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ASSOCIATION OF ACTIN WITH CHROMAFFIN GRANULE MEMBRANES AND THE EFFECT OF CYTOCHALASIN B ON THE POLARITY OF ACTIN FILAMENT ELONGATION *

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Summary

Membranes of chromaffin granules isolated from bovine adrenal medulla are shown to bind dihydrocytochalasin B with high affinity. These membranes also bound [^{3}H]actin in a time- and Mg^{2+}-dependent manner and electron microscopy showed the presence of membrane-attached actin filaments following addition of exogenous actin.

Binding of [^{3}H]actin was partially inhibited by cytochalasin B. Electron microscopic analysis of heavy meromyosin-decorated, membrane-attached filaments showed terminally (end-on) attached filaments with both possible polarities (i.e., filaments with arrowheads pointing both towards and away from the membranes). Treatment of samples with cytochalasin B preferentially inhibited growth of filaments with their 'barbed' ends pointing away from membranes. These results are discussed with respect to the role of actin in secretory granule function and the mechanism of cytochalasin action.

Introduction

The adrenal medulla is one of the sites for synthesis, storage and release of catecholamines in mammals. In this system, cellular epinephrine and norepinephrine are stored in membrane-bound organelles, the chromaffin granules, and are released into the bloodstream via exocytosis [1,2]. The events occurring during secretion have led to the theory of stimulus-secretion coupling [3],

* Part of this study has recently appeared in abstract form [19].

Abbreviations: SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

which is reminiscent of excitation-contraction coupling involving actin and myosin in muscle cells [4].

The chromaffin granule membrane has been found by several investigators to contain a polypeptide with the same electrophoretic mobility as actin in SDS-polyacrylamide gels [5]. The presence of actin in this preparation has been confirmed with the use of peptide fingerprinting and antibody precipitation techniques [6,7]. These findings have lent support to the idea that actin might be involved in the contractile events occurring during exocytosis. Indeed, several laboratories have reported dramatic reductions in secretory activity of adrenal medullary [8] and neuronal [9] cells upon treatment with cytochalasin B, a drug which has been shown to affect the structure and assembly of actin filaments (F-actin) in vitro [10—16].

The above considerations lead to the question of how actin is associated with the chromaffin granule membrane. In a preliminary report, Burridge and Phillips [5] showed that F-actin could reassociate with these membranes in the presence of muscle myosin in vitro. More recently, experiments performed in this laboratory have demonstrated the presence of stable, actin-containing complexes (originally referred to as motility-related cytochalasin binding complexes) associated with the cytoplasmic side of human erythrocyte membranes [11] and in extracts of bovine brain [17] and human platelets [17]. These structures have the ability to stimulate actin filament formation in vitro by acting as nuclei in the actin polymerization reaction. It is possible, therefore, that such complexes may play a role in assembly of actin filaments and in their attachment to cellular membranes in vivo.

In this report, we show that actin in the form of stable oligomers can be found on membranes isolated from chromaffin granules. These molecules can serve as nuclei in stimulating assembly of actin filaments that become attached to the membranes. In addition, we present evidence that cytochalasin B partially inhibits this type of filament formation by selectively affecting the addition of G-actin onto the preferred end for polymerization (i.e., the barbed end of an actin filament following decoration with heavy meromyosin [18]).

Methods

Materials

N-[^{3}H]Ethylmaleimide was obtained from New England Nuclear. Labelled and unlabelled dihydrocytochalasin B were synthesized from [^{3}H]cytochalasin B as previously described [20]. Unlabelled cytochalasin B was obtained from Aldrich. All other chemicals used were of reagent grade.

Preparation of chromaffin granule membranes

Chromaffin granules were prepared by a method similar to those used previously (e.g., Ref. 21). For a typical preparation, 12 bovine adrenal glands were transported to the laboratory on ice from a local slaughterhouse. All procedures were carried out at 4°C. Following removal of cortex tissue, medullae were chopped into small slices with scissors and homogenized in 10 vol. of a buffer containing 320 mM sucrose, 25 mM Hepes, 10 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (homogenization buffer).

