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Phalloidin unzips nebulin from thin filaments in skeletal myofibrils

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SUMMARY

Fluorescent phallotoxins such as rhodamine-phalloidin take hours to bind uniformly to thin filaments of skeletal myofibrils, after fast initial binding to both ends of thin filaments. Observation of this process in skeletal and cardiac myofibrils and of the resulting re-distribution of nebulin using anti-nebulin antibody showed that: (1) rhodamine-phalloidin binds uniformly to actin in cardiac myofibrils within minutes, in contrast to skeletal myofibrils; (2) overnight pre-incubation of skeletal myofibrils with phalloidin results in uniform initial binding of rhodamine-phalloidin and a changed nebulin localization; (3) pre-incubation of skeletal myofibrils with Ca^{2+}-calmodulin results in uniform initial binding of rhodamine-phalloidin; (4) the binding of rhodamine-phalloidin to actin in skeletal myofibrils is unidirectional, i.e. the fluorescence of incorporated rhodamine-phalloidin moves from the pointed ends where it is bound initially toward the barbed end at the Z-band; (5) the unidirectional binding of rhodamine-phalloidin results in redistribution of nebulin, i.e. the initial fluorescent bands associated with the epitopes of bound nebulin antibody change to a single band located close to Z-line. These results indicate that nebulin inhibits rhodamine-phalloidin binding to actin and suggests that the unidirectional rhodamine-phalloidin binding may be due to cooperative competitive binding, i.e. phalloidin 'unzips' nebulin starting from the pointed ends of the thin filaments.

Key words: cardiac, rhodamine, fluorescence microscopy, immunofluorescence

INTRODUCTION

It has been reported that the binding of fluorescent phallotoxins to actin in thin filaments of skeletal myofibrils is not uniform (Bukatina et al., 1984; McKenna et al., 1985; Wilson et al., 1987; Greaser and Schnasec, 1990; Szczesna and Lehrer, 1993). Fluorescence microscope images show fast initial binding to both ends of the actin thin filament, in the Z-band and at the ends distal from the Z-band (pointed end). The fluorescence pattern becomes uniform only after several hours of incubation (Szczesna and Lehrer, 1993). In contrast, purified F-actin binds phalloidin at every subunit within 20 minutes incubation (Huang et al., 1992; Allen and Janmey, 1994; De La Cruz and Pollard, 1994). These effects can be explained either by an intrinsic difference between actin subunits at the ends compared to the bulk of the filament or the presence of actin binding proteins which inhibit phalloidin binding.

Nebulin, a protein which spans most of the length of the actin thin filament in skeletal myofibrils (Wang and Wright, 1988) is absent in cardiac myofibrils (Hu et al., 1986; Locker and Wild, 1986). Nebulin has been shown to stabilize actin from depolymerizing (Chen et al., 1993), a similar effect to that of phalloidin (Estes et al., 1981). The similar effects of nebulin and phalloidin suggested that the inhibition of rhodamine-phalloidin (Rh-Ph) binding may be due to competition between nebulin and phalloidin binding to actin subunits via an allosteric or a direct effect.

To explore these possibilities, we studied the binding of fluorescent phallotoxins to cardiac myofibrils and found that the binding was uniform at all times, indicating random binding as occurs in purified F-actin filaments (De La Cruz and Pollard, 1994). This suggested that nebulin which is present in skeletal myofibrils could be inhibiting Rh-Ph binding. We found that this putative inhibition could be removed by overnight pre-incubation of skeletal myofibrils with unlabeled phalloidin and short time incubation with Ca^{2+}-calmodulin. Studies of the kinetics of the binding of Rh-Ph using the changing fluorescence pattern, indicated that the Rh-Ph binding takes place in a directional manner beginning from the pointed ends. This vectorial binding suggests a cooperative deinhibition process, i.e. an 'unzipping' of nebulin from actin. The release of nebulin from actin by Rh-Ph was verified by the observed changed location of nebulin epitopes within the sarcomere.

