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Interaction of smooth muscle caldesmon with phospholipids

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Taking into account the perimembrane localization of caldesmon [(1986) Nature 319, 68] and its ability to participate in the regulation of receptor clusterization [(1989) J. Biol. Chem. 264, 496], we studied the interaction of duck gizzard caldesmon with soybean phospholipids (azolectin). By using four independent methods, i.e. light scattering, gel-electrophoresis, gel-filtration and ultracentrifugation, we showed a Ca-independent complex formation between caldesmon and azolectin. Interacting with caldesmon, calmodulin is shown to dissociate the caldesmon-azolectin complex. It is supposed that the caldesmon-phospholipid interaction may affect caldesmon phosphorylation by Ca-phospholipid-dependent protein kinase. This effect may be important for various cell motility processes.

Caldesmon; Phospholipid; Phosphorylation

1. INTRODUCTION

Caldesmon is a multifunctional actin- and calmodulin-binding protein [1,2]. Caldesmon is supposed to be involved in the regulation of smooth muscle contraction [3]. Recently published data indicate that caldesmon plays a role in chromaffin granule secretion [4] as well as in the capping of receptors on the surface of lymphoma T cells [2,5]. In certain cells caldesmon is located close to the surface membrane [4]. Moreover, many actin-binding proteins (profilin, gelsolin, myosin-1) are able to interact with phospholipids [6-8]. These results prompted the present investigation into the interaction of duck gizzard caldesmon with a mixture of soybean phospholipids (azolectin).

2. MATERIALS AND METHODS

Duck gizzard caldesmon and bovine brain calmodulin were isolated according to earlier described methods [9,10]. Chymotryptic cleavage of caldesmon was performed as described by Fujii et al. [11]. Ca-phospholipid-dependent protein kinase (protein kinase C) was isolated from rat brain [12]. The phospholipid-independent form of the enzyme was obtained by two cycles of freezing and thawing of the partially purified protein kinase C. The chymotryptic peptide map indicates that both enzymes phosphorylate the same sites in caldesmon. Phosphorylation of caldesmon by protein kinase C and the phospholipid-independent form of the enzyme was performed under earlier described conditions. Phospholipid suspension (2-10 mg/ml) was obtained after 3-fold sonication (30 s) of azolectin (Serva) in 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA.

In experiments with light scattering, the azolectin suspension (10-20 mg/ml) in 50 mM imidazoles-HCl, pH 7.0, and 0.1 mM $CaCl_2$ was titrated with caldesmon. Light scattering was measured in a 1 ml cell at 25°C on a Hitachi F-3000 fluorometer setting the wavelength of emission and excitation at 340 nm.

The method of electrophoresis under non-denaturating conditions [13] was used for investigation of the caldesmon/azolectin interaction. Caldesmon (0.1-0.3 mg/ml) was mixed with the azolectin suspension (0-0.2 mg/ml) in the presence of 2 mM EGTA or 0.1 mM $CaCl_2$ and after 10-15 min incubation at room temperature was loaded on the gel.

Gel-filtration was performed at 4°C on a Sepharose 4B column (450 × 16 mm) equilibrated with buffer A (10 mM Tris-HCl, pH 7.2, containing 100 mM NaCl, 0.1 mM NaN_3, 3 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 1 μg/ml leupeptin and either 2 mM EGTA or 0.1 mM $NaCl_2$). Caldesmon (0.6-0.8 mg/ml) was mixed with azolectin (0-6 mg/ml) and incubated in buffer A for 15-30 min at room temperature. The sample (0.5-1.0 ml) was loaded on the column, and after completion of experiment aliquots of each fraction were monitored for protein content by the Spector method [14]. For experiments performed by ultracentrifugation intact caldesmon or a mixture of chymotryptic peptides of caldesmon (0.1-0.5 mg/ml) in 20 mM Tris-HCl pH 7.5, 0.1 M NaCl in the presence of 2 mM EGTA or 0.4 mM $CaCl_2$ were mixed with azolectin (0-0.9 mg/ml), incubated for 15-30 min at room temperature and subjected to centrifugation at 105 000 × g (1 h, 4°C). Aliquots of the pellet and supernatant were analyzed by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate [15]. To prevent the non-specific adsorption in several experiments bovine serum albumin (0.3-0.6 mg/ml) was added to the incubation mixture.

