Volume 216, number 1, 45–50 FEB 04688 May 1987

Lipocortin-like anti-phospholipase A_2 activity of endonexin

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Received 5 February 1987

Endonexin (protein II, 32.5 kDa) has been purified to homogeneity from bovine liver in the following steps: selective extraction by EGTA from membranes precipitated with Triton X-100/calcium; chromatography on DEAE-TSK 545 at pH 7.0, endonexin being eluted at 0.1 M NaCl; affinity chromatography on polyacrylamide-immobilized phosphatidylserine; gel filtration on TSK 3000. The amino acid composition was essentially similar to that previously reported. Using [^{3}H]oleic acid-labelled *Escherichia coli* membranes as substrate, endonexin inhibited phospholipase A_2 from pig pancreas. Maximal inhibition was 55 and 70%, whereas 50% inhibition occurred at 480 and 120 nM endonexin and lipocortin II, respectively. These data could be related to common features shared by both lipocortins/calpactins and endonexin, i.e. the presence of a consensus sequence and the ability to bind to anionic phospholipids in a calcium-dependent manner.

Endonexin; Lipocortin; Phospholipase A_2; Ca^{2+}; Phosphatidylserine

1. INTRODUCTION

Lipocortins (LCs) have been described as a group of proteins exerting anti-inflammatory effects through an inhibitory effect against PLA_2 [1–6] (see also [7–9] for reviews and nomenclature). Considerable progress was recently made in this field by immunological approaches [10–13], but mainly by obtaining sequence information from either purified LC preparations [14,15] or the corresponding cDNA [16,17]. These studies revealed two striking features: (i) two types of LC exist (I and II), present in a monomeric or tetrameric form, and identical to calpactins II and I, respectively [17–19]. Both proteins display a 50% sequence homology of the 35–36 kDa chain and LC II (protein I or p36) was recently described as an inhibitor of PLA_2 [20]. (ii) A 17 amino acid residue consensus sequence has been identified not only in LCs, but also in a group of other proteins, including 34 and 67 kDa calelectrins as well as endonexin, a 32.5 kDa protein isolated from bovine adrenal gland and liver ([21–24]; recent reviews [25,26]). Finally, some sequence homology was also noticed between LC and *ras* gene proteins [27].

Although all of these proteins behave as calcium- and phospholipid-dependent membrane-binding proteins, their physiological significance still remains obscure. Fragmentary data accumulated so far concern anti-PLA_2 activity of LCs [1–6,14,17,20], their phosphorylation by tyrosine kinases [12,13,15,28] or serine/threonine protein kinases [29–32], related to some possible regulation of anti-PLA_2 activity [28–32]. There are also some reports on the enhancement of secretory granule aggregation by calelectrins or endonexin

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Abbreviations: PLA_2, phospholipase A_2; BSA, bovine serum albumin

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00145793/87/$3.50

[33] and on the subcellular localization of these proteins [34–38].

One of the questions raised by this information is whether this anti-PLA_2 activity is specific for LCs or is also shared by other related proteins like calelectrins or endonexin. The present study provides evidence for an anti-PLA_2 activity of endonexin.

2. MATERIALS AND METHODS

[^{3}H]Oleic acid (8.9 Ci/mmol) was from NEN (Dreieich). Phosphatidylserine (bovine brain), fatty acid-free BSA and PLA_2 (pig pancreas, 900 U/mg) were from Sigma (St. Louis, MO).

2.1. *Purification of endonexin*

An EGTA extract was prepared from bovine liver as in [37]. After overnight dialysis against buffer A (10 mM Hepes, pH 7.4, 0.5 mM dithiothreitol, 1 mM NaN_3) and clarification by centrifugation (100000 × *g*, 60 min, 4°C), the extract was loaded onto a column of DEAE-TSK 545 (21.5 × 150 mm) equilibrated with buffer A. The

