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EFFECT OF CYTOCHALASIN B ON FORMATION AND PROPERTIES OF MUSCLE F-ACTIN

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SUMMARY

Cytochalasin B stimulated polymerization and decreased the concentration of G-actin remaining in equilibrium with F-actin filaments. Polymerization in the presence of cytochalasin B gave rise to a smaller increase of viscosity but to the same increase in light scattering, compared to polymerization in the absence of cytochalasin B. Cytochalasin B reduced the viscosity of F-actin and caused the appearance of ATP hydrolysis by F-actin. The cytochalasin B-induced ATPase activity was inhibited by concentrations of KCl higher than 50 mM. The cytochalasin B-induced ATPase activity was enhanced by ethyleneglycol bis(α-aminoethyl ether)-*N,N'*-tetraacetic acid and reduced by $MgCl_2$ at concentrations higher than 0.75 mM. The findings suggest that the stability of actin filaments is reduced by cytochalasin B.

INTRODUCTION

The alkaloid cytochalasin B, a metabolite of the fungus *Helminthosporium dematioideum*, inhibits a variety of cellular functions. Actin-like microfilaments are believed to participate in many of these functions (for review see refs 1 and 2). This prompted the work of Spudich, who found that cytochalasin B decreases the viscosity of muscle F-actin and alters the electron microscopic appearance of actin [3, 4]. The viscosity attained after addition of cytochalasin B to F-actin was higher than that of G-actin and was the same as that seen after the polymerization of G-actin in the presence of cytochalasin B. These findings demonstrate that cytochalasin B does not inhibit actin polymerization but alters the structure of the actin polymer. More recently we have shown that cytochalasin B induces F-actin to hydrolyze ATP at a rate similar to that seen during ultrasonic vibration [5]. In the present paper the conditions under which cytochalasin B alters the structure of F-actin are characterized more fully. We present evidence that the ability of cytochalasin B to destabilize F-actin is inversely proportional to the initial stability of the actin filament.

Abbreviation: EGTA, ethyleneglycol bis-(α-aminoethyl ether)-*N,N'*-tetraacetic acid.

MATERIAL AND METHODS

Actin, which according to sodium dodecyl sulfate-polyacrylamide electrophoresis was free from other proteins, was prepared as described elsewhere [6] ("pellet actin"). This procedure is based on partial polymerization of actin in 0.7 mM $MgCl_2$ followed by ultracentrifugation. Actin prepared in this way, when polymerized, forms filaments of apparently varying lengths, as evidenced by the instability in the extent of light scattering. To avoid this apparent heterogeneity of the F-actin solutions we used, for many measurements, actin immediately after extraction from the acetone powder with H_2O at 0 °C. 5 mM Tris · HCl (pH 8.0) and ATP or ADP were added immediately after extraction. The F-actin solutions prepared from these G-actins ("extract actin") exhibit stable values of light scattering, although a somewhat lower viscosity than "pellet actin". "Extract actin" sometimes shows in sodium dodecyl sulfate-polyacrylamide gel electrophoresis traces of the tropomyosin-troponin com-

