Calponin induces actin polymerization at low ionic strength and inhibits depolymerization of actin filaments

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Calponin from chicken gizzard induced polymerization of actin in the presence of 10 mM KCl. Only 2 min after the addition of KCl in the presence of a 0.0625–0.25:1 molar ratio of calponin to actin, a Poisson-type length distribution (with an average length of approx. 0.7 μm) was observed with formed actin filaments. This result suggests that calponin–actin complexes served as nuclei for rapid elongation. Calponin caused a rapid polymerization of actin even in G-buffer (2 mM Tris/HCl, pH 8.0) which is usually used for depolymerization of actin filaments. Binding of calponin at a level of up to 1.25 mol per mol of actin was observed in the actin filaments formed in the presence of calponin at very low ionic strengths. When actin filaments were exposed to 3.3 mM KCl, by dilution with G-buffer, a rapid depolymerization occurred. Addition of calponin greatly retarded the depolymerization process and, in the presence of an equimolar ratio of calponin to actin, depolymerization hardly occurred. In the presence of calmodulin, this inhibitory effect on depolymerization was reversed by Ca^{2+}, releasing calponin from actin filaments.

INTRODUCTION

Calponin is a thin filament-associated smooth-muscle protein that has been implicated in playing a role in the Ca^{2+}-regulation of smooth-muscle contraction [1–5]. Interaction of calponin with actin has been extensively studied to reveal the regulatory role of calponin in smooth-muscle contraction [6,7]. We have also reported that calponin binding to an actin filament causes conformational changes in the actin molecules and results in a drop of fluorescence intensity in pyrenyl actin [8]. On the other hand, it has been suggested that calponin and actin interactions may have some other roles. Localization studies suggest that calponin and caldesmon bind to distinct thin filaments [9] and calponin is localized in both the contractile apparatus and the cytoskeleton in smooth-muscle cells [10]. The roles of calponin in differentiation and cell-matrix adhesion of smooth-muscle cells have been reported [11]. Dynamic redistribution of calponin from cytosolic to primarily surface-cortex-associated was observed in vascular smooth-muscle cells upon stimulation with an agonist (phenylephrine) activating protein kinase C [12]. Furthermore, a 'non-muscle isoform' of calponin was identified, and it was reported that this type of calponin also interacts with actin [13]. In this paper, we describe that calponin induces polymerization of actin at a low ionic strength (at which the actin filament is usually depolymerized) and also inhibits this depolymerization at low ionic strengths.

MATERIALS AND METHODS

Preparation of proteins

Calponin was prepared from chicken gizzard according to Takahashi et al. [1] and purified by HPLC gel chromatography using TSK gel G 2000 SW. The molecular mass was 34000 Da [1]. Actin was prepared from rabbit skeletal muscle [14] and further purified by gel filtration using a Sephadex G-150 column; its molecular mass was 42300 Da [15]. Calmodulin was purchased from Sigma and was of molecular mass 16700 Da [16].

Fluorescence measurements

Actin was labelled at Cys-374 with *N*-(1-pyrenyl)iodoacetamide following the method of Cooper et al. [17] based on the report by Kouyama and Mihashi [18]. Pyrenyl actin was mixed with unlabelled actin to give a final concentration of 10% labelled actin. Fluorescence intensity of pyrenyl actin was measured at 365 nm (excitation) and at 407 nm (fluorescence) in a Hitachi fluorescence spectrophotometer 650–60 at 25 °C.

