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Purification and characterization of phospholipase A_2 inhibitory proteins from pig thyroid gland

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A 32 kDa phospholipase A_2 inhibitory protein was isolated from pig thyroid gland after calcium precipitation and fast protein liquid anion-exchange chromatography. SDS-polyacrylamide gel electrophoresis revealed the purity of the protein. The protein activity was assessed by the inhibition of pancreatic phospholipase A_2 on [^{3}H]oleic acid-labelled *Escherichia coli* membranes as substrate and on the prostaglandin E_2 production of cultured thyroid cells. The amino acid composition and the isoelectric point were quite similar to those of endonexin previously described in other tissues or cells. The cross-reactivity of a polyclonal antibody against a 32 kDa lipocortin from human peripheral blood mononuclear cells with our thyroidal 32 kDa protein confirmed its lipocortin nature. Before the purification by fast protein liquid chromatography, the Ca^{2+} pellet contained lipocortin I (35 kDa and its core protein 33 kDa) identified by its cross-reactivity with a polyclonal antibody.

Lipocortin; Endonexin; Phospholipase A_2; Thyroid

1. INTRODUCTION

Lipocortins have been initially described as anti-inflammatory proteins acting through an inhibitory effect on phospholipase A_2 (PLA_2) [1–5]. They have been described in several tissues and cells: rat macrophages [2], rabbit neutrophils [3], rat renomedullary interstitital cells [5], human fibroblasts [6], mouse and bovine thymus [7], human monocytes [8]. Since these previous works, lipocortins have been cloned and sequenced [9–11] and related to a group of calcium- and phospholipid-binding proteins named calpactins I and II, calelectrin, endonexin, calcimedin, chromobindin [12–20]. All these proteins presented a 17 amino acid sequence homology [16] also shown in ras gene proteins [21]. Lipocortins exist in two types I and II identical to calpactin II and I, respectively [11,22], and are phosphorylated by tyrosine kinases [12,23–25] or by protein kinase C [4,26,27]. Moreover, their anti-PLA_2 activity is regulated by phosphorylation [4,25,26].

In previous works, we described the important role played in the thyroid tissue by the phospholipase A_2 activity and its regulation by thyrotropin [28–31]. The present study provides the evidence that a 32 kDa phospholipase A_2 inhibitory protein exists in thyroid tissue and that it has an inhibitory effect on thyroid cellular phospholipase A_2.

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Abbreviations: PLA_2, phospholipase A_2; PMSF, phenylmethylsulfonyl fluoride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

2. MATERIALS AND METHODS

[^{3}H]Oleic acid (4.2 Ci/mmol) was purchased from Amersham, France; porcine pancreatic PLA_2 from Boehringer, Mannheim; fatty acid free bovine serum albumin from Sigma.

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2.1. *Purification of PLA_2 inhibitory proteins*

2.1.1. Ca^{2+} purification

50 g pig thyroid tissue was hashed after elimination of the connective and fat tissues and homogenized using a Polytron with 250 ml of a pH 7.6 buffer containing 10 mM Hepes, 100 mM NaCl, 5 mM EDTA, 200 μM PMSF and 1000 U/ml aprotinin.

The homogenate was sonicated 3 × 10 s at 4°C and centrifuged at 20000 × *g* for 30 min (Sorvall RC2 superspeed centrifuge, Dupont Instruments). 6 mM of $CaCl_2$ was added to the supernatant and left for 30 min at 4°C. After centrifugation at 39000 × *g* for 30 min, the pellet was washed with a pH 7.4 buffer containing 10 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$ and centrifuged again at 39000 × *g* for 30 min. The pellet was resuspended in 13 ml of 10 mM Hepes, 10 mM EGTA, 200 μM PMSF and 1000 U/ml aprotinin, pH 7.4, buffered solution. After centrifugation at 100000 × *g* for 30 min (Beckman L265B Ultra-centrifuge) the supernatant was used for further purification.

2.1.2. Fast protein liquid chromatography

The purification by fast protein liquid chromatography (Pharmacia, Uppsala, Sweden) was carried out as described by Rothhut et al. [8] on a strong anion-exchange resin mono Q HR 5/5 at a flow rate of 1 ml/min except that the initial elution buffer was 100 mM Tris-HCl, pH 8, containing 1 mM PMSF. The injected volume was 200 μl (300 μg protein) and the proteins were eluted with a NaCl gradient.

