Does Calponin Interact with Caldesmon?*

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The roles of calponin and caldesmon and their interaction in regulation of smooth muscle contraction are controversial. Recently, strong binding between these two proteins has been reported (Graceffa, P., Adam, L. P., and Morgan, K. G. (1996) J. Biol. Chem. 271, 30336–30339). Results in this paper fail to confirm their data and are consistent with the concept of independent functions for calponin and caldesmon.

To examine the ability of duck gizzard caldesmon to interact with calponin, three caldesmon derivatives, each containing a different sulfhydryl-specific reporter probe (6-acryloyl-2-dimethylaminonaphthalene, N-(1-pyrenyl)iodoacetamide, and N-iodoacetetyl-N'-5-sulfo-1-naphthyl)ethylenediamine) attached to a single cysteine located in the C-terminal domain, were synthesized. Addition of calponin to labeled caldesmon at both low and physiological salt concentrations did not induce any changes in fluorescence intensity or maximum shift. Under the same conditions, calmodulin and tropomyosin (known to bind to the C terminus of caldesmon) produced substantial changes in these spectral parameters. Gel filtration of an equimolar caldesmon-calponin mixture on a fast protein liquid chromatography Superose-12 column revealed two baseline-separated peaks, the first containing only caldesmon and the second only calponin, thus confirming the lack of any interaction between these two proteins. Also, the addition of calponin did not change the fluorescence parameters of labeled caldesmon in complexes with F-actin and F-actin-tropomyosin.

The primary mechanism for smooth muscle regulation, necessary to initiate contraction, involves phosphorylation of the 20-kDa myosin light chains by a specific Ca2+/calmodulin-dependent myosin light chain kinase (for reviews see Refs. 1–3). It is postulated that the specific thin filament-associated proteins caldesmon and calponin take part in the secondary mechanism of regulation, probably during the relaxation phase. This view is supported by in vitro experiments showing that both of these proteins inhibit actin-activated ATPase activity of myosin, mobility of actin filaments over immobilized myosin in motility assay, and skinned muscle contraction. This inhibition can be reversed by Ca2+/calmodulin, or other Ca2+-binding proteins (like S100 or caltrpin), and by phosphorylation with protein kinase C or casein kinase II (Refs. 3–6 and references therein).

Studies by Makuch et al. (7) revealed that calponin and caldesmon compete for the binding sites on actin filament, calponin being more effective at displacing caldesmon than vice versa. Similar results were obtained in the Chalovich laboratory (8). This suggested that calponin and caldesmon do not form a mutual complex on actin and reside on different populations of thin filaments in vivo. Immunofluorescence and immunogold electron microscopy confirmed this suggestion showing that, whereas caldesmon is present exclusively in the contractile domain, calponin is primarily, although not exclusively, located in the cytoskeletal domain of chicken gizzard muscle (9, 10). This result implied that calponin and caldesmon are segregated in different thin filaments, as postulated earlier (11) on the basis of immunoprecipitation by anti-caldesmon and anti-filamin antibodies of the two different subsets of thin filaments.

In view of these results, the results showing a strong, specific interaction of calponin and caldesmon (12) were unexpected. Moreover, a very weak association of these two proteins was earlier reported and was considered to be too weak to affect the function of each of the two proteins (13). These controversial results (12) sparked our interest in resolving the problem.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—Caldesmon was prepared from duck gizzards according to the method of Bretscher (14) with some modifications (15). Calponin was purified from chicken gizzard by the procedure of Takahashi (16). Chicken gizzard tropomyosin was obtained as described earlier by Dąbrowska et al. (17). Rabbit skeletal muscle actin was isolated from acetone-dried muscle powder and purified as described by Spudich and Watt (18). Calmodulin was isolated from bovine brain by the method of Gopalakrishna and Anderson (19).

