Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 349-355

INFLUENCE OF CALTROPIN ON THE CALDESMON INDUCED POLYMERIZATION OF G-ACTIN

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Received June 28, 1995

Summary: The effect of caltropin (CaT) on the caldesmon (CaD)-G-actin interaction was monitored by viscosity measurements, bioassays measuring the release of inorganic phosphate (Pi) following G-actin polymerization and fluorescence studies using acrylodan labelled G-actin. CaD induced polymerization of G-actin into filaments in the absence of salt was accompanied by an increase in relative viscosity. This effect of CaD was essentially abolished by CaT in the presence of Ca^{2+}. In bioassays the rate of Pi release was reduced significantly in the presence of Ca^{2+}/CaT. Acrylodan labelled G-actin when excited at 375 nm exhibited an emission maximum at 478 nm. Polymerization of G-actin resulted in shifting the emission maximum to 465 nm. When CaD was added to G-actin containing Ca^{2+}/CaT, the rate of G-actin polymerization was reduced considerably, suggesting that CaT interferes in the CaD-G-actin interaction.

Caldesmon (CaD) is an actin binding protein found in smooth muscle and many non-muscle cells (1,2). Binding of CaD to F-actin inhibits the actomyosin ATPase activity *in vitro* (3-5). This inhibition is not Ca^{2+}-sensitive but can be rendered Ca^{2+}-dependent by a suitable Ca^{2+}-binding regulatory protein (Calmodulin (CaM) or CaT) which, by competing with actin for binding to CaD is able to reverse CaD's inhibition (6). Simultaneous binding of CaD to actin and myosin may provide an additional role for CaD in stabilizing the structure of the contractile apparatus, which is particularly important in non-muscle cells where both the actin and myosin filaments are in transient structures (7). Thus CaD is an important regulator of both motile activities and cell architecture. The latter function can be fulfilled by CaD mediated control of the actin assembly/disassembly process.

CaD is known to affect actin polymerization (8). In the absence of salt CaD can influence the polymerization of G-actin. It also interacts with F-actin to form a network or bundles of filaments (1). Both these processes, like the effect of CaD on actin-myosin interaction, are reversed by Ca^{2+}/CaM (8-10). But CaT is much more potent than CaM in reversing CaD's inhibition on the actin activated myosin ATPase activity (6). For this reason in the present study we utilized bioassays for measuring the release of Pi during actin polymerization, viscometry and fluorescence measurements to study the effect of CaT on the CaD-G-actin interaction.

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0006-291X/95 $12.00

MATERIALS AND METHODS

Protein purification: CaT (11) and CaD (12) were isolated from chicken gizzard. G-actin was prepared from rabbit skeletal muscle (13). Protein concentrations were determined using the following values of extinction co-efficients:

$$\text{CaD } E^{1\%}_{1\,cm\ 278\ nm} = 3.8\ (14);\quad \text{G-actin, } E^{1\%}_{1\,cm\ 290\ nm} = 6.3\ (15);\quad \text{CaT } E^{1\%}_{1\,cm\ 278\ nm} = 6.8\ (6)$$

Actin was specifically labelled at cysteine 374 using Acrylodan (Molecular Probes, Eugene, OR), a thiol specific fluorescent probe following the procedure of Marriott *et al.* (16). The ratio of label to protein was determined to be 0.95, suggesting that the active sulfhydryl group (Cys-374) in actin is indeed labelled under these conditions. Fluorescence measurements were carried out on a Perkin Elmer model MPF-44 spectrofluorometer (17).

Measurement of Pi release: Rate of ATP hydrolysis during actin polymerization induced by CaD was monitored by measuring the amount of Pi liberated. Samples of G-actin (0.5 mg/ml) in G-buffer were incubated for 5 min at 37°C with and without CaD. The amount of Pi liberated was determined according to Itaya and Ui (18) using the basic dye malachite green. Viscosity measurements were made with a Cannon-Manning semi-micro type viscometer. The charge volume of the viscometer was 1.0 ml and the flow time for the solvent corresponded to 82 sec.

RESULTS

ATP hydrolysis during actin polymerization: The rate of Pi release from G-actin in the absence of salt was taken as the control value (Table 1). Addition of CaD accelerated the G—F actin transformation and this rate exceeded the observed rate of polymerization with 100 mM KCl. However, if Ca^{2+}/CaT was added to G-actin prior to the addition of CaD then the observed rate of polymerization is very slow and is comparable to the control value *i.e.,* CaD's effect on actin polymerization was now essentially abolished.