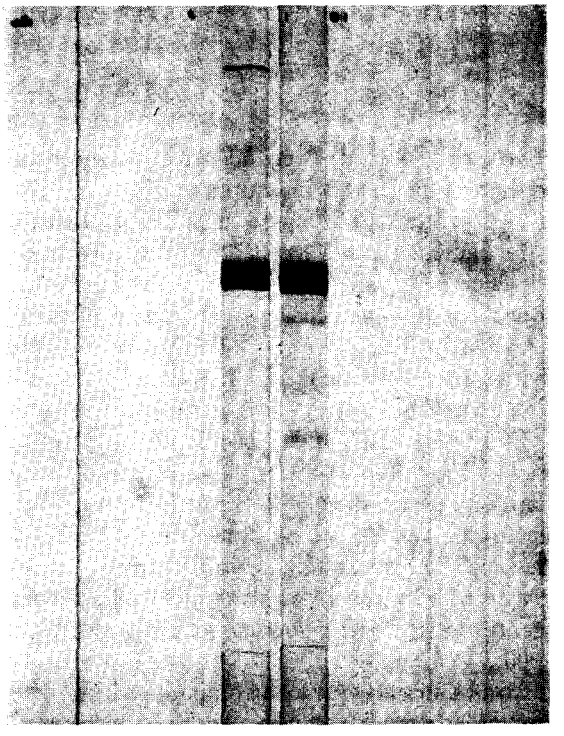

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the actin preparations used. Left, pellet actin; right, extract actin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in tubes with gels containing 10 % polyacrylamide and 0.1 % sodium dodecyl sulfate. The gel and electrode buffer was 0.1 M Tris/bicine (pH 8.2) containing 0.1 % sodium dodecyl sulfate. The gels were fixed in 20 % sulfosalicylic acid, stained with 0.25 % Coomassie brillant blue and destained in 10 % acetic acid containing 2 % methanol. The gels were photographed with a polaroid camera. Prior to application to the gels the proteins were dissolved in the electrode buffer plus 10 % saccharose and 0.14 M mercaptoethanol and heated to 80 °C for 30 min. About 50 μg of protein were applied to the gels.

TABLE I

ATPase ACTIVITY, INDUCED BY CYTOCHALASIN B, OF PURIFIED "PELLET ACTIN" AT DIFFERENT CONCENTRATIONS OF ACTIN, $MgCl_2$, AND EGTA

Pellet actin (free from minor protein components) was dissolved in 0.2 mM ADP and 5 mM Tris · HCl (pH 7.4). From this solution samples were taken and allowed to repolymerize overnight. The samples contained either 0.6 mg (13 nmol) actin in 0.5 ml ($MgCl_2$: 0.75 mM) or 1.2 mg actin in 1 ml ($MgCl_2$: 0.38 mM or 0.75 mM). The next day the samples were adjusted to 1.05 ml and to the following concentrations: 1 mM ATP, 0.2 mM cytochalasin B and the concentrations of actin and $MgCl_2$ indicated in the table. EGTA was, if present, 0.2 mM.

Actin concentration (μM)	Cytochalasin B-induced ATP splitting (mol P_i · mol actin^{-1} · 30 min^{-1})			
	−EGTA		+EGTA	
	0.38 mM $MgCl_2$	0.75 mM $MgCl_2$	0.38 mM $MgCl_2$	0.75 mM $MgCl_2$
13	8.8	7.4	13.6	9.1
26	4.6	3.2	6.5	4.2

plex (Fig. 1). It will be shown that the results of this paper are not caused by such protein impurities.

Measurements of ATPase activity and viscosity were identical to those described earlier [5, 7, 8]. ATP hydrolysis by F-actin was measured at 30 or 35 °C at various ionic conditions (see legends) in 0.7–1.0 mM ATP. Each value of cytochalasin-induced ATPase activity indicated in the figures and in Table I is calculated from the amount of ATP hydrolyzed in the presence of cytochalasin B minus the amount of ATP hydrolyzed in the absence of cytochalasin B under otherwise identical conditions. The latter values were about 1 mol ATP hydrolyzed per mol actin in 30 min. The ATPase reactions were stopped with trichloroacetic acid and inorganic phosphate (P_i) was determined as described by Marsh [9]. Viscosity was measured with 2 ml samples in spirally formed capillary viscosimeters at 23 °C. Specific viscosity (η_{spec}) denotes the expression $(t/t_0 - 1)$, where t_0 is the outflow time of the solvent and t is the outflow time of the solution. Light scattering perpendicular to the incident beam was measured with a Hitachi-Perkin-Elmer Fluorescence Spectrophotometer MPF 2 A at 400 nm. Changes in light scattering intensity are expressed in arbitrary units.

Protein concentration was determined with the Lowry method [10]. Calculations involving the amounts or concentrations of actin are based on a molecular weight for the actin monomer of 45 000.

Cytochalasin B was purchased from Aldrich Chemical Company, Milwaukee, U.S.A., and dissolved in dimethylformamide in stock solutions with a concentration of either 1 mg/ml or 10 mg/ml.

