Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 12-17

INTERACTION BETWEEN CALTROPIN AND THE C-TERMINAL REGION OF SMOOTH MUSCLE CALDESMON§

Shaobin Zhuang[1], Rajam S. Mani[2], Cyril M. Kay[2], and C.-L. Albert Wang[1,*]

[2]M.R.C. Group in Protein Structure and Function, Dept. of Biochemistry, Univ. of Alberta, Edmonton, T6G 2H7, Canada

[1]Muscle Research Group, Boston Biomedical Research Institute, Boston, MA 02114

Received February 5, 1995

SUMMARY: Caltropin (CaT) binds caldesmon (CaD) in a Ca^{2+}-dependent manner with an affinity higher than that of calmodulin (CaM). Photo-crosslinking between CaT and a benzophenone-labeled C-terminal CaD fragment (27K) results in a 35-kDa protein that corresponds to the 1:1 adduct between CaT and 27K. In the absence of Ca^{2+}, no crosslinking is obtained. This result is similar to that obtained with CaM and 27K. The apparent affinity of CaM for GS17C, a CaM-binding peptide of CaD, is weakened by CaT, suggesting CaT competes with CaM for the peptide. In contrast to CaM, CaT does not induce changes in the tryptophan fluorescence of GS17C. Thus although the two Ca^{2+}-binding proteins behave similarly, there are differences in their interactions with CaD.

Caldesmon (CaD) is an actin-binding protein found in smooth muscle and many non-muscle cells (1). Binding of CaD to F-actin inhibits the actomyosin ATPase activity in vitro (2, 3). Inhibition by ~90% is commonly observed with fewer than one CaD molecule bound to every 7 actin monomers (4). In the presence of Ca^{2+}, CaM reverses the inhibitory action of CaD by interacting with CaD and thereby weakening its association with the actin filament (5, 6). The interaction between CaM and CaD, however, is rather weak ($K_d = 10^{-6}$ M); nearly 10-fold molar excess of CaM is required to completely neutralize CaD's effect. It has been suggested that other Ca^{2+}-binding proteins may be responsible for regulating the effect of CaD in vivo. One of such candidates is caltropin (CaT) (7, 8).

CaT is present in significant amount in chicken gizzard ($\geq$ 10 mg/350 g of wet tissue, see 7). It has been found that CaT binds to CaD, as evidenced by the observation that CaD is retained by a CaT-Sepharose affinity column in the presence of Ca^{2+}. Like Ca^{2+}/CaM, Ca^{2+}/CaT reverses the inhibitory effect of CaD on the actomyosin ATPase activity (8) and decreases binding

§ This work was supported by Medical Research Council of Canada and by grants from National Institutes of Health (HL-41411 and AR41637).

* To whom correspondence should be addressed.

0006-291X/95 $5.00

between CaD and smooth muscle heavy meromyosin (9). In contrast to CaM, however, only one mol of CaT per mol of CaD is sufficient to reverse CaD's action. The affinity of CaT to CaD is at least 10 times higher than that between CaM and CaD. Similar interaction has also been found between CaT and calponin (10).

CaT is in fact the smooth muscle isoform of calcyclin (CaCY), which is a member of the S-100 protein family. Like all S-100 proteins, CaCY has two EF-hand type Ca^{2+}-binding sites. CaCY is found in many non-muscle cells, in particular, a tumor-related cell line 2A9 (11, 12). The physiological function of CaCY remains yet unknown. Since its gene is activated in the G_0 to S transition of fibroblast (13) and vascular smooth muscle cells (14), CaCY is thought to be involved in the cell cycle regulation and cytoskeletal assembly. The mRNA level of CaCY is considerably elevated in proliferating human adenocarcinoma cells upon stimulation by phorbol esters, suggesting that CaCY plays a role in the protein kinase C-mediated signaling process (15). In view of the fact that non-muscle CaD is also involved in the process of cytokinesis (16), it is quite possible that CaD's function in these cellular processes is modulated by Ca^{2+}/CaCY. Thus a better characterization of the CaT-CaD interaction in the smooth muscle system may provide a means to elucidate the roles of both proteins in other non-muscle cells.

