Interaction of smooth muscle calponin with phospholipids

Natalia V. Bogatcheva, Nikolai B. Gusev*

Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, Russian Federation

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Abstract **Analyzing the primary structure we predicted that calponin may interact with phospholipids. In order to check this suggestion we investigated the interaction of calponin with phospholipids by ultracentrifugation, light scattering, vesicles leakage and differential scanning calorimetry. In agreement with our prediction calponin interacts with acidic phospholipids and the phospholipid-binding site was located in the short (13 kDa) N-terminal chymotryptic peptide of calponin. The apparent dissociation constant of calponin-phospholipids complex was less than 0.2 μM and calmodulin competes with phospholipids for calponin binding. Although the interaction of calponin with phospholipids decreases at high ionic strength, calponin binds phospholipids even in the presence of 100–150 mM of the salt. Under certain conditions calponin induced leakage of phospholipid vesicles and affected the cooperativity of lipid phase transition. It is concluded that both electrostatic and hydrophobic interactions provide for calponin-phospholipid complex formation.**

Key words: Calponin; Phospholipid; Calmodulin; Differential scanning calorimetry; Light scattering; Ultracentrifugation

1. Introduction

Calponin is an actin- and calmodulin-binding protein detected in smooth muscle and a number of non-muscle cells [1–3]. Calponin is supposed to be involved in the regulation of smooth muscle contraction [4,5]. On the other hand a rather high content and wide distribution of calponin among different tissues may indicate that this protein also plays other functional roles. Calponin was detected in dense bodies and adhesion plaques of smooth muscle cells [6]. Stimulation of vascular smooth muscle is accompanied by redistribution of calponin and its accumulation close to the cell membrane [7]. Calponin was detected in the cytoskeletal domain of smooth muscle [6] and calponin-containing actin filaments were located just under the cell membrane [2]. All these facts may indicate that calponin plays an important structural role and like many actin-binding proteins (such as α-actinin, filamin, gelsolin, profilin, caldesmon) may interact with phospholipids and by this means anchor actin filaments to the cellular membrane.

Analyzing the primary structure of calponin we found that the segment restricted to residues 85–95 may form the so-called surface seeking amphiphilic α-helix which may contribute to the phospholipid binding. Therefore we put forward the hypothesis that calponin may tightly interact with phospholipids [8]. This paper deals with the experimental verification of this hypothesis. By means of ultracentrifugation, light scattering, fluorescence spectrophotometry and differential scanning calorimetry we have shown that calponin interacts with phospholipids with high affinity and that the phospholipid-binding site is located somewhere between residues 7–144 of calponin.

2. Materials and methods

Calponin from duck gizzard and calmodulin from bovine brain were prepared by earlier described methods [1,9]. Limited chymotrypsinolysis of calponin was performed as described by Mezgueldi et al. [10].

Small unilamellar vesicles (SUVs) were prepared by suspending of phospholipids (0.5–10 mg/ml) in appropriate buffer with subsequent sonication (3–5 times, 30 s each) on ice under argon. Synthetic phospholipids (DPPS, DPPE and DSPA) were sonicated under similar conditions at 55°C. For preparation of SUVs filled with calcein (2′,7′-bis[di(carboxymethyl)aminomethyl]fluorescein) phosphatidylserine (bovine brain extract type 5; Sigma) (2.8 mg/ml) was sonicated in 5 mM Tris-HCl (pH 7.5) containing 20 mM of calcein. SUVs were separated from non-encapsulated calcein by gel-filtration on Sephadex G-50 column equilibrated by 5 mM Tris-HCl, containing 100 mM NaCl.

Multilamellar vesicles (MLVs) were prepared as described earlier [11]. Stock solution of DPPS in chloroform/methanol, 2:1 (v/v) mixture was placed in the glass tube and evaporated. The lipid film was dispersed in buffer containing 30 mM HEPES/NaOH (pH 7.3), 45 mM NaCl and 1 mM EGTA by vortexing and equilibrated overnight at 54°C.

