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Caldesmon–phospholipid interaction

Effect of protein kinase C phosphorylation and sequence similarity with other phospholipid-binding proteins

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Recently published data [Vorotnikov & Gusev (1990) FEBS Lett. 277, 134–136] indicate that smooth muscle caldesmon interacts with a mixture of soybean phospholipids (azolectin). Continuing this investigation, we found that duck gizzard caldesmon interacts more tightly with acidic (phosphatidylserine) than with neutral (phosphatidylcholine) phospholipids. A high concentration of Ca^{2+} (50 μM) decreased the interaction of caldesmon with phosphatidylserine. Among chymotryptic peptides of caldesmon, only those having molecular masses of 45, 40, 23, 22 and 20 kDa were able to specifically interact with phospholipids. These peptides, derived from the *C*-terminal part of caldesmon, contained the sites phosphorylated by Ca^{2+}/phospholipid-dependent protein kinase, and phosphorylation catalysed by this enzyme decreased the affinity of these peptides for phospholipids. In the presence of Ca^{2+}, calmodulin competed with phospholipids for the interaction with the caldesmon peptides. The *C*-terminal part of caldesmon contains three peptides with a primary structure similar to that of the calmodulin- and phospholipid-binding site of neuromodulin. These sites may be involved in the interaction of caldesmon with calmodulin and phospholipids.

INTRODUCTION

Caldesmon is an ubiquitous multifunctional protein detected in smooth muscle and non-muscle cells (Burgoyne *et al.*, 1986; Sobue *et al.*, 1988; Walker *et al.*, 1989). Caldesmon interacts with actin, tropomyosin, calmodulin and myosin (Hayashi *et al.*, 1991; Wang *et al.*, 1991) and is thought to be involved in the Ca^{2+}-dependent regulation of actin–myosin interactions (Marston & Smith, 1985). It has been shown that, in certain non-muscle cells, caldesmon is located close to the outer cell membrane (Burgoyne *et al.*, 1986; Yamakita *et al.*, 1990; Takeuchi *et al.*, 1991) and plays a significant role in receptor clustering (Walker *et al.*, 1989) and exocytosis (Burgoyne *et al.*, 1986; Linstedt & Kelly, 1987). Taking into account these data and the well-known ability of some actin-binding proteins (such as profilin, myosin I, gelsolin, vilin, destrin, cofilin etc.) to interact with phospholipids, we previously investigated the interaction of smooth muscle caldesmon with a mixture of soybean phospholipids (azolectin) (Vorotnikov & Gusev, 1990). Although we demonstrated a direct interaction of caldesmon with phospholipids, many important details of this interaction remained unknown. The present paper details further investigations into the caldesmon–phospholipid interaction.

MATERIALS AND METHODS

Protein isolation

Caldesmon was isolated from frozen duck gizzards according to Vorotnikov & Gusev (1991), and was subjected to chymotrypsin-treatment as described by Fujii *et al.* (1987). Briefly, caldesmon (about 3 mg/ml) in 10 mM-imidazole (pH 7.0) containing 100 mM-NaCl was hydrolysed with chymotrypsin (~ 1000:1, w/w) for 5–10 min at 30 °C. The reaction was stopped by addition of phenylmethanesulphonyl fluoride up to a final concentration of 0.5 mM. Calmodulin was isolated from bovine brain by the method of Gopalakrishna & Anderson (1982). The 68 kDa protein calcimedin was isolated from frozen duck gizzards according to Kobayashi & Tashima (1990).

Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) was isolated from rat brains and its activity was determined by previously described methods (Vorotnikov *et al.*, 1988*a*). Briefly, the tissue was homogenized in buffer containing $CaCl_2$, the pellet obtained after centrifugation (40000 *g*, 15 min) was extracted with buffer containing a mixture of EDTA and EGTA. The extract containing protein kinase C was subjected to ion-exchange chromatography on Whatman DE-52 cellulose. Ca^{2+}/phospholipid-dependent histone kinase activity was eluted from the column by a linear gradient of NaCl (0–0.25 M). Final purification was achieved by Ca^{2+}-dependent chromatography on a mixture of phosphatidylserine and cholesterol immobilized in polyacrylamide gel, as described by Uchida & Filburn (1984). The specific activity of the enzyme thus obtained was equivalent to 0.2–0.4 μmol of phosphate transferred on histone H-1/min per mg of enzyme at 30 °C. The apparent molecular mass of enzyme determined by SDS/PAGE was 80 kDa. The Ca^{2+}/phospholipid-independent form of protein kinase C was obtained by two cycles of freezing and thawing of the partially purified enzyme obtained after chromatography on DEAE-cellulose. After these procedures, the activity of enzyme was only slightly dependent on the presence of phospholipids and Ca^{2+}, and the apparent molecular mass was 67 kDa. Thus this enzyme seems to be similar to the proteolytic fragment of protein kinase C possessing Ca^{2+}/phospholipid-independent protein kinase activity described by Girard *et al.* (1986). The pattern of chymotryptic and cyanolytic peptides of caldesmon that were phosphorylated by the native and Ca^{2+}/phospholipid-independent forms of protein kinase C were identical. This means that both enzymes phosphorylate similar or identical sites located in the *C*-terminal part of caldesmon.

Preparation of phospholipid vesicles

Phospholipids [azolectin (Serva), phosphatidylserine (bovine brain extract type 5; Sigma), and egg phosphatidylcholine] were suspended in 20 mM-Tris/HCl, pH 7.5, containing 0.1 mM-EDTA at a final concentration of 2–10 mg/ml. The suspension

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was sonicated on ice under a stream of argon for 3–5 × 30 s, with 1 min intervals between sonications, in a Soniprep (MSE) ultrasound disintegrator, and then stored on ice under argon.

Light-scattering experiments

These were performed using a Hitachi F-3000 spectrofluorimeter. The suspension of phosphatidylserine (20 μg/ml), in buffer containing 50 mM-imidazole/HCl, pH 7.0, or 50 mM-imidazole/HCl, pH 7.0, with 100 mM-NaCl, was titrated with caldesmon or calcimedins. The intensity of light scattered was measured at 90° at excitation and emission wavelengths of 340 nm. The method of Nelsestuen & Lim (1977) was used for the determination of the apparent binding constants and the number of phospholipid molecules interacting with the protein.

Caldesmon phosphorylation

This was performed in buffer containing 20 mM-Tris/HCl, pH 7.5, 4 mM-$MgCl_2$, 20–100 μM-[γ-^{32}P]ATP [(0.2–1) × 10^5 c.p.m./μl of final incubation mixture], 4 mM-2-mercaptoethanol, and either 0.2 mM-$CaCl_2$ and 0.1 mg of azolectin/ml for protein kinase C) or 0.5 mM-EGTA (for Ca^{2+}/phospholipid-independent form of the enzyme). The caldesmon concentration varied from 1 to 1.7 mg/ml, that of the enzyme varied from 5 to 30 μg/ml. After incubation for 2–3 h at 30 °C the incubation mixture was boiled for 3 min. The pellet (if any) containing the denatured enzyme was discarded and the supernatant containing phosphorylated caldesmon was collected.

Ultracentrifugation

Caldesmon (or chymotryptic peptides of caldesmon) (0.6–0.8 mg/ml) in 50 mM-Tris/HCl, pH 7.5, containing 12 mM-mercaptoethanol was mixed with the suspension of phospholipids, the final concentration of which varied from 0.1 to 0.7 mg/ml. The incubation mixture was completed by addition of either 1 mM-EGTA or 50 μM-$CaCl_2$. The ionic strength of the probe was adjusted to the desired value by addition of 1 M-NaCl. After incubation at room temperature for 10–15 min the samples (total volume 50–100 μl) were centrifuged in the LP-42 Ti rotor of a Beckman L-8-55 ultracentrifuge for 30 min at 105000 *g* at 25 °C. The aliquots of initial incubation mixture and of supernatant and pellet were analysed by SDS/PAGE.

