Interaction of caldesmon with endoplasmic reticulum membrane: effects on the mobility of phospholipids in the membrane and on the phosphatidylserine base-exchange reaction

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We have previously demonstrated by tryptophan fluorescence the interaction of caldesmon with anionic phospholipid vesicles [Czuryło, Zborowski and Dąbrowska (1993) *Biochem. J.* **291**, 403–408]. In the present work we investigated the interaction of caldesmon with natural-membrane (rat liver endoplasmic reticulum) phospholipids by co-sedimentation assay. The results indicate that 1 mol of caldesmon binds approx. 170 mol of membrane phospholipids with a binding affinity constant of $7.3 \times 10^6 \text{ M}^{-1}$. The caldesmon–membrane phospholipid complex dissociates with increasing salt concentration and in the presence of Ca^{2+} /calmodulin. As indicated by EPR measurements of

membrane lipids labelled with 5-doxyl stearate and TEMPO-phosphatidylethanolamine, binding of caldesmon results in an increase in mobility of the acyl chains (in the region of carbon 5) and a decrease in polar headgroup mobility of phospholipids. Interaction of caldesmon with phospholipids is accompanied by inhibition of phosphatidylethanolamine synthesis via a phospholipid base-exchange reaction, with phosphatidylserine as substrate. This shows that, of the endoplasmic reticulum membrane phospholipids, the main target of caldesmon is phosphatidylserine.

INTRODUCTION

Caldesmon is an actin-, tropomyosin-, myosin- and Ca^{2+} /calmodulin-binding protein that is expressed in smooth-muscle and non-muscle cells and located *in situ* on the actin filaments (for a review see [1–3]). It is believed to take part in the regulation of motile phenomena as well as in the organization and dynamics of the actin cytoskeleton. In some non-muscle cells, caldesmon is located in the subplasmalemmal region: in patches at the internal periphery of adrenal chromaffin cells [4], directly beneath cups of concanavalin A receptor [5,6], and in the leading edge of fibroblast membrane [7]. This localization suggested its direct interaction with membranes. Moreover, subsequent studies on the interaction of caldesmon with liposomes composed of various phospholipids indicated complex-formation with anionic phospholipids, particularly phosphatidylserine (PS) [8–10]. The binding affinity of PS for caldesmon is around 10^5 M^{-1} . Interaction of caldesmon with PS is reversed by either Ca^{2+} /calmodulin [8,9] or phosphorylation of caldesmon with protein kinase C [9], and in this respect it is similar to caldesmon–actin interaction [11,12]. This reversal is due to co-localization of calmodulin- and PS-binding sites as well as phosphorylation sites on the caldesmon molecule [8–10,13]. PS-liposome-bound caldesmon can still bind actin filaments and attach them to the liposome [13] which suggests that PS–caldesmon interaction may play a role *in vivo* in the organization of cytoskeletal/membrane structure.

The aim of the present study was to analyse the ability of caldesmon to bind to natural membranes such as endoplasmic reticulum (ER), a major site of phospholipid synthesis in the cell, and to investigate the structural and functional implications of this binding. It may be supposed that reversal of the binding of caldesmon to the surface of a membrane that is involved in the

subplasmalemmal dynamics of the actin cytoskeleton that accompany motile phenomena also affects the dynamics of the membrane. To obtain information about changes in the mobility of phospholipid chains of biological membranes on binding of caldesmon, we used ER membrane vesicles devoid of cytoskeletal elements and applied the EPR technique. We also considered the possibility that the relatively strong binding of caldesmon to PS [8–10] might affect its participation in various processes taking place in membranes, such as, for example, the Ca^{2+} -dependent phospholipid base-exchange (PLBE) reaction that occurs in ER membrane with PS as substrate [14]. It is known that the protein-facilitated phospholipid motion across ER membranes and Ca^{2+} -mediated changes in configuration of the polar headgroups of phospholipids can regulate synthesis of phosphatidylethanolamine (PE) from PS by the PLBE reaction [15]. The results indicate that binding of caldesmon to ER membranes affects their mobility, in the region of the acyl chain at carbon 5 as well as in the region of the phospholipid polar headgroups. Moreover, the interaction of caldesmon with the membrane significantly inhibits PLBE reaction with PS as substrate.

