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Effect of 67 kDa calcimedin on caldesmon functioning

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Interaction of smooth muscle caldesmon with calmodulin, troponin C, S-100 protein and 67 kDa calcimedin was analyzed. Native gel electrophoresis and crosslinking revealed the complex formation between caldesmon and three EF-hand Ca-binding proteins, whereas calcimedin did not interact with caldesmon. In the presence of Ca^{2+}, calcimedin binds to actin-tropomyosin without affecting the interaction of caldesmon with this complex. Although calcimedin reversed the inhibitory action of caldesmon on the actomyosin ATPase activity at a lower concentration than three other Ca-binding proteins, this effect only slightly depends on Ca^{2+} and was observed at the concentration of calcimedin comparable to that of actin. It is concluded that calcimedin itself cannot be responsible for Ca-dependent regulation of caldesmon functioning, but actin bundling induced by calcimedin (or by other actin binding proteins) decreases the inhibitory action of caldesmon on the actomyosin ATPase activity.

Caldesmon; Calcimedin; Calmodulin; Troponin C; S-100

1. INTRODUCTION

Caldesmon is an actin- and calmodulin-binding protein playing an important structural and regulatory role in smooth muscles and certain non-muscle cells [1,2]. Caldesmon inhibits the ATPase activity of actomyosin and this inhibition is reversed only after addition of a large excess of Ca-saturated calmodulin [1,3]. Therefore it was supposed that calmodulin mimics the effect of another much more effective Ca-binding protein which regulates caldesmon activity [3]. A partially purified fraction of Ca-binding proteins containing calmodulin, S-100 protein and calcimedin was more potent than isolated calmodulin in reversal of caldesmon induced inhibition of the actomyosin ATPase activity [3]. S-100 protein [4–6] and caltropin [7] interact with caldesmon and reverse its inhibitory action on ATPase of actomyosin. At the same time, in the case of smooth muscle actin, the effectivity of S-100 protein does not differ significantly from that of calmodulin [6]. Therefore the search for another Ca-binding protein involved in the regulation of caldesmon action was continued [8]. Since calcimedin was one of the constituents of the protein mixture effectively reversing caldesmon inhibition [3] it was desirable to analyze the effect of 67 kDa Ca-phopholipid binding protein (calcimedin) on caldesmon functioning.

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Abbreviations: DFDNB-1,3-difluoro-4,6-dinitrobenzene; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; PMSF, phelylmethanesulfonylfluoride; SDS, sodium dodecyl sulphate.

2. MATERIALS AND METHODS

Caldesmon and tropomyosin were isolated from duck gizzard by earlier described methods [9]. Actin and myosin were prepared from rabbit skeletal muscles [10,11]. Calmodulin and S-100 protein were obtained from bovine brain [12,13] and troponin C from rabbit skeletal muscle [14]. 67 kDa calcimedin was purified from duck gizzard according to Kobayashi and Tashima [15] with slight modifications.

The interaction of Ca-binding proteins with caldesmon was measured either by the method of native gel electrophoresis [4] or by using the cross-linking technique. Zero-length crosslinking was performed according to Grabarek and Gergely [16]. Ca-binding proteins (about 0.3 mg/ml) in 0.1 M MES (pH 6.0), 100 mM NaCl, 0.1 mM $CaCl_2$ were mixed with EDC and NHS (final concentrations 2 and 5 mM, respectively) and incubated for 15 min at 25°C. Reaction was quenched by mercaptoethanol (20 mM) and caldesmon (final concentration 0.3–0.6 mg/ml) was added to the incubation mixture. After 30–60 min incubation at 25°C reaction was stopped by addition of SDS-containing sample buffer and the protein composition was analyzed by 7–15% gradient polyacrylamide gel electrophoresis in the presence of SDS [17]. Alternatively, caldesmon was activated by EDC and NHS by the same procedure and mixed with Ca-binding proteins. The mixture of caldesmon (0.6 mg/ml) and Ca-binding proteins (0.6–1 mg/ml) in 50 mM MOPS pH 8.0, containing 0.1 mM $CaCl_2$ was also subjected to crosslinking by 1 mM DFDNB. After incubation for 30 min at 30°C the reaction was stopped either by addition of glycine (up to 70 mM) or mercaptoethanol (50 mM). The protein composition was analyzed by SDS-gel electrophoresis.