2.1.3. SDS-polyacrylamide gel electrophoresis

Gradient-SDS (5–15% acrylamide) gels were run using BRL (Bethesda Research Laboratories) apparatus or (10–15% acrylamide) gels when the PHAST-system was used. Proteins were visualized by Coomassie blue or by silver staining.

2.1.4. Isoelectric focusing

Isoelectric focusing was performed with different pH gradients using the PHAST-system.

2.2. *Antibody recognition*

Different fractions were analyzed by Western blot analysis described by Towbin et al. [32] and modified by Rothhut et al. [8].

2.3. *Phospholipase (PLA_2) inhibitory properties*

The porcine pancreatic PLA_2 inhibitory assay was performed as described by Rothhut et al. [33]. Prostaglandin E_2 biosynthesis was assessed on cultured pig thyroid cells.

Pig thyroid cells were cultured as previously described [34]. After a 4 day culture, cells were washed by 200 × *g* centrifugation with Earle Hepes, pH 7.4, buffered solution and resuspended in an RPMI solution in the presence or absence of PLA_2 inhibitory proteins; at various times, the prostaglandin E_2 content of the incubation medium was determined with the Institut Pasteur kit.

2.4. *Amino acid analysis*

Amino acid composition analysis was performed on 200 μg of the 32 kDa protein on a Beckman analyser (Multichrom model B4255) using a W1 (56 × 0.9 cm) column resin Beckman as described by Spackman et al. [35]. Values were expressed as mol% ± SD.

2.5. *Protein measurement*

Proteins were measured as described by Lowry et al. [36].

3. RESULTS

3.1. *Purification of a 32 kDa protein from pig thyroid gland*

In the chromatographic conditions described in section 2, the elution profile on Mono Q of the proteins is presented in fig.1. The single peak eluted at 150 mM NaCl has an apparent molecular mass of 32 kDa (fig.2, lane C). Before the Mono Q purification, the 32 kDa protein was the major protein of the extract but several other proteins were present (fig.2, lane B). Using isoelectric focusing, the 32 kDa protein has an isoelectric point of 4.85–4.90 (not shown).

3.2. *PLA_2 inhibitory properties*

When tested with 0.1 μg porcine PLA_2 on the *E. coli*-labelled substrate, 14 μg of the crude extract, before the Mono Q purification, gave a 50% inhibition (fig.3, left) and 5.8 μg of the purified 32 kDa protein gave a 50% inhibition (fig.3, right). In a previous study [28], we demonstrated that in pig thyroid, the prostaglandin E_2 biosynthesis was closely related to the cellular PLA_2 activity. So, to test the cellular PLA_2 activity we measured the prostaglandin E_2 biosynthesis of cultured pig thyroid cells in the presence or absence of the 32 kDa protein. 3.75 μg of the 32 kDa protein decreased the prostaglandin E_2 biosynthesis of thyroid cells by 35% ($P < 0.01$) in a five hour in-

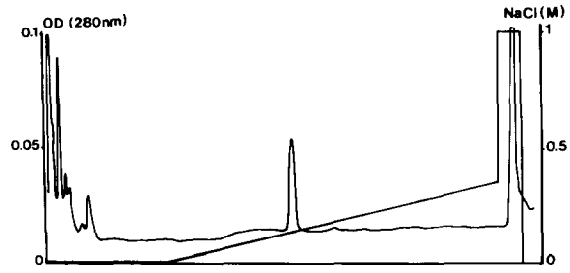

Fig.1. Chromatographic profile of the Ca^{2+} precipitated proteins on Mono Q. The sample was applied to the column in a pH 8 buffered 0.1 M Tris-HCl solution and the proteins were eluted with a NaCl gradient and detected by absorbance at 280 nm.