MATERIALS AND METHODS

Proteins and Peptides: CaT (7) and CaD (17) were isolated from chicken gizzard. CaM was isolated from bovine brain (18). Actin was from rabbit skeletal muscle (19). The recombinant C-terminal fragment (27K) of CaD was expressed by E. coli. as a cII fusion protein; the CaD portion begins from Lys-579 and ends at Pro-756 (20). GS17C, which contains the CaM-binding sequence of CaD (21), was chemically synthesized on an automated peptide synthesizer (ABI 431A) and purified by reversed phase HPLC. Labeling of 27K and GS17C was carried out as follows: The CaD fragment/peptide was first reduced with dithiothreitol, followed by dialysis using a low molecular weight (1000 Da) cut-off dialysis tubing, then reacted with thiol-directed reagent (photo-crosslinker or fluorescence probe), followed by dialysis.

Crosslinking Experiments: Photo-crosslinking was carried out using a Rayonet Photochemical Reactor with a fixed irradiation wavelength of 350 nm.

Fluorescence Spectroscopy: Fluorescence measurements were carried out on a Perkin Elmer MPF-4 fluorometer. For NBD emission, λ_{exc}=490 nm; λ_{em}=540 nm.

Actin Binding Measurements: Co-sedimentation with F-actin was done by centrifugation in a Beckman Airfuge at the speed of 100,000 x g for 30 min.

RESULTS AND DISCUSSION

CaT has been shown previously to reverse the inhibitory effect of CaD on the acto-S1 ATPase activity (8). To test whether the de-inhibition is due to a weakened affinity of CaD for actin, as in the case of CaM, we have examined the binding of CaD to F-actin by co-sedimentation in the presence and absence of Ca^{2+}/CaT. We found that CaT, like CaM, is capable of dissociating CaD from actin filament in the presence of Ca^{2+}. No such an effect was seen when Ca^{2+} was absent (Fig. 1). Thus binding of CaD to F-actin can be modulated by the Ca^{2+}-dependent interaction between CaT and CaD.

To determine the region of interaction between CaT and CaD, we have carried out photo-crosslinking experiments between CaT and a 27-kDa C-terminal fragment of CaD, 27K. The

single cysteine residue (Cys-580) of 27K was labeled with a photo-crosslinker, benzophenone maleimide (BP-Mal). The resulting $27K_{BP}$ was then mixed with CaT in the presence or absence of Ca^{2+}, and irradiated with UV light for 30 min before subjected to SDS-PAGE analysis. Parallel experiments were carried out with CaM. In both cases there was clearly a Ca^{2+}-dependent crosslinking between $27K_{BP}$ and CaT or CaM, as evidenced by the appearance of the higher molecular weight bands in the presence of Ca^{2+}, but not in the absence of Ca^{2+} (Fig. 2). The apparent molecular weights of the crosslinked products were 35 kDa and 43 kDa for the 1:1 complexes of CaT-27K and CaM-27K, respectively. Thus the interactions are very similar between 27K and the two Ca^{2+}-binding proteins, viz. both CaT and CaM bind to 27K (and therefore to CaD) at a site that is topographically near the probe attached to Cys-580.

The interaction between CaT and 27K was further investigated by fluorescence spectroscopy. Initial experiments showed that CaT, unlike CaM, had no effect on the tryptophan fluorescence of 27K regardless of the presence or absence of Ca^{2+}, similar to the case of intact CaD (8). We then labeled 27K with IANBD at the cysteine residue in the hope that binding of CaT would change the emission intensity of the probe. The NBD fluorescence typically undergoes a 5- to 10-fold enhancement upon binding of CaM in the presence of Ca^{2+}. Under the same condition, on the contrary, CaT resulted in no detectable changes in the extrinsic fluorescence intensity (data not shown). This is rather surprising, because the probe is attached to the same residue (Cys-580) which, as indicated by the previous fluorescence titration using acrylodan-labeled CaD (8) and the results of the crosslinking experiment (see above), should be close to the CaT-binding site. One possible explanation for the lack of fluorescence change is that the NBD probe attached to Cys-580

