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Isolation of two 67 kDa calcium-binding proteins from pig lung differing in affinity for phospholipids and in anti-phospholipase A_2 activity

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Two 67 kDa proteins adsorbed to membranes in the presence of Ca^{2+} have been purified to homogeneity from pig lung using conventional procedures, followed by calcium-dependent affinity chromatography on polyacrylamide-immobilized phosphatidylserine. The two proteins were, respectively, excluded (67E) and retained (67R) on the column in the presence of Ca^{2+}. On the basis of amino acid composition and isoelectric point, 67R was identified as 67 kDa calelectrin/calcimedin, whereas 67E could be differentiated from albumin, calregulin, 67 kDa fragment of protein kinase C and surfactant-associated proteins. Only 67R was slightly phosphorylated by protein kinase C, reacted with an antibody raised against 32.5 kDa endonexin and inhibited pig pancreas phospholipase A_2 in a way similar to that of lipocortin or endonexin. These data bring further support to the view that inhibition of phospholipase A_2 by lipocortin or other related proteins involves interaction with the lipid/water interface. They also provide evidence for a new kind of Ca^{2+}-binding protein (67E), whose role still remains to be determined.

Ca^{2+}-binding protein; Lipocortin; Phospholipase A_2; Phosphatidylserine; (Lung)

1. INTRODUCTION

The anti-inflammatory action of steroids is thought to involve induction of proteins able to inhibit PLA_2, a key enzyme in the biosynthesis of icosanoids and of platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or PAF-acether) [1]. The anti-phospholipase A_2 activity of these proteins, called lipocortins [2–4], could also be regulated by phosphorylation involving either serine/threonine protein kinases [5] or oncogene/growth factor tyrosine kinases [6,7].

Greater knowledge of these proteins was recently obtained, mainly through data on their amino acid sequence [3,8,9]. These revealed the existence of two different lipocortins detected respectively as a 35–37 kDa monomer (LC I), or as a heterotetramer containing two copies of a 36 kDa chain displaying 50% homology with LC I, together with two copies of a 10 kDa chain related to S100 proteins (LC II) [10]. Moreover, complementary studies revealed that LCs I and II are identical to calpactins II and I, respectively, defined by their ability to bind to polymerized actin and to anionic phospholipids in a Ca^{2+}-dependent manner [11]. Finally, some degree of homology has also been found between calpactins/LCs and a

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Abbreviations: PLA_2, phospholipase A_2; LCs, lipocortins; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; 67R and 67E, 67 kDa proteins retained and excluded, respectively, during calcium-dependent affinity chromatography on phosphatidylserine

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group of other Ca^{2+}- and phospholipid-binding proteins, which contain a 17 amino acid residue consensus sequence [12]. These include 32.5 kDa endonexin, also referred to as protein II, 35 kDa and 67 kDa calelectrins (the latter also called protein III), which are able to modulate aggregation of chromaffin secretory granules (other name: chromobinding [13]). In the same group, other Ca^{2+}-binding proteins (calcimedins) seem to be identical to calelectrins and to 68–73 kDa protein from lymphocytes (reviews [14,15]). These observations led us to emphasize some possible common properties of these related proteins. In this respect, we recently found that endonexin from bovine liver displays an anti-PLA_2 activity very similar to that of LC, as studied in an in vitro test [16]. The present study extends the observation to the case of 67 kDa calelectrin/calcimedin from pig lung and gives further support to the view that anti-PLA_2 activity of these proteins is closely related to their phospholipid binding properties. In addition, a new type of Ca^{2+}-binding protein without affinity for phospholipid is described.

2. MATERIALS AND METHODS

All chemicals used were from Sigma (St Louis, MO). LC I was obtained from pig lung according to De et al. [17]. During the same procedure, fractions from an AcA 44 column containing 67 kDa proteins were pooled and submitted to affinity chromatography on a polyacrylamide-immobilized phosphatidylserine column [18]. Polyacrylamide gel electrophoresis in the presence of SDS was performed under reducing conditions according to Laemmli [19]. Isoelectric points of the proteins were determined by analytical focusing in polyacrylamide gel using an LKB 2117 multiphor II apparatus and ampholine polyacrylamide gel plates (pH 3.5–9.5). Amino acid analysis was carried out according to [16] and protein determined as in [20].

Rabbit polyclonal antibody was raised against bovine liver endonexin [16] by conventional procedures. Immune blotting was performed using an LKB 2117-250 Novablot electrophoretic transfer kit according to [21].

In vitro phosphorylation of proteins was determined using protein kinase C purified from rat brain according to [22]. Autoradiography of dried gels was performed at −80°C, using Kodak X-OMat films.