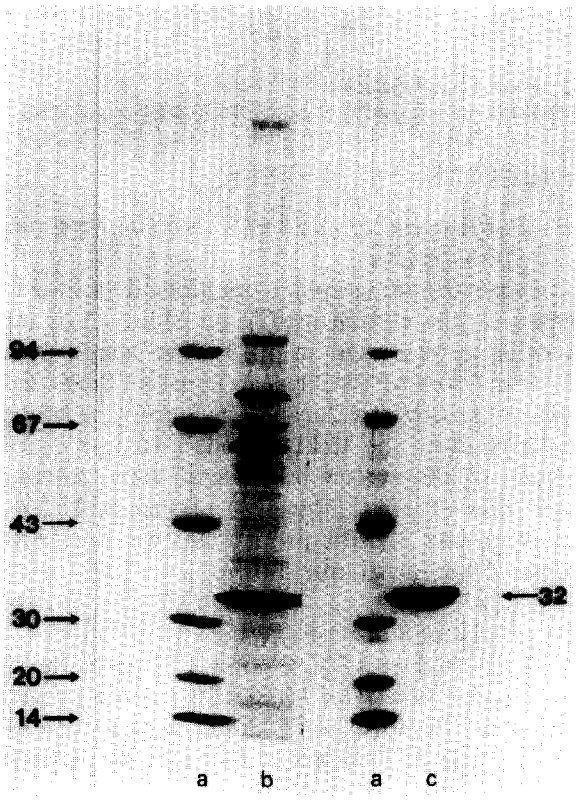

Fig.2. SDS-PAGE of protein fractions at different purification steps. Samples of protein fractions were analyzed by SDS-PAGE (5–15% gradient acrylamide gels) before (lane b) and after (lane c) the Mono Q purification and visualized by Coomassie blue. Arrows on the left indicate the molecular mass markers (kDa), phosphorylase *b* (94); bovine serum albumin (67); ovalbumin (43); carbonic anhydrase (30); trypsin inhibitor (20) and lactalbumin (14), shown in lanes a. On the right, the relative molecular mass of the purified protein is indicated.

cubation. (The prostaglandin E_2 content of control thyroid cells was 115 ± 5 nmol for 6×10^5 cells.)

3.3. *Recognition by antibodies*

Immunoreactive proteins were detected by Western blotting (fig.4). The polyclonal antibody raised against a 32 kDa lipocortin from human peripheral blood mononuclear cells (fig.4, left) recognized the 32 kDa component of the purified fraction (lane 2) and of the calcium precipitate fraction (lane 1). Moreover an anti-serum against recombinant lipocortin I antibody (fig.4, right) recognized the 33 kDa and 35 kDa proteins in the calcium-precipitate fractions (lane 1). These proteins were absent in the purified Mono Q fraction (lane 2).

3.4. *Amino acid composition*

Table 1 shows the amino acid analysis of the 32 kDa protein.

4. DISCUSSION

In this paper we describe the purification and the characterization of a 32 kDa PLA_2 inhibitory protein from pig thyroid gland. This protein belongs to a group of calcium-precipitable proteins described previously in many tissues and cells [2,3,5–8]. Biochemical studies (molecular relative mass, isoelectric point and amino acid composition) show that the 32 kDa thyroid protein shares common properties with endonexin [20,37]. Nevertheless we could demonstrate that this protein was recognized by a polyclonal antibody raised against a lipocortin indicating that this 32 kDa thyroid protein belongs to the lipocortin family. The protein inhibits the pancreatic porcine PLA_2 activity and the in vivo cellular thyroid PLA_2 activity. These results indicate that the cellular PLA_2 activity was accessible from the outside of the cells or that the protein interacted with the membrane phospholipids leading to an inhibition of the

Table 1

Amino acid composition of the 32 kDa protein

Amino acid	Thyroid 32 kDa (mol%)	Monocyte lipocortin (mol%)
Asp	10.9 ± 0.7	9.9 ± 1.0
Thr	6.9 ± 0.5	6.0 ± 0.3
Ser	6.6 ± 0.1	8.3 ± 1.6
Glu	12.8 ± 0.5	13.8 ± 0.9
Pro	1.1 ± 0.5	2.2 ± 0.2
Gly	7.7 ± 0.3	9.3 ± 1.2
Ala	8.1 ± 0.3	8.2 ± 0.5
Val	4.3 ± 0.2	4.0 ± 0.2
Met	3.1 ± 0.7	1.2 ± 0.6
Isoleu	5.5 ± 0.3	4.5 ± 0.1
Leu	13.2 ± 0.2	10.7 ± 0.9
Tyr	2.3 ± 1.8	3.8 ± 0.6
Phe	3.9 ± 0.1	4.0 ± 0.4
Lys	7.0 ± 0.3	7.6 ± 1.1
His	1.1 ± 0.1	1.5 ± 0.4
Arg	6.1 ± 0.9	5.7 ± 0.7