Homogenization was performed in a Sorvall Omnimixer for 1 min at a setting of 7. The suspension was then diluted to approx. 300 ml with homogenization buffer and centrifuged at 1500 × *g* for 15 min. The supernatant fractions were saved and the pellets were suspended in approx. 250 ml of homogenization buffer. The suspension was homogenized as above for 15 s and was centrifuged at 1500 × *g* for 15 min. The pellets from this centrifugation were discarded and the supernatant fractions were combined with those from the initial centrifugation. The combined supernatant fraction was again centrifuged at 1500 × *g* for 20 min to remove any remaining cellular debris. The supernatant fraction was then centrifuged at 10 000 × *g* for 25 min to sediment primarily mitochondria and chromaffin granules. The pellet fractions from this step (which also contain lysosomes, plasma membrane fragments and broken chromaffin granules) were resuspended in approx. 50 ml of homogenization buffer. 8-ml aliquots of this suspension were then layered onto each of six discontinuous sucrose density gradients consisting of equal volumes (15 ml) of 1.6 and 0.8 M sucrose containing 50 mM KCl, 1 mM dithiothreitol and 10 mM Hepes, pH 7.0. Gradients were then centrifuged for 1 h at 96 000 × *g* in an SW27 rotor at 4°C. The pellets of these gradients (consisting mostly of chromaffin granules) were washed gently with ice-cold homogenization buffer to remove a loosely adhering layer of mitochondria. Analysis of chromaffin granules isolated by this method on SDS-polyacrylamide gels indicated the presence of substantial amounts of chromogranin A, a soluble protein of the chromaffin granule [22].

Membranes were prepared from chromaffin granules by hypotonic lysis. Pellets from sucrose gradients were resuspended in approx. 10 ml of a buffer consisting of 10 mM Hepes, 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4, in order to lyse the granules. This suspension was allowed to stand overnight. On the following day, the suspension was centrifuged at 50 000 × *g* for 30 min. The supernatant fraction containing released catecholamines, nucleotides and proteins was discarded. The pellet, consisting of chromaffin granule membranes and partially lysed granules, was resuspended in approx. 10 ml of 10 mM Hepes, 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4, and is referred to throughout this paper as chromaffin granule membranes. Analysis of membranes on SDS-polyacrylamide gels showed a considerable reduction in the amount of chromogranin A when compared to intact granules.

Cytochalasin binding assays

Binding of [^{3}H]dihydrocytochalasin B was measured using a method similar to the centrifugation assay described previously [23]. Briefly, chromaffin granule membranes were suspended in 0.5 ml of 10 mM Hepes, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4, and incubated with appropriate concentrations of [^{3}H]dihydrocytochalasin B. A 100-fold excess of unlabelled dihydrocytochalasin B was added to some samples in order to determine displaceable (high-affinity) binding present as opposed to nonspecifically bound dihydrocytochalasin B. Following a 5 min incubation, samples were centrifuged at 4000 × *g* for 20 min. After careful removal of supernatant fractions, pellets were dissolved in 0.3 ml of Protosol (New England Nuclear). Aliquots of supernatant fractions and pellets were then assayed for radioactivity by liquid

scintillation counting at an efficiency of 30%. Binding data were analyzed according to the method of Scatchard [24].