MATERIALS AND METHODS

Myofibrils were obtained from glycerinated rabbit psoas muscle fibers by homogenization as described earlier (Szczesna and Lehrer, 1992). Cardiac myofibrils were obtained from both fresh and glycerinated rabbit hearts by a similar homogenization with the inclusion of 0.1% Triton X-100 in the homogenization buffer. Chicken calmodulin prepared by recombinant DNA methods and rabbit muscle troponin C were provided by Dr A. C.-W. Wang and Dr T. Tao, respectively. Ghost myofibrils (myosin and regulatory protein-depleted) were

obtained by irrigating myofibrils attached to the surface of a microscope sample cell with a concentrated salt solution (Szczesna and Lehrer, 1993) and then rinsing with rigor buffer (0.05 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 0.01% NaN_3, 20 mM Hepes, pH 7.2). Rhodamine-phalloidin (Molecular Probes, Eugene, OR), at concentrations of 1-10 μM in rigor buffer containing an oxygen-removing enzyme system (0.3% D-glucose, 0.16 mg/ml glucose oxidase and 0.016 mg/ml catalase) to minimize photo bleaching, was introduced into the cell containing intact or ghost myofibrils. For the immunofluorescence study, the ghost rabbit skeletal myofibrils attached to a microscope coverslip were incubated with 10 μg/ml of polyclonal goat anti-nebulin IgG (provided by Dr K. Wang) for 30 minutes, excess anti-nebulin was then rinsed with the rigor buffer followed by irrigation with rhodamine labeled anti-goat IgG (Sigma Immuno Chemicals, Product #T-6028, ×100 dilution) for another 30 minutes. Finally, images were obtained after excess fluorescent antibody was washed out with rigor buffer containing the oxygen-removing enzyme system.

A sensitive cooled CCD camera (PXL system, Photometrics, Tucson, AZ) coupled to a Zeiss Axiovert microscope was used for fluorescence and phase contrast imaging. A 100×/1.25 phase contrast achrostigmat objective was used to select myofibrils, to locate regions of the sarcomere, to verify loss of thick filaments when ghost myofibrils were prepared and to image the fluorescence patterns. With this optics, the distance between pixels corresponded to 64 nm. For fluorescence measurements, light was directed from a 50 W Hg lamp onto the sample through a Zeiss dichroic filter assembly for rhodamine (#14) and coumarin (#1), collected with the objective and imaged with the PXL camera using exposure times of 0.1-0.2 second. The digital data were transferred to a Macintosh computer and analyzed by using IPLab spectrum software (Signal Analytics, Vienna, VA). To determine the kinetics of the fluorescence intensity distribution along the axis of the myofibrils, image intensities taken from the same myofibril at different times were normalized to the background intensity of the free dye near the fibril. Densitometry patterns of the fluorescence from the same region of several sarcomeres at different times were obtained from a narrow rectangular area located in the middle of the fibril. The possibility of the contribution of non-specific binding of Rh-Ph via interaction with the fluorophor was eliminated by noting negligible fluorescence when a similar concentration of rhodamine (carboxytetramethylrhodamine mixed isomers) was introduced to the myofibrils. Photos of the fluorescence images were obtained from the computer screen with a 35 mm camera. The magnification of the photos varied somewhat but the distance between the Z-lines of each sarcomere was between 2.0 and 2.4 μm for all of the images.