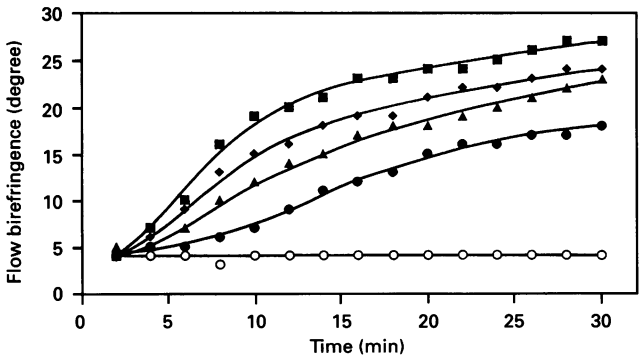

Figure 1 Induction of polymerization of actin at 10 mM KCl

Conditions: 0.3 mg/ml G-actin, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM dithiothreitol, 0.01% NaN_3 and 2 mM Tris/HCl, pH 8.0. Polymerization was initiated by adding 10 mM KCl at 25 °C. Molar ratio of calponin to actin: ○, actin alone; ●, 1:16; ▲, 1:8; ◆, 3:16; ■, 1:4. Degrees of flow birefringence were measured at a velocity gradient of 100 s^{-1}.

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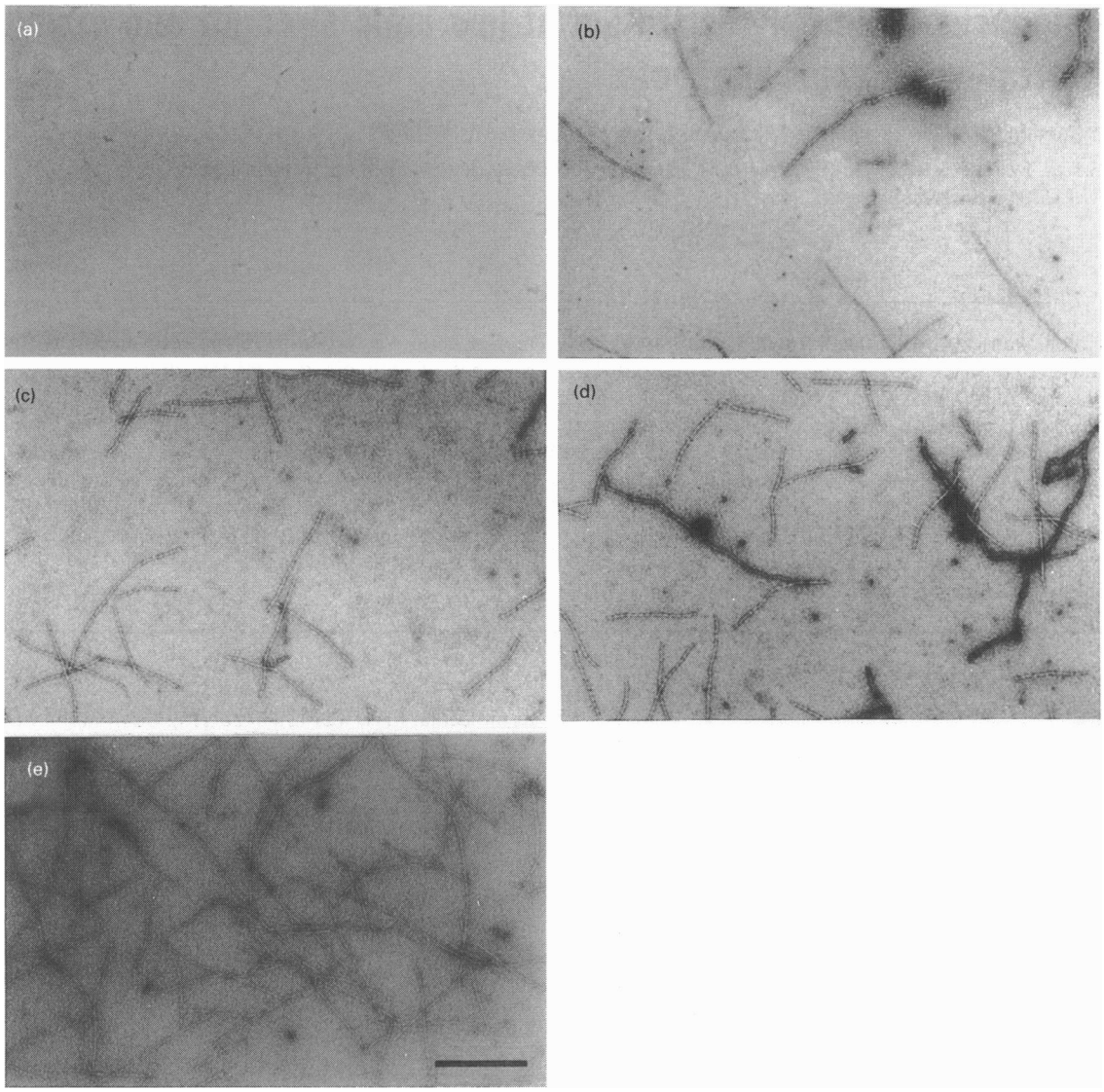