Electrophoresis

SDS/PAGE was performed in 12% (w/v)-polyacrylamide/0.5% (w/v)-methylene bisacrylamide slab gels according to the Laemmli (1970) method. Molecular masses were determined by using the low molecular mass calibration kit purchased from Sigma. Electrophoresis under non-denaturing conditions was performed by the method of Schaub & Perry (1969). The samples for electrophoresis (total volume 20–40 μl) were prepared as follows. Caldesmon (5–8 μg) in the sample buffer (8 mM-Tris/glycine, pH 8.3, 10% sucrose and 10 mM-mercaptoethanol, containing either 50 μM-$CaCl_2$ or 1 mM-EGTA) was mixed with phospholipid vesicle suspension (0.04–16 μg). After incubation for 15–30 min at room temperature these samples were subjected to electrophoresis in polyacrylamide gels containing either 50 μM-$CaCl_2$ or 1 mM-EGTA.

After routine procedures of staining, destaining and drying, the gels containing radioactivity were subjected to autoradiography under previously described conditions (Vorotnikov *et al.*, 1988*b*). Both Coomassie R-250-stained gels and corresponding autoradiograms were scanned on an LKB Ultroscan XL laser densitometer. Each track was scanned twice at two different positions of the light beam, and the mean value of the area under the peak was determined. As a rule, the difference between the two measurements did not exceed 10%. Under the conditions used the intensity of staining was proportional to the quantity of the protein (or peptide) loaded on to the column. For each gel two autoradiograms differing in time of exposure were prepared and scanned on the densitometer. This confirmed that the intensity of the autoradiograms was proportional to the radioactivity of the corresponding protein bands. The specific radioactivity was determined as the ratio of areas under the corresponding peaks on the autoradiogram and on the Coomassie R-250-stained gel.

Protein concentration

This was determined spectrophotometrically by taking $A^{1\%}_{278}$ to be equal to 3.3 for caldesmon (Graceffa *et al.*, 1988) and 2.0 for calmodulin (Szpacenko & Dabrowska, 1986), or by the method of Spector (1978). The molecular mass of caldesmon was taken to be 87 kDa (Bryan *et al.*, 1989), and that of the phospholipids was 750 Da. The numbering of caldesmon amino acid residues is that introduced by Bryan *et al.* (1989).

RESULTS

Interaction of caldesmon with different phospholipids and the effect of Ca^{2+} on this interaction

We have previously shown that smooth muscle caldesmon interacts with a mixture of soybean phospholipids (Vorotnikov & Gusev, 1990). In order to determine which phospholipids are involved in this interaction, we investigated the interaction of caldesmon with phosphatidylserine and phosphatidylcholine. Under non-denaturating conditions, caldesmon has a low electrophoretic mobility (Fig. 1, slot 1). Addition of phosphatidylserine resulted in a decrease in the intensity of the protein band corresponding to isolated caldesmon, and in the accumulation of protein at the top of the gel (Fig. 1*a*, slots 2–11). These effects are due to the formation of large protein–phospholipid complexes which are unable to enter the gel. The band of isolated caldesmon disappeared at a protein/phospholipid molar ratio of 1:20 or 1:40. When caldesmon was mixed with phosphatidylcholine, the band of isolated protein was visible even at a protein/phospholipid molar ratio of 1:600. The data presented indicate that smooth muscle caldesmon preferentially interacts with acidic phospholipids, and its interaction with neutral phospholipids is

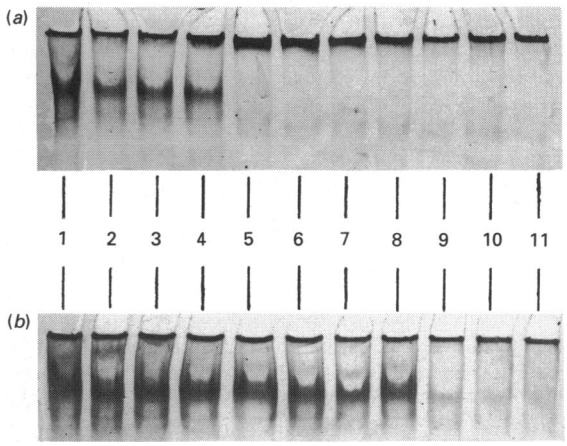

Fig. 1. Interaction of caldesmon with phosphatidylserine (*a*) and phosphatidylcholine (*b*)

Isolated caldesmon (slot 1) or a mixture of caldesmon and phospholipids (slots 2–11) was subjected to 7% polyacrylamide gel electrophoresis under non-denaturing conditions. The molar ratio of caldesmon/phospholipids was equal to 1, 2, 10, 20, 40, 60, 80, 100, 200 and 600 for slots 2–11 respectively.

rather weak. In the case of azolectin used in our previous investigation, the main acidic phospholipid is phosphatidylinositol, which seems to be responsible for the interaction with caldesmon.