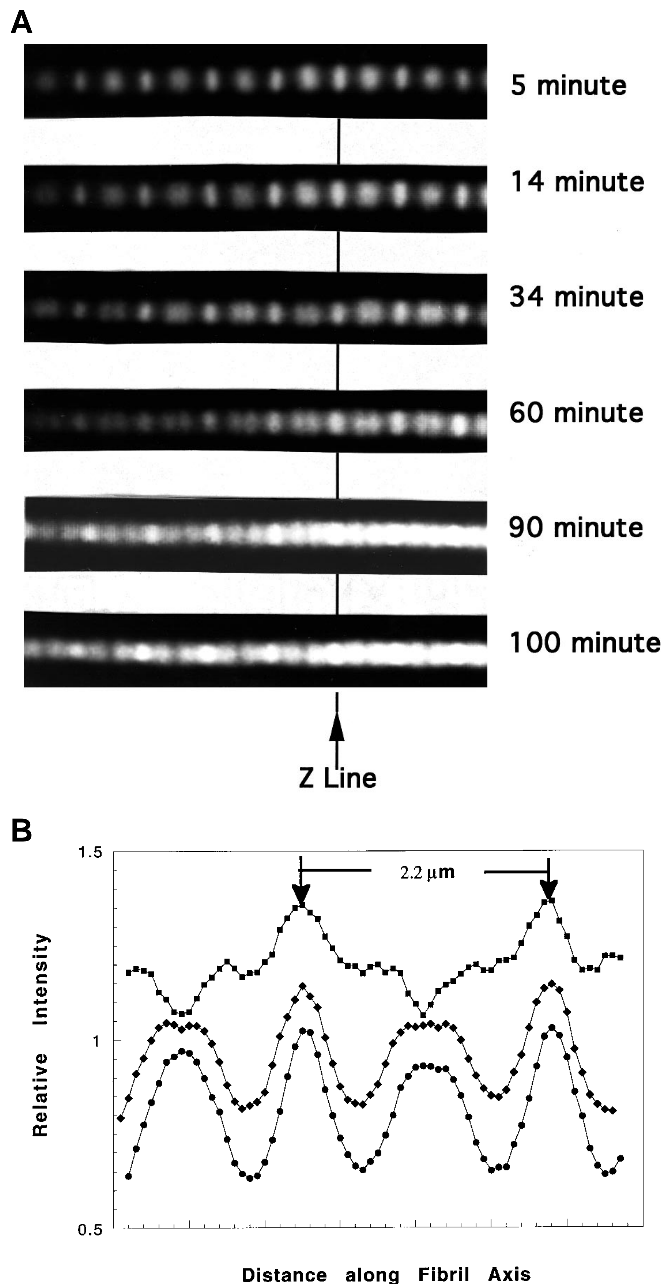


Fig. 1. (A) Fluorescence patterns of rhodamine-phalloidin (Rh-Ph) incorporation into actin filaments of a representative skeletal myofibril at different times after introduction of excess Rh-Ph. The sample was incubated with 6.6 μM Rh-Ph in rigor buffer (0.05 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 0.01% NaN_3, 20 mM Hepes, pH 7.2) at room temperature. (B) Relative fluorescence intensity distribution across two typical sarcomeres in A at incubation times of 5 minutes (●), 14 minutes (◆) and 100 minutes (■). The middle of the Z-band is indicated by arrows. The apparent decrease in Rh-Ph fluorescence intensity in the middle of the sarcomere at the longer times is not due to loss of Rh-Ph binding or depolymerization of actin. It is due to the preferential imaging of the greater fluorescence in the bulk of the thin filaments at the longer times which de-emphasizes the less intense fluorescence at the pointed ends. The images of the short-time pattern at the pointed ends results in one apparent overlapped band due to resolution broadening.

RESULTS

Fluorescence images of a representative single intact skeletal myofibril taken at different times after introduction of rhodamine-phalloidin (Rh-Ph) are shown in Fig. 1A. There is an initial preferential binding to the ends of thin filaments in the myofibril which becomes more uniform after long times as previously noted (Szczesna and Lehrer, 1993). Data from the present study shows that, after the initial binding, slow binding to the rest of the actin filament is unidirectional from the pointed end toward Z-band. For the 2.2 μm sarcomere length myofibril shown here, the pointed ends of the 1 μm long thin filaments are close together, resulting in a broad fluorescent band at the pointed ends in the pattern taken after 5 minutes incubation (Fig. 1A). With increasing incubation time, the fluorescent band broadens toward the Z-line (Fig. 1B, middle pattern). The width of the Z-band remains essentially

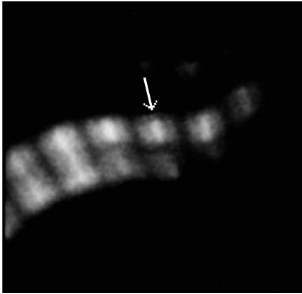


Fig. 2. Fluorescence image of rabbit cardiac myofibrils taken 4 minutes after the introduction of excess Rh-Ph. Specimen conditions same as for Fig. 1.