Figure 2 Electron micrographs of actin filaments formed by calponin at 10 mM KCl

Conditions are as described in Figure 1. Observations were made 2 min after the addition of 10 mM KCl. Molar ratio of calponin to actin: A, actin alone; B, 1:16; C, 1:8; D, 3:16; E, 1:4. Scale bar represents 0.5 μm.

Flow birefringence measurements

The degrees of flow birefringence were measured in a Wakenyaku Micro FBR Mark II at a velocity gradient of 100 s^{-1} at 25 °C.

SDS/PAGE

SDS/PAGE was carried out on 12.5% polyacrylamide gels according to the method of Laemmli [19]. Gels were stained with Coomassie Brilliant Blue R-250 and scanned with a Shimadzu densitometer (CS-9000).

Electron microscopy

Actin filaments were negatively stained with 1% (w/v) uranyl acetate and observed under a JEOL 100S electron microscope. Calibration for magnification was carried out using a carbon grating (2610/mm).

RESULTS

Polymerization of actin induced by calponin in the presence of 10 mM KCl

At pH 8.0 and 25 °C, KCl concentrations higher than 30 mM are usually required for the polymerization of actin. Certainly, 10 mM KCl is not enough to induce polymerization, except for a very high concentration of actin. However, in the presence of calponin, actin was rapidly polymerized, when monitored by flow birefringence measurements. Just a ratio of 1:16 mol of calponin/mol of actin was enough to induce polymerization, as shown in Figure 1. Larger amounts of calponin up to a 1:4 molar ratio to actin accelerated the rate of polymerization. Electron microscopic observations of the actin filaments formed in the