By using two independent methods, light scattering and ultracentrifugation, we determined the parameters of the caldesmon–phosphatidylserine interaction. The apparent dissociation constant was 0.1–0.4 μM, and 400–480 molecules of phospholipid were bound per mol of caldesmon at room temperature. These values are comparable with the corresponding parameters for the interaction of brush border myosin I (Hayden *et al.*, 1990), myosin I from Acanthamoeba (Adams & Pollard, 1989) and profilin (Isenberg, 1991) with phospholipids. We compared the phospholipid-binding properties of caldesmon with the corresponding properties of a typical Ca^{2+}/phospholipid-binding protein, calcimedin (68 kDa), isolated from duck gizzard. The apparent dissociation constant for calcimedin–phosphatidylserine interaction was of the same order as for caldesmon. At the same time, the number of phospholipid molecules interacting with calcimedins was smaller (about 100–150). Furthermore, in contrast to caldesmon, calcimedin interacts with phospholipids only in the presence, but not in the absence, of Ca^{2+}. The data presented indicate that the phospholipid-binding properties of caldesmon are similar to those of a number of proteins which interact strongly with phospholipids.

Using the phospholipid mixture we failed to observe any effect of Ca^{2+} on the caldesmon–phospholipid interaction. When caldesmon was mixed with phosphatidylserine in the presence of 1 mM-EGTA the band representing the isolated protein disappeared at a protein/phospholipid molar ratio of 1:20 or 1:40. At the same time, in the presence of 50 μM-$CaCl_2$ the band of isolated caldesmon was visible even at a protein/phospholipid ratio of 1:80 or 1:100. Thus in the presence of Ca^{2+} the caldesmon–phospholipid interaction is diminished. This may be due either to Ca^{2+}-induced aggregation of phospholipid vesicles or to the direct effect of Ca^{2+} on the caldesmon–phospholipid interaction. Similar effects of bivalent cations on the interaction of cofilin (Yanezawa *et al.*, 1990) and neuromodulin (Houbre *et al.*, 1991) with acidic phospholipids have been described. The data presented here indicate that caldesmon interacts mainly with acidic phospholipids, and under certain conditions Ca^{2+} interferes with this interaction. In order to determine the site of caldesmon involved in the interaction with phospholipids, we turned to the investigation of the interaction of caldesmon fragments with phospholipids.

Interaction of chymotryptic peptides of caldesmon with phospholipids, and effect of calmodulin on this interaction

In full agreement with previous data (Szpacenko & Dabrowska, 1986; Fujii *et al.*, 1987), limited chymotrypsinolysis of caldesmon led to the formation of a number of peptides with apparent molecular masses of 110–120, 70–80, 60, 45, 40, 25–27 and 19–22 kDa. Among these peptides only five, having molecular masses of 45, 40, 23, 22 and 20 kDa, were co-sedimented with azolectin vesicles (Fig. 2). These peptides are derived from the *C*-terminal part of caldesmon, which is enriched in positively charged amino acid residues and contains the sites of caldesmon interaction with calmodulin, actin and tropomyosin (Szpacenko & Dabrowska, 1986; Fujii *et al.*, 1987; Katayama, 1989; Bryan *et al.*, 1989). Taking into account that all of these peptides have a net positive charge and that caldesmon interacts mainly with acidic phospholipids, we propose that electrostatic interactions play an important role in caldesmon–phospholipid interactions. Indeed, when the ionic strength of the incubation mixture was increased from 40 to 120 mM-NaCl, the quantity of peptides co-