Caldesmon Labeling—Labeling of caldesmon cysteine with acrylodan1 (Molecular Probes) was carried out as described by Graceffa et al. (12) at a probe to caldesmon molar ratio of 5. The degree of labeling determined using the acrylodan molar extinction coefficient of 1.29 × 104 M−1 cm−1 at 360 nm (20) was 0.70–0.90 mol of acrylodan/mol of caldesmon.

Labeling of caldesmon with pyrenyl (Molecular Probes) was carried out according to Koyama and Mihashi (21) at a probe to caldesmon molar ratio of 4.5. The degree of labeling determined using the pyrenyl molar extinction coefficient of 2.2 × 104 M−1 cm−1 at 344 nm was 0.57–0.63 mol of pyrenyl/mol of caldesmon.

Labeling of caldesmon with AEDANS (Sigma) was performed as described in Tawada et al. (22) at a probe to caldesmon molar ratio of 7.5. The degree of labeling determined using the AEDANS molar extinction coefficient of 0.61 × 104 M−1 cm−1 at 337 nm (23) was 0.50–0.65 mol of AEDANS/mol of caldesmon.

Each labeling procedure was repeated twice for different caldesmon preparations. Labeling reactions were stopped by the addition of excess of DTT and followed by an exhaustive prolonged dialysis with fluorescence control of the diastase (80%) mixture with ethanol (20%) for the presence of the DTT derivative of each dye. The actomyosin ATPase activity assay (7) was used to control whether the described

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1 The abbreviations used are: acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; pyrenyl, N-1-pyrenyl)iodoacetamide; AEDANS, N'-iodoacetyl-N'-5-sulfo-1-naphthyl)ethylenediamine; DTT, dithiothreitol; PAGE, polyacylamide gel electrophoresis; FPLC, fast protein liquid chromatography.
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RESULTS AND DISCUSSION

Duck gizzard caldesmon is a single thiol protein; its Cys residue is located in the C-terminal part of the molecule at a position corresponding to 580 in the amino acid sequence of chicken gizzard caldesmon (30). Since the C-terminal sequences of avian isoforms of caldesmon are similar (31), and the site of the interaction between caldesmon and calponin was ascribed to just its C-terminal domain (12), duck gizzard caldesmon appeared to be convenient for fluorescent studies after modification with sulphydryl-specific reporter probes.

As shown in Fig. 1, the fluorescence spectrum of acrylodan-duck gizzard caldesmon adduct has a very broad peak with a maximum at 517 nm. This means that the acrylodan label is located in a polar environment (20). The fluorescence maximum does not alter upon a change of excitation wavelength from 360 to 390 nm. Our spectra were blue shifted by 5 nm as compared with those reported for acrylodan-labeled porcine stomach caldesmon (12). According to our observations, the DTT derivative of acrylodan could only be removed after an extensive and prolonged dialysis possibly because it fits into the molten globules located at both ends of the caldesmon molecule (32). An insufficient volume of dialysis buffer may be an explanation why Graceffa et al. (12) obtained a labeling ratio of up to 1.1 for porcine stomach caldesmon which is unexpected for a single thiol protein, as discussed in (20).

Addition of calponin to acrylodan-labeled caldesmon (at a 1:1 molar ratio) did not affect its fluorescence spectrum at 50 and 150 mM NaCl (Fig. 1). Under the same conditions, calmodulin, a protein with a similar hydrophobicity as calponin, induced marked changes in fluorescence spectra parameters (intensity and maximum blue shift) of acrylodan-labeled caldesmon. It is also noteworthy that the calponin to caldesmon binding constant (12) is of the same range as we observed earlier for the calmodulin-caldesmon complex (15). Significant changes in acrylodan-labeled caldesmon were also evoked by tropomyosin (data not shown) though its binding constant to caldesmon is 2 orders of magnitude lower (15) than that reported for the calponin-caldesmon complex (12). The shape of the fluorescence spectrum of AE-DANS-labeled caldesmon is very similar to that presented in Fig. 1. In this case, we also did not observe any effect of calponin on the caldesmon spectra (data not shown).