Ultracentrifugation of a mixture of phospholipids and calponin (or calponin peptides) was performed at both low and high ionic strength conditions. In the first case the buffer contained 20 mM Tris-HCl, pH 7.4, 6 mM mercaptoethanol, and in the second case this buffer was supplemented by 150 mM NaCl. In all ultracentrifugation experiments performed in the presence of calmudolin the above mentioned buffer contained either 1 mM EGTA or 0.2 mM $CaCl_2$. Calponin (or its peptides) (0.2–0.8 mg/ml) was mixed with SUVs of phospholipids (1–2 mg/ml) and after incubating for 10–15 min at room temperature was subjected to ultracentrifugation at 120,000 × *g* for 1 h at 20°C. Protein composition of the supernatant and pellet was analyzed by SDS-gel electrophoresis [12].

Light scattering was measured on a Hitachi F-3000 spectrofluorometer. 1 ml of phospholipid suspension (SUVs at the concentration of 8–40 μM) in a buffer (20 mM Tris-HCl, pH 7.4, containing different concentrations of KCl ranging from 0 to 100 mM) was titrated with calponin solution in a thermostated cell at 25°C. The total volume of calponin added was less than 50 μl. The excitation (slit 5 nm) and emission (slit 1.5 nm) monochromators were set at 340 nm and the light scattering was registered at 90°. In separate experiments the above mentioned buffer without phospholipids was titrated by calponin and light scattering induced by isolated protein was subtracted from the corresponding value of protein-phospholipid mixture.

Calponin induced leakage of phospholipid vesicles was measured by an enhancement of calcein fluorescence. The suspension of phospholipid vesicles (40 μM of PS) filled with calcein at self-quencing concentration (20 mM) was titrated by calponin in a buffer containing 5 mM Tris-HCl (pH 7.4) and 12–100 mM NaCl. The fluorescence of calponin-

*Corresponding author. Fax: (7) (95) 939 39 55.
E-Mail: Gusev@Gusev.bio.msu.su

Abbreviations: Azo, azolectin; PC, phosphatidylcholine; DPPE, L-α-phosphatidyl-ethanolamine, dipalmitoyl; DPPS, D,L-phosphatidyl-L-serine, dipalmitoyl; DSPA, L-α-phosphatidic acid, distearoyl; PC, phosphatidylcholine; PS, phosphatidylserine; MLVs, multilamellar vesicles; SUVs, small unilamellar vesicles; DSC, differential scanning calorimetry.

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phospholipid mixture was excited at 485 nm (5 nm slit width) and registered at 520 nm (1.5 nm slit width). Calponin induced leakage of phospholipid vesicles is accompanied by liberation of encapsulated calcein and increase of its fluorescence. Calponin induced leakage was expressed as a percentage of a maximal liberation caused by addition of 0.5% sodium cholate to calcein filled phospholipid vesicles.

Calorimetric measurements were performed on a differential adiabatic scanning microcalorimeter DASM-4 (Russia). Samples (0.8 ml) containing 0.2–0.5 mg/ml of DPPS multilamellar vesicles and different concentration of calponin (0–0.3 mg/ml) were injected into the sample cell, preequilibrated at 4°C and scans were performed at a rate 60° C/h. Quantification of the phospholipid content of the sample was achieved by the inorganic phosphate determination. We have not observed any heat capacity transitions when isolated calponin (1.2 mg/ml) was heated from 45 up to 80°C. This means that all transitions observed in this temperature range belong to phospholipids.