RESULTS

Polymerization of actin in the presence of cytochalasin B

We previously found that in the absence of added nucleotides actin did not polymerize when cytochalasin B was present [7, 11]. When nucleotides are added, however, actin does polymerize in the presence of cytochalasin B (Fig. 2, compare

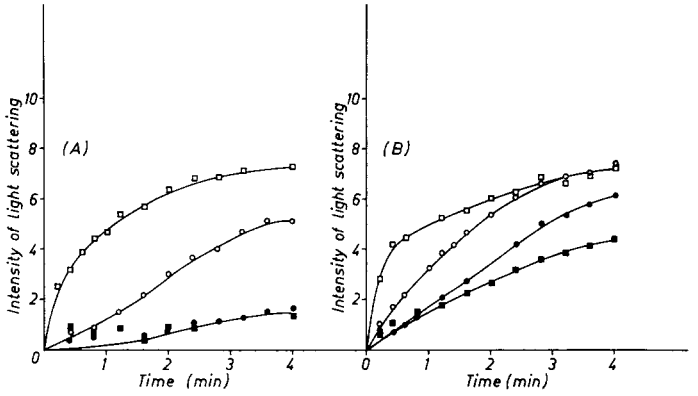

Fig. 2. Stimulation of actin polymerization by cytochalasin B. The respective G-actins (1.3 mg/ml) originated either from extract actin (A), which contained 0.1 mM ATP and 10 mM Tris · HCl buffer (pH 8.0) or from pellet actin (B) which was depolymerized overnight in 0.1 mM ATP/Tris · HCl (pH 8.0). Polymerization was started by adding 1 mM $MgCl_2$ at zero time and was followed by the increase of light scattering intensity. The 2 ml samples contained besides the concentrations already mentioned the following components: (●) 0.1 mM $CaCl_2$, 5 μl dimethylformamide; (■) 1 mM EGTA, 5μl dimethylformamide; (○) 0.1 mM $CaCl_2$, 50 μg (104 nmol) cytochalasin B in 5μl dimethylformamide; (□) 1 mM EGTA, 50 μg cytochalasin B in 5 μl dimethylformamide.

also Spudich [4]). Quite unexpectedly, cytochalasin B enhanced actin polymerization. In the "extract actin", when there was only little polymerization in the control reaction polymerization was much enhanced by cytochalasin B (Fig. 2A). In the presence of calcium, polymerization was less stimulated by cytochalasin B than in its absence (due to the presence of ethyleneglycol bis(α-aminoethyl ether)-*N*,*N*′-tetra-acetic acid (EGTA)). In "pellet actin" a similar effect of cytochalasin B was observed, although the difference between polymerization in the presence and absence of cytochalasin B was less pronounced because appreciable polymerization occurred in the absence of cytochalasin B. Furthermore the difference between the effect of cytochalasin B in the presence and absence of calcium was less in "pellet actin" though this difference was clearly visible and a consistent feature of all experiments. (The small stimulation of polymerization by calcium in the absence of cytochalasin B did not occur in all experiments.)

The stimulation of actin polymerization by cytochalasin B was visible only when the polymerization of the control actin was low. This can be seen also in Fig. 3, where values of light scattering and viscosity of actin solutions of different concentrations are compared. The increase of viscosity and light scattering began only when actin concentration had reached a critical value. Measurements like those of Fig. 3 were frequently used to determine the critical concentration for actin polymerization, that is the concentration of G-actin, which remains in equilibrium with the F-actin filaments (for recent discussion of this phenomenon see refs 12–14). The critical concentrations are usually inferred from those actin concentrations where the lines relating degree of polymerization to actin concentration intersect the abscissa. Viscosity and light scattering data of Fig. 3 would, however, lead to different critical concentrations, so that the true value cannot be deduced. (The higher concentration deduced from the viscosity data suggests that there may exist a critical filament