MATERIALS AND METHODS

Purification of caldesmon and calmodulin

Chicken gizzard caldesmon was prepared as described by Bretscher [16]. Calmodulin was purified from bovine brain as described by Gopalakrishna and Anderson [17] with an additional purification step of Affi-Gel 501 (Bio-Rad) [18]. Protein concentration was determined by measuring UV absorbance using the following absorption coefficients: caldesmon, $A_{280}^{1\%} = 3.8$ [19]; calmodulin, $A_{278}^{1\%} = 2.0$ [17].

Abbreviations used: ER, endoplasmic reticulum; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PLBE, phospholipid base exchange; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; TEMPO-PE, N-4-TEMPO-thiocarbamyl-PE.

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Preparation of ER membranes

Rat liver ER membranes were obtained as described previously [20]. Briefly, adult male Wistar rats weighing 150–180 g were killed by decapitation. Livers (3.5–4.0 g wet weight) were excised and rinsed, and 10% homogenate was prepared in 3 mM Hepes, pH 7.4, containing 225 mM mannitol, 75 mM sucrose and 0.5 mM EGTA. Large debris, nuclei and mitochondria were removed by stepwise centrifugation, with the final step at 10000 *g* for 10 min, and light and disrupted mitochondria were removed by additional centrifugation of postmitochondrial supernatant at 15000 *g* for 20 min. The microsomal fraction was obtained by centrifugation of the supernatant at 105000 *g* for 1 h. The resulting pellet was resuspended in 3 mM Hepes buffer, pH 7.4, containing 250 mM sucrose, at a protein concentration of 10–20 mg/ml and stored at –70 °C. All procedures were carried out at 4 °C. Contamination of microsomes by mitochondrial and plasma membranes was determined by measurement of PS decarboxylase and 5'-nucleotidase activities, as described in detail in [15], and was no higher than 5 and 10% respectively. Membrane protein concentration was determined by the method of Bradford [21] with BSA as a standard. Phospholipids were extracted from the membrane as described by Bligh and Dyer [22] and quantified by phospholipid phosphorus analysis by the method of Rouser et al. [23]. Total phospholipid content in ER membrane preparations was 550 nmol/mg of membrane protein.

Binding of caldesmon to ER membranes

Binding of caldesmon to microsomes of rat liver was analysed by co-sedimentation assay. Samples containing caldesmon alone or caldesmon, calmodulin and microsomes (1 mg of protein/ml) in 40 mM Hepes (pH 7.4)/1 mM CaCl₂ were incubated for 20 min at 4 °C and ultracentrifuged at 100000 *g* for 60 min. Caldesmon remaining in the supernatant was quantified by analysis of 12.5% gels after SDS/PAGE [24] using a Molecular Dynamics computing densitometer and subtracted from total caldesmon in the sample.

Spin-labelling technique

EPR spectroscopy was performed using spin-labelling of the ER membranes with 5-doxyl stearate and N-4-TEMPO-thiocarbamyl-PE (TEMPO-PE). Labelling with 5-doxyl stearate (Aldrich) was achieved by 30 min incubation at room temperature of the membrane suspension in 40 mM Hepes buffer, pH 7.4, containing the amounts of caldesmon indicated in the Figure legends, in a glass test tube with a dry film of the spin-label probe. TEMPO-PE was synthesized by incubating equimolar amounts of 4-isothiocyanato-TEMPO (Aldrich) and egg yolk PE (Lipid Products) in 0.5 ml of chloroform/methanol (1:1, v/v) containing 50 µl of 100 mM Na₂CO₃ at room temperature in the dark for 24 h. The product was then concentrated by evaporation of the solvent and subjected to TLC as described by Yao and Rastetter [25]. TEMPO-PE was extracted from the gel with chloroform/methanol (1:1, v/v). Concentrated product was resubjected to TLC to ensure purification. Purity of TEMPO-PE was confirmed by electrospray ionization MS using a TSQ 700 Finnigan spectrometer. Labelling of ER membranes with TEMPO-PE (final concentration 0.3 µM) was achieved by incubating the suspension in the absence and presence of caldesmon (at the concentrations indicated in the Figure legends).