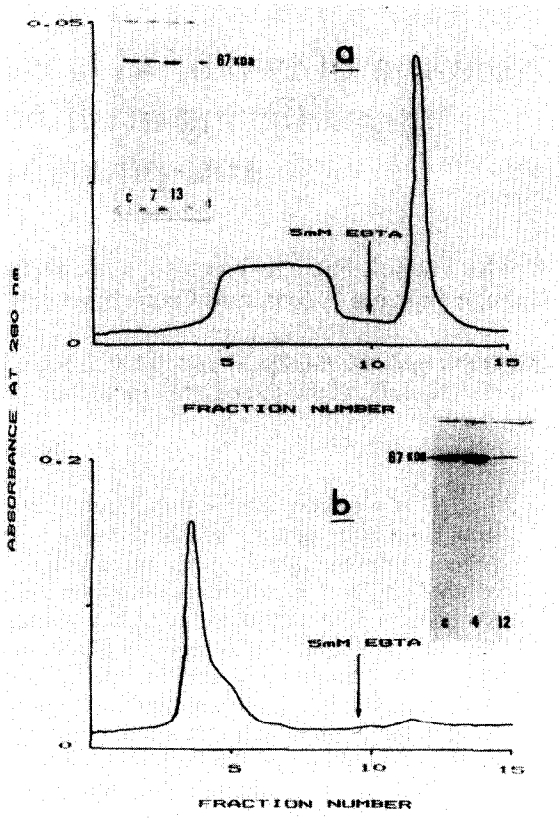

Fig.1. Affinity chromatography on polyacrylamide-immobilized phosphatidylserine of 67 kDa proteins (a) and of pig serum albumin (b). 67E and 67R were eluted from the column with buffer (100 mM KCl, 10 mM Hepes, pH 7.4) containing 1 mM $CaCl_2$ or 5 mM EGTA, respectively. (Inset) SDS-PAGE of various fractions detected by silver staining. (a) Lane c, 67 kDa proteins from AcA 44 column chromatography; lanes 7,13, fractions 7 and 13 from polyacrylamide-immobilized phosphatidylserine column. (b) Lane c, pig serum albumin; lanes 4,12, fractions 4 and 12 from polyacrylamide-immobilized phosphatidylserine column.

Anti-phospholipase A_2 activity was assayed as in [16] using [^{3}H]oleic acid-labelled *Escherichia coli* membranes (3×10^5 dpm/nmol phospholipid) as substrate of pancreatic phospholipase A_2.

3. RESULTS

Proteins extracted with EGTA from pig lung membranes previously sedimented in the presence of Ca^{2+} were gel filtered onto an AcA 44 column. Fractions eluting with an apparent molecular mass of 63 kDa appeared homogeneous upon SDS-PAGE, the estimated molecular mass being in that case 67 kDa. However, two different proteins could then be resolved from this pool using Ca^{2+}-dependent affinity chromatography on polyacrylamide-immobilized phosphatidylserine. As shown in fig.1a, two proteins displaying identical molecular masses upon SDS-PAGE were recovered in roughly equal amounts (0.731 and 0.741 mg, respectively, from 100 g tissue) in the flow-through fraction (67E) and in the EGTA eluate (67R). Such an effect of the phosphatidylserine chromatography appeared rather specific, since pig serum albumin was not adsorbed onto the column (fig.1b).

The isoelectric point of 67R and 67E was 6.4 and below 4.9, respectively. Moreover, amino acid analysis revealed some striking differences between the two proteins, especially in the level of Glx (11.8 and 23.3% in 67R and 67E, respectively). As reported in table 1, some significant differences were noted for other less abundant amino acids such as Ser, Met, Ile, Leu, Tyr, Lys and Arg. Table 1 also lists the amino acid composition of porcine serum albumin, which differed from the two proteins. Two attempts to sequence intact protein 67R remained unsuccessful, suggesting that the N-terminal end is probably blocked, as already found for endonexin [16].

As shown in fig.2, 67R reacted with a polyclonal antibody raised in rabbit against bovine endonexin, whereas 67E was not recognized. In vitro phosphorylation by protein kinase C was also checked. As illustrated in fig.3, 67R, but not 67E, was slightly phosphorylated in the presence of purified protein kinase C. In contrast to lipocortin I, which became heavily labelled (fig.3), 67R phosphorylation was hardly dependent on the presence of phosphatidylserine and Ca^{2+}, suggesting that some phospholipid might remain adsorbed onto the protein.