Viscosity measurements: The relative viscosity (η_{rel}) of G-actin increased in the presence of CaD indicating polymerization (Fig. 1). CaD and actin were mixed in 1:5 mole ratio, respectively. However, when CaD was added to G-actin containing Ca^{2+}/CaT, there was no significant increase in the relative viscosity value suggesting that CaD was not effective in inducing

TABLE 1

Rate of ATP hydrolysis during actin polymerization. Samples of G-actin were incubated for 5 min at 37°C in 5 mM Tris, pH 8.0, 0.2 mM ATP, and 0.2 mM $CaCl_2$. Mole ratio of caldesmon to G-actin used was 1:4. Caldesmon and caltropin were mixed in 1:2 mole ratio, respectively.

	mols of Pi/mol of actin
G-actin	0.10
G-actin + 100 mM KCl	0.57
G-actin + CaD	1.40
G-actin + CaT + CaD	0.17

actin polymerization in the presence of Ca^{2+}/CaT. CaD and CaT were added in a 1:1 mole ratio using a molecular weight of 87000 and 21000 for CaD and CaT, respectively. Polymerization of G-actin induced by CaD and 2 mM $MgCl_2$ are shown in Fig. 1. Polymerization of G-actin by 2 mM $MgCl_2$ exhibited an initial lag phase and the addition of CaD shortened this lag phase. In addition the concentration of CaD now required to induce G-actin polymerization was significantly less (mole ratio of CaD to G-actin used was only 1:14). This indicates a strong nucleating effect of CaD on G-actin polymerization. The recorded flow times (*i.e.*, the time it took for the protein solution to flow through the viscometer) for G-actin, G-actin-CaD and G-actin-CaT-CaD as a function of time of incubation at 15°C is shown in the insert in Fig. 1. For example, the observed flow times after 20 min of incubation corresponded to 115, 152 and 120 sec for G-actin, G-actin-CaD, and G-actin-CaT-CaD, respectively. The interaction between G-actin and CaD is virtually abolished in the presence of CaT. This effect of CaT was noticeable only in the presence of calcium. If calcium in the solvent (0.2 mM) was replaced by 1 mM EGTA, CaT addition had no significant effect on the G-actin-CaD viscosity values, suggesting that CaD could interact with G-actin even in the presence of CaT if Ca^{2+} was not present.

Acrylodan labelled G-actin, when excited at 375 nm, exhibited an emission maximum around 480 nm, and after the completion of the polymerization process in F buffer the maximum shifts to 465 nm (16). Hence using this environmentally sensitive probe, one can accurately

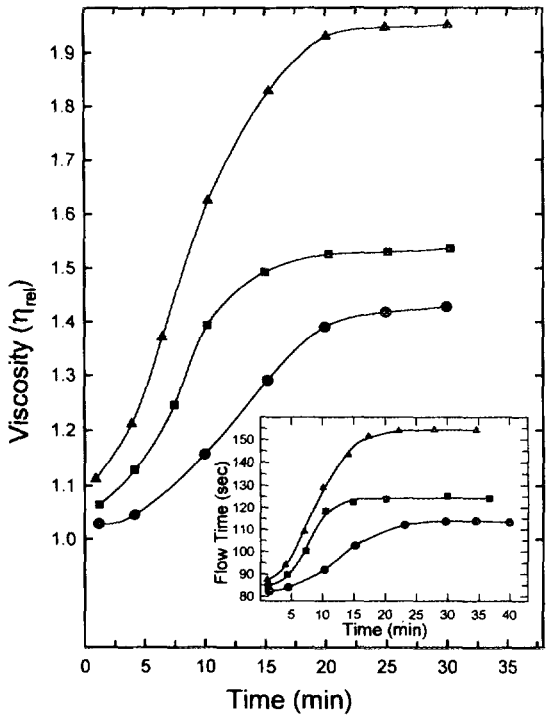

Fig. 1. Relative viscosity of G-actin (●), G-actin plus caldesmon (▲) and G-actin-caltropin-caldesmon ternary complex (■) in 5 mM Tris,pH 8.0, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.2 mM ATP at 15°C. G-actin concentration was 0.5 mg/ml.

follow the polymerization process. Addition of CaD to actin in G-buffer produced an increase in fluorescence intensity and the emission maximum was now centered at 465 nm (Fig. 2). Thus CaD is able to polymerize G-actin in the absence of salt and this process seems to level off in about 30 min (Fig. 3). However, when CaD was added to G-actin containing Ca^{2+}/CaT, the rate of polymerization was reduced considerably suggesting that CaT interferes in the G-actin-CaD interaction. This effect of CaT was observed only in the presence of Ca^{2+}. Rate of actin polymerization was calculated by dividing the initial slope of the polymerization curve (Fig. 3) by the final equilibrium value of fluorescence upon the completion of polymerization. G-actin polymerized at a low rate (7.4×10^{-4} S^{-1}, curve a) and the addition of CaD accelerated this process (1.4×10^{-3} S^{-1}, curve b) nearly 2 fold. However, in the presence of Ca^{2+}/CaT, CaD was not very effective in polymerizing G-actin since the observed rate of polymerization was only 8.5×10^{-4} S^{-1} (curve c).