The interaction of calcimedin with phospholipids and actin-tropomyosin or actin-tropomyosin-caldesmon complex was analyzed by ultracentrifugation. The mixture of azolectin (1.1 mg/ml) and calcimedin (0.3 mg/ml) in buffer A (10 mM Tris-HCl, pH 7.5, 0.5 mM mercaptoethanol, 0.1 mM PMSF) was ultracentrifuged for 1 h at 116,000 × *g* and the protein composition of supernatant and pellet was analyzed by SDS-gel electrophoresis. The mixture of actin (4 μM) and tropomyosin (0.8 μM) in buffer B (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM ATP and 1 mM mercaptoethanol) was

titrated with calcimedin (final concentration 1–10 μM) and after a 15 min incubation at room temperature, ultracentrifuged at 130,000 × g for 40 min. Competition of calcimedin and caldesmon for binding to actin-tropomyosin was analyzed by titrating the mixture of actin, tropomyosin and caldesmon by calcimedin or by titrating the mixture of actin, tropomyosin and calcimedin with caldesmon. After ultracentrifugation, the protein composition of the pellet and supernatant was determined by quantitative scanning of Coomassie brilliant blue stained polyacrylamide gels [18].

The ability of Ca-binding proteins to detach caldesmon from the actin-tropomyosin complex was analyzed by ultracentrifugation. The mixture of actin (5 μM), tropomyosin (0.8 μM) and caldesmon (0.5 μM) in the buffer containing 15 mM imidazole (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2 mM ATP and 2 mM mercaptoethanol was titrated by different Ca-binding proteins (final concentration 1–7 μM). After ultracentrifugation the content of caldesmon in the supernatant and pellet was determined by SDS-gel electrophoresis.

The ATPase activity of actomyosin was determined in the buffer containing 15 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 0.3 mM mercaptoethanol, 0.1 mM $CaCl_2$ and 2 mM ATP. The incubation mixture contained 1 μM actin, 0.2 μM tropomyosin and 0.3–0.4 μM myosin. Under these conditions the ATPase activity measured at 30°C by inorganic phosphate liberation [19] was equal to 300 nmol P_i/min/mg of myosin. Addition of caldesmon (0.4 μM) induced 60–70% inhibition of actomyosin ATPase. The inhibition induced by caldesmon was reversed by different Ca-binding proteins (1–8 μM) which were added to the reconstructed actomyosin. Protein concentration was determined either by molar extinction coefficients published in the literature or by the method of Spector [20] using bovine serum albumin as a standard.

3. RESULTS

Slightly modified procedure of Kobayashi and Tashima [15] was used for purification of 67 kDa calcimedin from duck gizzard. The complex of calcimedins with membranes and cytoskeleton which was formed in the presence of Ca^{2+} was recovered after ultracentrifugation and calcimedin enriched fraction was extracted with EGTA. The thus obtained extract contained filamin, actin, tropomyosin and 67 and 32–34 kDa calcimedins. Further purification was achieved by ion-exchange chromatography on DEAE-Toyopearl where calcimedins were eluted at 0.1–0.15 M NaCl immediately after filamin. Final purification and separation of 67 and 32–34 kDa calcimedins was performed on the Ultrogel AcA 44 column. The yield of 67 kDa calcimedin was equal to 3–3.5 mg from 100 mg of duck gizzard and was slightly higher than that reported in the literature. In the presence (but not in the absence) of Ca^{2+}, 67 kDa calcimedin interacted and was coprecipitated with azolectin (mixture of soybean phospholipids). This agrees well with the data of Kobayashi and Tashima [15]. In the presence of Ca^{2+} 67 kDa calcimedin interacts with actin-tropomyosin complex. The half-maximal binding was observed at 4–6 μM calcimedin and saturation was achieved when about 0.5 mol of calcimedin were bound per mol of actin. Thus, all properties of 67 kDa duck gizzard protein were similar to those of 67 kDa calcimedin isolated from chicken gizzard.

