Actin polymerization induced by calspectin, a calmodulin-binding spectrin-like protein

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We have purified from a membrane fraction of bovine brain a calmodulin-binding protein (calspectin) that shares a number of properties with erythrocyte spectrin: It has a heterodimeric structure with M_r 240 000 and 235 000 and binds to (dimeric form) or crosslinks (tetrameric form) F-actin. We show that calspectin (tetramer) is capable of inducing the polymerization of G-actin to actin filaments by increasing nucleation under conditions where actin alone polymerizes at a much slower rate. Thus, brain calspectin behaves in the same manner as erythrocyte spectrin, supporting the idea that, in conjunction with actin oligomers it comprises the cytoskeletal meshwork underlying the cytoplasmic surface of the nerve cell.

Calspectin *Actin polymerization* *Calmodulin-binding protein* *Spectrin analogue* *Cytoskeletal network* *Synapse function carbol*

1. INTRODUCTION

In 1978, using a rat brain homogenate, Teshima and Kakiuchi [1] demonstrated a Ca^{2+}-dependent calmodulin-binding activity of particulate nature which was able to associate with 100 mg calmodulin/kg brain: ~ 25% the concentration of total EGTA-extractable calmodulin in brain tissue. This particle-associated calmodulin-binding activity was present in all rat tissues examined, with the highest concentration in brain followed by adrenal gland [2]. Subsequently, this binding activity was solubilized from a microsomal fraction of brain homogenates with 6 M urea [3] or a low ionic strength medium [4] and then purified to homogeneity by various column chromatographic steps [3,4]. The calmodulin-binding protein was termed calspectin since it shares the following characteristic properties with erythrocyte spectrin [4,5]:

(i) Like spectrin, it has a heterodimeric structure with subunits of M_r 240 000 (α subunit) and 235 000 (β subunit) as estimated on SDS–polyacrylamide gel electrophoresis [4,5];

(ii) Both spectrin [6] and calspectin [3–5] bind to calmodulin in the presence of Ca^{2+};

(iii) Both spectrin [7,8] and calspectin [5] undergo dimer–tetramer interconversion depending upon the ionic strength of the medium;

(iv) Both dimeric and tetrameric forms of calspectin are capable of binding to F-actin, but only the tetramer can crosslink F-actin to form a viscous gel [4,5]. Similar properties have been reported for spectrin [9,10];

(v) The molecular form of calspectin revealed by a low angle rotary-shadowing technique appears to be an elongate, flexible rod of ~200 nm (tetramer) in length [5], which is very similar to that of spectrin [11];

(vi) Calspectin is mainly associated with membranes [1–3], from which it can be solubilized with 6 M urea [3] or a low ionic strength medium [4] but not with Triton X-100 [3]. Its location on the membrane and its solubilities in these agents are almost identical to those of spectrin [12,13].

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Abbreviations: EGTA, ethylene glycol bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate

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Other laboratories have independently obtained evidence for the same protein. Davies and Klee [14] and Shimo-oka and Watanabe [15] partially purified the protein from the supernatant fraction and a microtubule-associated protein fraction, respectively, of brain homogenates. Willard et al. recognised the same protein as an axonally transported protein [16] and later identified it as a specific component of the cortical cytoplasm of many cells including neurons [17]. They termed the protein fodrin. Subsequently, Glenney et al. purified the same protein from chicken brain [18] and pig brain [19] and carried out detailed biochemical and morphological studies which are in general agreement with our own data. The same, or similar proteins, have been demonstrated in various tissues and cultured cells by using immunological methods [17,18] and gel overlay techniques [20,21].

It has been thought that spectrin is specifically located in mammalian erythrocytes because attempts to detect spectrin or spectrin-like proteins in different cell types by immunological techniques were unsuccessful [22,23]. However, the results from several laboratories provide evidence for a family of proteins which share common features with erythrocyte spectrin in many cell types including neuronal cells. Here we show that purified brain calspectin (tetramer) is capable of accelerating the polymerization of G-actin to actin filaments, another important property of spectrin that has been clarified [24]. This result, together with findings [5,19] that calspectin tetramers can crosslink actin filaments, is consistent with the view that, like spectrin in erythrocyte (see section 3) calspectin and actin oligomers comprise the cytoskeletal network underlying the cytoplasmic surface of the nerve cell.