3. RESULTS

Titration of azolectin suspension by caldesmon leads to an increase in light scattering (Fig. 1). The titration curve was hyperbolic and the saturation was observed at the caldesmon:azolectin weight ratio equal to 5. Taking the average molecular weight of the phospholipid equal to 750 kDa and that of caldesmon to 87 kDa [16,17], one can estimate that one molecule of caldesmon interacts with 20-25 molecules of phospholipid. Thus, experiments on light scattering indicate that caldesmon can interact with azolectin.

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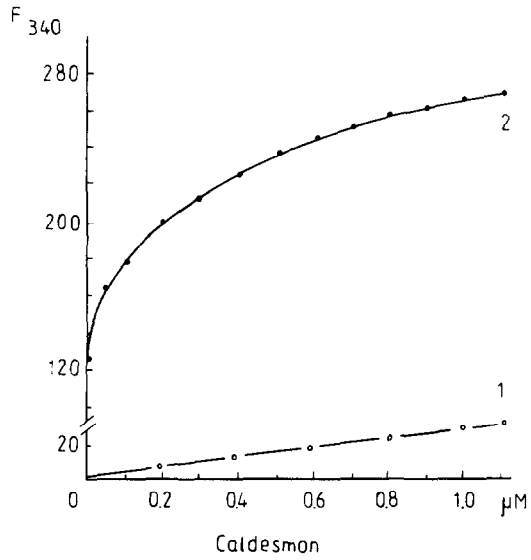

Fig. 1. Caldesmon induced increase in the light scattering of azolectin suspension. The incubation mixture (50 mM imidazole/HCl, pH 7.0, 0.1 mM $CaCl_2$) did not contain azolectin (curve 1) or contained 20 μg/ml of azolectin (curve 2).

This conclusion was checked by the method of gel-electrophoresis. Caldesmon has rather a low electrophoretic mobility at alkaline pH values in the absence of denaturating agents (Fig. 2). Addition of small quantities of azolectin results in the formation of a new band having a lower electrophoretic mobility than isolated caldesmon. In the presence of greater quantities of azolectin the band of isolated caldesmon completely disappears and the protein does not enter the gel (Fig. 2). These data indicate that under the conditions used caldesmon strongly interacts with azolectin and is thus unable to enter the gel. To verify this assumption we used the method of gel-filtration.

Isolated vesicles of azolectin are eluted from the column of Sepharose 4B in excluded volume (fractions 11–13 on Fig. 3). Isolated caldesmon enters the gel and is eluted from the column in fractions 21–23 (Fig. 3). When the caldesmon/azolectin mixture was loaded on the column a part of the protein was eluted in the excluded volume. The amount of caldesmon bound to azolectin depends on the initial concentration of the protein and phospholipid in the sample subjected for gel-filtration (cf. Fig. 3A and B). These experiments were performed in the presence of 0.1 mM $CaCl_2$ and 2 mM EGTA. The formation of caldesmon-phospholipid complex was observed both in the presence and absence of Ca^{2+}.

The method of ultracentrifugation in addition to the three previously described approaches (light scattering, gel-electrophoresis and gel-filtration) was used to obtain further data on the caldesmon/phospholipid interaction. In these experiments rather low concentrations of phospholipid and caldesmon were used. Even at caldesmon concentration of 0.3 mg/ml and

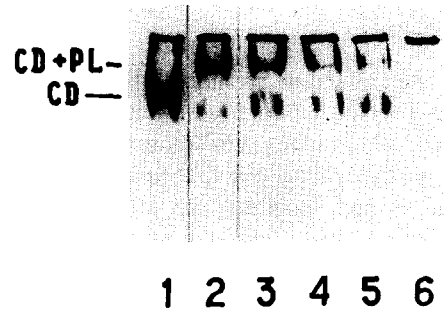

Fig. 2. Effect of azolectin on the electrophoretic mobility of caldesmon. The concentration of caldesmon in the sample loaded on the gel was 0.1 mg/ml; that of azolectin was equal to 0 (slot 1), 30 (slot 2), 60 (slot 3), 100 (slot 4), 150 (slot 5) and 200 μg/ml (slot 6), respectively.

that of azolectin 0.8 mg/ml, about 20% of the protein was bound to azolectin and sedimented upon ultracentrifugation. Worth noting is that addition of bovine serum albumin (up to 0.3–0.6 mg/ml) to the incubation mixture did not prevent the interaction of caldesmon with phospholipids. When the mixture of chymotryptic peptides of caldesmon and azolectin was subjected to ultracentrifugation, the pellet formed thereby was enriched in the peptides with M_r of 40 and 20000, whereas the supernatant contained higher quantities of peptides with M_r of 25000–27000 and 60000. Taking into account the literature data [12,17,18], we may suppose that the phospholipid-binding site of caldesmon is located in the C-terminal chymotryptic peptide of caldesmon, i.e. close to the sites involved in the caldesmon interaction with actin and calmodulin.