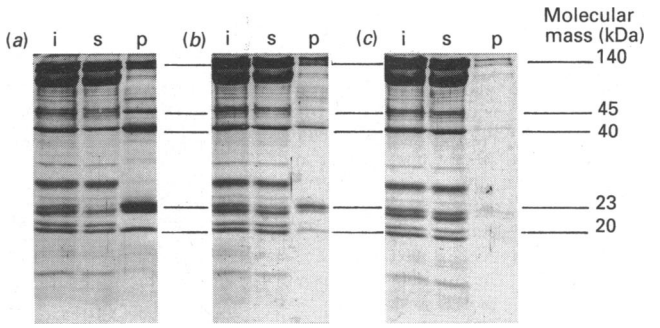

Fig. 2. Interaction of caldesmon-derived peptides with phospholipids

Chymotryptic peptides of caldesmon (0.6 mg/ml) were mixed with a suspension of azolectin vesicles (0.45 mg/ml) in the presence of 1 mM-EGTA and different concentrations of NaCl (*a*, 40 mM; *b*, 120 mM; *c* 200 mM), and subjected to ultracentrifugation. The composition of the initial mixture (i), supernatant (s) and pellet (p) were analysed by SDS/PAGE. A scale of apparent molecular masses (in kDa) is given on the right.

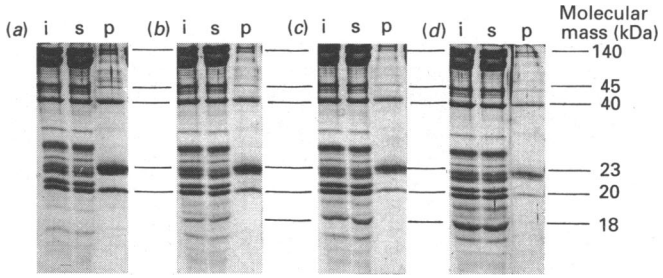

Fig. 3. Effect of calmodulin on the interaction of caldesmon-derived peptides with azolectin

Chymotryptic peptides of caldesmon (0.8 mg/ml) were mixed with azolectin (0.65 mg/ml) in the presence of 0.1 mM-$CaCl_2$ and different quantities of calmodulin (mg/ml: *a*, 0; *b*, 0.24; *c*, 0.48; *d*, 0.80). After ultracentrifugation, the supernatant and pellet were subjected to SDS/PAGE. i, initial mixture; s, supernatant; p, pellet. A scale of apparent molecular masses (in kDa) is given on the right.

sedimented with azolectin was diminished by more than 50% (Fig. 2). A similar effect was observed in the case of native caldesmon. These data indicate that caldesmon and its peptides electrostatically interact with the negatively charged groups of phospholipids located on the surface of azolectin vesicles.

As mentioned earlier, the peptides interacting with azolectin were derived from the *C*-terminal part of caldesmon, which contains the sites that are involved in the caldesmon–calmodulin interaction. We proposed that calmodulin may interfere with the interaction of caldesmon peptides with azolectin (Vorotnikov & Gusev, 1990). To check this assumption, we analysed the effect of calmodulin on the interaction of chymotryptic peptides of caldesmon with phospholipids. As can be seen in Fig. 3, calmodulin decreased the quantity of caldesmon peptides co-sedimented with azolectin. Thus, in the presence of Ca^{2+}, calmodulin competes with phospholipids for the interaction with caldesmon.

Effect of caldesmon phosphorylation on its interaction with phospholipids

Previously published data indicated that all sites phosphorylated by protein kinase C are located in the *C*-terminal part of caldesmon (Vorotnikov *et al.*, 1988*b*; Vorotnikov & Gusev, 1991). Indeed, protein kinase C phosphorylates two sites

Table 1. Effect of protein kinase C-catalysed phosphorylation on the interaction of caldesmon and its peptides with azolectin

Caldesmon phosphorylated by protein kinase C in the presence of [γ-^{32}P]ATP was digested with chymotrypsin, and the peptides thus obtained (0.8 mg/ml) were mixed with azolectin (0.6 mg/ml) in the presence of 0.1 mM-EGTA and 50 mM-NaCl. After ultracentrifugation, the protein compositions of the pellet and supernatant were analysed by SDS/PAGE followed by autoradiography. The specific radioactivity of certain bands was determined as described in the Materials and methods section. Results are means ± S.D. The numbers in parentheses indicate the numbers of independent experiments.