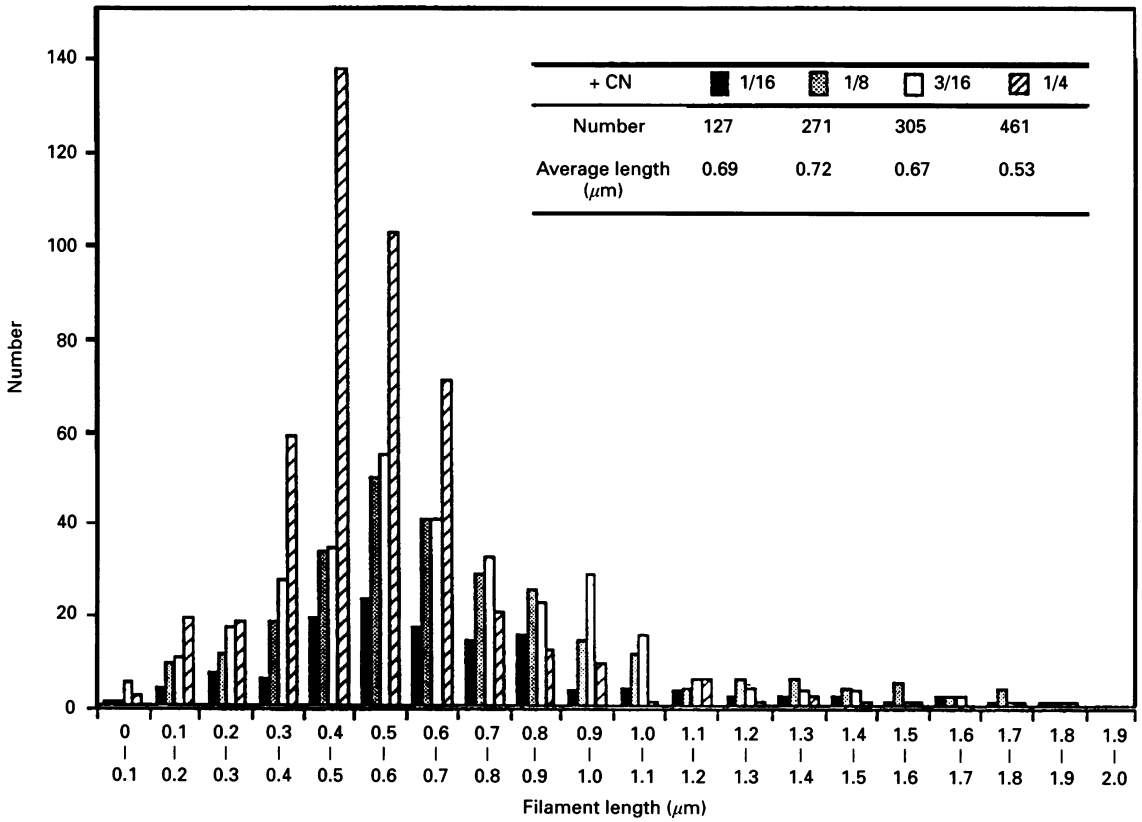

Figure 3 Length distribution of actin filaments formed under the influence of calponin at 10 mM KCl

Conditions are as described in Figure 2. Two min after the addition of 10 mM KCl, samples were treated with 1% (w/v) uranyl acetate. Molar ratio of calponin to actin: solid column, 1:16; shadowed column, 1:8; open column, 3:16; oblique line column, 1:4.

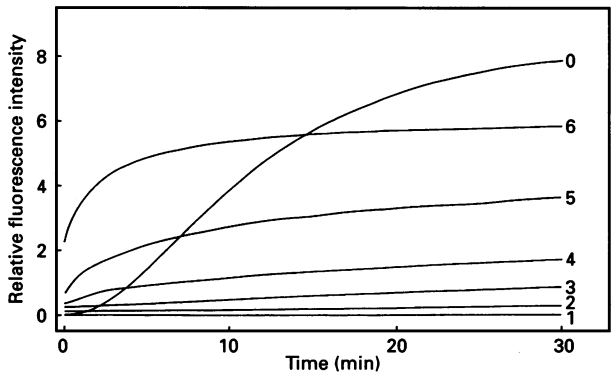

Figure 4 Polymerization of actin induced by calponin in G-buffer as monitored by the increase in fluorescence intensity

Conditions: 0.3 mg/ml pyrenyl actin; 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM dithiothreitol, 0.01% NaN_3 and 2 mM Tris/HCl, pH 8.0. Curve 0, actin polymerized by 100 mM KCl. Molar ratio of calponin to actin: curve 1, actin alone; curve 2, 1:4; curve 3, 1:2; curve 4, 3:4; curve 5, 1:1; curve 6, 5:4.

presence of calponin, 2 min after the addition of 10 mM KCl, revealed that there were rather short, approximately 0.5 μm long, actin filaments present, but they were not present at all in the absence of calponin (Figure 2). Therefore, the filament lengths of the actin filaments formed were measured. As shown in Figure 3, the length distribution was of the Poisson type, rather than the usual exponential type seen when actin is polymerized by the addition of salt alone [20]. The average lengths were 0.69, 0.72 and 0.67 μm in the presence of 1:16, 1:8 and 3:16 molar ratios of calponin/actin respectively. It somewhat decreased to 0.53 μm in the presence of a 1:4 ratio. It should be noted that the number of the actin filaments detected under an electron microscope increased as the amount of calponin added was increased.