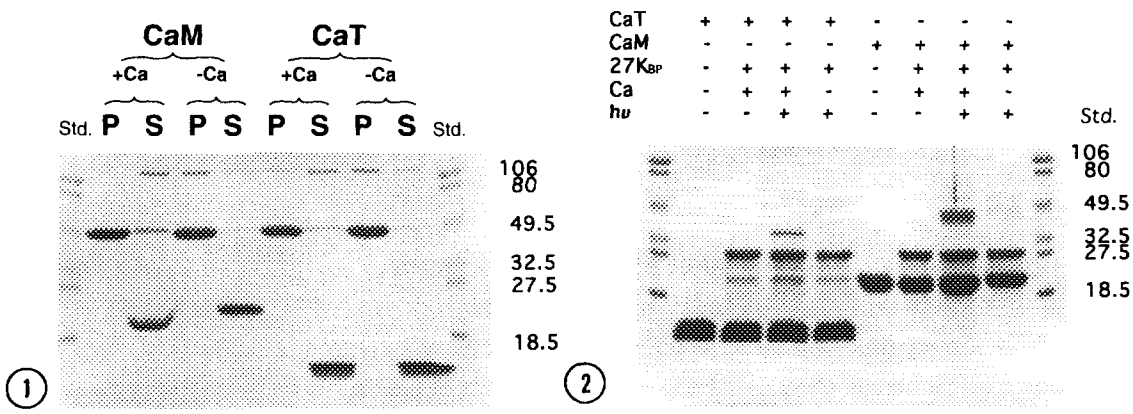

Figure 1. Effect of CaT or CaM on the binding of CaD to F-actin. Chicken gizzard CaD was incubated with F-actin (in 10 mM Tris, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$ and 0.4 mM ATP) and CaT or CaM in the presence and absence of Ca^{2+}, followed by centrifugation. Both the supernatant and pellet fractions were subjected to SDS gel electrophoretic analysis.

Figure 2. Photo-crosslinking between 27K and CaT or CaM. BP-Mal-labeled CaD fragment ($27K_{BP}$) was incubated with either CaT (left panel) or CaM (right panel) in the presence and absence of 1 mM $CaCl_2$. The mixtures were irradiated with UV light for 30 min before addition of DTT to quench the reaction. Aliquots of the reaction mixtures were then taken for SDS gel electrophoretic analysis.

remains exposed to the solvent in the CaT-27K complex as in 27K alone, and therefore, the environment of the probe does not change appreciably, although the BP moiety in $27K_{BP}$ may be sufficiently close to the bound CaT molecule to form a covalent linkage.

We have tried to monitor the CaT-$27K_{NBD}$ interaction indirectly by carrying out fluorescence titrations of $27K_{NBD}$ with CaM in the presence and absence of CaT. When CaT was included in the solution containing $27K_{NBD}$, it required a higher amount of CaM to induce a given level of fluorescence enhancement than in the case where there was no CaT (Fig. 3). The binding of initially added CaM to $27K_{NBD}$ apparently was inhibited by the presence of CaT, suggesting that CaM is indeed competing with CaT for the CaD fragment; this in turn implies that CaT does interact with 27K. We have estimated the binding constants by a nonlinear regression curve fitting program (BIND2), assuming the stoichiometry remains unchanged, to be $7.31 \times 10^6\ M^{-1}$ and $1.75 \times 10^5\ M^{-1}$ for the titrations without and with CaT, respectively. Thus the presence of 5-fold molar excess of CaT over $27K_{NBD}$ resulted in a 40-fold decrease in the apparent affinity between CaM and $27K_{NBD}$, consistent with the previous finding that the intrinsic affinity of CaT for CaD is 5-10 times stronger than that of CaM (8). But since binding of CaT to $27K_{NBD}$ does not increase the fluorescence of the labeled 27K, the mode of interaction with the CaD fragment must be different between CaM and CaT.