	Specific radioactivity (arbitrary units)	
Protein species	Supernatant	Pellet
Native caldesmon	1.80 ± 0.19	1.13 ± 0.18 (5)
40 kDa fragment	3.47 ± 0.48	1.73 ± 0.25 (8)
23 kDa fragment	5.73 ± 1.77	1.07 ± 0.19 (5)
22 kDa fragment	3.28 ± 0.64	1.64 ± 0.07 (3)

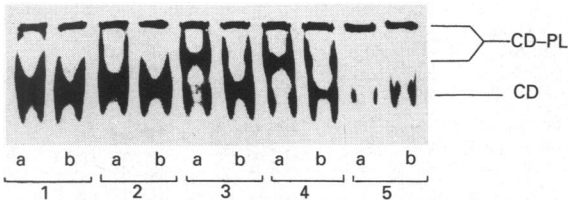

Fig. 4. Effect of caldesmon phosphorylation on its interaction with phospholipids

Unphosphorylated (slots a) and phosphorylated (0.9 mol of phosphate per mol of protein; slots b; phosphorylation was by the Ca^{2+}-independent form of protein kinase C) caldesmon was mixed with different quantities of azolectin in the presence of 0.1 mM-EGTA and 50 mM-NaCl and subjected to electrophoresis under non-denaturing conditions on a 5% polyacrylamide gel. The caldesmon concentration was 0.2 mg/ml, and that of azolectin was 0, 0.06, 0.10, 0.14 and 0.20 mg/ml for the pairs of slots labelled 1–5 respectively. Arrows indicate the positions of isolated caldesmon (CD) and caldesmon–phospholipid complexes (CD–PL).

in turkey gizzard caldesmon (Ikebe & Hornick, 1991) and three sites in mammalian smooth muscle caldesmon (Adam & Hathaway, 1990). The primary structure of these sites corresponds to Ser-587, Ser-600 and Ser-726 of chicken smooth muscle caldesmon (Bryan *et al.*, 1989; Adam & Hathaway, 1990; Ikebe & Hornick, 1991). Knowing that the phospholipid-binding site is located in the *C*-terminal part of caldesmon, we proposed that phosphorylation of caldesmon may affect its interaction with phospholipids (Vorotnikov & Gusev, 1991).

Caldesmon was phosphorylated by protein kinase C in the presence of [γ-^{32}P]ATP; the reaction was stopped by boiling for 3 min and caldesmon remaining in the supernatant was hydrolysed with chymotrypsin. Peptides thus obtained were mixed with a suspension of azolectin and subjected to ultracentrifugation (105000 ***g***, 30 min). Radioactive bands with molecular masses of 45, 40, 23 and 22 kDa detected in the pellet correspond to peptides derived from the *C*-terminal part of caldesmon. The comparison of the Coomassie-stained gel with the corresponding autoradiogram indicated that the peptides co-sedimented with azolectin were less radioactive than the same peptides remaining in the supernatant. The quantitative estimation presented in Table 1 indicates that the specific radioactivity of native caldesmon and of its peptides with molecular masses of 40, 23 and 22 kDa remaining unbound to azolectin is significantly higher than the specific radioactivity of the corresponding protein species that were co-sedimented with azolectin. The data presented indicate that phosphorylation decreases the affinity for azolectin both of native caldesmon and especially of its short *C*-terminal peptides of molecular mass 40, 23 and 22 kDa.