3. Results

When isolated calponin (or its proteolytic fragments) were subjected to ultracentrifugation the protein remained in the supernatant. At the same time ultracentrifugation of calponin with azolectin or PS (bovine brain extract type 5) results in cosedimentation of the protein with phospholipids. At low ionic strength about 80% of calponin was cosedimented with Azo, whereas in the presence of 150 mM NaCl about 30% of calponin were detected in the pellet. When the mixture of calponin chymotryptic peptides with phospholipids was subjected to ultracentrifugation the large N-terminal 22 kDa peptide (residues 7–182) was pelleted at both low and high ionic strength, whereas the short C-terminal 13 kDa fragment (residues 183–292) was detected in the pellet only under low ionic strength conditions and was not cosedimented with phospholipids in the presence of 150 mM NaCl (Fig. 1A). After extensive chymotryptic hydrolysis 22 kDa fragment is converted to a shorter N-terminal 13 kDa fragment (residues 7–144) and the C-terminal 13 kDa fragment is completely degraded [10]. In the mixture of deeply hydrolyzed calponin peptides we found that the N-terminal 13 kDa peptide was able to interact with phospholipids and cosedimented with Azo or PS both at low and high ionic strength (Fig. 1A). The data presented indicate that calponin interacts with phospholipids and that in good agreement with our prediction [8] the phospholipid-binding site is located somewhere between residues 7 and 144. If this suggestion is correct then we may expect that calmodulin interacting with the same region of calponin [4,10] will affect the calponin–phospholipid interaction. Indeed we found that calmodulin added at 3- to 6-fold molar excess over calponin significantly diminished its interaction with azolectin or PS in the presence of 0.2 mM $CaCl_2$ (Fig. 1B) and was much less effective in the presence of 1 mM EGTA. Thus results from ultracentrifugation experiments mean that calponin interacts with phospholipids and that this interaction is modulated by calmodulin.

In order to check this assumption by another method we analyzed the calponin-phospholipid interaction by light scattering. Titration of the suspension of SUVs by calponin is accompanied by a large (often more than 3-fold) increase in the light scattering. In order to analyze the mechanism of calponin-phospholipid interaction we plotted $\sqrt{(I/I_0}$ (where I and I_0 are intensities of light scattering in the presence and in the absence of calponin) against calponin/phospholipids weight ratio [13]. Since all experimental points were above the line (1+(calponin/phospholipid)) we conclude that the light scattering induced by calponin is mainly due to the aggregation of phospholipid vesicles induced by calponin. When calponin was added to the suspension of azolectin at low ionic strength the titration curve was sigmoidal (with Hill coefficient close to 2). Increase of ionic strength by addition of 50 mM KCl is accompanied by about 2-fold decrease in the amplitude of the light scattering and