Actin binding assays

[^{3}H]Actin was prepared by incubating unlabelled actin prepared by the method of Spudich and Watt [25] with *N*-[^{3}H]ethylmaleimide essentially as described by Cohen et al. [26], with the exception that the reaction and subsequent dialysis steps were performed in a buffer containing 5 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, pH 8.0. (The dialysis buffer also contained 0.5 mM β-mercaptoethanol.) [^{3}H]Actin prepared by this method contained 0.8—1.0 mol *N*-[^{3}H]ethylmaleimide per mol actin. The critical concentration of [^{3}H]actin was indistinguishable from that of unlabelled actin (data not shown). The specific radioactivity of [^{3}H]actin ranged between 1 and $1.5 \cdot 10^4$ dpm/μg protein. Binding of [^{3}H]actin to membranes was assayed in the following way. Chromaffin granule membranes were incubated at a protein concentration of 1 mg/ml and fragmented erythrocyte ghosts (prepared by using the method of Cohen et al. [26]) at a protein concentration of 0.5 mg/ml with various concentrations of [^{3}H]actin for a specified length of time at room temperature in a buffer containing 5 mM NaH_2PO_4, 1 mM ATP, and 0.75 mM β-mercaptoethanol, pH 6.5 (actin binding buffer). Additions were made as indicated in the figure legends. The total volume of the assay was 0.5 ml. After incubation, 0.2 ml samples were withdrawn from the tubes and carefully layered onto 0.5 ml cushions of 20% sucrose in actin binding buffer which had been added to 1.5-ml polypropylene microcentrifuge tubes. The microcentrifuge tubes were immediately centrifuged at $35\,000 \times g$ in a Beckman JA20 rotor for 30 min at 4°C. The supernatant fractions from these tubes were carefully withdrawn and pellets were dissolved in 0.3 ml Protosol. Samples were then counted for radioactivity by liquid scintillation spectrometry at an efficiency of approx. 30%. Quantitative recovery of membrane protein was confirmed by assaying for total protein in the pellets by using the method of Hartree [27].

Electron microscopy

Samples of chromaffin granule membranes were incubated and prepared in a similar manner to that described for actin binding assays. Granule membranes were incubated with 400 μg/ml unlabelled actin for 1 h in actin binding buffer containing 0.4 mM $MgCl_2$. Following centrifugation through 20% sucrose, pellets of membranes were gently resuspended in 0.1 ml of 10 mM Hepes, 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. Aliquots of 10 μl of the samples were withdrawn and applied to carbon-coated electron microscope grids, and incubated for 1 min. The grid was blotted with filter paper and 10 μl of a 0.3 mg/ml solution of heavy meromyosin (generously provided by Drs. Michael Rogers and William Harrington) were applied to the grid. After another 1 min incubation, the grid was rinsed with 20 mM NaH_2PO_4, pH 7.0, and negatively stained with 1% aqueous uranyl acetate. All specimens were viewed at 80 kV in a JEOL model 100B electron microscope.

Results

Isolation of chromaffin granule membranes

Chromaffin granules were isolated from bovine adrenal medulla and a membrane preparation was obtained from these organelles as described in Methods. Examination of the isolated membranes by electron microscopy showed a population of vesicular structures ranging between 0.2 and 0.5 μm in size. No actin filaments were seen in this preparation, reflecting the fact that the membranes were isolated under low ionic strength conditions which favor depolymerization of F-actin. However, electrophoresis in SDS-polyacrylamide gels showed that the membranes did contain a detectable amount of a protein with the mobility of muscle actin (less than 5% of the total membrane proteins). It is possible, therefore, that the isolated membranes contained actin in the form of monomers or short oligomers which were not detected by electron microscopy.

As stated in the Introduction, erythrocyte membranes contain actin-containing complexes that can stimulate actin filament assembly in vitro by serving as nuclei, or seeds, in the polymerization reaction (Refs. 11 and 28; and Lin, D.C., unpublished observations). These complexes are also characterized by their ability to bind cytochalasin with high affinity [11]. Subsequent work showed that these drugs bind tightly to the polymeric form of actin [12,13]. In order to determine whether chromaffin granule membranes contain actin oligomers resistant to the low ionic strength conditions used in the isolation procedure, we measured the binding of [^{3}H]dihydrocytochalasin B [23] to the membranes. Scatchard plot analysis of the binding data showed that a significant number of high-affinity binding sites are associated with the membranes; the x-intercept and the slope of the plot correspond to 6 pmol of sites per mg of protein and a dissociation constant of 6 nM, respectively (Fig. 1).