The shape of the fluorescence spectrum of pyrenyl-labeled duck gizzard caldesmon (Fig. 2) differs from the fluorescence spectrum of pyrenyl-protein adducts often presented in the

![Image](image.png)

**Fig. 1.** Fluorescence spectra of acrylodan-labeled duck gizzard caldesmon in the absence and presence of calponin or calmodulin. Labeled caldesmon itself (1 μM, dotted line) and upon addition of 1.1 μM calponin (CaP) or calmodulin (CaM) (solid lines). Spectra were measured at 20 °C in buffer T (see “Experimental Procedures”) containing 50 mM (A) or 150 mM (B) NaCl. Excitation wavelength was 380 nm; both slits were adjusted to 5 nm.

**Fig. 2.** Fluorescence spectra of pyrenyl-labeled duck gizzard caldesmon in the absence and presence of calponin or calmodulin. Caldesmon-pyrenyl adduct itself (1 μM, dotted line) and upon addition of 1.1 μM calponin (CaP) or calmodulin (CaM) (solid lines). Excitation wavelength was 342 nm. Other conditions were as in Fig. 1. A, 50 mM NaCl; B, 150 mM NaCl.
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Fig. 3. Elution profiles of calponin-caldesmon mixture from gel filtration FPLC Superose-12 column. Calponin-caldesmon mixture (1.7 μmol each), in 200 μl of buffer T containing 50 (curve a) or 150 (curve b) mM NaCl, was applied on the column and eluted with loading buffer. To make the picture clearer, curve b was shifted up by 0.01 absorbance unit. Flow rate was 0.1 ml/min, and fraction size was 0.5 ml.

Inset, 10% SDS-PAGE gel of the peaks eluted from the column; the last lane represents the caldesmon-caldesmon mixture applied on the column.

The fluorescence studies indicate that calponin does not induce any conformational changes in the spatial vicinity of the probe attachment to the caldesmon molecule and points to a lack of an interaction between calponin and this part of caldesmon.

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Table I

| Sample composition      | 50 mM NaCl | 150 mM NaCl |
|------------------------|------------|-------------|
|                        | λ<sub>max</sub> | Q<sub>rel</sub> | λ<sub>max</sub> | Q<sub>rel</sub> |
| CaD                    | 517        | 0.41        | 517        | 0.56        |
| CaD + Ac (1:7)         | 490        | 1.00        | 500        | 1.00        |
| CaD + Ac + TM (1:7:1)  | 489        | 0.93        | 493        | 1.01        |
| CaD + Ac + TM + CaP (1:7:1:1) | 490 | 0.93        | 499        | 0.93        |
| CaD + Ac + CaP (1:7:1) | 493        | 0.90        | 503        | 0.94        |
| CaD + Ac + CaP + TM (1:7:1:1) | 490 | 0.92        | 501        | 0.97        |
| CaD + Ac + CaP + TM (1:7:1) | 488 | 1.02        | 499        | 1.06        |
| CaD + Ac (1:14)        | 489        | 1.00        | 499        | 1.00        |
| CaD + Ac + TM (1:14:1) | 488        | 1.01        | 498        | 1.01        |
| CaD + Ac + TM + CaP (1:14:1:1) | 489 | 1.00        | 499        | 0.95        |
| CaD + Ac + TM + CaP (1:14:1:1) | 489 | 1.00        | 499        | 0.95        |
| CaD + Ac + CaP (1:14:1) | 490        | 0.92        | 500        | 0.94        |
| CaD + Ac + CaP + TM (1:14:1:1) | 489 | 1.01        | 498        | 0.93        |
| CaD + Ac + CaP + TM (1:14:1) | 488 | 1.02        | 497        | 0.96        |

The fluorescence studies indicate that calponin does not induce any conformational changes in the spatial vicinity of the probe attachment to the caldesmon molecule and points to a lack of an interaction between calponin and this part of caldesmon. This confirms our fluorescence data and extends the conclusion about the lack of an interaction with the entire caldesmon molecule.