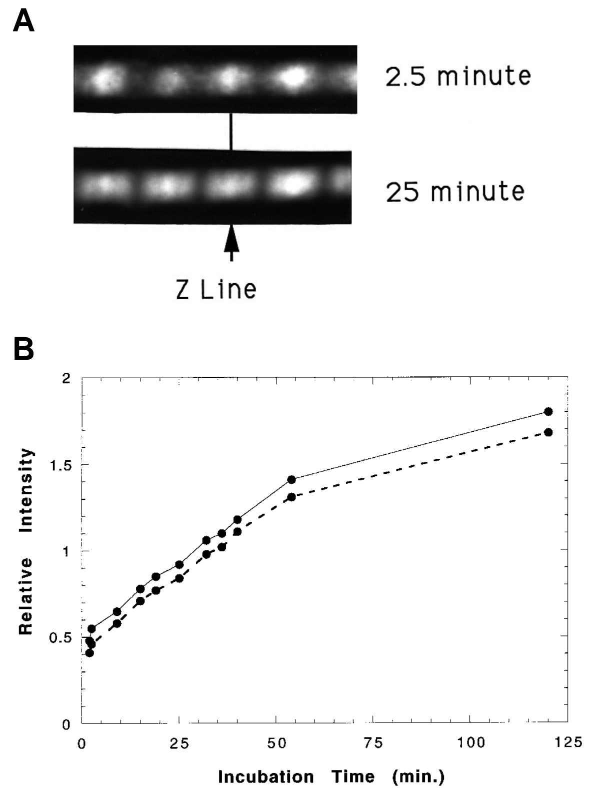


Fig. 3. (A) Fluorescence distribution within sarcomere of single skeletal myofibrils at 2.5 minutes and 25 minutes after introduction of Rh-Ph to the myofibrils pre-incubated overnight with excess unlabeled phalloidin (10 μM). The free phalloidin was washed away with the rigor buffer before an excess of 6.6 μM Rh-Ph was introduced and incubated for the times indicated. (B) Comparison of the kinetics of fluorescence increase associated with Rh-Ph binding to actin in the Z-band (solid line) compared to actin in the rest of the sarcomere (broken line) in A.

unchanged, indicating that Rh-Ph binding does not proceed from the ends of the thin filaments attached to the Z-band matrix. A similar time-dependent change in intensity distribution was observed with ghost myofibrils (myosin and regulatory protein depleted), indicating that the presence of myosin or the regulatory proteins was not the cause of the selective inhibition (Szczesna and Lehrer, 1993). Some ghost myofibrils and sarcomeres of a given ghost myofibril showed much faster phalloidin uptake relative to intact myofibrils. This may indicate some alteration in the structure that occurred in the preparation of the more fragile ghost myofibrils.

To test the possibility that nebulin inhibits the binding of phalloidin we studied the time dependence of the binding distribution of Rh-Ph within the sarcomeres of rabbit cardiac myofibrils which lack nebulin. There was an initial uniform binding of Rh-Ph within each cardiac sarcomere (Fig. 2), in contrast to the slow directional binding for the skeletal system. The initial intensity distribution pattern within cardiac sarcomeres (Fig. 2) was similar to the pattern at long incubation times within skeletal sarcomeres (Fig. 1A). The large difference in binding kinetics of the two types of myofibrils does not appear to be due to differences in the diffusion rates of Rh-Ph to actin in these systems in view of the similar protein composition and organization of cardiac and skeletal myofibrils (Schwartz et al., 1990). The main difference is that the thin filaments in cardiac myofibrils have a broader length distribution than in skeletal myofibrils (Robinson and Winegrad, 1977) which appears to be due to the absence of nebulin (Wang and Wright, 1988; Labeit et al., 1991; Kruger et al., 1991). It is most likely, therefore, that nebulin inhibits the binding of phalloidin to actin in skeletal myofibrils.