EPR spectra of the spin-labelled samples were obtained using a Bruker ESP 300E spectrometer equipped with a temperature-

control device. Order parameter values were calculated as described by Sefton and Gaffney [26] and rotational correlation times (τ_c) as described by Swartz [27]. Means of two independent measurements (differing by less than 10%) are presented.

Synthesis of PE from PS by the PLBE reaction

The ER membranes (1 mg of protein/ml) were preincubated at 4 °C for 20 min in 40 mM Hepes buffer, pH 7.4. Microsomal PS was labelled via the PLBE reaction started by addition to the membranes of [2-¹⁴C]serine (specific radioactivity 1.5 mCi/mmol) and CaCl₂ to 50 µM and 1 mM (final concentrations) respectively. After 20 min of incubation, the reaction was terminated by the addition of EGTA (final concentration 2 mM) and cooling the sample to 4 °C. ER membranes were sedimented by centrifugation at 100000 *g* and resuspended in 40 mM Hepes, pH 7.4; incorporation of radiolabelled substrate into PS was assessed by scintillation counting in a Beckman LS 6000TA counter. Blank samples (not incubated after the addition of substrate or incubated in the presence of 1 mM EGTA) were used to measure non-specific binding of [2-¹⁴C]serine to microsomes, and the values obtained were subtracted from the respective experimental data. Non-specific binding of [2-¹⁴C]serine to microsomes never exceeded 0.5–1.0% of the total incorporation. The ethanolamine base-exchange reaction with microsomal radioactively labelled serine in PS was performed at a microsomal membrane protein concentration of 1 mg/ml in medium containing 40 mM Hepes, pH 7.4, 1 mM CaCl₂, 100 µM ethanolamine and caldesmon at concentrations indicated in the Figure legends. After incubation at 37 °C for various lengths of time as indicated in the Figure legends, the reaction was terminated by the addition of 4.0 ml of chloroform/methanol (1:2, v/v) to 0.5 ml of sample. Phospholipids were extracted as described by Bligh and Dyer [22] and ethanolamine base-exchange activity was calculated from the decrease in PS radioactivity in the membranes. Control experiments did not show any decrease in radioactivity in the absence of ethanolamine.

RESULTS

Binding of caldesmon to ER membranes and its reversal by Ca²⁺/calmodulin

The capacity of chicken gizzard caldesmon to bind to natural-membrane phospholipids was determined by co-sedimentation with microsomes of rat liver and subsequent quantification of the bound caldesmon by SDS/PAGE. As shown in Figure 1, binding saturation occurred at approx. 6 nmol of caldesmon/µmol of phospholipid. Scatchard analysis of the data in terms of a one-site model indicated about 1 nmol of caldesmon bound/approx. 170 nmol of total ER phospholipids, with a binding affinity constant of $7.33 \times 10^6 \text{ M}^{-1}$. The absence of Ca²⁺ did not significantly change either binding affinity constant or maximum binding of caldesmon to the membrane (results not shown).

Binding of caldesmon to the membrane was inhibited in the presence of Ca²⁺/calmodulin, with a half-maximum effect at a molar ratio of the two proteins of 1:1 (Figure 2). Caldesmon binding to ER membrane also decreased with increasing NaCl concentration (Figure 3), which indicates its mostly ionic character. However, in the physiological range of ionic strength, about 70% of caldesmon still remained bound to the membrane. Moreover, the plateau on the binding curve in the range 100–250 mM NaCl may suggest involvement of hydrophobic forces in the stability of the caldesmon–membrane complex.

Figure 1 Binding of caldesmon to ER membranes

Samples containing various amounts of chicken gizzard caldesmon and microsomal fraction corresponding to 140 nmol of rat liver membrane phospholipids in 40 mM Hepes buffer (pH, 7.4)/1 mM CaCl_2 were sedimented, and pelleted caldesmon was quantified by SDS/PAGE of supernatant samples as described in the Materials and methods section. Results are means $\pm$ S.D. for five experiments. The inset shows a Scatchard representation of the data; the correlation coefficient of linear regression is 0.975.

