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Influence of Calmodulin on the Human Red Cell Membrane Skeleton†

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ABSTRACT: The calcium receptor calmodulin interacts with components of the human red cell membrane skeleton as well as with the membrane. Under physiological salt conditions, calmodulin has a calcium-dependent affinity for spectrin, one of the major components of the membrane skeleton. It is apparent from our results that calmodulin inhibits the ability of erythrocyte spectrin (when preincubated with filamentous actin) to create nucleation centers and thereby to seed actin polymerization. The gelation of filamentous actin induced by spectrin tetramers is also inhibited by calmodulin. The inhibition is calcium dependent and decreases with increasing pH, similar to the binding of calmodulin to spectrin. Direct binding studies using aqueous two-phase partition indicate that calmodulin interferes with the binding of actin to spectrin. Even in the presence of protein 4.1, which is believed to stabilize the ternary complex, calmodulin has an inhibitory effect. Since calmodulin also inhibits the corresponding activities of brain spectrin (fodrin), it appears likely that calmodulin may modulate the organization of cytoskeletons containing actin and spectrin or spectrin analogues.

The mechanical properties of the human red blood cell are determined by a network of proteins attached to the cytoplasmic surface of the membrane. It is also believed that the network is involved in maintaining the lipid asymmetry and unique biconcave shape of the cell. This network or membrane skeleton is composed of spectrin, actin, and proteins 4.1 and 4.9, the latter being a minor component. The cytoskeleton is attached to the membrane by ankyrin which possesses binding sites for both spectrin and the transmembrane protein band 3 (anion channel) (Cohen, 1983; Gratzer, 1983; Marchesi, 1984; Bennett, 1985). In addition, protein 4.1 appears to have a polyphosphoinositide-dependent affinity for glycophorin (Anderson & Marchesi, 1985).

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In the cell, spectrin occurs predominantly as a tetramer (Ungewickell & Gratzer, 1978; Liu & Palek, 1980) formed by head-to-head association of two heterodimers. The head region contains the binding site for ankyrin (Cohen, 1983; Gratzer, 1983; Bennett, 1985) whereas the region at the distal ends of the tetramer contains binding sites for actin and protein 4.1 (Tyler et al., 1979). The spectrin tetramer is therefore divalent with respect to actin and protein 4.1.

In the cytoskeleton, actin is organized into short protofilaments, each containing about 12 actin monomers (Pinder & Gratzer, 1983; Shen et al., 1986). Recently, it was inferred from electron microscopy of cytoskeletons (Byers & Branton, 1985; Shen et al., 1986) that each actin protofilament is typically associated with five to eight spectrin tetramers. The association between actin filaments and spectrin is strengthened by protein 4.1; the dissociation constant of the ternary complex is about 1 μM[2] compared to a value of 0.2 mM for the binary complex between actin and spectrin (Ohanian et al., 1984). Thus, in the continuous network, actin protofilaments provide junctions for spectrin tetramers, and the tetramers bridge adjacent protofilaments whereas protein 4.1 confers stability on the network. The function of other cytoskeletal proteins is unknown, although it has been shown that protein 4.9 has actin-bundling activity (Siegel & Branton, 1985).

In addition, the red cell contains proteins that may interact with the cytoskeleton, although they are not yet regarded as cytoskeletal components. For instance, it has been suggested that tropomyosin, due to its physical dimension and abundance, may coat almost every actin protofilament and thereby both stabilize and determine the length of the actin filaments (Fowler & Bennett, 1984; Shen et al., 1986).

The calcium binding protein calmodulin interacts with components of the membrane cytoskeleton as well as with the membrane itself. It has been shown that, under physiological salt conditions, calmodulin has a calcium-dependent affinity for purified spectrin (Berglund et al., 1984; Husain et al., 1984) and isolated membrane skeletons (Burns & Gratzer, 1985). Since the affinity of calmodulin for spectrin and the cytoskeleton is similar (about 10 μM) and the number of sites for calmodulin in the cytoskeleton corresponds to the concentration of spectrin, it appears likely that spectrin constitutes the major binding site for calmodulin in intact cytoskeletons. Recently, a protein was discovered that may indirectly connect calmodulin to the cytoskeleton. This protein (which is a heterodimer with subunit molecular weights of 103 000 and 97 000) associates tightly with the cytoskeleton and binds calmodulin with an affinity of 230 nM (Gardner & Bennett, 1986; Ling et al., 1986). The binding protein is present at about 30 000 copies per cell, compared to about 200 000 spectrin dimer molecules per cell (Pinder & Gratzer, 1983).