Polymerization of actin induced by calponin in G-buffer

It is well known that so-called G-buffer (0.1 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM dithiothreitol, 0.01% NaN_3 and 2 mM Tris/HCl, pH 8.0) keeps actin in a monomeric form or depolymerizes an actin filament into monomers. It turned out that a high concentration of calponin, e.g. an almost equal molar ratio to actin (i.e. 1:1) induced a rapid polymerization of actin (Figure 4, curve 6). In doing the experiments, calponin in a solution containing 20 mM KCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.01% NaN_3, 20 mM Tris/HCl, pH 7.0, had been dialysed against the G-buffer overnight and then clarified for 1 h by ultracentrifugation at 150000 ***g***. At a half molar ratio of calponin to actin (0.5:1), actin polymerization was very slow (Figure 4, curve 3) in contrast to the polymerization in the presence of 10 mM KCl (Figure 1). To get a full polymerization within 10 min, calponin in a 5:4 molar ratio to actin was required (Figure 4, curve 6). The lower level of fluorescence intensity at the maximum level in curve 6, compared with that in curve 0 in

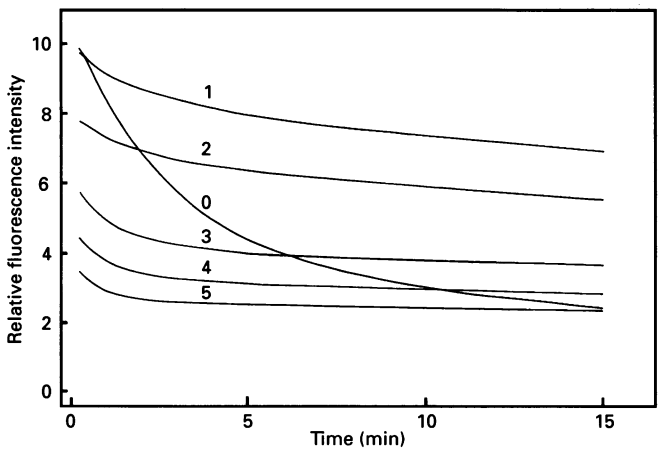

Figure 5 Effects of calponin on depolymerization of F-actin at 3.3 mM KCl as monitored by the decrease in fluorescence intensity

Pyrenyl F-actin, 1.5 mg/ml, polymerized for 5 h in the presence of 50 mM KCl was diluted 15-fold with G-buffer at 25 °C in the presence of various molar ratios of calponin to actin; curve 0, actin alone; curve 1, 1:8; curve 2, 1:4; curve 3, 1:2; curve 4, 3:4; curve 5, 1:1.

Figure 4, was due to the drop in intensity caused by calponin binding to the C-terminal region of an actin molecule [8,21].

Electron microscopic observations showed that a typical actin filament was formed when polymerized by calponin in G-buffer (results not shown). After 48 h of incubation, the solution was ultracentrifuged to sediment actin filaments. The amount of calponin bound to F-actin increased as the amount of calponin added was increased. No saturation of the amount of sedimented F-actin was observed up to 1.25:1 molar ratio of calponin to actin, and co-sedimented calponin with actin was approx. 1.25 mol of calponin to actin in the presence of 1.25 mol of calponin per mol of actin (results not shown).

Inhibition of actin depolymerization by calponin at low ionic strengths

Actin filaments in 50 mM KCl were quickly depolymerized when the KCl concentration was lowered to 3.3 mM by dilution with G-buffer, as seen in Figure 5 (curve 0). Addition of calponin before dilution at a molar ratio to actin as low as 1:8 remarkably inhibited the depolymerization (Figure 5, curve 1). The decrease in the initial level of fluorescence intensity was due to the binding of calponin to an actin filament [8]. Even 48 h after dilution, actin filaments were present in the presence of 0.125 mol of calponin per mol of actin. Sedimentation of actin filaments was accompanied by co-precipitation of nearly all the calponin added, up to a 1:1 calponin/actin ratio. The amount of sedimented F-actin increased linearly with the molar ratio of calponin added to actin, and became saturated at a ratio of approx. 1 mol of calponin to 1 mol of actin (results not shown). This inhibitory effect was not influenced by the addition of smooth-muscle tropomyosin (results not shown).