Membrane-stimulated actin filament assembly

Cohen et al. [26] have previously shown that assembly of G-actin into filaments that are attached to erythrocyte membranes can be monitored by measuring the incorporation of radioactively labelled actin monomers into the

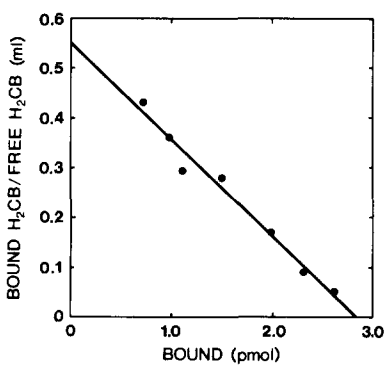

Fig. 1. Scatchard plot analysis of high-affinity dihydrocytochalasin B binding to chromaffin granule membranes. Chromaffin granules (0.5 mg of protein) were incubated with various concentrations of [^{3}H]-dihydrocytochalasin B (H_2CB). Binding was evaluated as described in Methods.

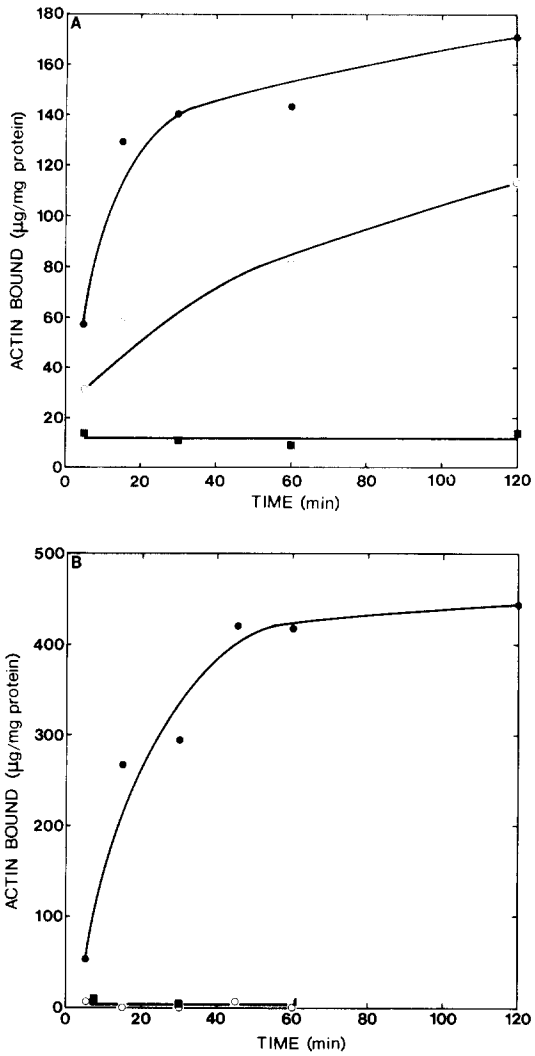

Fig. 2. Time course of [^{3}H]actin binding to chromaffin granule membranes and to fragmented erythrocyte membranes. (A) Binding of [^{3}H]actin to chromaffin granule membranes in the presence (●) and absence (■) of 0.4 mM $MgCl_2$ and in the presence (○) of 1 μM cytochalasin B. (B) Binding of [^{3}H]actin to fragmented erythrocyte membranes prepared by using the method of Cohen et al. [25], in the presence (●) or absence (■) of 0.4 mM $MgCl_2$ or in the presence (○) of 1 μM cytochalasin B.

membranes. Using this technique, we found that chromaffin granule membranes bind [^{3}H]actin in a time-dependent manner, approaching a maximum level of about 160 μg actin/mg of membrane protein (Fig. 2A). The amount of Mg^{2+} in the assay medium used in this experiment is 0.4 mM, a concentration which allows only a very slow rate of actin polymerization when actin nuclei are not added to the system [11]. These conditions were used to ensure that any stimulatory effects of the added membranes on actin polymerization would be readily seen. When Mg^{2+} was omitted from the assay buffer, the amount of [^{3}H]actin bound to the membranes dropped to a very low level (Fig.