Although the concentration of calmodulin is relatively high in the red cell [it has been estimated at 2–3 μM (Foder & Scharff, 1981; Kakiuchi et al., 1982)], the only function established is the activation of the membrane (Ca^{2+} + Mg^{2+})-ATPase (Larsen & Vincenzi, 1979; Graf & Penniston, 1981). Since nanomolar calmodulin fully activates this enzyme (Agre et al., 1983), the bulk of calmodulin should be available for other functions.

Despite the relatively weak binding to spectrin, a substantial fraction of calmodulin may be bound to the cytoskeleton due to the high spectrin concentration close to the membrane (Morrow et al., 1981). Therefore, if calmodulin has other functions besides its activation of the calcium pump, it seems rational that these imaginable functions could involve the membrane skeletal complex. In view of this possibility, we have attempted to resolve whether binding of calmodulin to spectrin exerts any influence on the cytoskeleton and its organization.

From the experimental results, we conclude that calmodulin decreases the ability of spectrin, even in the presence of protein 4.1, to fragment actin and inhibits the gelation process. We conclude further than actin filaments reduce, in a concentration-dependent manner, the binding of calmodulin to spectrin.

Materials and Methods

Protein Purification. Actin was extracted from acetone powder of rabbit back and leg muscles by using a modification (Eisenberg & Kielly, 1974) of the method of Spudich and Watt (1971) except that the buffer used to depolymerize actin contained 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] 0.2 mM ATP, 0.2 mM $CaCl_2$, and 0.5 mM 2-mercaptoethanol, pH 8.0 (buffer A). Further purification of monomeric actin was achieved by gel filtration on Sephadex G-100 (Pharmacia) (1.6 × 70 cm). Actin was stored on ice for up to 1 week or lyophilized and stored at −20 °C. Lyophilized actin was dissolved as described previously (Strömqvist et al., 1985).

Erythrocyte spectrin and protein 4.1 were prepared according to Ohanian and Gratzer (1984) from fresh human blood. After chromatography on Sepharose CL-4B (Pharmacia) (2.5 × 130 cm), spectrin was dialyzed against 10 mM Tris-HCl, pH 7.5, and protein 4.1 against 10 mM Tris-HCl/0.01% Tween 20, pH 7.5. To ensure that spectrin was dimeric, it was incubated at 37 °C for 15 min after ammonium sulfate precipitation. Spectrin dimer was partially converted to tetramer by incubation in 10 mM Tris-HCl and 150 mM KCl, pH 7.5, at 37 °C for 2 h. Proteins were stored on ice and used within 2 weeks. Brain spectrin was prepared according to Bennett et al. (1986) with some minor modifications.

Calmodulin was prepared from bovine brain (Gopalakrishna & Anderson, 1982) and radiolabeled by reductive methylation (Dottavio-Martin & Ravel, 1978) using [^{14}C]formaldehyde (New England Nuclear). Gel electrophoresis (Laemmli, 1970) was used to determine the purity of the protein preparations.

Buffers. The binding of calmodulin to erythrocyte spectrin is very dependent on the pH (Burns & Gratzer, 1985); above pH 8, only a fraction is bound compared to the amount bound at pH 7.0. We have therefore used the following buffers: 5 mM imidazole hydrochloride, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 0.5 mM 2-mercaptoethanol, pH 7.0 (Ca buffer); 5 mM imidazole hydrochloride, 0.2 mM ATP, 0.2 mM $MgSO_4$, 1.0 mM EGTA, and 0.5 mM 2-mercaptoethanol, pH 7.0 (Ca-free buffer). Proteins were transferred into the used buffer by dialysis overnight at 4 °C.

Fluorescence Measurements. Actin was labeled with *N*-(3-pyrenyl)maleimide (Fluka) as described previously (Strömqvist et al., 1984). The fluorescence enhancement upon polymerization of pyrenyl-labeled actin was measured by using a Perkin-Elmer 512 fluorometer with excitation and emission wavelengths of 366 and 405 nm, respectively.

Gelation Experiments. Many different approaches were tested for measuring the degree of cross-linking or gelation: rolling ball viscometry, time for an air bubble to travel up through the solution, etc. However, the most reproducible

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ATP, adenosine 5′-triphosphate; kDa, kilodalton(s).