Effect of calmodulin and Ca^{2+} on the action of calponin

It is known that calmodulin induces the release of calponin from an actin filament in the presence of Ca^{2+} [6]. Therefore, we tested the effects of calmodulin on calponin-modulated depolymerization of F-actin in the presence or absence of Ca^{2+}. Addition of calmodulin did not affect the inhibitory effect of calponin on the depolymerization process of F-actin at all in the presence of

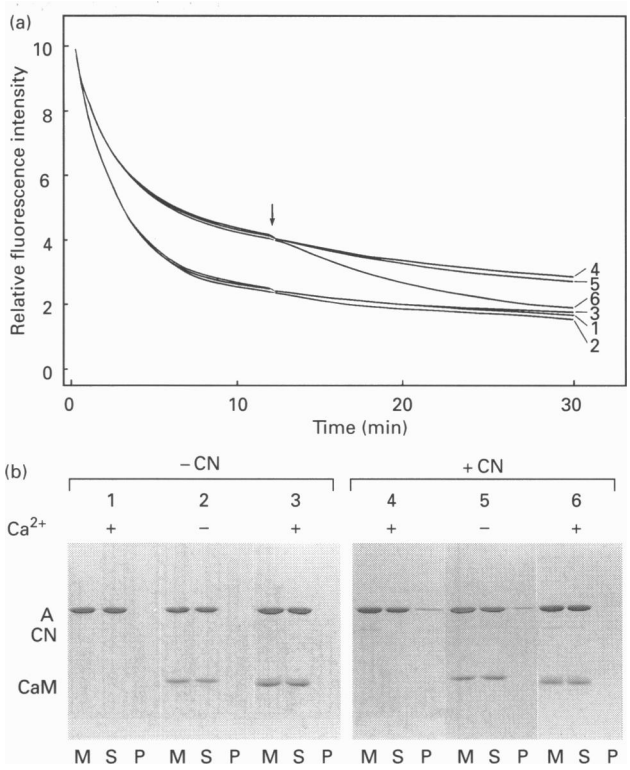

Figure 6 Effects of Ca^{2+}-calmodulin on the actin depolymerization-inhibitory action of calponin

Conditions are as described in Figure 5, except in the presence of 0.2 mM $CaCl_2$ (1, 3, 4 and 6) or 0.5 mM EGTA (2 and 5). Molar ratio of calponin to actin: 1, 2 and 3, actin alone; 4, 5 and 6, 1:16. The samples were ultracentrifuged for 1 h at 100000 ***g*** 48 h after dilution. (**a**) Fluorescence intensity changes after dilution. Calmodulin (20:1, molar ratio to calponin) was added at the time indicated by the arrow (2, 3, 5 and 6). (**b**) SDS/gel electrophoresis patterns of the supernatant (S), precipitate (P) and the mixture before ultracentrifugation (M) with (+CN) or without (−CN) calponin. 1, 2, 3, 4, 5 and 6, as in (**a**).

0.5 mM EGTA (Figure 6a, curve 5). However, in the presence of 0.1 mM $CaCl_2$, depolymerization proceeded markedly (Figure 6a, curve 6), up to the level seen without calponin. This depolymerization corresponded to the dissociation of calponin from an actin filament induced by Ca^{2+}-calmodulin (Figure 6b). It should be mentioned that both calmodulin and Ca^{2+} did not affect the actin depolymerization at all when calponin was not present (Figure 6a, curves 1, 2 and 3). Electron microscopic observations revealed that actin filaments stabilized by calponin disappeared in the presence of Ca^{2+}-calmodulin, but some actin filaments remained, even 48 h after dilution, in the presence of calmodulin at low Ca^{2+} concentrations (0.5 mM EGTA), as can clearly be seen in Figure. 7.