2A). In addition, 1 μM cytochalasin B inhibited the amount of bound [^{3}H]actin by about 50%. This result is in contrast to those obtained with human erythrocyte membranes. As shown in Fig. 2B, 1 μM cytochalasin B completely blocked binding of [^{3}H]actin to these membranes under identical conditions to those of the previous experiment. This result is in agreement with those reported by Cohen et al. [26], who measured [^{3}H]actin binding to erythrocyte membranes in a medium containing 2 mM Mg^{2+}.

To characterize further the binding of actin to granule membranes, we studied the binding reaction as a function of actin concentration. As shown in Fig. 3A, actin binding after 2 h of incubation increased as the concentration of actin in the assay medium increased, and did not reach a saturating level even at 1 mg actin/ml. This result, along with the Mg^{2+} and time dependency of actin binding (Fig. 2A), indicates that actin polymerization was occurring during the assay and that the majority of the actin bound to the membranes is in the filamentous form. On the other hand, when Mg^{2+} was omitted from the assay medium, the level of bound actin at all actin concentrations was drastically reduced. The residual level of actin binding under these conditions may reflect binding of G-actin to the membranes. In support of this conjecture, we found that the amount of actin bound to the membranes in 0.4 mM Mg^{2+} at zero incubation time is as low as that measured in the absence of Mg^{2+} after 2 h of incubation. In a related set of experiments, we found that 1 μM cytochalasin B partially inhibited actin binding to granule membranes at all actin concentrations (Fig. 3B). In addition, we performed a 'control' experiment in which membranes were omitted from the assay mixture. At actin concentrations above 200 μg/ml, the amount of actin sedimented by centrifugation during the

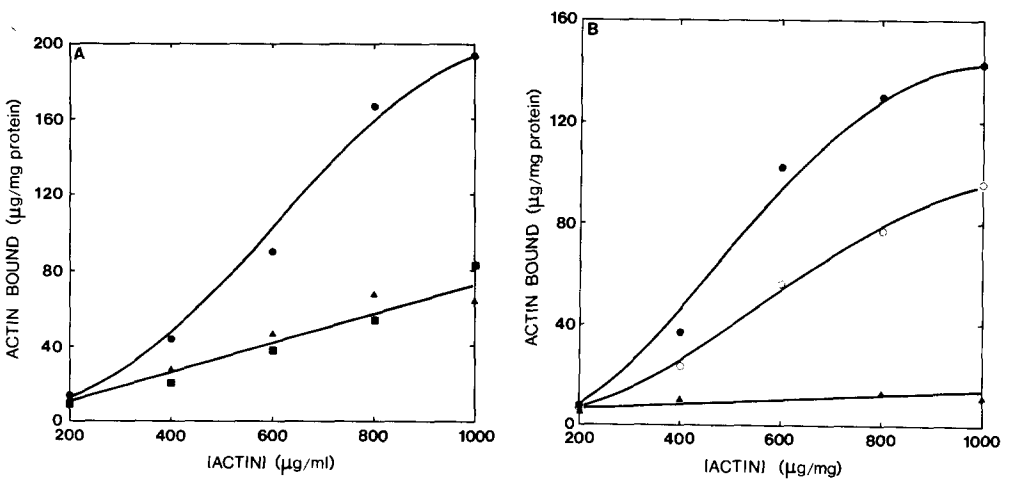

Fig. 3. Concentration dependence of [^{3}H]actin binding to chromaffin granule membranes. (A) Chromaffin granule membranes were incubated for 2 h at room temperature in actin binding buffer with the indicated concentrations of [^{3}H]actin in the presence (●) or absence (■) of 0.4 mM $MgCl_2$. Alternatively, [^{3}H]actin was incubated with 0.4 mM $MgCl_2$ in the absence of chromaffin granule membranes followed by addition of membranes (▲) (see text). (B) Chromaffin granule membranes were incubated as described above with the indicated concentrations of [^{3}H]actin; control (●), control + 1 μM cytochalasin B (○). Alternatively, [^{3}H]actin was incubated as above, but in the absence of granule membranes (▲).