Figure 2 Effect of Ca^{2+} /calmodulin on the binding of caldesmon to ER membranes

Sedimentation of chicken gizzard caldesmon–rat liver microsome complex (obtained from 0.75 nmol of caldesmon and microsomal fraction corresponding to 140 nmol of membrane phospholipids) with various amounts of calmodulin was performed in the same buffer as in Figure 1. Bound caldesmon was quantified as described in the Materials and methods section. The amount of caldesmon bound in the absence of calmodulin (100%) was 4.4 nmol/μmol of membrane phospholipid. Results are means $\pm$ S.D. for three experiments.

Changes in ER membrane lipid mobility evoked by their interaction with caldesmon

Changes in the mobility of ER membrane lipids on interaction with caldesmon were studied by the spin-labelling technique. Since the results presented above indicated predominantly ionic surface interaction of phospholipids with caldesmon, we looked for conformational changes in these regions of the lipid molecules using spin probes: 5-doxyl stearate and TEMPO-PE. The first probe gives information about fatty acid acyl-chain mobility in

Figure 3 Effect of NaCl concentration on the binding of caldesmon to ER membranes

Conditions and procedure were as described in Figure 2. The amount of caldesmon bound in the absence of NaCl (100%) was 4.4 nmol/μmol of membrane phospholipid. Results are means $\pm$ S.D. for three experiments.

Figure 4 Effect of caldesmon on the order parameter of ER membrane lipids labelled with 5-doxyl stearate at various temperatures

(A) Dependence of the order parameter on temperature. Samples contained 30 μg/ml labelled rat liver microsomes without (○) or with (●) 10 μM chicken gizzard caldesmon in 40 mM Hepes buffer, pH 7.4. (B) Effect of caldesmon concentration on the order parameter. Measurements were performed at 23 °C (■) and 37 °C (▲). Results are means $\pm$ S.D. for two experiments. The differences between curves (in A and B) are significant ($P < 0.01$; Student's *t* test for paired comparisons).

the region relatively close to the polar headgroup of the phospholipid bilayer, whereas the second should enable direct changes in mobility of the polar headgroups to be monitored. The results show that 10 μM caldesmon decreased by 2–3.5% the order parameter of ER membrane labelled with 5-doxyl stearate over the temperature range 23–37 °C (Figure 4A). A concentration of 1 μM also decreased the values of this parameter, but the effect was less pronounced (results not shown). The caldesmon concentration-dependence of the order parameter is shown in Figure 4(B). At both 23 and 37 °C the order parameter decreased even in the presence of relatively low concentrations of caldesmon. These data indicate a small but significant increase in the mobility of fatty acids in the region of carbon 5, i.e. close to the polar headgroups of the phospholipids, on binding of caldesmon.

Further EPR studies were performed using synthesized TEMPO-PE. When this compound was dispersed in the buffer, the ratio of high-field to middle-field line intensities was 0.995–

Figure 5 Effect of caldesmon on rotational correlation time (τ_c) of TEMPO-PE label incorporated into ER membrane

Samples containing labelled rat liver microsomes (30 $\mu\text{g}/\text{ml}$) were preincubated without caldesmon ($\circ$) or with 1 μM ($\blacktriangle$) or 10 μM ($\bullet$) caldesmon in 40 mM Hepes buffer, pH 7.4. Results are means $\pm$ S.D. for two experiments. The differences between curves for both 1 μM and 10 μM caldesmon and that in the absence of caldesmon are significant ($P < 0.01$; Student's t test for paired comparisons).

Figure 6 Effect of caldesmon on PE synthesis from PS by the PLBE reaction

(A) Time course of PLBE reaction in the absence ($\circ$) or presence ($\bullet$) of 8 μM caldesmon. (B) Effect of caldesmon concentration on PE synthesis by the PLBE reaction. PE synthesis was performed for 20 min and calculated from the decrease in PS radioactivity as described in the Materials and methods section. Values are means $\pm$ S.D. for five experiments.

1.05, which corresponds to rotational correlation times from 5×10^{-13} to 3.7×10^{-11} s. When liver microsomes were incubated in the mixture with spin-label, this ratio changed to 0.6–0.8, giving an average rotational correlation time of 5.4×10^{-11} s. Addition of caldesmon increased the rotational correlation time to 9.3×10^{-11} s at 1 μM caldesmon and to 1.5×10^{-10} s at 10 μM caldesmon (Figure 5). In all cases the rotational correlation time for the TEMPO-PE spin label was practically independent of temperature in the range investigated. The increase in rotational correlation time of TEMPO-PE on binding of caldesmon to ER membrane phospholipids indicates that it immobilizes phospholipid polar headgroups.