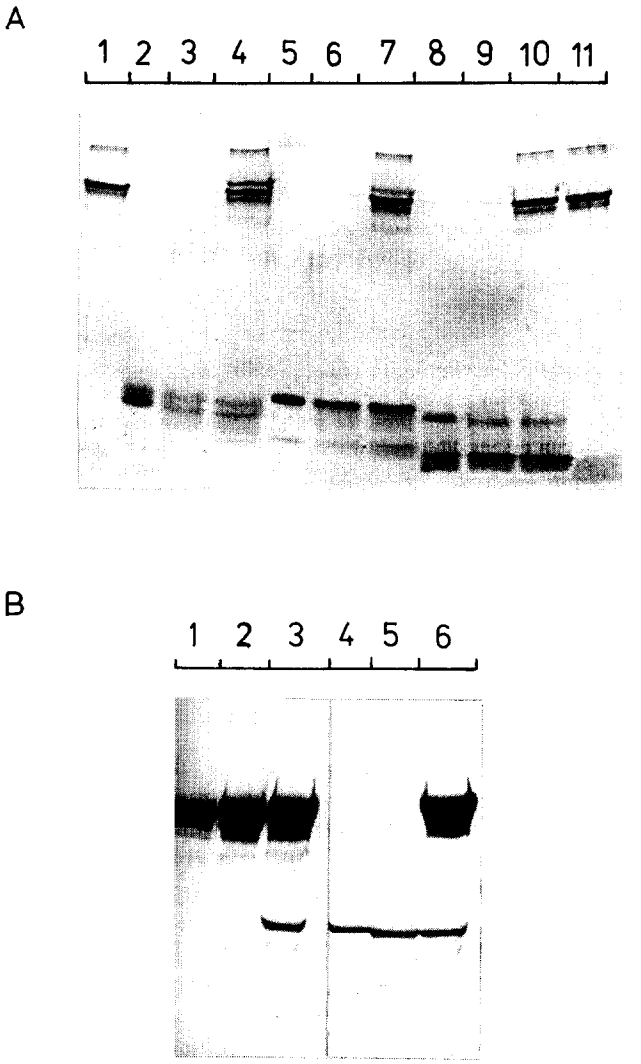

Fig. 1. Crosslinking of caldesmon with different Ca-binding proteins (A) Zerolength cross-linking of caldesmon with calmodulin (2–4), troponin C (5–7) and S-100 protein (8–10). 1 and 11, isolated caldesmon; 2, 5 and 8, isolated Ca-binding proteins; 3, 6 and 9, Ca-binding proteins after activation with NHS and EDC; 4, 7 and 10, mixture of caldesmon with activated Ca-binding proteins after crosslinking. (B) Zero-length cross-linking of caldesmon and calcimedin. 1, isolated caldesmon; 2, caldesmon activated by EDC and NHS; 3, the mixture of calcimedin with activated calcimedin; 4, isolated calcimedin; 5, calcimedin activated by EDC and NHS; 6, the mixture of caldesmon with activated calcimedin.

The interaction of different Ca-binding proteins with caldesmon was analyzed by two different methods. As described earlier [4] under nondenaturating conditions, the complex of caldesmon with calmodulin, troponin C or S-100 protein has higher electrophoretic mobility than isolated caldesmon. Although the complex of caldesmon with the three above mentioned proteins was easily detected we failed to observe any complex formation between caldesmon and calcimedin.

We also used the method of zero-length crosslinking for analyzing the interaction of caldesmon with Ca-binding proteins. The data of Fig. 1A indicate that after

activation with EDC and NHS calmodulin and troponin C can be crosslinked to caldesmon in the presence of Ca^{2+}. An earlier and similar method was successfully used for analysis of interaction of calmodulin and its peptide with caldesmon [21]. We were unable to crosslink activated S-100 protein or activated calcimedin to caldesmon (Fig. 1). The same negative results were obtained when activated caldesmon was mixed with non-activated calcimedin (Fig. 1B) or S-100 protein. Using a short rigid bifunctional reagent DFDNB we crosslinked calmodulin and troponin C with caldesmon. At the same time we failed to detect crosslinking products when caldesmon was mixed with S-100 protein or calcimedin.

Both caldesmon and calcimedin interact with actin-tropomyosin complex, therefore it seems reasonable to analyze whether these proteins can compete for actin