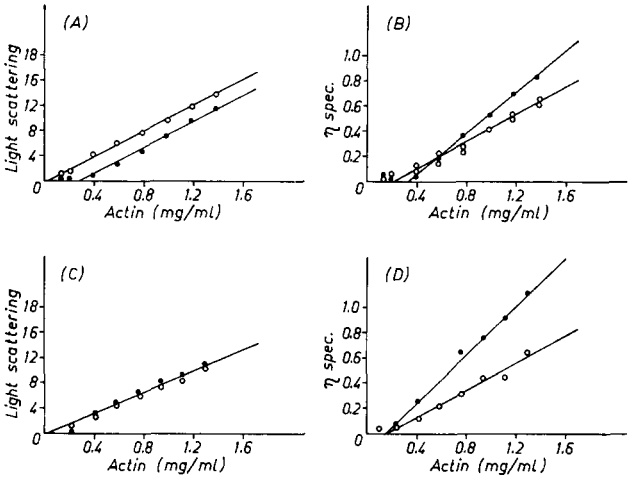

Fig. 3. Viscosity and light scattering as influenced by cytochalasin B after the polymerization of different actin concentrations. Solutions of "extract actin" which contained actin (originally as G-actin) at the concentrations indicated at the abscissa were polymerized for 20 h (3 h at 23 °C and 17 h at 4 °C) and then measured. Two experiments are depicted in Fig. 3: A and B show light scattering and specific viscosity of the one experiment and C and D of the second experiment. Light scattering and viscosity were measured at 23 °C with the same probes. In the experiment of A and B actin was polymerized with 0.7 mM $MgCl_2$, in the experiment of C and D the concentration of $MgCl_2$ was 1 mM. The pH was 7.2 (Tris/maleate buffer, 10 mM). (●) Without cytochalasin B; (○) polymerization in the presence of 50 μM cytochalasin B. When in the case of cytochalasin B-actin (B) two values are indicated at a given actin concentration, the higher value refers to the second viscosimeter run, the lower value refers to the 5th viscosimeter run. In the other cases the 5th run was used for calculating specific viscosity. In an experiment with "pellet actin" performed identically to those shown in this figure ($MgCl_2$ concentration: 0.7 mM) the slopes of the lines relating specific viscosity to actin concentration were as follows: absence of cytochalasin B, 0.95 ml/mg; presence of 50 μM cytochalasin B, 0.4 ml/mg.

length which must be reached until viscosity begins to increase.) Nevertheless in the particular experiment of Figs 3A and 3B the concentrations at which both light scattering and viscosity began to increase are lower in the presence of cytochalasin B. This suggests that cytochalasin B has decreased the critical concentration of G-actin in this experiment. In the case of the other experiment (at a higher concentration of $MgCl_2$) the critical concentration was very low in the absence of cytochalasin B so that no shift could be observed (Figs 3C and 3D).

Actin filaments formed in the presence of cytochalasin B differed from those formed in its absence as is evident from the different dependence of light scattering and viscosity on actin concentration. Whereas cytochalasin B did not affect the concentration dependence of light scattering, it changed that of the viscosity. The curves relating viscosity to actin concentration have a lower slope in the presence (about 0.5–0.6 ml/mg) than in the absence (about 1 ml/mg) of cytochalasin B. This indicates that the incorporation of a given amount of G-actin into a filament population leads to the same increase in light scattering both in the presence and in the absence of cytochalasin B, whereas the increase in viscosity is smaller when actin is incorporated in the presence of cytochalasin B. These findings also suggest that actin filaments formed in the presence of cytochalasin B are less rigid and less

extended (giving rise to a lower viscosity) than those formed in the absence of cytochalasin B.

The effect of cytochalasin B on F-actin

Fig. 4 shows that addition of cytochalasin B to F-actin induces an ATPase activity. At the higher actin concentration (26 μM) the ATPase could be maximally activated already by low concentrations of cytochalasin B. At the lower actin concentration (13 μM) the specific ATPase activity rose above the value observed at the lower actin concentration when the concentration of cytochalasin B was increased above the value which induced maximal ATPase activity at the higher actin concentration. It is a matter of further investigation whether this is due to different kinds of binding sites for cytochalasin B at different actin concentrations. Higher concentrations of KCl (Fig. 4B) depressed the cytochalasin-induced ATPase activity. This agrees well with our earlier observation [5] that cytochalasin B does not lower F-actin viscosity when KCl is present.

Increasing the concentration of $MgCl_2$ had an effect similar to that of increasing actin concentration. Concentrations of $MgCl_2$ above 0.75 mM (see Fig. 5B) inhibited ATP hydrolysis, such that higher concentrations of cytochalasin B were required for optimal ATPase activity (Fig. 5A). The optimal $MgCl_2$ concentration for ATP hydrolysis was about 0.4 mM (Fig. 5B).

In the presence of EGTA the ATPase activity induced by cytochalasin B was much higher than in its absence, particularly when the $MgCl_2$ concentration was low.