To further clarify the mechanism of inhibition of phalloidin binding, we pre-incubated skeletal myofibrils overnight with unlabeled phalloidin. Rh-Ph was then introduced into the sample cell after removing excess unlabeled phalloidin. Although the binding of the fluorescent Rh-Ph was slow due to the necessary exchange with unlabeled phalloidin, the pattern of fluorescence was uniform at all times and looked very similar to the cardiac system (Fig. 3A). The time dependence of the relative intensities at the Z-band and in the rest of the sarcomere taken from the same fibril during the 2 hour period, increased in parallel (Fig. 3B), indicating that the same kinetic process is taking place in all regions of the sarcomere. If the non-uniform initial pattern of Rh-Ph binding to the thin filaments is intrinsic to skeletal actin in myofibrils, the same initial pattern of binding of Rh-Ph would be expected after pre-incubation with phalloidin. If, however phalloidin removes a binding inhibition caused by an actin binding protein, the re-introduction of labeled phalloidin would result in uniform binding. Since we observed uniform binding kinetics, it appears that pre-incubation with phalloidin releases a binding inhibition by some component, probably nebulin, allowing the newly introduced labeled phalloidin to bind uniformly to skeletal myofibrils as it does to cardiac myofibrils which lack nebulin.

It has been reported that a nebulin peptide inhibits myosin-propelled actin velocity in an in vitro motility assay and that calmodulin reverses this effect (Root and Wang, 1994). To study the effect of calmodulin on Rh-Ph binding, skeletal ghost myofibrils were pre-incubated with 50 μM calmodulin in the presence of either 1 mM Ca^{2+} or 5 mM EGTA before 5 μM Rh-Ph was introduced (Fig. 4). In the presence of Ca^{2+}, a fast uniform binding pattern was seen (Fig. 4a), similar to the pattern at long incubation times in the skeletal myofibril (Fig. 1). In contrast, in the absence of Ca^{2+}, no effect of calmodulin was seen (Fig. 4b) since there was little difference in the time dependence of Rh-Ph uptake compared to the untreated skeletal system. Thus, pre-incubation with Ca^{2+}-calmodulin releases the phalloidin binding inhibition. To further clarify that the effect is specific for calmodulin, a control experiment was done with Ca^{2+}-troponin C under the same conditions, and no release of inhibition was observed (Fig. 4c). These myofib-

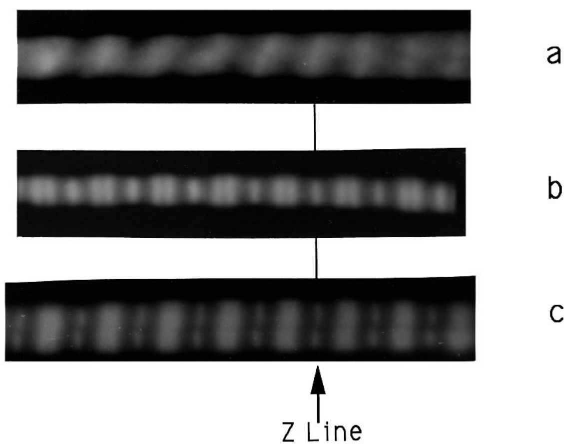


Fig. 4. Fluorescence patterns of single and double myofibrils shortly after introduction of 5 µM Rh-Ph to rabbit skeletal myofibrils pre-incubated for 30 minutes with: (a) 50 µM calmodulin, 1 mM $CaCl_2$, observed 5 minutes after addition of Rh-Ph; (b) 50 µM calmodulin, 5 mM EGTA, observed 20 minutes after addition of Rh-Ph; (c) 50 µM troponin C, 1 mM $CaCl_2$, observed 20 minutes after addition of Rh-Ph. All situations in rigor buffer.

rils have long sarcomeres which produced a double fluorescence band in the image at the pointed ends.