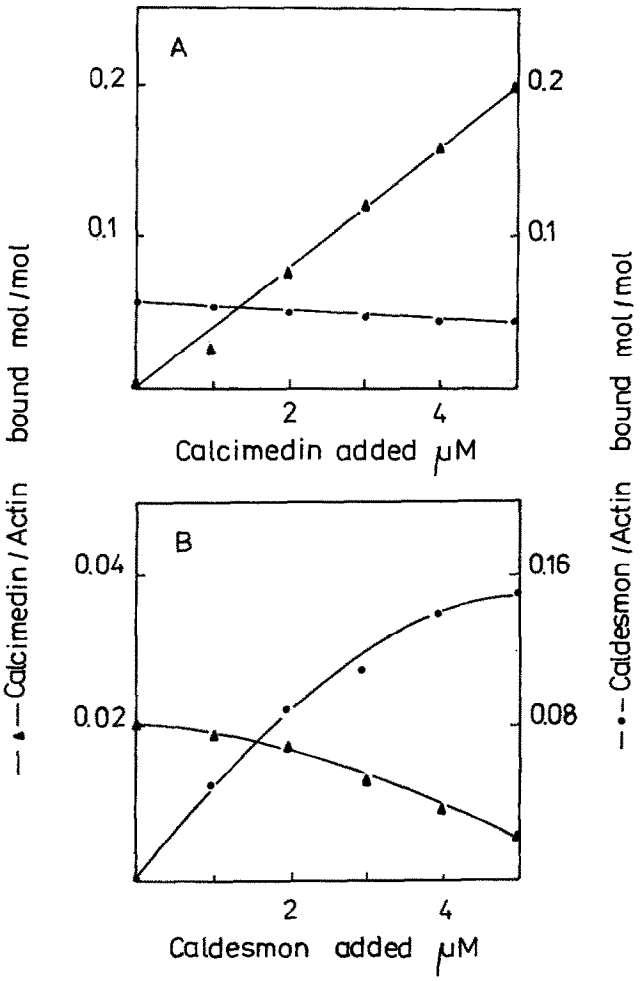

Fig. 2. (A) Effect of calcimedin on caldesmon binding to actin-tropomyosin. 14 μM actin and 7 μM tropomyosin containing 1 μM caldesmon were titrated with calcimedin and the protein composition of the pellet obtained after ultracentrifugation was analyzed by SDS-gel electrophoresis. (B) Effect of caldesmon on the binding of calcimedin to actin-tropomyosin. 14 μM actin and 7 μM tropomyosin containing 1 μM calcimedin were titrated with caldesmon and proteins bound to actin were determined after ultracentrifugation.

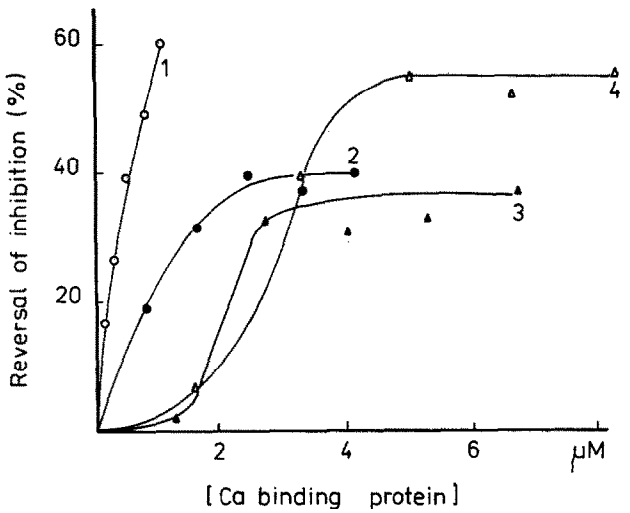

Fig. 3. Effect of Ca-binding proteins on the ATPase activity of skeletal muscle actomyosin inhibited by caldesmon. Final concentration of proteins was: actin, 1 μM; myosin, 0.4 μM; tropomyosin, 0.14 μM; caldesmon, 0.4 μM. 60% inhibition of actomyosin ATPase activity induced by caldesmon was reversed by addition of calcimedin (1), S-100 protein (2), troponin C (3) and calmodulin (4).

binding. The data of Fig. 2A indicate that binding of up to 0.2 mol of calcimedin per mol of actin does not decrease the quantity of caldesmon bound to actin. On the contrary when the actin-tropomyosin-calcimedin complex was titrated by caldesmon we observed more than two-fold decrease in calcimedin content (Fig. 2B). Thus, caldesmon displaced calcimedin from its complex with actin. Continuing these experiments we analyzed the ability of different Ca-binding proteins to detach caldesmon from actin-tropomyosin. Under the conditions used (5 μM actin, 0.8 μM tropomyosin and 0.5 μM caldesmon), the ratio of caldesmon remaining free and bound to actin was equal to 1. Addition of 5 μM of calcimedin did not change this ratio, whereas addition of 6 μM calmodulin, S-100 protein or troponin C increased this value up to 1.7–2.5. This means that in the presence of a large excess of EF-hand Ca-binding proteins caldesmon was detached from actin filaments.