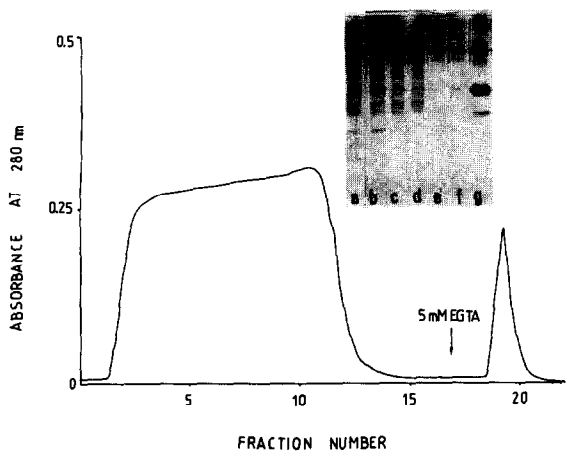

Fig.1. Affinity chromatography of salt gradient DEAE-TSK fractions on polyacrylamide-immobilized phosphatidylserine. After washing the column with buffer A containing 100 mM NaCl and 1 mM $CaCl_2$, proteins retained on the column were eluted as shown at the arrow by the same buffer containing 5 mM EGTA instead of $CaCl_2$ (flow rate 15 ml/h; fraction volume 3 ml). Inset: SDS-polyacrylamide gel electrophoresis of various fractions detected by silver staining. Lanes: a, EGTA extract; b, protein pool after salt gradient on DEAE-TSK column; c,d, fraction numbers 5 and 10 non-retained on polyacrylamide-immobilized phosphatidylserine; e–g: fraction nos 18–20 eluted with 5 mM EGTA.

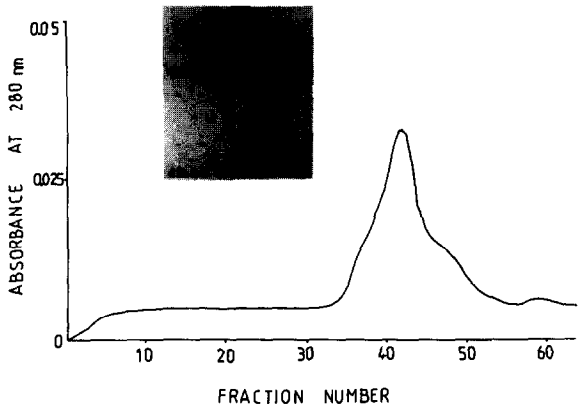

Fig.2. Elution profile of protein from TSK gel G3000 SW column chromatography (flow rate 2 ml/min, fraction volume 2 ml). Inset: SDS-polyacrylamide gel electrophoresis of fractions detected by silver staining.

Table 1

Amino acid composition of 32.5 kDa protein

Amino acid	mol%[a]	mol%[b]
Asx	10.2	11.4
Thr	4.6	5.7
Ser	9.8	6.4
Glx	11.7	13.6
Pro	2.3	1.8
Gly	10.5	7.9
Ala	8.9	8.6
Val	3.7	3.9
Met	2.0	2.1
Ile	5.9	5.7
Leu	8.6	10.7
Tyr	2.7	3.2
Phe	3.1	3.6
His	2.3	1.4
Lys	7.7	7.1
Arg	6.1	5.4
Cys	ND	1.4

[a] Values are expressed as mol% and are extrapolated to zero time from two analyses performed after 20 and 70 h hydrolysis

[b] From Südhof et al. [33]

ND, not determined

flow-through fractions were discarded and the column was developed with a 0–0.3 M NaCl gradient. Fractions containing endonexin eluted at around 0.1 M NaCl. They were pooled and loaded onto a polyacrylamide-immobilized phosphatidylserine column prepared as in [39] and equilibrated with buffer A containing 100 mM KCl and 1 mM $CaCl_2$. The major part of the proteins was not retained by the column, except for a small peak eluted by replacing $CaCl_2$ by 5 mM EGTA. The purified fractions were further submitted to gel filtration on a column of TSK 3000 (21.5 × 600 mm) equilibrated with buffer A containing 100 mM KCl. Protein purity was checked at the various steps of the procedure by SDS-polyacrylamide gel electrophoresis [40], proteins being detected by silver staining, using a kit from Biorad (Richmond, CA).