As shown in fig.4, protein 67R promoted a dose-dependent inhibition of PLA_2 similar to that of LC, whereas 67E was inactive. Maximal inhibition was 50 and 60% for 67R and LC I, respectively. On a molar basis, 50% inhibition of PLA_2 activity

Table 1

Amino acid composition of 67 kDa proteins from pig lung and of pig serum albumin

Amino acid	67R		67E		Pig serum albumin	
	mol%	Molar ratio	mol%	Molar ratio	mol%	Molar ratio
Asx	9.6	60	1.1	68	9.6	51
Thr	5.1	32	6.0	37	5.7	30
Ser	8.9	56	12.7	78	5.7	30
Glx	11.8	74	23.3	143	15.1	80
Gly	15.0	94	13.4	82	4.2	22
Ala	7.0	44	8.0	49	9.6	51
Val	3.8	24	4.6	28	5.8	31
Met	2.5	16	0.5	3	0.2	1
Ile	5.3	33	2.9	18	3.6	19
Leu	9.1	57	5.2	32	11.9	63
Tyr	4.1	26	2.0	12	4.7	25
Phe	3.3	21	2.3	14	5.3	28
His	2.1	13	2.6	16	3.2	17
Lys	6.8	43	3.9	24	10.4	55
Arg	5.6	35	1.8	11	5.1	27

Values are extrapolated to zero time from two analyses (3 for 67E) performed after 20 h hydrolysis and one analysis after 70 h hydrolysis

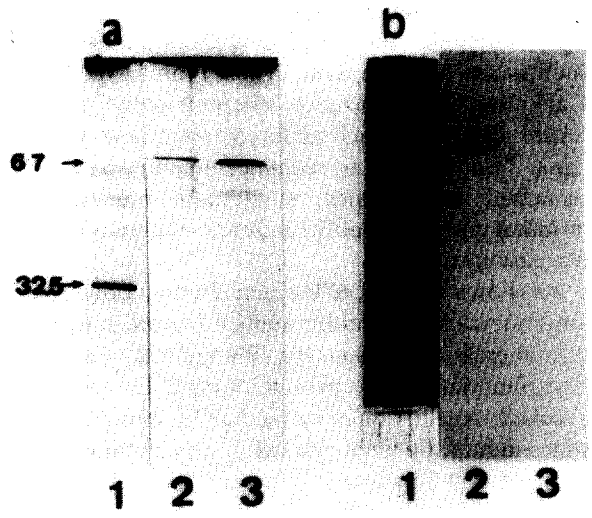

Fig.2. Immune blotting experiments with anti-endonexin antiserum. (a) SDS-PAGE slab stained with silver reagents; lane 1, 32.5 kDa liver endonexin; lane 2, 67 kDa R; lane 3, 67 kDa E. (b) Immune blot of a second gel identical to that of panel a after reaction with antiserum to 32.5 kDa endonexin and peroxidase-labelled anti-immunoglobulins. With 3-amino-9-ethyl-carbazole, a red insoluble precipitate characterizes the antigen-antibody complex. Molecular mass (kDa) is shown on the left.

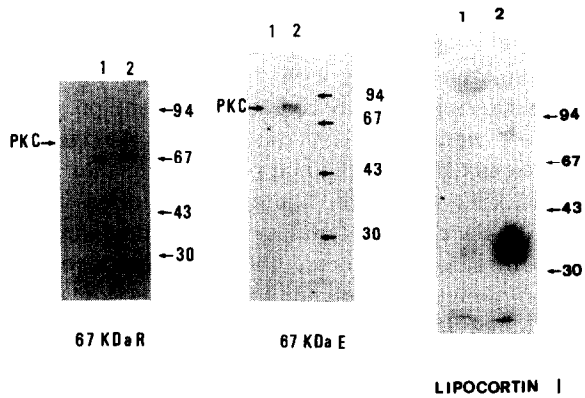

Fig.3. Phosphorylation of purified 67 kDa proteins by protein kinase C in vitro. Autoradiograms of the SDS-PAGE of the reaction mixtures resulting from the incubation of protein kinase C (5 units) in the presence of [γ-^{32}P] ATP and 1 μg of each protein with and without the phospholipid-calcium mixture; lanes 1 and 2, respectively. Phosphorylated material at higher apparent molecular size than 67 kDa proteins was attributed to self-phosphorylated protein kinase C (84 kDa). 1 unit of protein kinase C activity was defined as 1 pmol ^{32}P incorporated per min at 30°C, into histone III-S type as a standard protein substrate. Molecular mass (kDa) indicated on the right for each panel.

was attained at 250 nM and 316 nM of 67R and LC, respectively. This is essentially in the same order as the values previously reported for endonexin [16].