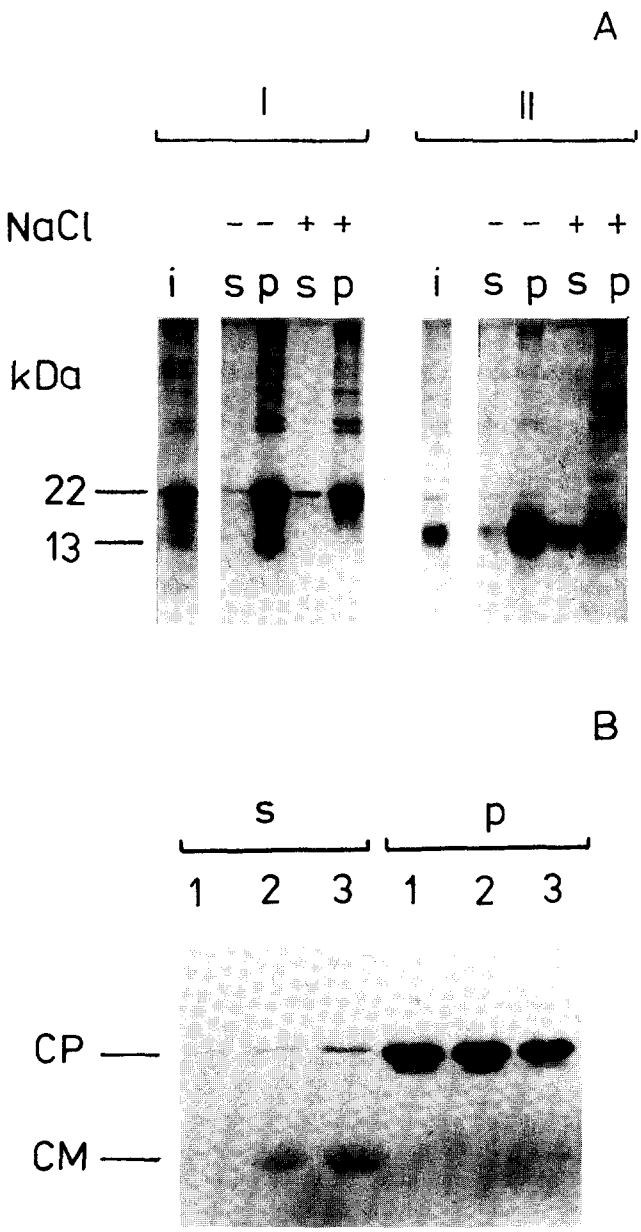

Fig. 1. Interaction of calponin and its proteolytic fragments with azolectin studied by ultracentrifugation. (A) Calponin peptides (0.8 mg/ml) obtained after limited (I) and extensive (II) chymotrypsinolysis were mixed with azolectin (2 mg/ml) and subjected to ultracentrifugation in the absence (–) or in the presence (+) of 150 mM NaCl. Protein composition of initial mixture (i), supernatant (s) and pellet (p) was analyzed by SDS-gel electrophoresis. (B) Effect of calmodulin on the interaction of calponin with azolectin. Calponin (0.24 mg/ml) was mixed with azolectin (2 mg/ml) in the absence (1) or in the presence of 0.36 (2) or 0.72 mg/ml (3) of calmodulin in low ionic strength buffer containing 0.2 mM $CaCl_2$. Protein composition of supernatants (s) and pellets (p) obtained after ultracentrifugation was analyzed by SDS-gel electrophoresis. Positions of calponin (CP) and calmodulin (CM) are marked by arrows.

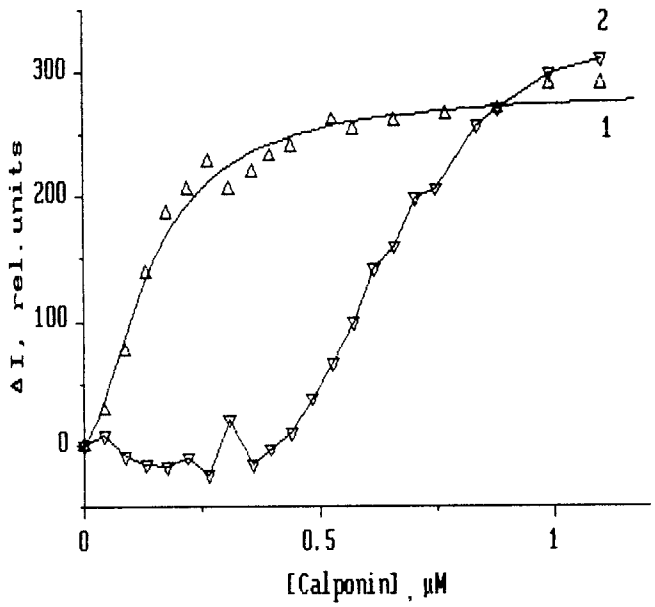

Fig. 2. Effect of calmodulin on calponin induced light scattering of azolectin vesicles. SUVs of azolectin (40 μM) in the buffer containing 20 mM Tris-HCl pH 7.4, 0.1 mM $CaCl_2$ were titrated by calponin in the absence (curve 1) or in the presence (curve 2) of 6.5 μM of calmodulin. The increase of the light scattering at 340 nm is plotted against the total calponin concentration.

decrease of the Hill coefficient. A further increase of the ionic strength (up to 100–150 mM) does not significantly affect the calponin-phospholipid interaction. These data mean that although electrostatic forces play an important role in calponin-phospholipid interaction, calponin may interact with phospholipids even at rather high ionic strength.

If this conclusion is correct we might expect that divalent cations will also affect calponin-phospholipid interaction. We found that addition of 0.1 mM $CaCl_2$ decreases the amplitude of the calponin-induced increase of the light scattering of azolectin vesicles and a half-maximal increase in the light scattering was observed at 0.15–0.20 μM of calponin (Fig. 2). When calmodulin was present in the incubation mixture, increase in the light scattering was observed at much higher concentration of calponin (0.6–0.7 μM) (Fig. 2). This agrees with our results obtained by ultracentrifugation and means that calmodulin may compete with phospholipids for interaction with calponin.

