lysophospholipase assays

Phospholipase A<sub>1</sub> and lysophospholipase activities were assayed in

a reaction mixture containing, in a total volume of 100  $\mu$ l, 25 mM glycine/NaOH, pH 9.5, 5 mM  $\text{CaCl}_2$ , different concentrations (approximately 1 or 100  $\mu$ M) of sonicated radioactively labeled substrate (phosphatidylserine, phosphatidylcholine, lysophosphatidylserine or lysophosphatidylcholine), and 0.5–10 ng purified HL. Incubation was carried out at 37°C for various periods of time (5–30 min). Termination of the reaction depended on the substrate used. As for the [ $^{14}$ C]LPC substrate, the radioactive fatty acids released were extracted according to the method of van den Bosch and Aarsman [19], and quantitatively determined by means of a scintillation spectrometer. With [ $^{14}$ C]PC, [ $^{14}$ C]PS or [ $^{14}$ C]LPS as substrates, the incubation was terminated by adding 10  $\mu$ l of 100 mM  $\text{Na}_2\text{EDTA}$ , 10  $\mu$ l of 200 mM HCl and lyophilisation. The residue was taken up in 120  $\mu$ l 80% (v/v) ethanol and 10  $\mu$ l of 1 M HCl and left at room temperature for 1 h. After centrifugation, the residue was washed 2 times each with 50  $\mu$ l 80% (v/v) ethanol. The resulting supernatants were subjected to thin-layer chromatography for separating radioactive substrates (phosphatidylserine, phosphatidylcholine or lysophosphatidylserine) and their metabolites. This was performed on precoated silica gel TLC plates, with a solvent mixture of chloroform/methanol/triethylamine/water (30:35:34:8, by vol.).  $R_f$  values of 0.48, 0.25 and 0 were obtained for phosphatidylserine, lysophosphatidylserine and glycerophosphorylserine, and of 0.42, 0.23 and 0 for phosphatidylcholine, lysophosphatidylcholine and glycerophosphorylcholine, respectively. Catalytic activities with the [ $^{14}$ C]PC, [ $^{14}$ C]PS and [ $^{14}$ C]LPS substrates were calculated from percent radioactivity recovered in the substrate and deacylated metabolite fractions, after correction for non-enzymatic substrate hydrolysis. All assays gave rates of reaction that were constant throughout the specified periods of incubation. Enzyme activities were proportional to the protein concentrations used within the specified ranges, and were independent of small changes in the substrate concentration. The enzyme activities calculated from the initial reaction rates are expressed in U/mg ( $\mu$ mol of substrate hydrolysed per min per mg protein at 37°C).

### 2.3. Triacyl glycerol lipase assay

Triacyl glycerol lipase activity was measured according to Jensen et al. [14] as modified by Kresse et al. [17]. Briefly, the standard reaction mixture contained, in a total volume of 100  $\mu$ l: 200 mM Tris-HCl (pH 9.0), 0.5% (w/v) arabic gum, 0.004% (w/v) Triton N-101, 1% (w/v) BSA, 10 mM  $\text{CaCl}_2$ , 0.3 ng purified enzyme and [ $^3\text{H}$ ]TO (approximately 1  $\mu$ M). After incubation at 37°C for 5 min, the reaction was terminated and the catalytic activity determined as described recently [17].

### 2.4. Determination of the binding of annexin II to HL

In order to determine the potential binding of annexin II to HL, an immunosorbent gel was prepared first. The IgG fraction was isolated from decomplexed (10 mM  $\text{Na}_2\text{EDTA}$ ) rabbit anti-HL polyclonal antiserum against rat liver HL [17] by chromatography on protein A Sepharose. 270 mg of this material was coupled to 25 ml CNBr-activated Sepharose 4B, as recommended by the manufacturer. 500  $\mu$ g purified rat liver HL were exposed to an aliquot of 1.5 ml immunosorbent gel equilibrated with buffer A (buffer medium for measuring phospholipase A<sub>2</sub> activity, i.e. 5 mM  $\text{CaCl}_2$  in 25 mM glycine/NaOH, pH 9.5) in a total volume of 3 ml, at 0°C for 3 h. The gel was washed 3 times each with 5 ml ice-cold buffer A. Under these conditions the immunosorbent was capable of binding approximately 10  $\mu$ g purified HL/ml gel. A 1 ml aliquot of the immunosorbent gel to which 10  $\mu$ g HL had been bound was filled into a column. 50  $\mu$ g annexin II, p11, in 25  $\mu$ l were applied to this column, and the column was subsequently washed with 10 ml buffer A. The eluents were analysed for protein content and for their capacity to inhibit HL activity with phosphatidylserine as a substrate.