2. MATERIALS AND METHODS

Calspectin was purified from a membrane fraction of bovine brain by a modification [25] of the method in [4,5]. Preparation of chicken gizzard actin was essentially as in [26]. After 3 cycles of polymerization and depolymerization, G-actin thus prepared was used either with or without the further purification by column chromatography, the preparations being referred to as column purified G-actin preparation and depolymerized actin preparation, respectively. For the former, the depolymerized actin preparation was applied to a column of Sephadex G-75. The column was then eluted with 2 mM Tris–HCl (pH 7.5), 0.2 mM ATP, 0.5 mM 2-mercaptoethanol and 0.2 mM $CaCl_2$. About 2–3% of the applied protein was eluted in a void volume fraction as actin oligomers and was separated from the peak of G-actin: the latter fraction was collected and used as the column purified G-actin preparation. Preparation of a DNase I–Sepharose column and immobilization of G-actin to this column were carried out as in [27]. DNase I was purchased from Sigma Chemical Co. For viscometry, proteins were incubated in the standard reaction mixture which consisted of 5 mM Tris–HCl (pH 7.5), 0.1 mM dithiothreitol, 0.1 mM ATP, 0.1 mM $CaCl_2$ and 0.3 mM $MgCl_2$ at 30°C, and the viscosity of the incubated solution was determined in an Ostwald type viscometer (Cannon-Manning semimicroviscometer, size 100) at 30°C. The specific viscosity was defined as (sample flow time – buffer flow time)/(buffer flow time). The buffer flow time was ~ 52–55 s. Electrophoresis of proteins was done in 7.5% polyacrylamide gels in a buffer system containing 0.1% SDS as in [28]. Protein concentrations were determined as in [29].

3. RESULTS AND DISCUSSION

In 1975, Pinder et al. [30] found that a crude preparation of spectrin promotes polymerization of muscle G-actin to actin filaments in a medium of relatively low ionic strength. Similar results were obtained in several laboratories [24,30,31]. The spectrin tetramer also crosslinks F-actin [9]. Combining these observations and taking a generally accepted concept of the head-to-tail polymerization of actin [33], Brenner and Korn [24] deduced that the actin-polymerizing activity of spectrin is due to the crosslink formation of spectrin tetramers with actin oligomers thereby providing stabilized actin nuclei with accessible net polymerizing ends and blocked net depolymerizing ends. Having found that the calspectin tetramers crosslinks F-actin [5], we therefore predicted that calspectin tetramer would also induce the polymerization of G-actin. In the following experiments using purified calspectin from bovine brain and actin preparations from smooth muscle, we were able to verify this prediction.

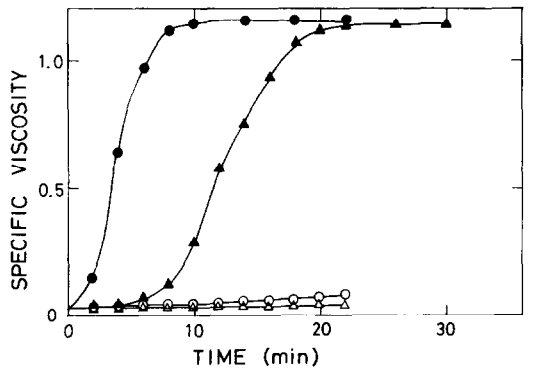

Fig.1. Effect of calspectin on the rate of actin polymerization. G-actin preparation (800 μg/ml), either the depolymerized actin preparation (●,○) or the column purified G-actin preparation (▲,△), was incubated with or without 90 μg/ml of purified brain calspectin in the standard reaction mixture at 30°C and the viscosity of the incubated solutions was determined as in section 2: (●,▲) with calspectin; (○,△) without calspectin.

Fig.1 demonstrates the calspectin-induced polymerization of smooth muscle actin, as measured by the increase in viscosity, in the presence of 0.3 mM $MgCl_2$. Under these conditions, calspectin existed in its tetrameric form [5]. Calspectin at 90 μg/ml gave a maximum effect (fig.2); at this [calspectin] polymerization of actin (800 μg/ml) at 30°C was complete within 10 min when a depolymerized actin preparation, which contained a trace (~ 2–3% of total actin) of actin oligomers, was used. With the column purified G-actin preparation (see section 2) in place of the depolymerized actin preparation, it required 20 min to complete polymerization and a lag period of 5–10 min was observed, although the extent of polymerization reached was the same level as with the depolymerized actin preparation (fig.1). Fig.3 shows that the presence of purified calspectin reduced the critical concentration for actin polymerization from the control value of 250 μg/ml to 80 μg/ml with the depolymerized actin preparation, or from 260 μg/ml to 105 μg/ml with the column purified G-actin preparation.