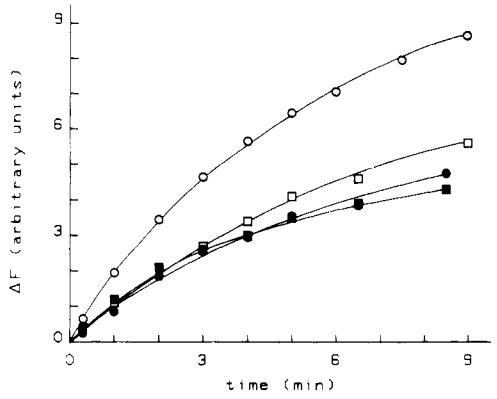

FIGURE 1: Effect of calmodulin and spectrin on actin polymerization. Unlabeled actin was polymerized by KCl (150 mM) overnight. The polymerized actin (3.8 μM final concentration) was mixed with either Ca buffer (●), spectrin [0.35 μM (○)], calmodulin [9.4 μM (■)], or spectrin (0.35 μM) and calmodulin (9.4 μM) (□) and incubated for 10 min at 20 °C. Each preincubated sample was added to an equal volume of 4.8 μM pyrenyl-labeled actin monomers, and the fluorescence enhancement at 20 °C was followed.

results were obtained with an Ubbelohde type viscometer with a wide capillary. The viscometer was filled with the actin-containing solution and left undistributed for 15–20 min. The flow time was thereafter determined, and immediately after measurement, the viscometer was refilled.

Binding Studies Using Aqueous Two-Phase Partition. The phase systems were prepared from a phase mix containing 16% (w/w) Ficoll 400, 12% (w/w) Dextran T40 (both Pharmacia), 2.1% (w/w) (trimethylamino)poly(ethylene glycol) 8000 (TMA-PEG 8000) (Johansson et al., 1973), 67 mM KCl, 2.7 mM $MgCl_2$, and 40 mM HEPES, pH 7.0. The phase mix was stirred continuously while aliquots of 0.75 g were weighed into plastic tubes; 250 μL of sample was then added to each phase system giving the final polymer concentrations given in Figure 5. Before addition to the phase system, actin was polymerized by addition of 2 mM $MgCl_2$ at pH 8.0. Spectrin and calmodulin were in 10 mM Tris-HCl, pH 7.5.

The samples were incubated in the phase systems for 30 min at 20 °C with end-over-end rotation. The phases were then separated by centrifugation at 2250*g* for 10 min. The concentration of [^{14}C]calmodulin was determined by counting 0.2 mL of upper and lower phase, respectively, in Lumagel scintillation fluid (Lumac). The concentrations of spectrin and actin in the phases were determined by the method of Bradford (1976). Since nearly all spectrin was recovered in the lower phase and thus the binding equilibria only occur in that phase, the concentrations of spectrin and actin used in the calculations and given in Figures 5 and 6 refer to the concentrations in the lower phase.

RESULTS

The rate at which filamentous actin incorporates pyrenyl-labeled actin monomers was significantly increased by a short preincubation of actin filaments with spectrin (Figure 1) as observed before (Strömqvist et al., 1985). This implies that spectrin increases the number of growing ends by fragmenting actin filaments. Inclusion of both spectrin and calmodulin in the preincubation mixture reduced the rate of incorporation of actin monomers. Incubation of filamentous actin with only calmodulin did not change the incorporation rate from the basal one.

The inhibitory effect of calmodulin on the fragmenting activity of spectrin was concentration dependent; with increased molar ratio of calmodulin to spectrin, the rate of incorporation of actin monomers decreased (Figure 2A) and approached the