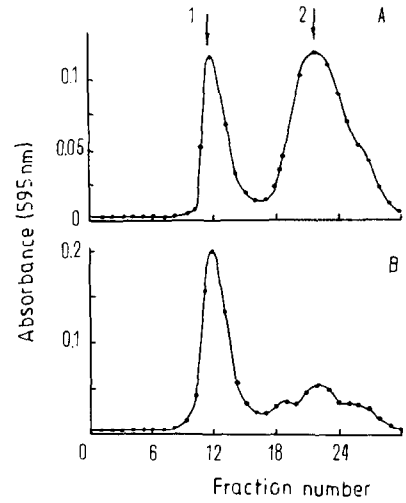

Fig. 3. Gel-filtration of azolectin/caldesmon mixture on a Sepharose 4B column. The sample (final vol. 0.96 ml) loaded on the column contained caldesmon (1.3 mg/ml) and azolectin – 1.52 mg/ml in A and 6.34 mg/ml in B. Ordinate: absorbance at 595 nm as determined after protein staining with Coomassie G-250. Arrows 1 and 2 indicate the position of separately chromatographed azolectin and caldesmon, respectively, whereas the profiles were obtained with the corresponding mixtures.

4. DISCUSSION

Using four different methods – light scattering, gel-filtration, gel-electrophoresis and ultracentrifugation – we succeeded in showing that smooth muscle caldesmon interacts with soybean phospholipids (azolectin). This fact seems to be important from many viewpoints. As was mentioned earlier, caldesmon may be located close to the membrane [4] and be involved in the regulation of exocytosis and receptor capping [2,5]. Caldesmon can regulate these processes because it is able to interact with actin and myosin [1,19] as well as with phospholipids (this publication). It is well known that cytoskeletal proteins preferentially interact with acidic phospholipids (phosphatidylserine and phosphoinositides) [6–8]. The mixture of phospholipids used in the present study (azolectin) alongside with phosphatidylcholine and phosphatidylethanolamine contains about 15% of phosphatidylinositols [20]. We may suppose that caldesmon primarily interacts with these acidic phospholipids. There are several facts supporting this assumption. Our preliminary data obtained through the method of gel-electrophoresis indicate that caldesmon interacts with phosphatidylserine more readily than with phosphatidylcholine. Moreover, ultracentrifugation data indicate that the C-terminal chymotryptic peptide of caldesmon interacts with azolectin. This peptide is enriched by positively charged amino acids and contains a fragment including residues 608–624 which seems to be able to form an amphiphilic α-helix [21,22]. It is supposed that this site is involved in the caldesmon/calmodulin interaction. At the same time it is obvious that the fragment containing a number of hydrophobic and positively charged residues may form a tight complex with phospholipids. For example, the fragment of gelsolin with these properties does interact with phospholipids [7]. If our assumption is correct, one may expect that calmodulin will compete with phospholipids for the interaction with caldesmon. Indeed, in experiments with gel electrophoresis we found that in the presence of Ca^{2+} calmodulin brings about the dissociation of the caldesmon-phospholipid complex. Under these conditions caldesmon interacts with calmodulin and penetrates the gel.

Ca-phospholipid-dependent protein kinase is able to phosphorylate caldesmon [12,23–25]. The sites phosphorylated by this enzyme are located in the C-terminal chymotryptic peptide of caldesmon [12,24,26]. Since both the caldesmon and protein kinase C are able to interact with the membrane these proteins may be located in the same compartment. This circumstance increases the probability of caldesmon phosphorylation by protein kinase C. Many well known in vitro substrates of protein kinase C strongly interact with phospholipids which affect the structure of this protein [27]. One may expect that phosphorylation will influence the interaction of proteins with phospholipids. Indeed our preliminary data indicate that phosphorylation of caldesmon (up to 0.9 mol of phosphate per mol of protein) by a phospholipid-independent form of protein kinase C results in a prominent decrease in the strength of the azolectin/caldesmon interaction. This is an indirect indication that the site of interaction with the phospholipid is located in the C-terminal fragment of caldesmon, i.e. close to the sites involved in the contact with actin and calmodulin.

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