2.2. *Purification of lipocortin*

LC II (calpactin I, 85 kDa) was isolated from bovine lung as in [37], omitting the gel filtration step. SDS-polyacrylamide gel electrophoresis revealed the presence of two bands at 37 and 10 kDa, corresponding to heavy and light chain of the tetramer, respectively, together with two proteolytic fragments at 33 and 22 kDa.

2.3. *Determination of PLA_2 activity*

This was performed as in [41] using [^{3}H]oleic acid-labelled *Escherichia coli* membranes isolated after autoclaving [42], with the following modifications: endonexin or LC was preincubated with PLA_2 (200 ng) at 4°C for 10 min, followed by addition of *E. coli* membranes and further incubation at 4°C for 20 min. Radioactivity of released [^{3}H]oleic acid was determined as in [41].

2.4. *Miscellaneous*

Protein was determined as in [43]. Amino acid hydrolysis of endonexin was performed at 110°C under vacuum in 6 M HCl for 20 or 70 h. Hydrolysates were analysed using a Beckman 6300 analyzer.

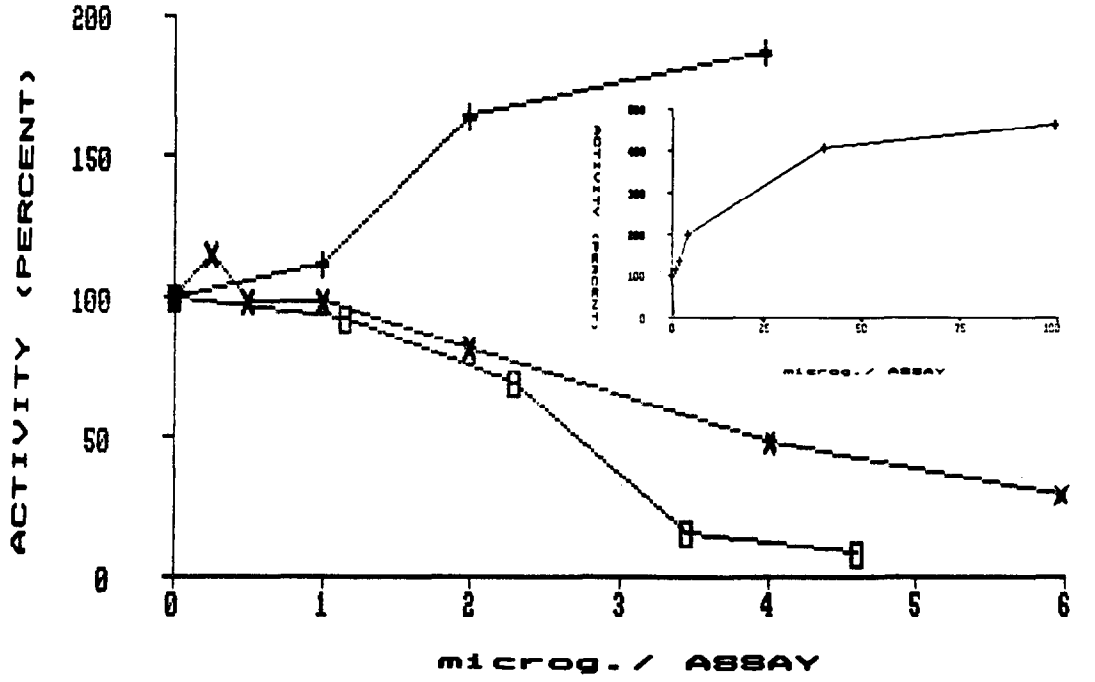

Fig.3. Effect of endonexin (×), lipocortin II (□) and bovine serum albumin (+) on phospholipase A_2 activity. Aliquots of different proteins were incubated with 200 ng pig pancreas phospholipase A_2 and activities were detected as described in section 2. Results are given as means from two determinations and are expressed as percentage of the phospholipase A_2 activity measured without proteins (100%).