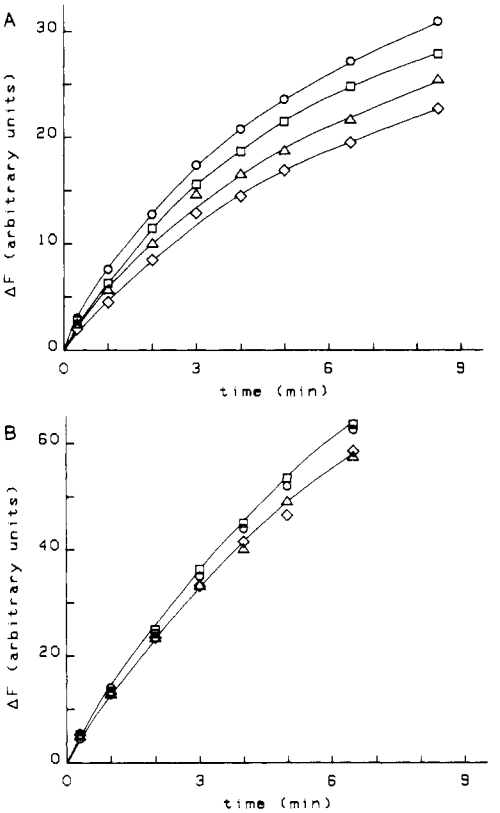

FIGURE 2: Effect of calmodulin concentration and calcium on spectrin-induced fragmentation of actin filaments. (A) Spectrin and calmodulin were added to unlabeled actin, polymerized as described in Figure 1. The final concentrations of actin and spectrin were 2.4 and 1.2 μM, respectively, and those of calmodulin were 0 (○), 8.1 (□), 16.2 (△), and 32.5 μM (◇). After incubation at 20 °C for 10 min, 150 μL of each preincubated sample was added to 8.3 μM pyrenyl-labeled actin (250 μL), and the fluorescence enhancement was followed. (B) Same procedure as in (A), except that proteins were dialyzed against Ca-free buffer to remove calcium from all solutions and the concentration of pyrenyl-labeled actin was 4.8 μM.

rate observed in the absence of spectrin. The calmodulin-dependent inhibition required calcium and consequently activated calmodulin since only minor effects were observed in calcium-free medium (Figure 2B). Unfortunately, the requirement of calcium-activated calmodulin could not be tested by doing the experiment in the presence of a calmodulin inhibitor (e.g., chlorpromazine) as the inhibitor caused actin to depolymerize (unpublished results).

In the erythrocyte, protein 4.1 is believed to confer stability on the membrane skeleton (Ohanian et al., 1984). Therefore, we wanted to determine whether Ca^{2+}–calmodulin inhibited the spectrin-induced fragmentation also in the presence of protein 4.1. Figure 3a shows, in accord with previous results (Strömqvist et al., 1985), that preincubation of actin with spectrin and protein 4.1 elevated the rate of actin monomer incorporation even further. When calmodulin was included in the preincubation mixture as well, the rate was reduced as in the case of spectrin alone (Figure 2). The reduction in incorporation rate was, however, dependent on the protein 4.1 concentration; at high molar ratios (above 2–3) of spectrin to protein 4.1, the rate was about the same as observed in the absence of protein 4.1 whereas at lower ratios the rate increased markedly though not as much as in the absence of calmodulin (Figure 3B).

The binding of calmodulin to spectrin is pH dependent; the association appears to be strongest around pH 6.5, and it is gradually reduced with increasing pH. At pH 8.5, the binding is about one-fourth of that observed at pH 6.5 (Burns &