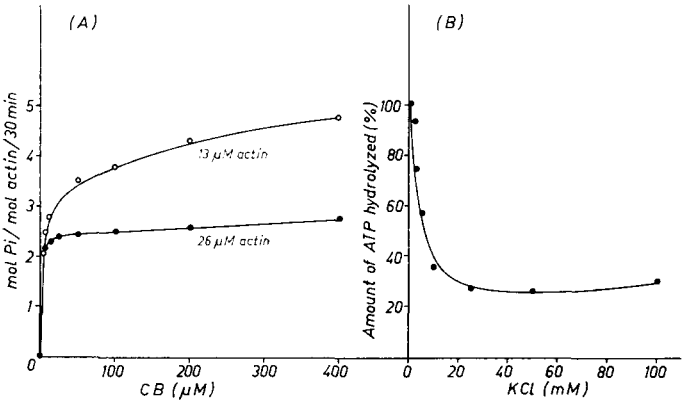

Fig. 4. ATP hydrolysis by F-actin, induced by cytochalasin B. (A) Dependence on the concentration of added cytochalasin B (CB). 0.6 mg or 1.2 mg G-actin (from extract actin) in 1 ml were polymerized overnight at 4 °C with 0.75 mM $MgCl_2$, 0.1 mM ADP and 5 mM Tris · HCl (pH 7.4). The next day ATP and cytochalasin B were added to the final concentrations of 1 mM (ATP) and the cytochalasin B concentrations indicated at the abscissa. The samples were then incubated for 30 min at 35° C. Then the reaction was stopped with 3 % trichloroacetic acid and the liberated P_i was measured. (B) Inhibition of the cytochalasin B-induced ATPase activity of F-actin by KCl. Samples of G-actin (from extract actin, 0.6 mg in 1 ml) were polymerized overnight as in A. During incubation with cytochalasin B (as in A) the samples contained the following concentrations: 0.38 mM $MgCl_2$, 1 mM ATP, 0.05 mM ADP, 0.2 mM EGTA, 0.2 mM cytochalasin B and the concentrations of KCl indicated. The amount of P_i liberated during 30 min incubation in the absence of KCl was taken as 100 % and was 8.75 mol P_i/mol actin. In an experiment with "pellet actin" performed identically to that of Fig. 4B the ATPase activity (7.4 mol P_i/mol actin in 30 min = 100 %) was inhibited by 25 mM KCl to 29 % of the original value.

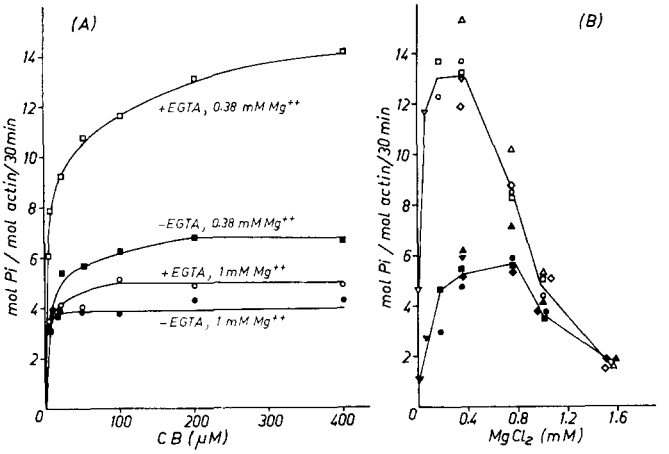

Fig. 5. Cytochalasin B-induced ATP hydrolysis by F-actin as dependent on the concentration of $MgCl_2$ and on the presence or absence of EGTA. (A) Samples of G-actin (from extract actin, 0.6 mg) were either polymerized in 0.5 ml containing 0.75 mM $MgCl_2$, 0.2 mM ADP, 5 mM Tris · HCl (pH 7.4) or in 1 ml containing 1 mM $MgCl_2$, 0.2 mM ADP, 5 mM Tris · HCl, (pH 7.4). For incubation with cytochalasin B (CB) for 30 min at 35 °C, all samples were adjusted to a volume of 1.05 ml containing the following final concentrations: 1 mM ATP, 0.2 mM ADP and the concentrations of $MgCl_2$ and cytochalasin B indicated in the figure. EGTA was, if present, 0.2 mM. (B) Inhibition by increasing concentrations of $MgCl_2$ of the activating effect of EGTA on the cytochalasin B-induced ATPase activity of F-actin. G-actin (from extract actin) was polymerized overnight at 4 °C at the concentrations of $MgCl_2$ indicated at the abscissa and 0.1 mM ADP. During incubation with 0.2 mM cytochalasin B, the samples (1 ml) contained 0.6 mg = 13 nmol actin and 1 mM ATP, 0.05 mM ADP, 5 mM Tris · HCl (pH 7.4) and $MgCl_2$ as indicated. EGTA was, if present, 0.2 mM (open symbols). Different kinds of symbol refer to different measurements.