On the basis of our observation that calspectin tetramer can crosslink F-actin [5] and by analogy with the mechanism of spectrin-induced actin polymerization discussed above, these results can be explained by the increase in nucleation due to the stabilization of actin oligomers, brought about by the crosslinking of calspectin at their net depolymerizing ends. When the column purified G-

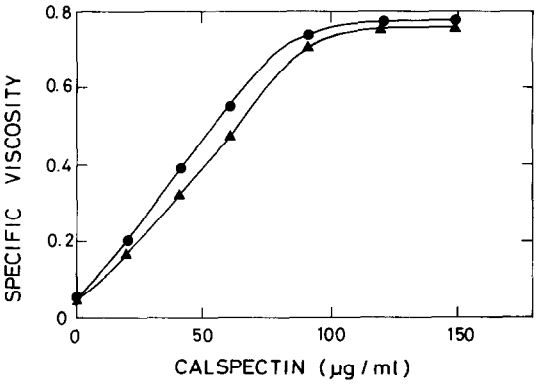

Fig.2. Effect of calspectin concentration on the rate of actin polymerization. G-actin preparation (600 μg/ml), either the depolymerized actin preparation (●) or the column purified G-actin preparation (▲), was incubated with various concentrations of calspectin as indicated in the figure in the standard reaction mixture at 30°C for 30 min and the viscosity of the incubated solutions was determined as in section 2.

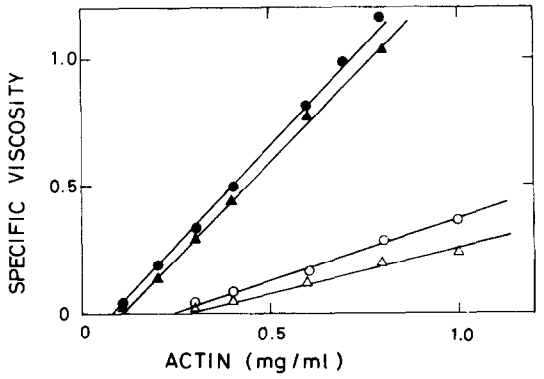

Fig.3. Effect of addition of calspectin on the critical concentration for actin polymerization. Various concentrations of actin were incubated with or without 90 μg/ml of calspectin in the standard reaction mixture at 30°C for 20 h and the viscosity of incubated solutions was determined as in section 2. NaN_3 was added to 0.1 mM final conc. to all tubes: (●,○) the depolymerized actin preparation; (▲,△) the column purified G-actin preparation; (●,▲) with calspectin; (○,△) without calspectin.

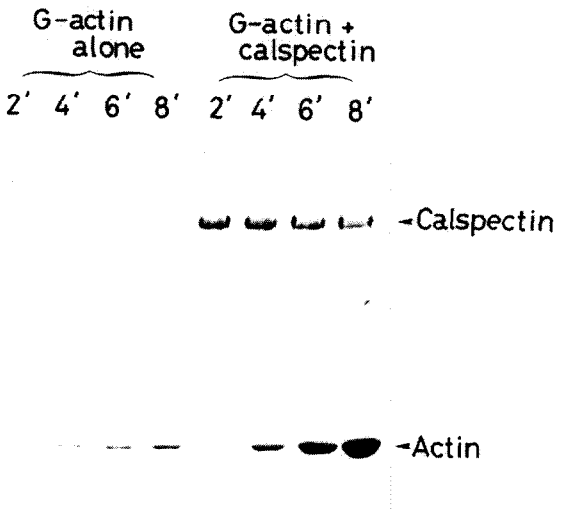

Fig.4. Formation of actin oligomer or calspectin–actin oligomer complex during the lag period of the incubation of G-actin with or without calspectin. The column purified G-actin preparation (800 μg/ml) was incubated with or without 90 μg/ml calspectin and in the standard reaction mixture (1.5 ml) at 30°C. At 2, 4, 6 and 8 min, aliquots of 0.3 ml were taken from the incubated solutions and then applied to Sephadex G-75 columns (0.5 × 25 cm) which had been equilibrated with the standard reaction mixture. The columns were then eluted with the same medium. High-M_r proteins eluted in a void volume fraction were electrophoresed in 7.5% polyacrylamide gel in the presence of SDS as in [28]. Proteins were then stained with Coomassie brilliant blue R-250.