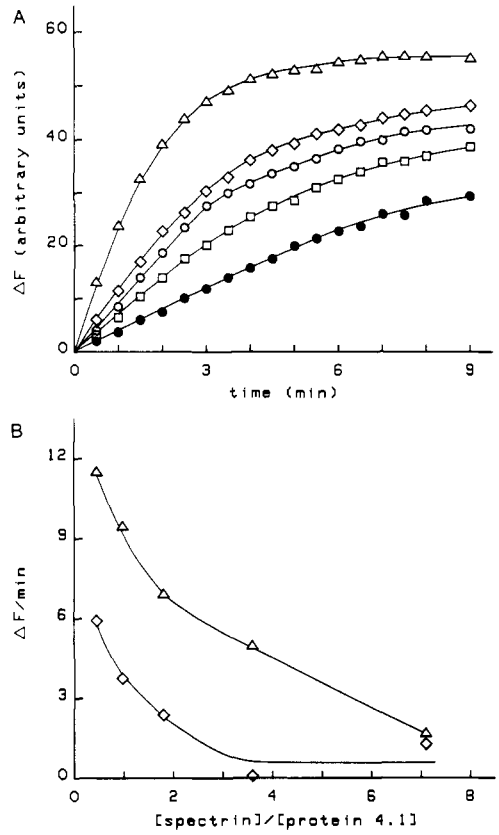

FIGURE 3: Effect of protein 4.1 on the calmodulin-inhibited fragmentation. (A) Actin, polymerized by KCl (100 mM) overnight, was mixed with spectrin, protein 4.1, and/or calmodulin. The final concentrations of actin and spectrin were 4.7 and 0.9 μM, respectively, and those of protein 4.1 and calmodulin were, respectively, 0, 0 (○); 0, 35 (□); 1, 0 (△); and 1, 35 μM (◇). Actin was also incubated in the absence of any other protein (●). After incubation at 25 °C for 10 min, 150 μL was added to 250 μL of 14.3 μM pyrenyl-labeled actin monomers, and the increase in fluorescence was monitored. (B) Same procedure as in (A) was used to determine the initial rate of polymerization at the indicated concentrations of protein 4.1 in the absence (△) or presence (◇) of calmodulin. The plotted values are the differences between the initial rates obtained in the presence and in the absence of protein 4.1. The initial rates determined, in the absence of protein 4.1, after preincubation of spectrin and actin with and without calmodulin were 10.8 and 7.8 arbitrary units/min, respectively.

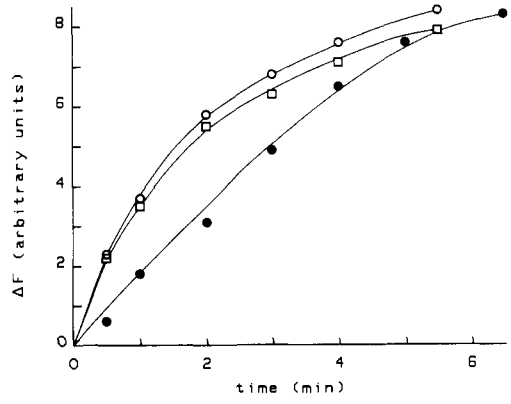

FIGURE 4: Effect of calmodulin on spectrin-induced fragmentation of actin filaments at pH 8.2. Filamentous actin (3.5 μM final concentration), polymerized by 100 mM KCl, was mixed with spectrin (1.6 μM) (○), spectrin (1.6 μM) and calmodulin [44 μM (□)], or buffer (●). After incubation for 10 min at 20 °C, 150 μL was mixed with 250 μL of 11.9 μM pyrenyl-labeled actin monomers, and the fluorescence enhancement was followed. Prior to the experiment, proteins were dialyzed against 5 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM 2-mercaptoethanol, pH 8.2.

Gratzer, 1985). We therefore repeated the fragmentation experiments described above at more basic pH. As can be seen

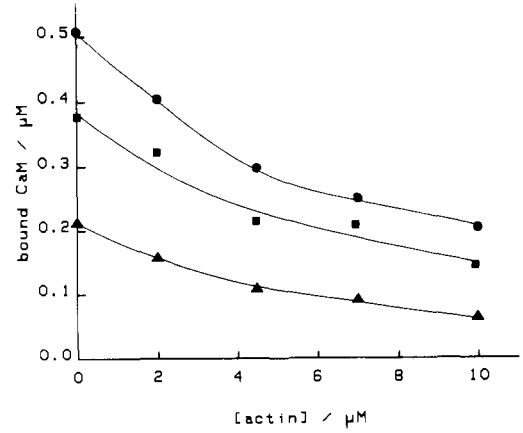

FIGURE 5: Binding of calmodulin to spectrin. Phase partition was used to determine the amount of spectrin-bound calmodulin in the presence of varying concentrations of filamentous actin. The concentration of calmodulin (in the total volume of the phase system) was 1.7 (●), 1.1 (■), or 0.5 μM (▲), respectively, and the concentration of spectrin in the lower phase was 2.7 μM. The composition of the phase system was 12% Ficoll 400, 9% dextran T40, 1.6% TMA-PEG 8000, 50 mM KCl, 2 mM $MgCl_2$, and 30 mM HEPES, pH 7.0. Due to addition of proteins, the system also contained 2.4 mM Tris-HCl, 17 μM $CaCl_2$, 17 μM ATP, and 40 μM 2-mercaptoethanol.

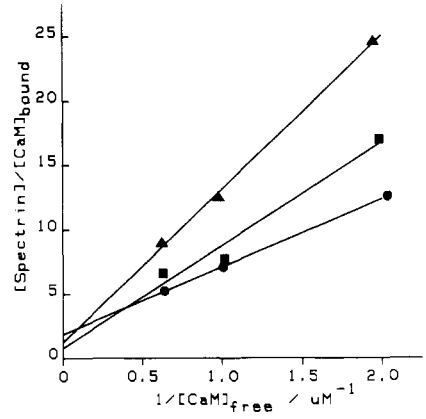

FIGURE 6: Double-reciprocal plot of calmodulin binding to spectrin. The data in Figure 5 were replotted as double-reciprocal plots. The concentrations of actin were 0 (●), 2 (■), and 4.5 μM (▲), respectively.

in Figure 4, actin preincubated with spectrin at pH 8.2 still increased the rate of polymerization whereas inclusion of calmodulin had only a minor inhibitory effect on the process. Thus, calmodulin affects the rate of incorporation of actin monomers into filaments only under conditions where it may associate with spectrin.