Caldesmon-induced inhibition of PE synthesis in ER membranes

The interaction of caldesmon with membrane phospholipids led to inhibition of PE synthesis from PS by the PLBE reaction.

Figure 6(A) shows the time course of PE formation from radioactive PS (labelled at the serine residue) in the absence and presence of 8 μM caldesmon. The efficiency of synthesis depended on caldesmon concentration; at low concentrations slight but significant ($P < 0.05$) activation and at high concentrations almost 80 % inhibition of PE synthesis was observed (Figure 6B). Since the PLBE reaction with PC as substrate was not affected by caldesmon (results not shown), it can be suggested that, on interaction with caldesmon, membraneous PS becomes inactive as a substrate for PE synthesis.

DISCUSSION

Interaction of cytoskeletal proteins with lipid molecules is known to play a key role in controlling the biological activity of both cytoskeletal components and the membranes [28,29].

The present results provide evidence that caldesmon interacts not only with liposomes, as was shown previously [8–10], but also with biological membranes. Binding of caldesmon to reconstituted phospholipid bilayers and to natural membrane, such as ER, has some common features: (1) it strongly depends on salt concentration, which indicates its mostly ionic character; (2) it is reversed by Ca^{2+} /calmodulin. The reversal of caldesmon binding to membrane phospholipids by calmodulin controlled by Ca^{2+} concentration appears to affect not only the dynamics of the subplasmalemmal cytoskeleton [13] but also those of the membrane. And indeed, spin-label studies revealed that caldesmon evokes small but significant changes in the mobility of the acyl chains (in the region of carbon 5) and in the polar headgroup region of the membrane phospholipids. The former effect could reflect the 'hydrophobic' component of caldesmon–lipid interaction. Similar changes have been observed on interaction of erythrocyte spectrin with erythrocyte membrane devoid of peripheral proteins [30]. However, another interpretation is that the increase in acyl-chain mobility on interaction with caldesmon is due to immobilization of the polar headgroups.

We have previously shown that caldesmon preferentially interacts with anionic phospholipid vesicles, particularly PS. The affinity constant for this phospholipid was much higher than for the others (around 10^5 M^{-1}) [9]. Rotary shadowing electron microscopy revealed the attachment of caldesmon (labelled with a monoclonal antibody against an epitope in the N-terminal region) to PS vesicles through the C-terminus [13]. As the affinity constant of caldesmon binding to ER membrane is relatively high ($7.3 \times 10^6 \text{ M}^{-1}$), it is probable that a polar head-group of PS, which is known to be located in the inner half of the plasma-membrane lipid bilayer [31], also provides the site of attachment of caldesmon to the membrane. However, the more than one order of magnitude higher binding constant for binding of caldesmon to the biological membranes than to PS vesicles may suggest that the other anionic phospholipids also take part in its binding to the membranes, which results in strengthening of the binding. Moreover, it cannot be excluded that some integral proteins contribute to the caldesmon binding.

The inhibition of PE synthesis in ER membrane via the PLBE reaction with PS as substrate can be accounted for by either the changes in PS molecule configuration or its steric hindrance by caldesmon. Perhaps the slight increase in PE synthesis at low caldesmon concentration corresponds to the primary rearrangement of phospholipid molecules via, e.g., a flip-flop movement which appears to regulate the PLBE reaction [15]. When the concentration of caldesmon increases, the protein attracts more substrate molecules and steric hindrance is responsible for the inhibition of the PLBE reaction. In conclusion, the inhibition confirms that the major target of caldesmon in the membranes is

PS. On the other hand, changes in the dynamics of the surface region of phospholipid bilayers on binding of caldesmon indicate that the mode of its binding to ER membrane is similar to that observed in the case of synthetic membrane [9]. A lack of increase in surface pressure (π) of phospholipid monolayers on addition of caldesmon (R. Makuch and A. F. Sikorski, unpublished work) shows that it does not penetrate the hydrophobic region of the membrane and thus further confirms the surface character of its interaction with membrane phospholipids.

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