To show that nebulin redistribution was caused by Rh-Ph binding, we used immunofluorescence microscopy. A polyclonal anti-nebulin goat IgG and rhodamine-labeled anti-goat IgG were applied to ghost rabbit skeletal myofibrils with and without overnight pre-incubation with excess phalloidin. The microscopic images of the control phalloidin-free myofibrils showed several fluorescent bands associated with several epitopes of nebulin attached to thin filaments in each half sarcomere (Fig. 5a) (Wang and Wright, 1988). After phalloidin pre-incubation, only one fluorescent band remained near the Z-line (Fig. 5b). Incubation of the ghost myofibrils with excess Ca^{2+}-calmodulin produced a similar changed immunofluorescence band pattern (Fig. 5c), indicating that calmodulin dissociated nebulin from actin.

To further study the relationship between nebulin dissociation and phalloidin uptake, we pre-incubated the myofibrils with coumarin-phalloidin (Cou-Ph) for 30 minutes to

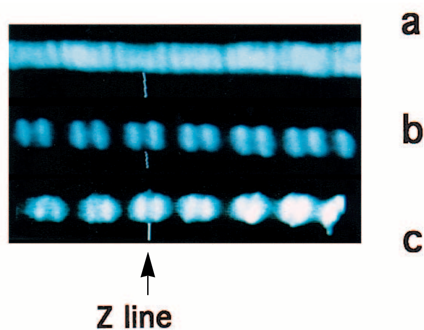


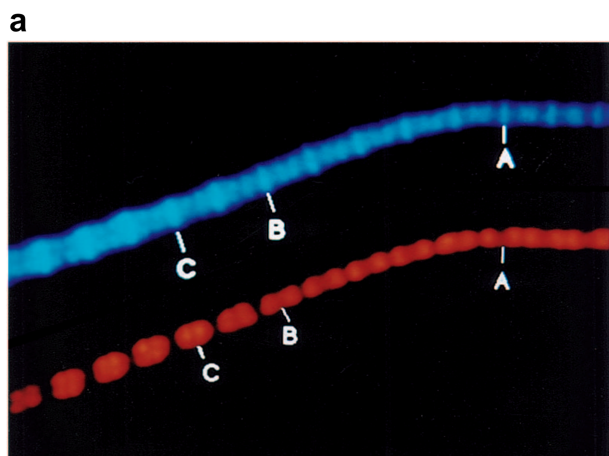
Fig. 5. Immuno-fluorescent visualization of nebulin epitopes in ghost rabbit skeletal myofibrils (a) before; and (b) after, overnight pre-incubation with 10 µM phalloidin in rigor buffer, (c) after 30 minutes pre-incubation with 50 µM calmodulin in the presence of 1 mM Ca^{2+}.

visualize the distribution of bound Cou-Ph (blue fluorescence) and then introduced labeled antibodies to locate nebulin (red fluorescence). We imaged the blue and red fluorescence from the same portion of a myofibril which had a fortuitous graded degree of Cou-Ph uptake from one end to the other, perhaps due to graded differences in local solute accessibility. Thus, the changed pattern from right to left represented in a spatial manner (Fig. 6) what was usually observed in a temporal manner. It can be seen that as the Cou-Ph binding increased from A to B to C (blue), the anti-nebulin fluorescence pattern which correspondingly changed from A to B to C (red) was dominated by a single broad band which moved closer to the Z-line (Fig. 6, red). This indicated that as more Cou-Ph bound, the structure of nebulin within the sarcomere correspondingly changed from an extended to a more collapsed structure. Clearly, as the uni-directional binding of phalloidin to actin took place, the anti-nebulin band moved uni-directionally from the pointed ends toward the Z-line.