Values are expressed as mol% ± SD

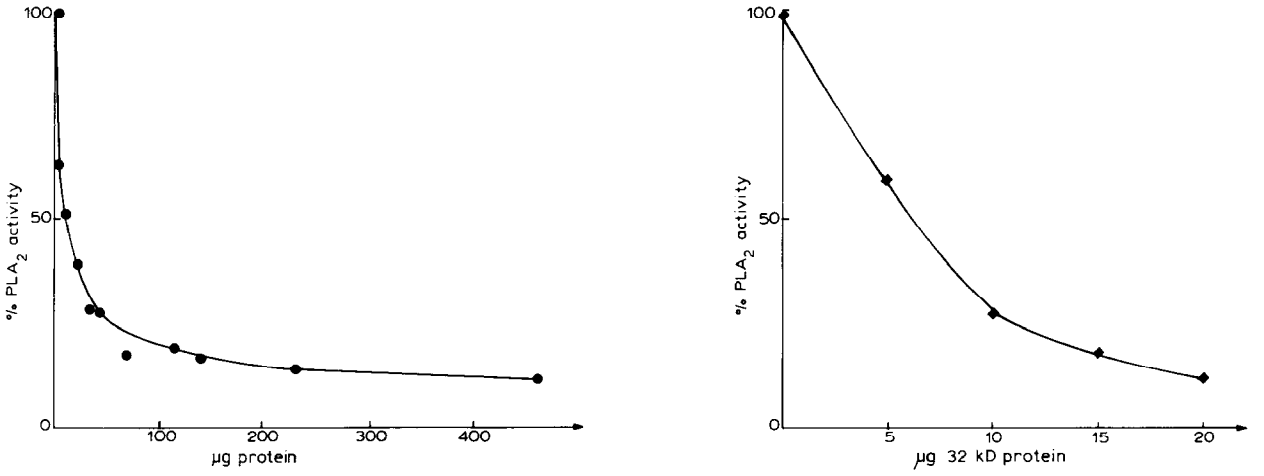

Fig.3. Dose-response curve of porcine pancreatic PLA_2 inhibition. Different concentrations of the purified proteins were incubated with 0.1 µg of porcine pancreatic PLA_2 using labelled *E. coli* as substrate. Results were expressed in % of PLA_2 activity. Left, proteins before the Mono Q purification; right, the 32 kDa protein.

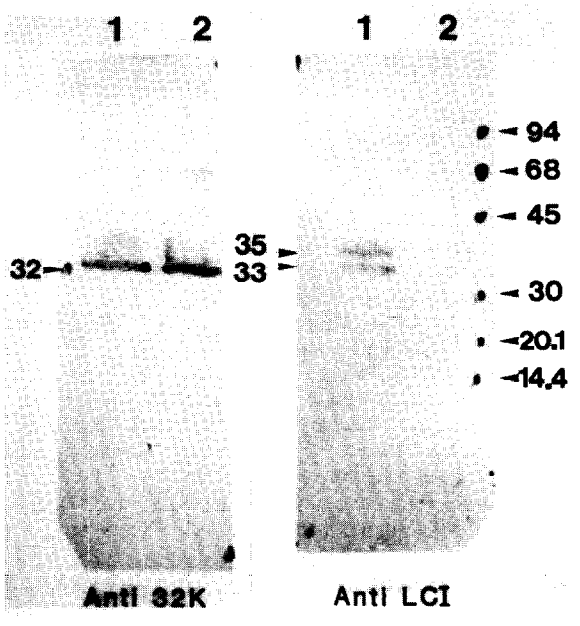

Fig.4. Western blot analysis of the proteins. (Left) Cross-reactivity with the 32 kDa lipocortin antibody. Lane 1, proteins before the Mono Q purification; lane 2, the purified 32 kDa protein. (Right) Cross-reactivity with the lipocortin I antibody. Lane 1, proteins before the Mono Q purification; lane 2, the purified 32 kDa protein. The arrowheads on the right indicate the molecular mass markers (kDa). The arrowheads on the left indicate the relative molecular mass of the immunoreactive proteins.

cellular enzyme previously described in other tissues and cells [38,39]. Moreover, before the purification on the Mono Q HR 5/5 column, Western blot analysis revealed in the Ca^{2+} pellet proteins the presence of lipocortin I. The 35 kDa protein and its core protein of 33 kDa were detected. The pig thyroid gland contains proteins of the lipocortin family able to bind calcium and to inhibit PLA_2. In previous works [28–31] we described the important role played in the thyroid by the PLA_2 activity, arachidonate and arachidonate metabolites. The PLA_2 activity of thyroid tissue and cells was under TSH regulation [28–31]. The biosynthesis and the phosphorylation of thyroidal lipocortins under chronic or acute treatment of cultured pig thyroid cells will be described in another paper.

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