DISCUSSION

The present work clearly demonstrated that calponin induces and accelerates polymerization of actin at very low ionic strengths and also inhibits depolymerization of actin at low ionic strengths. The dynamics of actin polymerization are affected by many actin-binding proteins, some of them are physiologically significant [22].

As Oosawa and Asakura [23] first described, actin polymerization consists of two main steps: nucleation and elongation of formed nuclei, i.e. most probably actin trimers. The rate of the nucleation step determines the rate of polymerization. In the

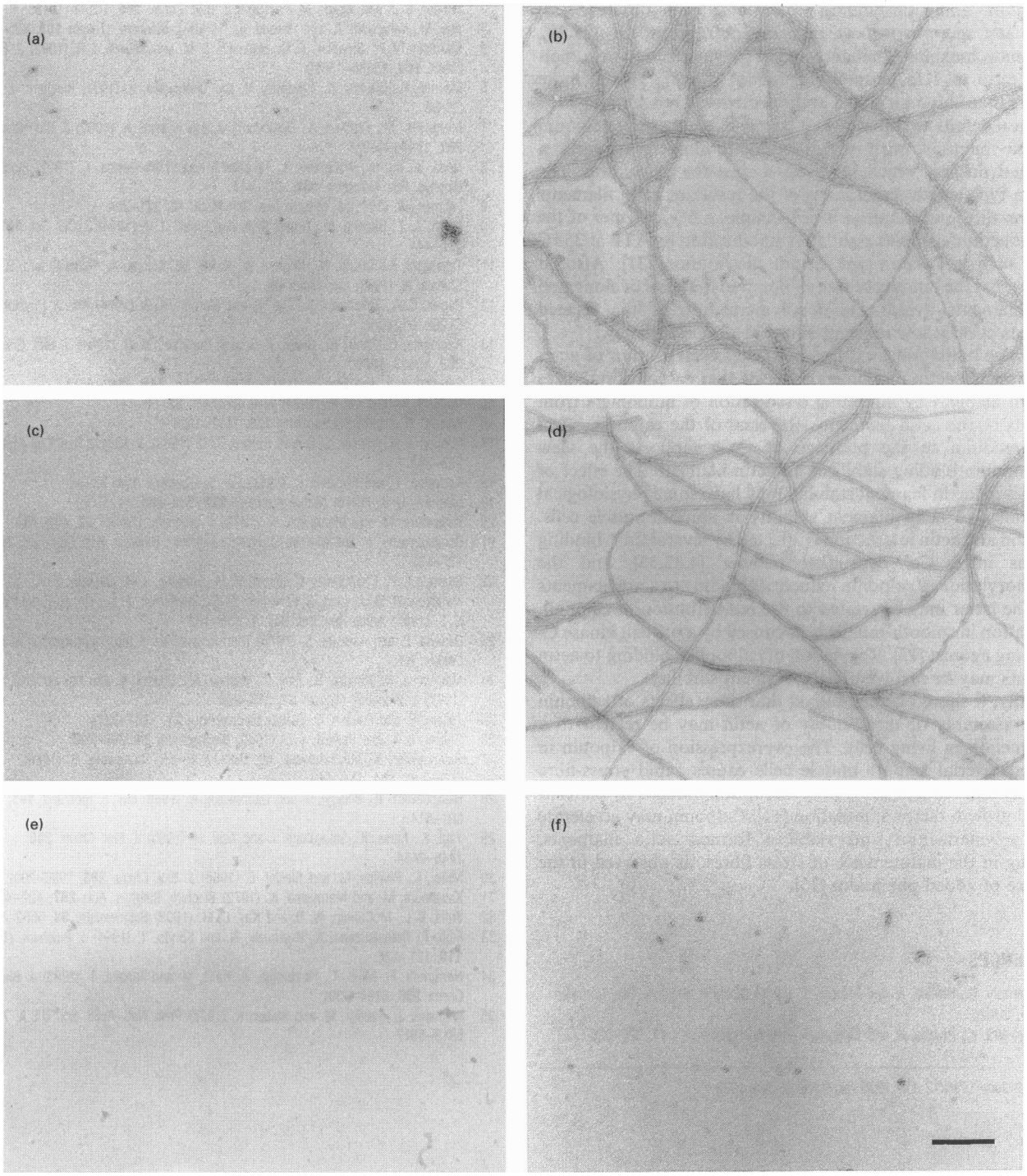

Figure 7 Electron micrographs of actin filaments treated with calponin and Ca^{2+}-calmodulin at 3.3 mM KCl

Conditions are as described in Figure 6(b). Samples were taken 48 h after dilution. A, actin (0.1 mM $CaCl_2$); B, actin and calponin (0.1 mM $CaCl_2$); C, actin and calmodulin (0.5 mM EGTA); D, actin, calponin and calmodulin (0.5 mM EGTA); E, actin and calmodulin (0.1 mM $CaCl_2$); F, actin, calponin and calmodulin (0.1 mM $CaCl_2$). Scale bar, 0.2 μm.

presence of ~ 50 mM KCl or ~ 0.5 mM $MgCl_2$, both steps proceed rapidly. Some actin-binding proteins, e.g. so-called capping proteins such as β-actinin (Cap Z) [24] and plasma gelsolin [25], accelerate the nucleation step by forming complexes with actin molecules [26]. Such complexes have, however, little tendency to elongate at very low ionic strengths. In the cases described above, the formed nuclei are easily depolymerized at low ionic strength and therefore the presence of salts is needed to get elongation. In the present study, elongation of the calponin–actin complex took place even in G-buffer (2 mM Tris/HCl, pH 8.0), where otherwise depolymerization occurred. This effect of calponin is similar to those of caldesmon (another