### 2.5. Chemical analysis

Inorganic phosphorus was determined after washing [20] as described by Chen et al. [21]. Protein content was determined according to Löffler and Kunze [22] using bovine serum albumin as standard.

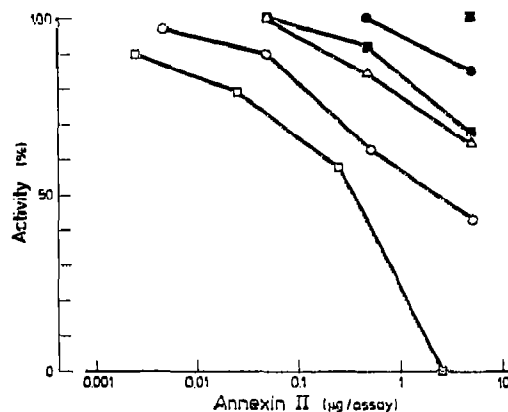


Fig. 1. Effects of annexin II<sub>p11</sub> on lipolytic activities of purified HL with different substrates. Phospholipase A<sub>2</sub> and lysophospholipase activities were determined as described under section 2. Hydrolysis of 1.3  $\mu$ M [ $^{14}$ C]LPC ( $\nabla$ ) was assayed with 5 ng HL and 20 min incubation time (annexin-free controls:  $0.19 \pm 0.01$  U/mg), of 1.4  $\mu$ M [ $^{14}$ C]LPS ( $\Delta$ ) with 5 ng HL and 20 min incubation time (annexin-free controls:  $0.36 \pm 0.04$  U/mg), and of 122  $\mu$ M [ $^{14}$ C]LPS ( $\blacktriangle$ ) with 20 ng HL and 30 min incubation time (annexin-free controls:  $1.88 \pm 0.12$  U/mg). Hydrolysis of 0.9  $\mu$ M [ $^{14}$ C]PC ( $\circ$ ) was determined using 2.5 ng HL and 30 min incubation time (annexin-free controls:  $0.33 \pm 0.02$  U/mg), and of 98  $\mu$ M [ $^{14}$ C]PC ( $\bullet$ ) with 20 ng HL and 30 min (controls:  $1.75 \pm 0.02$  U/mg). Hydrolysis of 1.2  $\mu$ M [ $^{14}$ C]PS ( $\square$ ) was assayed with 0.5 ng HL and 10 min incubation time (annexin-free controls:  $3.0 \pm 0.9$  U/mg), and of 102  $\mu$ M [ $^{14}$ C]PS ( $\blacksquare$ ) with 5 ng HL and 10 min (control  $9.2 \pm 2.8$  U/mg). Each data point represents the mean of triplicate determinations. S.D. did not exceed 9%.

## 3. RESULTS AND DISCUSSION

HL offers the unique opportunity to study potential effects of annexins on different lipolytic activities of one and the same enzyme including its catalytic action on phospholipid hydrolysis. Since inhibition by annexins of phospholipases has generally been observed only at substrate concentrations below  $10^{-5}$  M [3–6], we have used those conditions also in the present study.

Triacyl glycerol lipase activity of purified HL was independent of the presence of annexin II. When assayed with 0.3 ng HL and 5 min incubation time, hydrolysis of [ $^3\text{H}$ ]TO (1.1  $\mu$ M) was  $0.41 \pm 0.02$  U/mg, and in the presence of 5  $\mu$ g annexin II  $0.42 \pm 0.03$  U/mg. This indicates that even in the presence of calcium the triacyl glycerol-hydrolysing activity of HL is not affected by this annexin.