By using light scattering we compared the ability of different phospholipids to interact with calponin. Calponin induced only a small increase in the light scattering of amphyphilic phospholipids such as DPPE (Fig. 3), PC (data not shown) or of a mixture of predominantly neutral phospholipids (such as Azo) (Fig. 3). Calponin induced much higher effect on the light scattering of SUVs composed of DPPS or DSPA (Fig. 3). These experiments were performed in the presence of 100 mM KCl, and even under these conditions half-maximal increase in the light scattering was observed at calponin concentration less than 0.5 μM. This fact indicates a high affinity of calponin towards phospholipids.

In our previous publication [8] we supposed that calponin may interact with phospholipids by its amphyphilic α-helix formed by residues 85–95. If this suggestion is correct one can expect that calponin will affect the structure of the phospholipid bilayer. In order to check this suggestion we analyzed the effect of calponin on the leakage of SUVs filled with calcein and investigated effect of calponin on the DSC thermogram of DPPS MLVs. When SUVs filled with 20 mM calcein were titrated by calponin under hyper- or iso-osmotic conditions we were unable to observe their leakage. SUVs are rather resistant to the osmotic shock. Therefore isolated SUVs filled by 20 mM calcein retained their integrity even under low ionic strength conditions (5 mM Tris-HCl, 12 mM NaCl). Under these conditions addition of a very small quantities of calponin (less than 0.1 μM) induced the liberation of more than 60% calcein entrapped by 40 μM phosphatidylserine vesicles. In this case the efficiency of calponin seems to be increased by the difference in osmotic pressure inside and outside of vesicles. This effect observed under low ionic strength conditions has no physiological implications. Nevertheless, the leakage caused by calponin under certain conditions indicates that this protein is able to disturb the integrity of the phospholipid bilayer.

In complete agreement with this conclusion we found that calponin significantly affects the gel–liquid–crystal transition of DPPS MLVs. There were no peaks of thermal transitions when isolated calponin (1.2 mg/ml) was heated from 45 up to 80°C. Only one main sharp thermal transition around 54°C was observed for isolated DPPS MLVs (Fig. 4, curve 1). Addition of increasing quantities of calponin is accompanied by the appearance of a shoulder at a lower temperature and a decrease in the amplitude of the main phospholipid transition. (Fig. 4, curves 2,3). At the same time the transition enthalpy (ΔH_{cal}) was only slightly decreased from 31.5 kJ/mol for pure lipids to 30.1 kJ/mol at 1/20 protein/lipids molar ratio. A relative measure for the cooperativity of lipid system is the value of half-width ($\Delta T_{0.5}$) of the phase transition, i.e. the width at the half-height of the main transition. This value was equal to 2.1° for pure

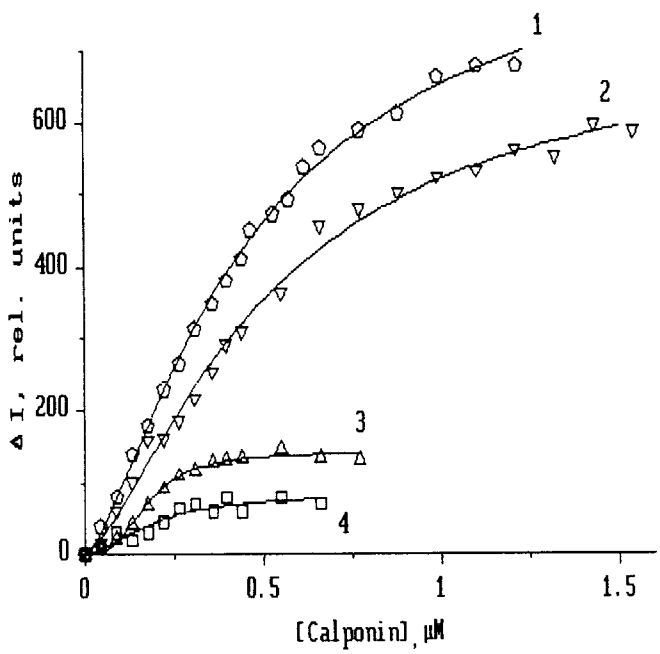

Fig.3. Interaction of calponin with different phospholipids measured by light scattering. Suspension of SUVs of DSPA (8 μM) (1), DPPS (8 μM) (2), Azo (40 μM) (3) or DPPE (40 μM) (4) in the buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM KCl was titrated by calponin and the increase of the light scattering (in relative units) was plotted against the total calponin concentration.