To assess whether calmodulin interferes with the binding of filamentous actin to spectrin, we did direct binding studies using the aqueous two-phase partition technique (Backman, 1985). This technique has proven to be well-suited for studying weak interactions, and we have previously used it to characterize the relatively weak association between calmodulin and spectrin (Berglund et al., 1984, 1986). It is clearly seen in Figure 5 that increasing amounts of filamentous actin reduced the fraction of calmodulin bound to spectrin; at 10 μM actin in the lower phase, 60–70% of bound calmodulin was released. When the data were analyzed, using double-reciprocal plots, a competitive type of binding was indicated (Figure 6). The slope, which equals the dissociation constant divided by the number of binding sites (K_d/n), increased 1.5- and 2.2-fold when the actin concentration was increased to 2 and 4.5 μM, respectively. Since filamentous actin, under the ionic conditions employed, partitioned not only between the bulk phases but also to the interphase, the data obtained at higher actin

Table I: Effect of Calmodulin on Gelation[a]

	apparent viscosity	
	−calmodulin	+calmodulin
−protein 4.1	0.95	0.49
+protein 4.1	0.44	0.44

[a] Gelation was measured as described in the text. The apparent viscosity in the absence of protein 4.1 was determined in Ca buffer containing 9.5 μM actin, 1.5 μM spectrin tetramers, and 41.2 μM calmodulin. Polymerization was started by adding KCl to 100 mM. In the presence of protein 4.1 (1.2 μM), the protein concentrations were 4.8 μM actin, 1.1 μM spectrin tetramers, and 29.4 μM calmodulin. The temperature was 20 °C, and the flow time was determined every 20 min until stable values were obtained.

concentrations were not accurate enough for this type of analysis. However, it is evident from this experiment that calmodulin interferes with the association between actin and spectrin.

Another way of studying the influence of calmodulin on the formation of actin–spectrin complexes is to monitor its effect on gelation. This process is, however, extremely difficult to quantify accurately when actin is involved since whenever the solution is perturbed some actin filaments will obviously break, thereby increasing the number of free ends and consequently also changing the steady-state level. One way, we believe, to obtain at least semiquantitative data is to let the actin-containing solution achieve steady-state before measurement. Therefore, we filled the viscometer with the solution containing actin and incubated it before the flow time was determined. Immediately after the measurement, the viscometer was refilled and left undisturbed until the next measurement. It should be noted that we fully realize that this method of measuring gelation excludes any absolute determinations of viscosity and that it should not be used for comparing solutions with small differences in viscosity. Anyhow, the results showed that the ability of spectrin tetramers and actin filaments to form a gel was reduced significantly in the presence of calmodulin. As distinguished from the fragmentation experiments, the effect of calmodulin on gelation was nearly abolished by protein 4.1 (Table I).

Like erythrocyte spectrin, brain spectrin (fodrin) appears to fragment actin filaments (Strömqvist, 1987). Brain spectrin also binds calmodulin, but with greater affinity than erythrocyte spectrin. Therefore, less calmodulin should be needed to inhibit the actin-fragmenting activity of brain spectrin. To test this hypothesis, we repeated the experiments described above. Although a direct comparison is very difficult, it is apparent that less calmodulin is required to produce a certain inhibition of brain spectrin as compared to erythrocyte spectrin (Figure 7a). Furthermore, under conditions when calmodulin does not associate with brain spectrin (i.e., in the absence of calcium), the presence of calmodulin has no effect on the ability of brain spectrin to increase the rate of incorporation of actin monomers into actin filaments (Figure 7B).