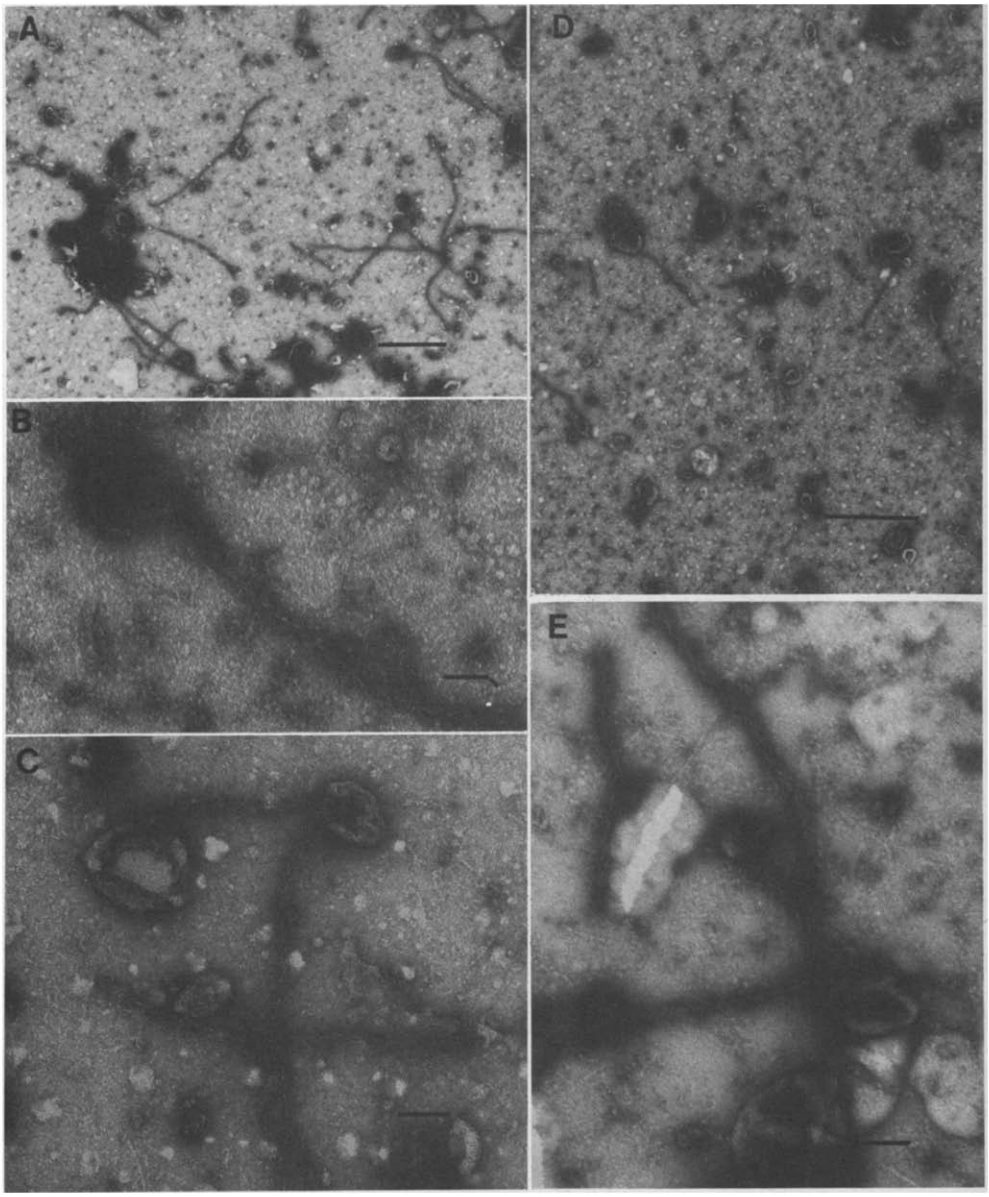

Fig. 4. Electron micrographs of membranes with heavy meromyosin-decorated actin filaments. (A) Control, low magnification view (×9330); (B) (×72 660), (C) (63 330) controls, higher magnification; (D) cytochalasin B-treated sample (note the difference in the length and number of filaments in D as compared with A, (×12 660); (E) cytochalasin B-treated sample (×66 000). Bars in A and D equal 1 μm. Bars in B, C and E equal 0.1 μm.

assay procedure and counted as 'membrane-bound' actin is negligibly low when compared to that measured in the presence of added membranes.

Effect of cytochalasin B on polarity of membrane-associated actin filaments

In order to correlate biochemical data with ultrastructural information, we

TABLE I

ASSOCIATION OF HEAVY MEROMYOSIN-DECORATED FILAMENTS WITH CHROMAFFIN GRANULE MEMBRANES

Samples were prepared for electron microscopy as described in Methods. Experiments I and II represent data obtained from samples prepared with two different granule membrane preparations. For all samples, between 100 and 125 filaments were scored. Filaments which were terminally attached, but the polarity of which could not be determined, were not counted.

Experiment	Terminally-attached filaments		Side attached (% of total)
	Filaments with arrowheads toward membranes (% of total)	Filaments with arrowheads away from membranes (% of total)	
I control	32	12	56
+ 1 μM cytochalasin B	14	42	44
II control	37	20	43
+ 1 μM cytochalasin B	17	50	33

incubated chromaffin granule membranes with unlabelled actin under the conditions used in the actin binding assay, and looked for membrane-associated filaments by electron microscopy. The micrographs shown in Fig. 4a—e are representative views of heavy meromyosin-decorated actin filaments associated with the membranes. It is apparent that most of the filaments associated with the membrane vesicles fall into one of the following categories:

(1) filaments that are attached sideways to the vesicles (side-attached), and

(2) filaments attached to the vesicles in an 'end-on' manner (terminally attached).