3. RESULTS

The inset to fig.1 illustrates the various steps used for endonexin purification. The EGTA extract from pig liver contained a large number of proteins (lane a), which was hardly decreased after chromatography on DEAE-TSK 545 (lane b). Upon loading onto a column of polyacrylamide-immobilized phosphatidylserine equilibrated with a buffer containing 1 mM $CaCl_2$, most of these proteins were recovered in the flow-through fractions (fig.1 and lanes c,d). This allowed a high degree of purification of the proteins retained on the column and eluted with EGTA. As shown in lanes e–g, the last fraction of the small peak was particularly enriched in two proteins (67 and 32.5 kDa), together with a 24 kDa fragment. Based on its molecular mass, its ability to bind to DEAE-TSK 545 at pH 7.0 and its calcium-dependent phospholipid-binding property, the 32.5 kDa protein was identified as endonexin [32,38].

This was further purified by gel filtration on TSK 3000. As shown in fig.2, no clear separation between the various proteins could be achieved, since the main peak at fraction 42 still contained both 67 and 32.5 kDa proteins, with some traces of the 24 kDa fragment. However, starting from fraction 46 until fraction 52, corresponding to the total volume of the column, endonexin was obtained in pure form, as revealed by polyacrylamide gel electrophoresis (fig.2, inset). These data indicate that some aggregation between the three proteins probably occurred, as well as some interaction with the TSK matrix. Fractions 48–52 were pooled and, again, it was verified that endonexin appeared as a single band upon SDS-polyacrylamide gel electrophoresis (not shown).

The amino acid analysis of purified endonexin is given in table 1. Our data are rather comparable to those of Südhoff et al. [33]. Two preliminary attempts to sequence endonexin remained unsuccessful, suggesting that the N-terminal end of the protein could be blocked. At present, no sequence data on endonexin N-terminal end are available in the literature.

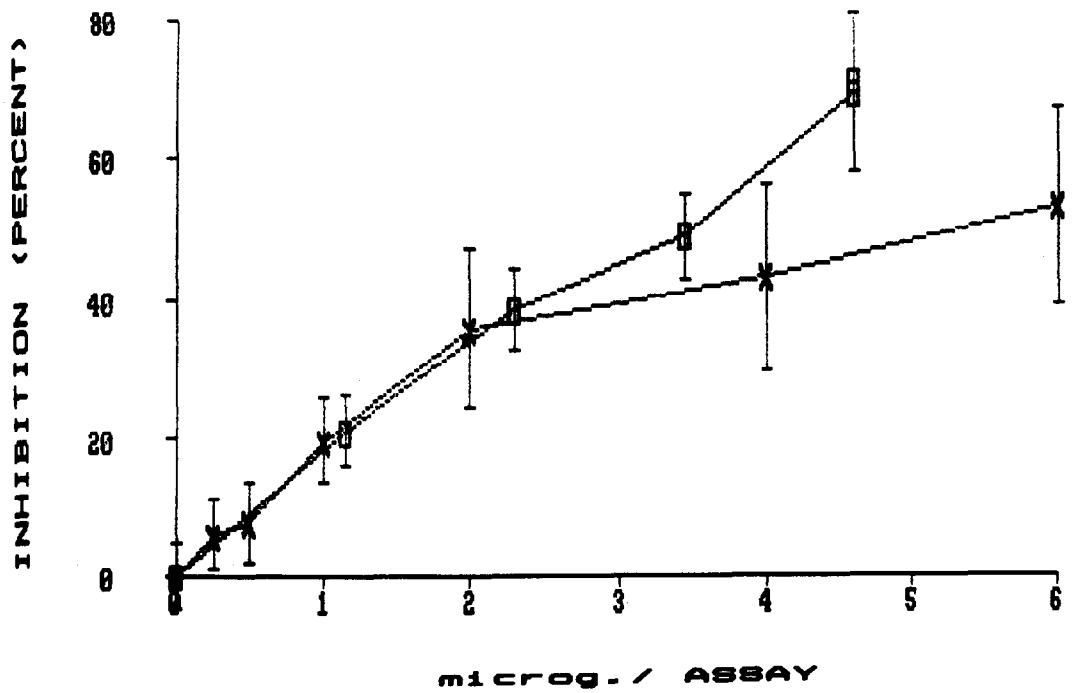

Fig.4. Dose-response curve of the inhibition of phospholipase A_2 activity by endonexin (×) and lipocortin II (□). Conditions as in fig.3, except that 50 μg bovine serum albumin was added to the incubation medium. Results are given as means ± SE from three experiments.