DISCUSSION

CaD is a major actin binding protein associated with thin filaments of smooth muscle (1) and non-muscle cells (2). CaD binds to actin and myosin with strong affinity (17). Several studies suggest that CaD inhibits the binding of myosin-ATP to actin possibly by competing with myosin for overlapping binding sites on actin (19,20). One proposed area of overlap between CaD and S_1-ATP is the N-terminus of actin involving the first seven amino acid residues. The N-

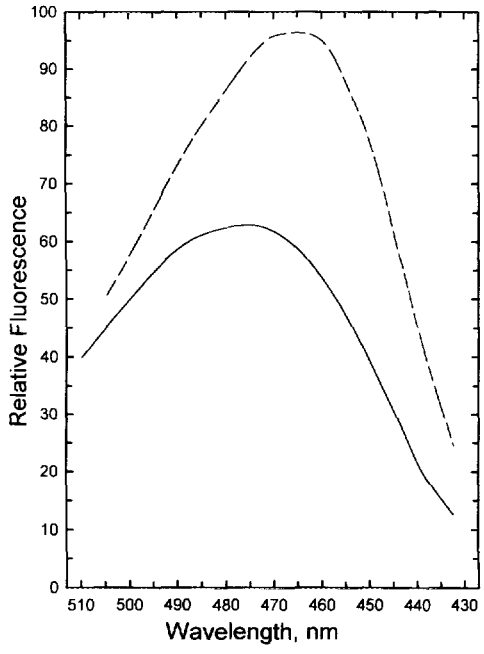

Fig. 2. Fluorescence emission sepctra of G-actin acrylodan (—) and G-actin-caldesmon complex (— —) in 5 mM Tris, pH 8.0, 0.2 mM $CaCl_2$ and 0.2 mM ATP at 20°C. The excitation wavelength was 375 nm. Concentration of G-actin acrylodan used was 0.4 μM.

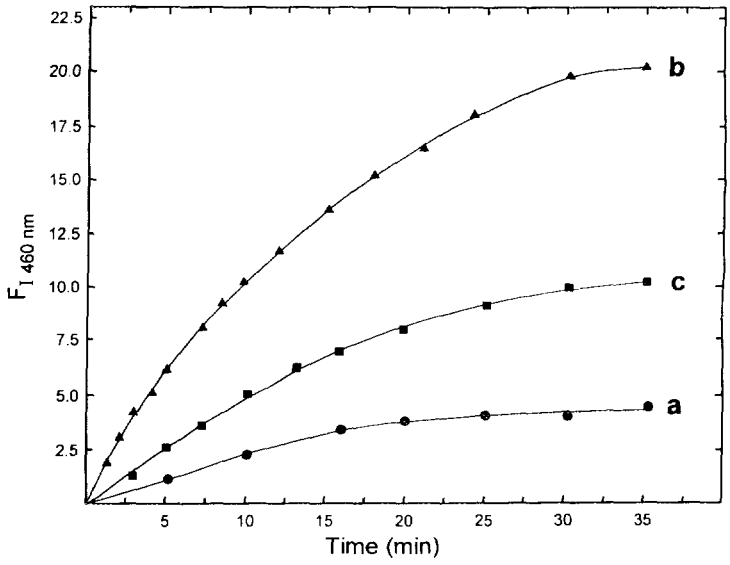

Fig. 3. Time-course of polymerization of G-actin acrylodan (●), G-actin-caldesmon (▲) and G-actin-caltropin-caldesmon (■) in 5 mM Tris,pH 7.5, 0.2 mM $CaCl_2$ and 0.2 mM ATP at 20°C. The excitation wavelength was 375 nm and the G-actin acrylodan concentration was 0.4 μM.

terminal acidic residues of actin are critical for the binding of CaD to G-actin and the polymerization induced by CaD (21). However, the N-terminal acidic residues of actin are not required for the binding of CaD to F-actin. This observed reduction in CaD binding to G-actin, in contrast with the binding of F-actin, highlights the existence of distinct interactions of CaD with G and F-actins. Thus CaD has multiple contact sites on actin and the other regions suggested include the C-terminus (22) and amino acid residues 18 → 28 in the N-terminal end (23). The link between the N-terminal charges on G-actin and its polymerization by CaD most likely results from charge-dependent binding of these proteins. CaD-G-actin interaction is important from a structural aspect because once actin is polymerized, CaD can crosslink F-actin and myosin (24) as it is localized in the actomyosin domain. Its structural function would be particularly useful in non-muscle systems where the contractile apparatus is not a permanent structure (25). The interaction between G-actin and CaD is also important for CaD's regulatory function since the observed CaD inhibition on acto-S_1, ATPase was drastically reduced when N-terminal yeast actin mutant devoid of acidic residues was used in the bioassays (21). Hence the polymerization of G-actin by CaD is important from both structural and regulatory functional aspects. In an earlier study we have shown that CaT in the presence of Ca^{2+} was effective in weakening the interaction between CaD and F-actin (17). In this paper we present evidence that CaT in the presence of Ca^{2+} can influence the interaction between CaD and G-actin as well. CaD might control the cytoskeleton not only by a direct effect on the G → F actin transformation but also by influencing the interaction of F-actin with other actin binding proteins like filamin, S_1 etc. In the presence of Ca^{2+}/CaT, CaD no longer can influence the interaction of actin with actin binding proteins. The CaD induced polymerization

of G-actin as well as the formation of supramolecular structures of actin filaments and their reversal by calcium binding proteins (CaM and CaT) in the presence of Ca^{2+} may be of physiological significance.

ACKNOWLEDGMENTS

We thank A. Keri and L. Hicks for their technical assistance.

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