In earlier work on calponin, Vancompernelle et al. (13) reported that caldesmon bound to a calponin affinity column at 5 mM KCl was eluted at 70 mM KCl. However, calponin is known as a protein extremely sensitive to ionic strength and even able to aggregate at low ionic strength. In our FPLC experiments performed at 20 mM NaCl, calponin was retarded on the column and eluted only after an extensive wash with buffer containing 200 mM NaCl (data not shown). Therefore, one may infer that in the experiment described by Vancompernelle et al. (13), caldesmon was possibly bound to a partially unfolded immobilized calponin and was released when the ionic strength became high enough to restore the native conformation of the immobilized protein.

The fluorescence spectra of acrylodan-labeled caldesmon complexed with F-actin show a 27-nm blue shift and an almost 150% increase in the quantum yield in 50 mM NaCl as well as a 17-nm blue shift and almost double the quantum yield in 150 mM NaCl when compared with the spectra of acrylodan-labeled caldesmon alone under the same conditions (Table I). These dramatic changes did not depend practically on the molar ratio of the proteins in the range studied. They might be caused by the acrylodan label transfer from a strongly polar (the surface of caldesmon molten globules) to a strongly hydrophobic (F-actin interior) environment. Binding of tropomyosin to this complex results in a further small (1–3 nm) blue shift and a very small increase in the quantum yield (see Table I). These small changes of the fluorescence parameters may be due to the structural changes in F-actin induced by the binding of tropomyosin and/or direct interaction of tropomyosin with caldesmon when bound to F-actin. Calponin binding to F-actin-caldesmon or F-actin-caldesmon-tropomyosin complexes causes a small (1–2 nm) red shift and a negligible decrease in the quantum yield. The largest decrease (10%) in the quantum yield was observed in the case of calponin binding to the F-actin-caldesmon complex (7:1 molar ratio) in 50 mM NaCl (see Table I). Conceivably this decrease might reflect the structural changes induced in F-actin by calponin binding; higher ionic strength or binding of tropomyosin reduces it by half. The structural changes in F-actin induced by calponin binding are rather local because they are ineffective when the quantity of F-actin in solution is doubled.

Taken together, the results presented above empower us to give a negative answer to the question “Does calponin interact with caldesmon?”