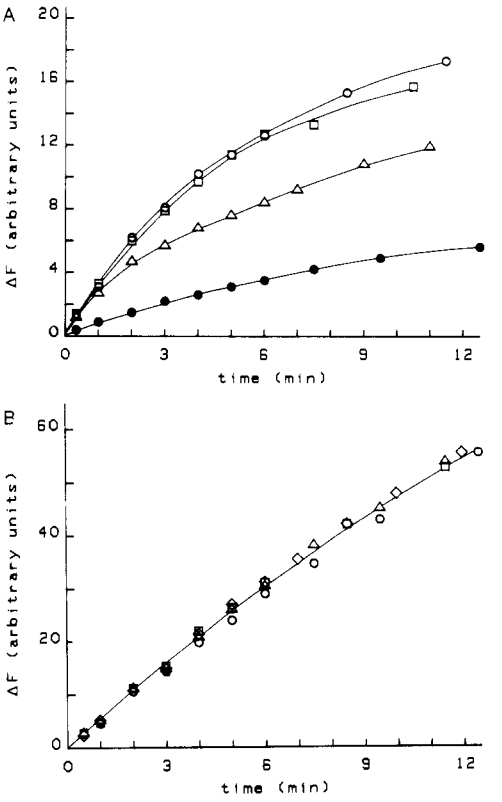

FIGURE 7: Effect of calmodulin on brain spectrin-induced fragmentation of actin filaments. The ability of calmodulin to inhibit actin fragmentation induced by brain spectrin in the presence and absence of calcium was investigated as described in Figure 3. (A) (In Ca buffer) To filamentous actin (4.7 μM), either Ca buffer (●), brain spectrin (0.16 μM) (○), or brain spectrin (0.16 μM) and calmodulin [2.1 μM (□) or 10.3 μM (△)] was added. After 10-min incubation at 20 °C, 150 μL was added to 250 μL of 14.3 μM pyrenyl-labeled actin monomers, and the fluorescence increase was followed. (B) (In Ca-free buffer) Filamentous actin (4.7 μM) and brain spectrin (0.16 μM) were preincubated for 10 min at 20 °C with calmodulin; 0 (○), 1.0 (□), 3.5 (△), and 10.3 μM (◇). Thereafter, an aliquot (150 μL) was added to 250 μL of 11.9 μM pyrenyl-labeled actin monomers, and the fluorescence enhancement was followed.

Discussion

Previous studies have shown that erythrocyte spectrin, in the presence or absence of protein 4.1 (Elbaum et al., 1984; Pinder et al., 1984; Strömqvist et al., 1985), influences actin polymerization; the critical concentration of actin decreases, and the lag phase is shortened in the presence of these proteins. It is also quite clear, from the present results as well as from previous work (Pinder et al., 1984; Strömqvist et al., 1985), that a short preincubation of filamentous actin with spectrin, with or without protein 4.1, markedly increases the number of nucleation centres, i.e., filament ends, compared to the number of ends of filamentous actin alone. Therefore, it is apparent that spectrin, either alone or together with protein 4.1, has the ability to fragment actin filaments.

Calmodulin, which is known to bind to erythrocyte spectrin (Berglund et al., 1984, 1986; Husain et al., 1984) and to other spectrins (Glenney et al., 1982), inhibits the fragmenting activity of spectrin on actin. The inhibition is calcium dependent since no reduction of fragmentation was observed when calcium was replaced by magnesium. This demonstrates that calmodulin must be activated; i.e., calmodulin must attain its calcium-induced conformation, in order to inhibit fragmentation. Although activated calmodulin is required to inhibit the formation of increased numbers of nucleation centers, it is not the only condition that must be fulfilled. At more basic pH (e.g., pH 8.2), we could not discern any significant effect of calmodulin on the system. Since the affinity of calmodulin for spectrin decreases markedly with increasing pH (Burns & Gratzer, 1985), it appears that calmodulin may only inhibit spectrin-induced fragmentation of actin filaments under conditions where calmodulin binds to spectrin.

In contrast to spectrin dimer, the tetramer, being divalent, can bind to and cross-link actin filaments (Ohanian et al., 1984). Therefore, spectrin tetramers may form an extensive gel with filamentous actin. From viscosity measurements (Table I), it is obvious that calmodulin reduces the extent of gel formation. This indicates that calmodulin either interferes

with the cross-linking of actin filaments by spectrin tetramers or shifts the dimer–tetramer equilibrium to favor the dimer. Direct binding studies using aqueous two-phase partition showed that spectrin-bound calmodulin is displaced by filamentous actin. That the double-reciprocal plot (Figure 6) indicates a competitive type of binding implies that calmodulin and filamentous actin bind to interfering binding sites on spectrin. We also tried to determine whether calmodulin influences the dimer–tetramer equilibrium using native gel electrophoresis. It was, however, not possible to analyze the gels accurately enough as large amounts of material stayed on top of the gel regardless of the buffer used, even at low protein concentrations [cf. Shahbakhti and Gratzer (1986)].