We have also carried out fluorescence titrations using a synthetic peptide of CaD, GS17C, as a target molecule for CaT. GS17C has the amino acid sequence corresponding to that of chicken gizzard CaD from Gly-651 to Ser-667. GS17C has been shown to bind CaM and actin (21) and to induce smooth muscle contraction (22). It is therefore reasonable to expect GS17C to bind CaT. Again, titrations of GS17C with CaT did not result in changes in the intensity of

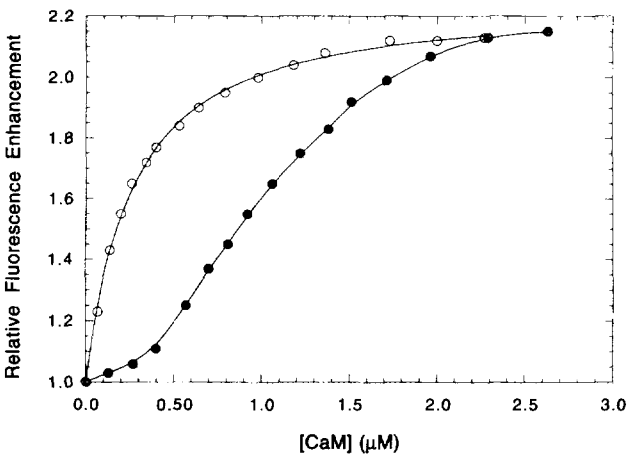

Figure 3. Fluorescence titration of $27K_{NBD}$ (0.2 μM) with CaM in the presence (closed circles) and absence (open circles) of 2 μM CaT (presumably in dimeric form). Conditions: 20 mM Tris (pH 7.5) 0.1 M KI and 1 mM $CaCl_2$. The curve for the titration without CaT was calculated based on the best fit parameters (see text); for the titration in the presence of CaT only a smooth curve was drawn through the data points.

tryptophan fluorescence, in contrast to CaM which induces a large fluorescence enhancement (21), also indicating differences between CaT and CaM in their interaction with the synthetic peptide. Competition experiments also showed the CaM-induced tryptophan fluorescence enhancement being impeded by the presence of CaT (Fig. 4); the apparent binding constant of CaM for GS17C was 3.13 x 10^6 M^{-1} in the absence of CaT, and was decreased to 6.62 x 10^4 M^{-1} when CaT was present, indicating that CaT and CaM compete against each other for the CaD peptide.

It is noteworthy that in the case of $27K_{NBD}$ (Figure 3) the titration curve with pre-added CaT was sigmoidal in shape, indicating some kind of cooperative interactions between CaM and CaT. In view of the fact that there exist two CaM-binding sites (Site A, from Met-658 to Ser-666, and Site B, from Asn-675 to Lys-695) within the sequence of 27K (21, 23, 24), the observed phenomenon can be explained as follows: CaT may only interact with one of the two sites (Site A) and such interaction may prevent CaM from binding to the same site; CaM, on the other hand, binds to both sites, and in the presence of CaT at Site A, it prefers binding to the other site (Site B). If one assumes that binding of CaM to Site B weakens the affinity of CaT for Site A, thereby facilitates subsequent displacement of CaT by CaM at Site A, then in the case of $27K_{NBD}$, CaM would eventually occupy both sites and enhance the fluorescence to the same level as in the absence of CaT (Fig. 3). Since GS17C contains only Site A, no such cooperative interactions are possible, and therefore, CaM competes less favorably with CaT owing to its weaker binding constant, the resulting fluorescence enhancement is much lower (Fig. 4).

CONCLUSIONS

CaT, similar to CaM, binds to CaD at its C-terminal region near Cys-580 in a Ca^{2+}-dependent manner. Although a photo-crosslinker (BP-Mal) attached to Cys-580 can reach to the

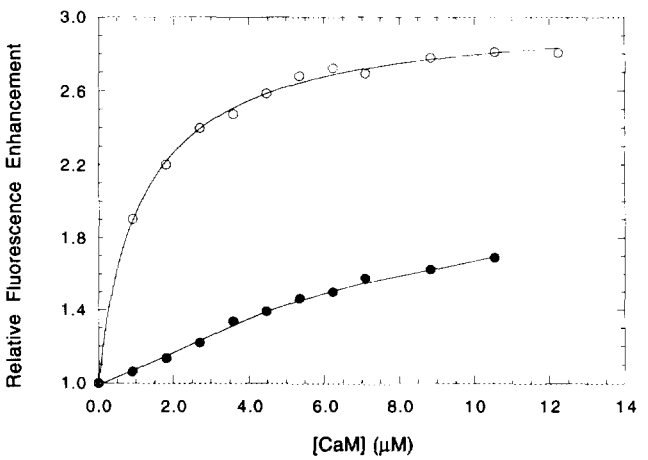

Figure 4. Fluorescence titration of $GS17C_{NBD}$ (2 μM) with CaM in the presence (closed circles, smooth curve) and absence (open circles, best fitted curve) of 7.5 μM CaT. Other conditions: same as in Fig. 3.