To obtain independent evidence on the effect of phosphorylation on caldesmon–phospholipid interactions, we phosphorylated caldesmon with the Ca^{2+}/phospholipid-independent form of protein kinase C and investigated the interaction of phosphorylated caldesmon with azolectin by means of electrophoresis under non-denaturating conditions. Fig. 4 indicates that phosphorylated and unphosphorylated caldesmon had identical electrophoretic mobilities. Addition of small quantities of azolectin led to the formation of a protein band having a lower electrophoretic mobility than isolated caldesmon. This band, representing the caldesmon–phospholipid complex, was formed only in the case of unphosphorylated caldesmon (see Fig. 4). At a high concentration of azolectin its complex with caldesmon has a very low electrophoretic mobility, and is located at the top of the gel. Even in this case (Fig. 4, lane 4) the quantity of protein remaining unbound was larger in the case of phosphorylated compared with unphosphorylated caldesmon. Thus the data presented indicate that phosphorylation with protein kinase C decreases the ability of caldesmon to interact with phospholipids.

DISCUSSION

There are many cytoskeletal and contractile proteins which are able to interact with phospholipids and biological membranes. In order to determine the phospholipid-binding site of caldesmon, we compared the structure and properties of caldesmon with those of some other phospholipid-binding proteins.

Certain cytosolic proteins which are able to interact with membranes (myosin IB, fodrin α-chain, phospholipase C, etc.) possess a common motif (the so-called A-box) in their primary structure (Rodaway *et al.*, 1989). Unfortunately, we were unable to find an A-box in the primary structure of caldesmon. The

Table 2. Putative calmodulin- and phospholipid-binding sites of caldesmon

The sequences were aligned with the sequences of calmodulin-binding peptides of myosin light chain kinases (Lin *et al.*, 1988), adenylate cyclase and Ca^{2+}/calmodulin-dependent protein kinase (Takagi *et al.*, 1989), and neuromodulin and neuroregulin (Houbre *et al.*, 1991). **Bold** letters represent identical or similar residues. The last line represent a consensus sequence, where ϕ are hydrophobic residues, + denotes positively charged residues and X denotes variable residues.

Caldesmon (residues 446–459)	**M-K-S-V-W-D-R-K-R-**G-**V-**P-E-Q
Caldesmon (residues 655–668)	**I-K-S-M-W-E-K-**G-N-**V-F-**S-**S-P**
Caldesmon (residues 718–731)	K-**R-N-L-W-E-K-**Q-S-**V-**E-K-P-**A**
Skeletal muscle myosin light chain kinase (residues 341–354)	**M-K-**R-**R-W-**K-**K-**N-F-**I-A-V-S-A**
Smooth muscle myosin light chain kinase (residues 493–506)	**A-R-**R-**K-W-Q-K-**T-G-**H-A-V-**R-**A**
Adenylate cyclase (residues 279–292)	**V-R-N-A-L-N-R-R-A-H-A-V-G-A**
Protein kinase IIβ (residues 341–354)	**A-K-S-L-L-N-K-K-A-**D-G-**V-**K-P
Neuromodulin (residues 42–55)	**F-R-G-H-I-**T-**R-K-K-L-K-**G-**G-**R
Neuroregulin (residues 35–48)	**F-R-G-H-M-**A-**R-K-K-I-K-**S-**G-**G
Consensus	ϕ + X ϕ ϕ X + + X ϕ ϕ X X X +

observed competition between calmodulin and phospholipids for the interaction with caldesmon might indicate that the sites of interaction of caldesmon with phospholipids and calmodulin are close to each other. Our experimental data indicate that both of these sites are located in a short (40 kDa) *C*-terminal portion of caldesmon. This part of the caldesmon molecule includes three Trp-containing peptides with rather similar primary structure (Table 2). The data of Takagi *et al.* (1989) indicate that peptides containing residues 446–459 and 718–731 of caldesmon are able to interact with calmodulin, whereas the data of Wang *et al.* (1991) indicate that a short peptide restricted by residues 659 and 666 is directly involved in the interaction with calmodulin.

Takagi *et al.* (1989) have shown that caldesmon-derived peptides containing residues 446–459 and 718–731 have sequence similarities to the weak calmodulin-binding sites of the calmodulin-dependent protein kinase and of *Bordetella pertussis* adenylate cyclase. On the other hand, these peptides of caldesmon are similar to the calmodulin-binding sites of neuromodulin and neuroregulin (Takagi *et al.*, 1989; Houbre *et al.*, 1991). Moreover, phospholipids also interact with these sites (Houbre *et al.*, 1991). This means that calmodulin competes with phospholipids for the interaction with neuromodulin and neuroregulin (Houbre *et al.*, 1991). In this respect, neuromodulin and neuroregulin are similar to caldesmon. Thus we may suppose that the three above-mentioned peptides of caldesmon can interact with both phospholipids and calmodulin.