Protein 4.1 is known to stabilize the binary association of filamentous actin and spectrin (Ohanian et al., 1984). It is apparent from our results that protein 4.1 also enhances the activity of spectrin to fragment actin and that this enhancement is reduced significantly by calmodulin (Figure 3B). At high spectrin to protein 4.1 ratios (above 3:1), calmodulin decreases the fragmenting activity to about the level observed in the absence of protein 4.1. At lower ratios, calmodulin still inhibits the fragmenting activity though not completely. If calmodulin and protein 4.1 were competing for the same binding site on spectrin, increasing concentrations of protein 4.1 should displace calmodulin and thus restore the activity. Therefore, it appears that their binding sites are distinct and that calmodulin reduces the binding of protein 4.1 to the spectrin–actin complex. It is also possible that binding of calmodulin to spectrin lowers the fragmenting activity of the ternary complex. A further possibility is that calmodulin binds to protein 4.1 and thereby reduces the affinity of protein 4.1 for the spectrin–actin complex, since it has been claimed that protein 4.1 binds calmodulin stronger than spectrin does (Husain et al., 1985; V. Bennett, private communication). The latter explanation appears, however, less likely as much less protein 4.1 than spectrin is retained by immobilized calmodulin under similar ionic conditions (L. Backman, unpublished observation).

We have previously obtained evidence that the α-subunit of human erythrocyte spectrin contains the major binding site for calmodulin (Berglund et al., 1986), in conformity with spectrin analogues from other sources (Glenney et al., 1982). By ultrastructural studies (Tsukita et al., 1983), it has even been shown that brain spectrin, which is very similar in structure to erythrocyte spectrin (Glenney & Glenney, 1984; Bennett, 1985), binds calmodulin at a site in the head region, close to the site for tetramer formation. In contrast, Sears et al. (1986) have found that the calmodulin binding activity of erythrocyte spectrin is associated with the β-subunit. They also suggested that the site is localized in a 10-kDa fragment of the tail region (β-IV domain), close to the binding site for actin and protein 4.1.

Like erythrocyte spectrin, brain spectrin both fragments (Figure 7) and gelates (not shown) actin filaments, and both these activities are inhibited by calmodulin. Therefore, the binding of calmodulin to brain spectrin, postulated to occur at the head region (Tsukita et al., 1983), must influence the actin binding site in the tail region nearly 100 nm away. This appears to be very unlikely, a more plausible explanation being that brain spectrin, and thus presumably all spectrins, does not contain one single binding site for calmodulin but rather two sites per dimer, one each in the head and tail regions. Such a model may also explain why the binding site of calmodulin on erythrocyte spectrin has been localized to both the α-chain (Berglund et al., 1986) and the β-chain (Sears et al., 1986). However, this matter requires further experimental work before it can be settled.

The only function of calmodulin in the human red cell, established so far, is the activation of the membrane-bound (Ca^{2+} + Mg^{2+})-ATPase. Other high-affinity sites also exist in the red cell (Agre et al., 1983; Gardner & Bennett, 1986), though the significance of these are not clear. Even if these proteins were to be saturated, a vast amount of calmodulin would be free and available for other functions, for instance, to influence the organization of the cytoskeleton.

To observe any effects on the spectrin-induced fragmentation and gelation, calmodulin concentrations much higher than the estimated cellular one are needed, probably due to the relatively weak association between calmodulin and spectrin. This does not, however, preclude the possibility that calmodulin may interfere with the binding of actin and/or protein 4.1 to spectrin and thereby influence the organization of the red cell membrane skeleton. It has recently been shown that even weak interactions (as determined in vitro) may have profound effects at the protein concentrations believed to occur in the cell (Strömqvist et al., 1984; Drenckhahn & Pollard, 1986) due to the excluded volume effect (Tanford, 1965). In nonerythroid cells, the conditions are quite different; spectrin analogues, such as brain and intestinal brush border spectrins, bind calmodulin much more strongly than erythrocyte spectrin does. It is therefore even more likely that calmodulin interacts with and participates in the organization of the cytoskeleton in these cells.

Added in Proof

During the revision of this paper, Anderson and Morrow (1987) have presented a paper that confirms our observations and conclusions.

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