At higher magnification (Fig. 4b and c), the terminally attached filaments can be further classified as those with arrowheads pointing towards the membrane, and those with arrowheads pointing away from the membrane. The relative distribution of the different types of membrane-attached filaments is given in Table I.

When cytochalasin B is added to the incubation mixture (Fig. 4d and e), the micrographs of the sample, compared with those of the control sample, showed a number of differences: (a) the total number of filaments attached to the membranes was reduced (Fig. 4d); (b) the filaments were shorter (Fig. 4d); and (c) the majority (75%) of the terminally attached filaments had arrowheads pointing away from the vesicles (in the control sample, 68% of the terminally attached filaments had arrowheads pointing toward the vesicles) (Table I). These results suggest that cytochalasin B preferentially inhibited the formation of the latter type of membrane-associated filaments.

Discussion

The present study was initiated to investigate the molecular nature of the interaction of actin with the chromaffin granule membrane in the hope of gaining insight into the role of cytoskeletal-contractile elements in secretory pro-

cesses. Since actin is known to be associated with chromaffin granule membranes, we decided to use cytochalasins as probes to determine whether this actin is in a form which could serve as starting points (i.e., nuclei) from which actin filaments can begin to form. In these experiments, granules and granule membranes were both prepared in low ionic strength media which would favor depolymerization of any F-actin that might be attached to the membranes in a specific or nonspecific way during the isolation procedures. Our finding that high-affinity cytochalasin binding sites are present in the isolated membranes indicates that they do contain a significant number of stable actin nuclei. These nuclei can stimulate the formation of membrane-attached actin filaments, as demonstrated by the [^{3}H]actin binding assay and by electron microscopy. In addition, we have obtained suggestive evidence that the membranes have the capacity to bind G-actin in a manner independent of filament formation.