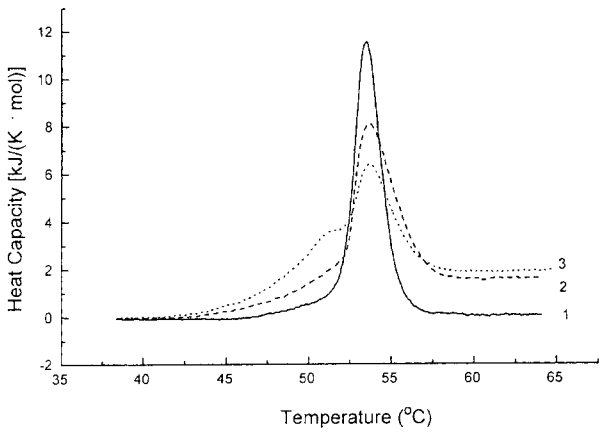

Fig. 4. DSC thermograms of pure DPPS multilamellar vesicles (1) and of the same vesicles containing calponin (2,3). The molar ratio calponin/phospholipid were equal to 0 (curve 1), 1/40 (curve 2) and 1/20 (curve 3). All experiments were performed in the buffer containing 30 mM HEPES/NaOH, pH 7.3, 45 mM NaCl, 1 mM EGTA.

DPPS and was increased up to 5.75° at protein/lipid molar ratio equal to 1/20. The decrease in sharpness of the main phase transition may indicate that calponin partially penetrates into and perturbs lipid bilayer.

4. Discussion

The data of this paper indicate that calponin interacts with different phospholipids. Acidic phospholipids are preferentially bound by calponin and this interaction is characterized by high affinity. Formation of a calponin–phospholipid complex is ionic strength-dependent, but even in the presence of 100–150 mM salt calponin interacts with phospholipids.

Using limited proteolysis we have found that the phospholipid-binding site is located somewhere between residues 7 and 144 of calponin. Since calmodulin competes with phospholipids for calponin binding we may suggest that the phospholipid-binding site is restricted to residues 52–144. This completely correlates with our prediction [8] that the amphyphilic α-helix formed by residues 85–95 can participate in phospholipid binding.

The interaction of calponin with phospholipids is accompanied by aggregation of phospholipid vesicles. This aggregation seems to be at least partly due to the electrostatic interaction of positively charged calponin with negatively charged phospholipids. At the same time this interaction was not completely abolished by the addition of 100–150 mM of the salt, therefore it cannot be completely nonspecific.

Under certain conditions calponin induces the leakage of phospholipid vesicles. This means that calponin may affect the integrity of phospholipid bilayer. Calponin decrease enthalpy and sharpness of the main thermal transition of DPPS. In this respect calponin is similar to myelin basic protein and cytochrome *c* [14]. All these proteins induce fluidization of the bilayer which is caused by a partial penetration of certain protein segments into the bilayer interior. Summing up, we may conclude that calponin interacts with phospholipids with high affinity and is able to affect both the outer and inner phases of phospholipid bilayer.

When this paper was already for publication we found the report of Fujii et al. [15] dealing with the same problem. By using low speed centrifugation and affinity chromatography these authors have shown that calponin preferentially interacts with acidic phospholipids, that this interaction is strongly dependent on the presence of $MgCl_2$, is completely abolished at 150 mM NaCl, and is significantly affected by actin and calmodulin. Thus, the main conclusions of Fujii et al. [15] correlate with our findings. On the other hand in a sharp difference to Fujii et al. [15] we have found that calponin interacts with phospholipids even at high ionic strength and that this interaction occurs even in the absence of magnesium. We defined the phospholipid-binding site of calponin more precisely and have shown that calponin is able to perturb the properties of the whole phospholipid bilayer. The physiological significance of calponin–phospholipid interaction will need further investigation.

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