calmodulin- and actin-binding protein of smooth muscle [27,28] and heavy meromyosin or myosin subfragment S1 [29,30]. Caldesmon maximally enhances actin polymerization at a molecular ratio of 1 (caldesmon): 3 (actin) [27,28]. This is more effective than calponin (1:0.8 in the present study). Although the structural details are unknown, it is very likely that one calponin molecule interacts with several actin monomers to form a stabilized nucleus which serves as a seed for elongation. The Poisson-type length distribution of the resultant actin filaments supports this interpretation. For example, a Mg polymer of the actin–β-actinin complex right after stabilization by ATP at 35 °C shows such a Poisson-type length distribution [31]. Also, it appears that the binding of free calponin to the sides of elongated actin filaments avoids the depolymerization of the formed filaments even at low ionic strengths as discussed below.

Effective inhibition by calponin of depolymerization of actin filaments into actin monomers suggests that calponin stabilizes an actin filament by inhibiting dissociation of monomers from the ends of the actin filaments. Reversal of the calponin effect by calmodulin in the presence of Ca^{2+} supports the view that calponin binding stabilizes an actin filament. The effect of calponin on actin filament stability may have some physiological significance to actin filament stability in smooth-muscle cells. Calponin and actin interaction is affected by several Ca^{2+}-binding proteins in a Ca^{2+}-dependent manner [4,32,33], and the phosphorylation of calponin reduces its binding to actin filaments [34]. The latter may be related to the redistribution of calponin localization in smooth-muscle cells caused by a protein kinase C-activating agonist [12]. Regulation of calponin's binding to actin filaments may be involved in the filament stability.

Finally, it should be mentioned that these effects of calponin on the assembly or disassembly of actin may be related to a phenomenon in living cells. The overexpression of calponin in cultured arterial smooth-muscle cells causes rapid stress-fibre assembly and reduces stress-fibre disruption induced by platelet-derived growth factor stimulation [11]. Calponin may accelerate actin polymerization and stabilize formed actin filaments, resulting in the maintenance of stress fibres, as observed in the presence of added phalloidin [35].

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