In the final stage of the investigation we analyzed the ability of different Ca-binding proteins to reverse caldesmon-induced inhibition of the actomyosin ATPase activity. As shown in Fig. 3 addition of S-100 protein, calmodulin or troponin C reversed the inhibitory action of caldesmon. This reversal was not complete and requred addition of a large excess of Ca-binding proteins. The effectivity of reversal decreased in the order S-100 protein > troponin C = calmodulin. Three out of four preparations of calcimedins reversed the inhibitory action of caldesmon at lower concentration than other Ca-binding proteins. The effect of calcimedin was rather variable, was observed at the concentration of calcimedin close or equal to that of actin and was only slightly dependent on the presence of Ca^{2+}. We failed to

detect any effect of calcimedin on the caldesmon-induced inhibition of acto-S-1 ATPase activity. Since calcimedin did not interact with caldesmon and is unable to displace caldesmon from actin filaments we suppose that the observed effect of calcimedin on the actomyosin ATPase activity is non-specific and is due to crosslinking or bundling of actin filaments.

4. DISCUSSION

We analyzed the interaction of different Ca-binding proteins with caldesmon. In good agreement with earlier published data [21,22] we found that calmodulin can be crosslinked to caldesmon in the presence of Ca^{2+}. Skeletal muscle troponin C can also be crosslinked to caldesmon (see Fig. 1), and troponin C and calmodulin are approximately equal in reversal of caldesmon induced inhibition of actomyosin ATPase activity (Fig. 3). S-100 protein belonging to the same family of EF-hand Ca-binding proteins interacted with caldesmon, but unlike calmodulin and troponin C we failed to crosslink S-100 to caldesmon. This may indicate that the sites of caldesmon interaction with S-100 protein are different from that of caldesmon-calmodulin interaction. In agreement with our earlier published data [4] we found that under certain conditions S-100 is more effective in reversal of caldesmon induced inhibition of skeletal actomyosin ATPase than calmodulin (Fig. 3). The effect of EF-hand proteins is explained by their direct interaction with caldesmon and their ability to cause partial detachment of caldesmon from actin-tropomyosin complex.

67 kDa calcimedin belongs to another family of Ca-binding proteins called annexins. The 67 kDa calcimedin did not interact with isolated caldesmon (Fig. 1). In the presence of Ca^{2+} calcimedin interacted with actin-tropomyosin complex and about 0.5 mol of calcimedin were bound per mol of actin. These data are similar to those described for lipocortin-85 [23]. Unlike lipocortin-85, the affinity of 67 kDa to actin-tropomyosin complex was rather low (the apparent dissociation constant was about 5 μM). Affinity of caldesmon to actin-tropomyosin is at least 5 times higher than that of calcimedin therefore under conditions used calcimedin did not compete with caldesmon for actin-tropomyosin binding (Fig. 2A), whereas addition of large quantities of caldesmon displaced calcimedin from its complex with actin (Fig. 2B). Thus, calcimedin did not directly interact with caldesmon and was not able to displace caldesmon from its complex with actin-tropomyosin. Nevertheless, some preparations of calcimedin reversed the inhibitory action of caldesmon at lower concentration than any other Ca-binding proteins (Fig. 3). The data presented do not mean that calcimedin is a more effective regulator of caldesmon action than calmodulin. The reversal of inhibition was observed when the concentration of calcimedin was close or equal to that of actin. Under these conditions lipocortin-85 [23] and other annexins [24] can crosslink and bundle actin filaments. Therefore we may suppose that the effect observed is at least partly due to the change in the state of actin filaments. This suggestion correlates with the observation that calcimedin was ineffective in reversal of caldesmon-induced inhibition of acto-S-1 ATPase activity. In this respect the effect of calcimedin is comparable with that of filamin which activated actomyosin ATPase activity but was unable to activate acto-S-1 ATPase activity [25]. It is worthwhile mentioning that after DEAE-Toyopearl chromatography calcimedin contained rather a large quantity of filamin and although gel-filtration on Ultrogel AcA-44 column was very effective it is difficult completely exclude contamination of filamin. Our preliminary data indicate that even at the molar ratio actin:filamin of 100:1, filamin prevents the inhibitory action of caldesmon on the ATPase of actomyosin. Therefore even 1% contamination of calcimedin by filamin will cause rather a strong effect on the ATPase activity under the conditions used (see Fig. 3). At present it is diffucult to explain the mechanism by which aggregation of actin filaments prevent the inhibitory action of caldesmon. In any case it is well-known that cross-linking of actin filaments strongly affects actin-myosin interaction and actomyosin ATPase [25–27].

The data presented together with a low content of 67 kDa calcimedin in smooth muscle cell [15] and its preferential localization close to sarcolemma and other membranes of the cell [28] make the direct involvement of calcimedin in the regulation of contraction very improbable. On the other hand we cannot completely exclude the effect of calcimedin (or other annexins possessing higher affinity to actin) on caldesmon action in certain cell compartments which are enriched in Ca-phopholipid binding proteins.

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