A question which remains unanswered is how actin nuclei are stabilized and attached to the chromaffin granule membrane. In the erythrocyte membrane, actin nuclei are stabilized by their association with two other proteins, spectrin and band 4.1, which also provide a link between the actin and the integral proteins in the membrane (Lin, D.C., unpublished observations; and Refs. 29—31). Whether a similar structural arrangement exists in the chromaffin granule membrane remains to be seen. Meyer and Burger [7] have shown that actin is probably not an integral membrane protein because it is not labelled by the lipophilic photoaffinity reagent 5-[^{125}I]iodonaphthyl azide. Preliminary results obtained in our laboratory have indicated that actin nuclei associated with the granule membrane can only be removed (or destroyed) from the membrane by prolonged dialysis in low ionic strength medium or by treatment with 0.6 M KI (unpublished results). α-Actinin, an actin binding protein [32], has previously been shown to be present in chromaffin granule membranes [33]. It is possible that this protein may play a similar role to spectrin and band 4.1 in the membrane association of actin in this type of membrane system.

We found that [^{3}H]actin binding to chromaffin granule membranes in 0.4 mM Mg^{2+} was only partially inhibited by cytochalasin B, whereas binding of the protein to erythrocyte membranes was completely blocked under these conditions. This result suggests that G-actin can be added onto the granule membranes in a cytochalasin-sensitive as well as a cytochalasin-insensitive manner. It has previously been demonstrated that actin filaments growing from the surface of inside-out erythrocyte membranes reconstituted with the spectrin-actin complex in vitro have a uniform polarity; after decoration with heavy meromyosin, all of the filaments have arrowheads pointing towards the membrane [34] indicating that nuclei in erythrocyte membranes have uniform polarity. In the case of granule membranes, the terminally attached filaments had arrowheads pointing towards as well as away from the point of attachment. However, when cytochalasin B was added to the system, the proportion of filaments with arrowheads pointing towards the membrane was significantly reduced. This result indicates that the chromaffin granule membrane contains nuclei which will stimulate polymerization of actin filaments with arrowheads pointing both towards and away from the membrane. It also indicates that cytochalasin B has a greater inhibitory effect on the rate of addition of G-actin

onto nuclei with their barbed ends pointing away from the membrane. Since the barbed end is the preferred end for monomer addition in filament elongation [18], this would also explain why the samples with cytochalasin had a smaller number of filaments and that these filaments are of shorter length when compared with those in the control samples. While this manuscript was in preparation, Pollard and coworkers [16,35] reported results which are in agreement with the above conclusion; they found that cytochalasin B preferentially inhibits the rate of actin monomer addition at the barbed end over that at the 'pointed' end of heavy meromyosin-decorated F-actin fragments and filament bundles obtained from the intestinal microvilli.

In addition to terminally attached filaments, we found a large number of filaments that were attached sideways to the chromaffin granule membranes. This class of filaments resembles filaments attached to the side of lysosomes in thin-section electron micrographs of polymorphonuclear leukocytes [36]. The presence of such filaments in our preparation is somewhat unexpected because of the experimental conditions. In this study, the Mg^{2+} concentration of the medium was sufficiently low that most of the filaments formed should have arisen from the addition of the membrane-associated actin nuclei. Therefore, the side-attached filaments may reflect addition of G-actin onto both ends of nuclei that were attached sideways to the membrane surface. Alternatively, these filaments could be the result of fragmentation or detachment of terminally attached filaments, followed by their reassociation with the membrane. In both of these cases, the association of the filaments with the membrane may be mediated by actin binding proteins located at the membrane surface; this question is currently under study.

The present work indicates that actin associates with the chromaffin granule membrane in a specific way. Cytoskeletal interactions with secretory organelles may be important not only for the contractile events which occur during exocytosis, but also for localizing the organelles at sites in the cell where exocytosis will occur. We showed here that cytochalasin B inhibits the formation of a class of chromaffin granule-associated actin filaments. This result along with those obtained by others [8,9] suggests that actin may play a central role in secretory phenomena.

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