As mentioned above, the putative phospholipid-binding sites are located in the *C*-terminal part of caldesmon in close vicinity to both the calmodulin- and actin-binding sites. Since the interaction of many actin-binding proteins (profilin, cofilin, destrin, gelsolin) with actin is affected by phospholipids (Kwiatkowski *et al.*, 1989; Goldschmidt-Clermont *et al.*, 1990; Yanezawa *et al.*, 1990); it will be interesting to investigate the effect of phospholipids on the interaction of caldesmon with actin.

Caldesmon is a good substrate for protein kinase C (Vorotnikov *et al.*, 1988*b*; Adam & Hathaway, 1990). It was proposed that phosphorylation of some proteins by protein kinase C depends on their ability to form tight complexes with phospholipids (mainly with phosphatidylserine) (Bazzi & Nelsestuen, 1987) and the site of phosphorylation is close to the phospholipid-binding site. This seems to be true for caldesmon. It is interesting to mention that one of the potential calmodulin- and phospholipid-binding site of caldesmon, i.e. the peptide containing residues 718–731, includes Ser-726, which is phosphorylated by protein kinase C. After phosphorylation the net positive charge of this peptide will be diminished and this will lead to a decrease in the electrostatic interaction with negatively charged phospholipids. Similar effects were recently described for neuromodulin, which is able to interact with calmodulin and phospholipids and is phosphorylated by protein kinase C (Houbre *et al.*, 1991).

There are two contradictory reports on the effect of caldesmon phosphorylation on its interaction with calmodulin. Tanaka *et al.* (1990) found that phosphorylation by protein kinase C decreases the affinity of caldesmon for both calmodulin and actin. On another hand, we failed to observe any effects of caldesmon phosphorylation on its interaction with calmodulin (Vorotnikov & Gusev, 1991). This contradiction can be resolved taking into account the ability of caldesmon to interact with phospholipids. Tanaka *et al.* (1990) used rather low concentrations of phosphatidylserine. Under these conditions phospholipids were weak competitors for calmodulin. Therefore Tanaka *et al.* (1990) measured mainly the effect of phosphorylation on the calmodulin–caldesmon interaction. In our work (Vorotnikov & Gusev, 1991), we used a higher concentration of mixed phospholipids (azolectin). Under these conditions a large portion of the caldesmon formed a complex with phospholipids and therefore it was difficult to observe a direct effect of phosphorylation on caldesmon–calmodulin interactions. It is worthwhile to mention that there is an unexpected dependence of the caldesmon–calmodulin interaction on the extent of caldesmon phosphorylation in the paper of Tanaka *et al.* (1990). The maximal decrease in caldesmon–calmodulin interaction was observed after incorporation of 1 mol of phosphate per mol (89 kDa) of caldesmon. Further phosphorylation, up to 1.6–2.1 mol of phosphate per mol of protein led to an increase in the affinity of caldesmon for calmodulin. This complicated pattern may be partly due to the fact that phosphorylation affects both caldesmon–calmodulin and caldesmon–phospholipid interactions.

In summary, we may conclude that caldesmon is able to interact with phospholipids, and this interaction is affected by calmodulin and depends on caldesmon phosphorylation. Since both light and heavy isoforms of caldesmon are often located close to the cell membrane (Sobue *et al.*, 1988; Walker *et al.*, 1989; Yamakita *et al.*, 1990), they can be readily phosphorylated by protein kinase C. Previously published data (Burgoyne *et al.*, 1986; Burgoyne, 1991) indicate that caldesmon belongs to the family of proteins interacting with chromaffin granules, and that protein kinase C and calmodulin may be involved in the process of exocytosis. Thus we may conclude that phosphorylation of caldesmon by protein kinase C plays an important physiological role in different cellular events, such as receptor capping or exocytosis.

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