4. DISCUSSION

In addition to the two LCs recognized so far [23] and to endonexin [16], our data provide evidence for a 67 kDa protein (67R) able to inhibit PLA_2, using [^{3}H]oleic acid-labelled *E. coli* membranes as a substrate. One common feature of these four proteins is their ability to bind anionic phospholipids such as phosphatidylserine [16,24]. The amino acid composition of 67R is very similar to that of 67 kDa calelectrin [13,25], 67 kDa calcimedin [26] and 68–73 kDa protein from lymphocytes [27], which are probably identical proteins [14]. However, 67R displayed some significant differences in the level of Gly and Lys, which were, respectively, higher and lower compared to other literature data. A similar, if not identical, amino acid composition has also been found for 32.5 kDa calelectrin/endonexin [16,25] as well as for 35 kDa calelectrin/calcimedin [28,29]. All these proteins share in common with

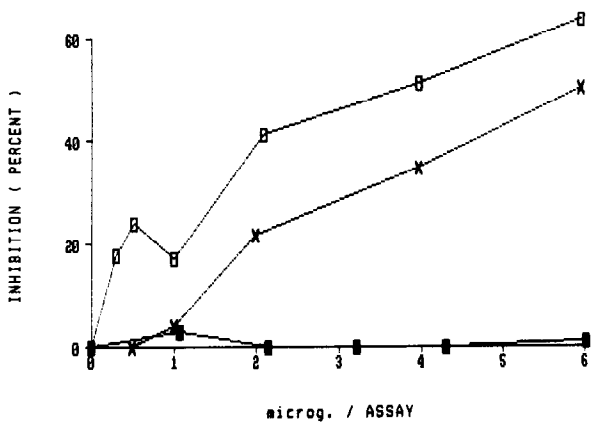

Fig.4. Inhibition of phospholipase A_2 activity by 67 kDa proteins (×—×, 67R; ■—■, 67E) and the lipocortin I (□—□). Results are means of three experiments.

LCs several repeats of a 17 amino acid residue consensus sequence, which probably explains the immunological cross-reactivity existing between these proteins [30] and illustrated herein. So, our data allow us to identify 67R as calelectrin/calcimedin.

Considering the common features of these various proteins, one can propose that the mechanism of PLA_2 inhibition probably involves some steric hindrance at the lipid/water interface, following their adsorption onto anionic phospholipids. A similar proposal was recently put forward by Davidson et al. [31], who also described a 73 kDa protein from bovine lung able to inhibit PLA_2 in a similar in vitro assay. Further studies are required to establish any relationship between 67R and this 73 kDa protein which was not further characterized [31].

Therefore, the number of Ca^{2+}-binding proteins including LCs, endonexin and calelectrin, able to inhibit PLA_2 in a non-specific manner is rapidly growing. It thus appears necessary to identify which ones are controlled by glucocorticoids, whose anti-inflammatory action seems to depend on PLA_2 inhibition [32]. On the other hand, the anti-PLA_2 activity of these proteins might also be an epiphenomenon simply reflecting their ability to adsorb onto phospholipids and not necessarily related to their biological function, which still remains obscure [15,29].

The anti-PLA_2 activity of lipocortin has been shown to be regulated by phosphorylation [32]. This is probably not the case for 67R, which was revealed as a poor substrate for protein kinase C. As recently shown by Touqui et al. [33], platelets contain, in addition to LC, a protein eluting at around 67 kDa upon molecular sieving on a TSK-G2000 column, whose activity is not modulated by protein kinase C activation. It is thus tempting to suggest some similarity if not identity between that platelet protein and the 67R protein from lung.

In contrast, the newly identified, glutamic acid-rich 67E Ca^{2+}-binding protein strongly differs from the protein family mentioned above. On the basis of amino acid composition, 67E is also different from albumin, calregulin [34], surfactant-associated proteins [35] as well as from a proteolytic cleavage product of protein kinase C identified in various tissues [36,37]. Moreover, the 67 kDa fragment of protein kinase C contains the catalytic site and can be (auto)phosphorylated, which does not appear from our phosphorylation experiments.

It must be recalled that 67 kDa calcimedin exists as a doublet, each protein of the pair displaying different expression depending on the tissue [29]. Since the phospholipid-binding properties of calcimedin were not previously checked, we suggest that the calcimedin doublet might correspond to the 67R and 67E identified here. So 67E appears as a new type of Ca^{2+}-binding proteins attaching to some membrane component different from phospholipids. Studies are now in progress to identify membrane target sites involved in this interaction as well as any possible biochemical or biological function of 67E.

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