Hence absence of calcium promoted not only the polymerizing affect of cytochalasin B (Fig. 2) but also the ATPase activity induced by cytochalasin B.

This suggests that cytochalasin B induces maximal ATPase activity only when certain calcium binding sites are free. Reduction of the effect of calcium removal at higher concentrations of $MgCl_2$ may be due either to an occupation of the calcium sites by magnesium or to a structural change in the actin polymer induced when magnesium binds to actin.

DISCUSSION

Two major findings are described in the present paper. (1) F-actin in the presence of cytochalasin B has a lower viscosity than usual F-actin but a similar degree of light scattering at 90°. (2) Under the influence of cytochalasin B F-actin hydrolyzes ATP at a rate similar to that when ordinary F-actin is sonicated. These findings suggest that under the influence of cytochalasin B actin polymers are destabilized and possess an increased flexibility. This destabilization of actin polymers does not depend on the presence of minor protein impurities as these phenomena were observed with both "pellet actin" (see Table I, Fig. 2 and legends of Figs 3 and 4) and "extract actin". The small differences found between the two actin preparations (compare Figs 2A and 2B) do not invalidate the principal conclusions of this study.

This destabilized form of actin can be reached from F-actin as well as from G-actin (cf. Spudich and Lin [3]). The finding that, under the same ionic conditions, polymerization is stimulated by cytochalasin B (as indicated by the results of Figs 2 and 3) suggests that structural constraints of the actin polymer are reduced by cytochalasin B so that monomers may be more easily incorporated into the filaments of destabilized F-actin.

Transformation of actin into a species which exhibits steady ATP hydrolysis has been reported previously. The "Mg-polymer" of actin from the slime mold *Physarum polycephalum* (Hatano et al. [15]) exhibits steady ATPase activity [16] and reduced viscosity compared to the ordinary F-actin of slime mold. According to Kamiya et al. [17] these Mg-polymers are caused by contaminations with the protein β-actinin because muscle F-actin can form Mg-polymers when β-actinin is present [17]. Pure muscle actin has also been transformed into a form showing steady ATPase activity by treatment with salyrgan [18] and ATP hydrolysis by muscle F-actin has been observed after termination of sonication at low concentrations of $MgCl_2$ [19, 20]. In all these cases, including the ATP hydrolysis during sonic vibration, a flexible structure of the actin filaments has been assumed although the true nature of the ATPase process is still a matter of speculation.

Conditions which favor actin polymerization (high concentrations of $MgCl_2$ and KCl, higher concentrations of actin) reduce the ability of cytochalasin B to induce ATP hydrolysis by F-actin. In other words, the more favorable the conditions for polymerization, the less the action of cytochalasin B. Since cytochalasin B does not cause actin to depolymerize, but rather destabilizes the actin polymer, one can deduce that conditions which favor the action of cytochalasin B are those which favor a form of the actin polymer which is more flexible than actin under conditions where the effects of cytochalasin B are reduced. According to current views on actin polymerization (for review see ref. 12), conditions under which actin is only incompletely polymerized are those under which the apparent affinity of monomers to the filaments is low (i.e. the critical concentration of G-actin is high). However, the increased sensitivity of actin to cytochalasin B under these conditions suggests also that the filaments formed under these conditions are already in the absence of cytochalasin B more flexible than filaments formed under conditions of complete polymerization.

EGTA favored the destabilization of actin induced by cytochalasin B, indicating that low concentrations of Ca^{2+} directly (not mediated by tropomyosin-troponin) affect the stability of actin filaments. It is possible that this effect of calcium is due to an action on the conformation of actin described many years ago by Katz [21]. It is possible that this calcium effect has physiological significance.

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