DISCUSSION

We have shown that: (1) cardiac myofibrils which do not contain nebulin (Hu et al., 1986; Locker and Wild, 1986), initially bind phalloidin fast and uniformly suggesting that the inhibition of phalloidin binding to thin filaments in skeletal myofibrils may be due to nebulin; (2) pre-incubation of skeletal myofibrils with phalloidin results in uniform binding of Rh-Ph, indicating that phalloidin removes a component of the thin filament which inhibits binding; (3) pre-incubation of skeletal myofibrils with calmodulin in the presence but not in the absence of Ca^{2+} releases the inhibition of phalloidin binding and changes the localization of nebulin, indicating that calmodulin binding to nebulin dissociates nebulin from thin filaments; (4) the lack of inhibition of Rh-Ph binding to actin in the Z-band and the pointed ends of the filaments indicates that nebulin is not present in those regions or if present, does not inhibit phalloidin binding; (5) the uni-directional binding of phalloidin to thin filaments is correlated with uni-directional nebulin dissociation and collapse.

Previous fluorescence imaging studies have shown preferential binding of labeled phalloidin to the ends of thin filaments in skeletal myofibrils (Bukatina et al., 1984; Greaser and Schnasec, 1990; McKenna et al., 1985; Szczesna and Lehrer, 1993; Wilson et al., 1987), postulated as either due to an inhibition by another protein (Bukatina et al., 1984; Greaser and Schnasec, 1990; Szczesna and Lehrer, 1993) or as an intrinsic difference between actin subunits at the ends and in the middle of filament (Cano et al., 1992; Szczesna and Lehrer, 1993). Although phallotoxins labeled with a fluorescent moiety bind quickly to every actin subunit in purified F-actin (Huang et al., 1992; Allen and Janmey, 1994; De La Cruz and Pollard, 1994), it has been noted that there seem to be kinetic differences in the rate of uptake of phalloidin by the ends compared to the bulk of the actin subunits in the second time scale (Cano et al., 1992) which led to the suggestion that this phenomenon, allowing for decreased accessibility to actin in myofibrils, could be a possible explanation (Szczesna and Lehrer, 1993). The uniform initial binding pattern to cardiac myofibrils observed in our study ruled out the intrinsic mechanism for the decreased accessibility to actin in skeletal myofibrils. Of course, there still could be preferential binding to