In detergent-free medium, rates of phospholipid hydrolysis catalysed by purified HL were optimal at 5 mM  $\text{CaCl}_2$  and pH 9.5, irrespective of the phospholipid species studied. Absolute values depended on the phospholipid type used. However, it has to be kept in mind that analyses of substrate specificity and kinetics are problematic due to the difficulty in controlling the interfacial quality of pure substrates [23], i.e. different phospholipids assume different physical structures depending on their molecular geometry and on the composition

of the aqueous environment [24,25]. Under our assay conditions, HL-catalysed hydrolysis of [ $^{14}$ C]PS significantly exceeded that of [ $^{14}$ C]PC (Fig. 1). The observed preference for phosphatidylserine over phosphatidylcholine was reversed, when these phospholipids were presented together with Triton X-100 in the form of mixed micelles (data not shown). The catalytic rates which had been measured in the absence of detergents differ slightly from those obtained in other laboratories [15,26]. This is most certainly due to different assay conditions, in particular pH, detergent and calcium concentrations.

In the absence of detergents, HL displayed intrinsic lysophospholipase activity. Pure [ $^{14}$ C]LPC and [ $^{14}$ C]LPS in micromolar concentrations were hydrolysed at rates slightly below those of [ $^{14}$ C]PC and [ $^{14}$ C]PS, respectively (Fig. 1). HL also catalyses deacylation of the 2-acyllysophospholipids which initially formed by its action on diacylphospholipids. Under standard assay conditions, about 69–78% of the metabolites formed from diacylphospholipids were identified as lysophospholipids.

HL activity with diacyl- as well as monoacylphospholipid (lysophospholipid) substrates was inhibited by annexin II *in vitro* (Fig. 1). The degree of this inhibition depended on the concentration of the phospholipid and on the nature of the phospholipid's polar head group. Thus, inhibition was low with the zwitterionic phosphatidylcholine and not detectable with the lysophosphatidylcholine substrate, but it became clearly evident with the acidic phosphatidylserine and lysophosphatidylserine (Fig. 1). Annexin II-induced inhibition of HL activity with each phospholipid substrate was increasingly reduced by raising the substrate concentration (Fig. 1).

It has been suggested that annexins generally interact only with the phospholipid substrate and not with the enzyme. This mode of inhibition, i.e. a substrate depletion by annexins, has been demonstrated for phospholipase A<sub>2</sub> [7,8] and phosphatidylinositol-specific phospholipase C [5]. In our experiments, the lack of inhibition of trioleoyl glycerol hydrolysis by annexin II in the presence of calcium already led us to assume that there is no HL/annexin II interaction. Direct evidence was obtained by experiments on HL immunoadsorbed to Sepharose. No binding of annexin II to this enzyme was detectable, since annexin II was completely recovered when the column flow-through was analysed for protein and inhibition of phospholipase A<sub>1</sub> activity (data not shown). Hence we conclude that the mechanism of inhibition of HL phospholipase A<sub>1</sub> and lysophospholipase activities by the annexin II<sub>2</sub>p11<sub>2</sub> complex is identical to that of other phospholipases, i.e. inhibition by inhibitor/ substrate interactions.

Rat liver HL in its mature form is a 59 kDa glycoprotein synthesised in hepatocytes and secreted into the blood stream [27,28]. Here it binds to the endothelial

plasma membranes of liver, adrenals and ovaries [28–30], where it obviously plays an important role in lipoprotein metabolism [31,32]. Glucocorticoid-induced alterations of some plasma lipoproteins [33] have been reported to be due to decreased HL activity [34,35]. Since it has been found that glucocorticoids inhibit HL biosynthesis and/or secretion [34,35], lipoprotein changes mediated by glucocorticoids [33] have been explained by this mechanism. Our *in vitro* studies now suggest that interference of glucocorticoid-induced annexins with HL activity may contribute to the generation of disturbed lipoprotein patterns. Lack of inhibition by annexin II of HL-catalysed triolein hydrolysis seems to be in contrast to the findings that glucocorticoids reduce lipoprotein triglyceride catabolism [33–35]. However, since phospholipids (and apolipoproteins) surround lipoprotein particles, phospholipid hydrolysis must precede triglyceride hydrolysis (otherwise the latter substrate does not become accessible to HL). In other words, hydrolysis of triglycerides is impaired, as soon as phospholipid hydrolysis is inhibited. Whether or not annexin-mediated HL inhibition also occurs *in vivo* remains yet to be clarified. Although some experimental evidence suggests that annexins are released into the blood stream [36,37], it is not yet clear whether (prolonged) treatment with glucocorticoids *in vivo* increases biosynthesis and secretion of annexins into the vascular lumen.

*Acknowledgements:* This work was supported by the Deutsche Forschungsgemeinschaft (Ku 588/4-1).

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