Note Added in Proof—In agreement with our findings, Drs. Graceffa, Adam, and Morgan have communicated to us that they have further verified a lack of strong interaction between caldesmon and calponin by analytical ultracentrifugation in collaboration with Dr. Walter Stafford.
| REFERENCES                                                                 |                                                                 |
|-------------------------------------------------------------------------|-------------------------------------------------------------------|
| 1. Somlyo, A. P., and Somlyo, A. V. (1994) Nature 372, 231–236           |                                                                 |
| 2. Allen, B. G., and Walsh, M. P. (1994) Trends Biochem. Sci. 19, 362–368|                                                                 |
| 3. Horowitz, A. Menice, C. B., Laporte, R., and Morgan, K. G. (1996)     |                                                                 |
| Physical Rev. 76, 967–1003                                              |                                                                 |
| 4. Marston, S. B., and Redwood, C. S. (1991) Biochem. J. 270, 1–16       |                                                                 |
| 5. Dąbrowska, A. (1994) in Airways Smooth Muscle: Biochemical Control    |                                                                 |
| of Contraction and Relaxation (Raeburn, D., and Giembycz, M. A., eds)  |                                                                 |
| pp. 31–59, Birkhauser Verlag, Basel, Switzerland                        |                                                                 |
| 6. Winder, S. J., and Walsh, M. P. (1990) Curr. Top. Cell. Regul. 34,   |                                                                 |
| 33–61                                                                    |                                                                 |
| 7. Makuch, R., Birkukov, K., Shirinsky, V., and Dąbrowska, R. (1991)    |                                                                 |
| Biochem. J. 280, 33–38                                                  |                                                                 |
| 8. Lu, F. W. M., Freedman, M. V., and Chalovich, J. M. (1995) Biochemistry 34, 11864–11871 |                                                                 |
| 9. North, A. J., Gimona, M., Cross, R. A., and Small, J. V. (1994) J. Cell Sci. 107, 437–444 |                                                                 |
| 10. Mabushi, K., Li, Y., Tao, T., and Wang, C-L. A. (1996) J. Muscle Res. Cell Motil. 17, 243–260 |                                                                 |
| 11. Lehman, W. (1991) J. Muscle Res. Cell Motil. 12, 221–224             |                                                                 |
| 12. Graceffa, P., Adam, L. P., and Morgan, K. G. (1996) J. Biol. Chem. 271, 30336–30339 |                                                                 |
| 13. Vancanemofolle, K., Gimona, M., Herzog, M., van Damme, J.,           |                                                                 |
| Vandekerckhove, J., and Small, J. V. (1996) FEBS Lett. 274, 146–150     |                                                                 |
| 14. Bretsher, A. (1984) J. Biol. Chem. 259, 12873–12880                 |                                                                 |
| 15. Czurylo, E. A., Emelyanenko, V. I., Permyakov, E. A., and Dąbrowska, R. (1991) Biophys. Chem. 40, 181–188 |                                                                 |
| 16. Takahashi, K., Hiwada, K., and Kokubu, T. (1986) Biochem. Biophys. Res. Commun. 141, 20–26 |                                                                 |
| 17. Dąbrowska, R., Nowak, E., and Drahiowski, W. (1980) Comp. Biochem. Physiol. 65B, 75–83 |                                                                 |
| 18. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871    |                                                                 |
| 19. Gopalakrishna, R., and Anderson, W. B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836 |                                                                 |
| 20. Prendergast, F. G., Meyer, M., Carlson, G. L., Iida, S., and Potter, J. D. (1983) J. Biol. Chem. 258, 7541–7544 |                                                                 |
| 21. Koyama, T., and Mihashi, K. (1981) Eur. J. Biochem. 114, 33–38        |                                                                 |
| 22. Tawada, K., Staubelin, T., and Gordon, J. (1978) Eur. J. Biochem. 88, 411–419 |                                                                 |
| 23. Hudson, E. N., and Weber, G. (1973) Biochemistry 12, 4154–4169       |                                                                 |
| 24. Laemmli., U. K. (1970) Nature 227, 680–685                           |                                                                 |
| 25. Czurylo, E. A., Venyaminov, S. Yu., and Dąbrowska, R. (1993) Biochem. J. 293, 365–368 |                                                                 |
| 26. Winder, S. J., and Walsh, M. P. (1990) J. Biol. Chem. 265, 10148–10155 |                                                                 |
| 27. Wolff, D. J., Poirier, P. G., Brustrom, C. O., and Brustrom M. A. (1977) J. Biol. Chem. 252, 4188–4117 |                                                                 |
| 28. Lehrer, S. S., Butteridge, D. R., Graceffa, P., Wong, S., and Seidel, J. C. (1984) Biochemistry 23, 1591–1595 |                                                                 |
| 29. Houk, W. T., and Ue, K. (1974) Anal. Chem. 62, 66–74                  |                                                                 |
| 30. Vorotnikov, A. V., and Gusev, N. B. (1991) Biochem. J. 273, 161–163   |                                                                 |
| 31. Collins, J. H., Leszyk, J., Mornet, D., and Audemard, E. (1991) Protein Sequences Data Anal. 4, 29–32 |                                                                 |
| 32. Czurylo, E. A., Hellweg, T., Eimer, W., and Dąbrowska, R. (1997) Biophys. J. 72, 835–842 |                                                                 |
| 33. Crossie, R. H., Chalovich, J. M., and Reisler, E. (1995) J. Muscle Res. Cell Motil. 16, 509–518 |                                                                 |