Upon incubation in the presence of pig pancreas PLA_2, endonexin promoted a dose-dependent decrease in enzyme activity very similar to that induced by LC (fig.3). In order to exclude any non-specific action of these proteins, their effects were compared to those of BSA. As also shown in fig.3, BSA actually stimulated PLA_2 activity about 5-fold. Further experiments were therefore performed in the presence of a BSA concentration (2.1 μM) allowing maximal PLA_2 activity. As shown in fig.4, both endonexin and LC still exerted the same inhibitory action against PLA_2. Endonexin appeared somewhat less active than LC, PLA_2 maximal inhibition being 55 and 70%, respectively. Moreover, 50% inhibition of PLA_2 was obtained at 120 and 480 nM LC and endonexin, respectively, taking 85 and 32.5 kDa as the corresponding molecular masses. In comparison, Khanna et al. [20] found half-maximal inhibition of PLA_2 by 223 nM LC II, vs 72 nM for LC I (37 kDa), as reported by Pepinsky et al. [14].

4. DISCUSSION

Our data present clear evidence that endonexin displays an anti-PLA_2 activity similar to that of LCs. Although the amino acid composition does not represent a definitive characterization of endonexin, the protein used here could be differentiated from LCs on the basis of a different charge (LCs are not retained by DEAE ion exchangers at pH 7.0) and a different molecular mass: 32.5 kDa for endonexin, vs 35–37 kDa for LC I or heavy chain of LC II [11,14,15,17,32,37,38]. Moreover, there is no risk that the protein purified herein could be a proteolytic cleavage product of LC, for instance, core protein, 33 kDa, from LC II or calpactin I, since the latter should also be detected in the flow-through fractions of DEAE-TSK chromatography [44]. Furthermore, liver has been shown to lack or to contain barely detectable amounts of LCs or their corresponding mRNA [11,14,16].

The anti-PLA_2 activity described here is rather specific since BSA, chosen as a negative control, actually stimulated PLA_2 activity. This might be due to the removal from the lipid interface of fatty acids produced by PLA_2. It should also be recalled that Khanna et al. [20] checked various calcium-binding proteins such as parvalbumin or calmodulin and did not find any anti-PLA_2 activity, except for LC II (also called p36 or protein I).

Our present finding is important to consider in the light of the known similarities between LCs/calpactins reported above, as well as the ability of these proteins to adsorb onto phospholipid surfaces in a calcium-dependent manner. It is thus tempting to suggest that the anti-PLA_2 activity described in vitro either involves a specific interaction between PLA_2 and these various proteins (possibly through the homologous part present in each of them), or is subsequent to some kind of steric hindrance occurring at the lipid/water interface, owing to the calcium-mediated adsorption of endonexin or LC. In this respect, it is worth recalling that *E. coli* membranes have a relatively high content of anionic phospholipids such as phosphatidylglycerol or cardiolipin [45], which are those favoring calcium-dependent interfacial adsorption of LC or endonexin.

Whatever be the molecular mechanism of the anti-PLA_2 action of endonexin, our observation raises some very intriguing questions concerning the biological significance of these various proteins. Since the first reports of glucocorticoid-induced protein inhibitors of PLA_2 [1–6], there is now evidence for the presence in various tissues, even in the absence of any glucocorticoid treatment, of at least three different proteins (LCs I and II, endonexin) displaying a similar in vitro anti-PLA_2 activity. Whether this reflects some reality of the in vivo situation still remains an open question and should warrant further studies.

ACKNOWLEDGEMENTS

We are grateful to Mrs J. Ragab for her help in preparing *E. coli* membranes and to Miss P. Vicendo for her participation in the purification of lipocortin. Thanks are also due to Mr P. Mansuelle and Mrs T. Brando for performing amino acid analysis as well as to Mrs Y. Jonquière for correcting the English manuscript.

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