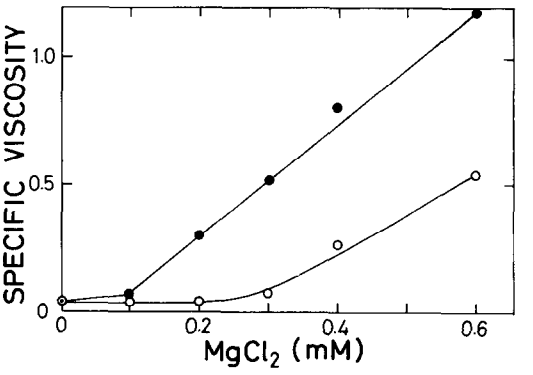

Fig.5. Dependency of actin polymerization on [$MgCl_2$]. The column purified G-actin preparation (580 μg/ml) was incubated with or without 80 μg/ml calspectin in 5 mM Tris–HCl (pH 7.5), 0.1 mM dithiothreitol, 0.1 mM ATP, 0.1 mM $CaCl_2$ and various [$MgCl_2$] as indicated in the figure at 30°C for 30 min and the viscosity of the incubated solutions was determined as in section 2: (•) with calspectin; (○) without calspectin.

actin preparation was used (fig.1), spontaneous self-nucleation occurred during the lag period with the aid of the stabilizing effect of calspectin. This was confirmed in fig.4: a time-dependent increase in the concentration of such actin oligomers (presumably in a form of calspectin–actin oligomer complex when calspectin was present) is seen on the polyacrylamide gel in which a high-M_r fraction of the incubated materials had been electrophoresed in the presence of SDS. With 0.3 mM $MgCl_2$, the formation of the oligomer was very slow, when actin monomers alone were incubated (see also fig.5). Addition of calspectin greatly enhanced the rate of the oligomer formation. The rate of actin polymerization in the presence and absence of calspectin, as measured by the increase in viscosity, is a function of the [$MgCl_2$] in the medium (fig.5). With [$MgCl_2$] > 0.3 mM, actin polymerization occurred regardless of whether or not calspectin was present, although with a slower rate in its absence (see also fig.4). When [$MgCl_2$] was < 0.1 mM, no polymerization occurred even in the presence of calspectin tetramer. This result is compatible with that obtained for spectrin: purified spectrin tetramer did not induce the polymerization of G-actin in the absence of $MgCl_2$ [9]. When [$MgCl_2$] was 0.1–0.3 mM, actin polymerization was absolutely dependent upon the presence of calspectin. A question one might ask is whether calspectin interacts directly with G-actin. We tested this possibility by using monomeric actin bound to DNase I immobilized on Sepharose. Calspectin applied to a G-actin–DNase I–Sepharose column was not retained on the column and was quantitatively recovered in the flowthrough fraction using a solution containing 2 mM Tris–HCl (pH 7.5), 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$ and various concentrations of $MgCl_2$ (0, 0.1, 0.3 and 0.5 mM). This indicated that calspectin does not interact directly with G-actin.

Thus, combining the present observation on actin polymerization and the previous result on F-actin crosslinking [5,19] both of which are induced by calspectin, we may conclude that, with regard

to its interaction with actin, brain calspectin behaves in the same manner as erythrocyte spectrin. As for the physiological significance of calspectin, it may perform functions analogous to those of spectrin in erythrocytes. Spectrin, accounting for ~ 75% of the cytoskeletal protein mass, forms a lining of the cytoplasmic surface of the erythrocyte membrane with actin oligomers (review [34]). Spectrin is thought to be responsible for the control of cell shape, cell deformability and the lateral mobility of transmembrane proteins, and hence the distribution of surface markers [13,34,35]. The same may be said for calspectin. In addition, on the basis of our findings that calspectin is specifically located in synaptic structures [3,4], calspectin might somehow be implicated in the synapse function possibly in Ca^{2+}-induced release of transmitter substances at the nerve terminals.

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