bound CaT or CaM and form a covalent linkage, the environment of a fluorescence probe (IANBD) attached to the same residue is altered by CaM, yet not significantly perturbed by CaT. CaT is able to, as is CaM, weaken the interaction between CaD and F-actin by binding to CaD. Both Ca^{2+}-binding proteins interact competitively with the C-terminal fragment of CaD, 27K, and a 17-residue peptide, GS17C; but, in contrast to CaM, CaT does not cause changes in either the intrinsic or the extrinsic fluorescence intensity of 27K and GS17C, suggesting the interaction is different from that of CaM. The significance of the differences in the mode of interaction between the two Ca^{2+}-binding proteins and CaD awaits further investigation.

REFERENCES

1. Marston, S. B. and Redwood, C. S. (1991) Biochem. J. 279, 1-16.
2. Marston, S. B. and Lehman, W. (1985) Biochem. J. 231, 517-522.
3. Ngai, P. K. and Walsh, M. P. (1984) J. Biol. Chem. 259, 13656-13659.
4. Smith, C. W. J., Pritchard, K. and Marston, S. B. (1987) J. Biol. Chem. 262, 116-122.
5. Smith, C. W. J. and Marston, S. B. (1985) FEBS Lett. 184, 115-119.
6. Horiuchi, K. Y., Miyata, H. and Chacko, S. (1986) Biochem. Biophys. Res. Commun. 136, 962-968.
7. Mani, R. S. and Kay, C. M. (1990) Biochemistry 29, 1398-1404.
8. Mani, R. S., McCubbin, W. D. and Kay, C. M. (1992) Biochemistry. 31, 11896-11901.
9. Mani, R. S. and Kay, C. M. (1993) Biochemistry. 32, 11217-11223.
10. Wills, F. L., McCubbin, W. D. and Kay, C. M. (1994) Biochemistry 33, 5562-5569.
11. Kuznicki, J., Filipek, A., Heimann, P., Kacsmarek, L. and Kaminska, B. (1989) FEBS Lett. 254, 141-144.
12. Tokumitsu, H., Kobayashi, R. and Hidaka, H. (1991) Arch. Biochem. Biophys. 288, 202-207.
13. Calabretta, B., Battini, R., Kaczmarek, L., Riel, J. K. and Baserga, R. (1986) J. Biol. Chem. 261, 12628-12632.
14. Kindy, M. S., Brown, K. E. and Sonenshein, G. E. (1991) J. Cell. Biochem. 46, 345-350.
15. Gong, Y., Alkhalaf, B., Murphy, L. J. and Murphy, L. C. (1992) Cell Growth Differ. 3, 847-853.
16. Yamashiro, S., Yamakita, Y., Ishikawa, R. and Matsumura, F. (1990) Nature. 344, 675-678.
17. Lynch, W. and Bretscher, A. (1986) Methods Enzym. 134, 37-42.
18. Dedman, J. R. and Kaetzel, M. A. (1983) Methods Enzymol. 102, 1-8.
19. Spudich, J. A. and Watt, S. (1971) J. Biol. Chem. 246, 4866-4871.
20. Wang, C.-L. A., Wang, L.-W. C., Xu, S., Lu, R. C., Saavedra-Alanis, V. and Bryan, J. (1991) J. Biol. Chem. 266, 9166-9172.
21. Zhan, Q., Wong, S. S. and Wang, C.-L. A. (1991) J. Biol. Chem. 266, 21810-21814.
22. Katsuyama, H., Wang, C.-L. A. and Morgan, K. G. (1992) J. Biol. Chem. 267, 14555-14558.
23. Marston, S. B., Fraser, I. D. C., Huber, P. A. J., Pritchard, K., Gusev, N. B. and Torok, K. (1994) J. Biol. Chem. 269, 8134-8139.
24. Mezgueldi, M., Derancourt, J., Calas, B., Kassab, R. and Fattoum, A. (1994) J. Biol. Chem. 269, 12824-12832.