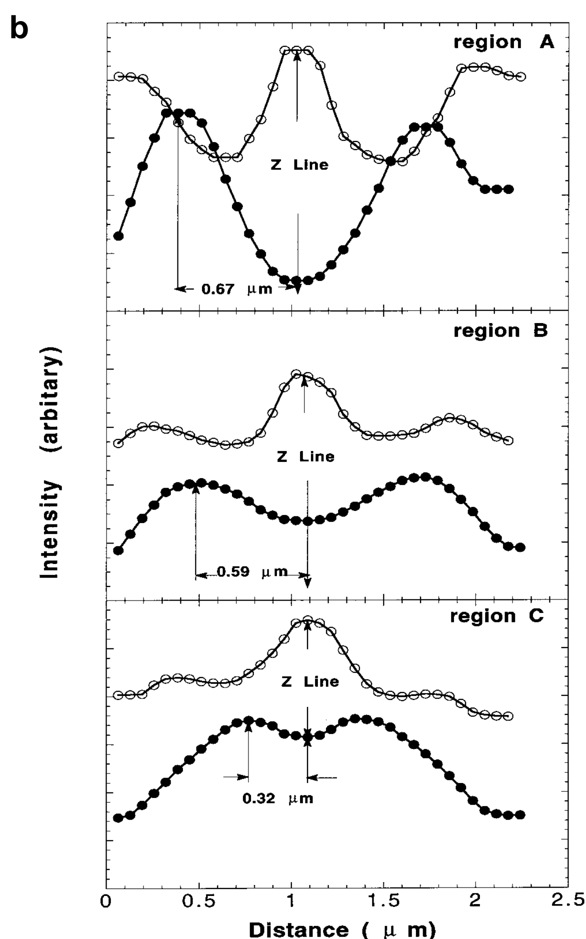


Fig. 6. Relationship between increased phalloidin binding and changed nebulin localization in different regions of a ghost rabbit skeletal myofibril. Ghost myofibrils attached to a microscope coverslip were irrigated with coumarin-phalloidin (Cou-Ph) for 30 minutes. A fibril with a continuous increase of Cou-Ph binding from one end to the other (right to left) was selected for imaging (blue fluorescence), rhodamine labeled antibodies were then introduced, and an image of the same fibril was obtained (red-orange fluorescence). Three regions representative of low (A), intermediate (B) and high Cou-Ph binding (C) were selected for densitometry. The lines in the photograph point to the Z-band in each region. (a) False colored Cou-Ph distribution (blue) and Nebulin localization (red-orange). (b) Densitometry of fluorescence in regions A, B, C. Cou-Ph (○), anti-nebulin (●). Note that as more directional phalloidin binding took place toward the Z-line, the dominant anti-nebulin fluorescent peak moved closer to the Z-line.

one or both ends on a fast time scale which would not be noted within the time scale of our experiments.

Nebulin is a huge protein of about 800 kDa which has been proposed to regulate the length of thin filaments in skeletal muscle (Kruger et al., 1991; Labeit et al., 1991, 1995; Wang and Wright, 1988). Partial amino acid sequence analyses have suggested that it is a modular protein of about 35 repeating residues which interact with actin subunits along the length of the thin filament (Labeit et al., 1991). Site specific monoclonal antibody binding observed by immuno-electron microscopy on treated skeletal fibers have verified that a single nebulin molecule can span the length of a thin filament from the Z-band to close to the pointed end of the actin filament (Wright et al., 1993). Fragments of nebulin constructed by recombinant DNA technology (Jin and Wang, 1991), have been shown to promote polymerization of actin and inhibit depolymerization, leading to the concept of nebulin as an actin ‘zipper’ (Chen et al., 1993), i.e. it provides a template for thin filament organization. Conformational studies of peptides of nebulin have suggested that the conserved region of the sequence of each module becomes helical when it interacts with actin subunits (Chen and Wang, 1994; Pfuhl et al., 1994), and a model for the location of nebulin domains between actin subunits of F-actin has been proposed (Pfuhl et al., 1994). It is noteworthy that phalloidin, which also stabilizes actin against depolymerization and binds between actin subunits although in a more limited region (Lorenz et al., 1993), appears to interact with some of the residues that interact with nebulin.

From the five types of experiments presented above and the information available on the mode of interaction between nebulin and actin, we are able to present a schematic model for a tentative mechanism of inhibition of phalloidin binding to actin in skeletal myofibrils (Fig. 7). Nebulin is shown to interact with every actin subunit outside of the Z-band except for one or two subunits at the pointed tip of the filament. It has been shown that tropomodulin binds to the amino terminus of tropomyosin at the pointed ends of thin filaments in skeletal myofibrils and appears to act to ‘cap’ thin filaments from elongating (Fowler et al., 1993). Our observations that Rh-Ph binds quickly to the pointed ends of thin filaments in both intact and ghost myofibrils indicate that tropomodulin does not inhibit phalloidin binding to thin filaments. On incubation with phalloidin, initial binding takes place to the more accessible actin sites, resulting in fluorescence at the tip of the thin filaments and in the Z-band where greater fluorescence is observed due to overlapping thin filaments (Szczesna and Lehrer, 1993). Displacement of a terminal domain at either end of nebulin from its actin site would be expected to be easier than an internal domain. Studies with nebulin modules of different size and of different composition have indeed indicated that the binding strength does depend on size (Jin and Wang, 1991). Our observation that competition with nebulin binding proceeds from the pointed end rather than the barbed end at the Z-disc, could be explained by a weaker nebulin-actin interaction at the pointed end. Additional actin-capping proteins at the Z-disc (Coluccio,

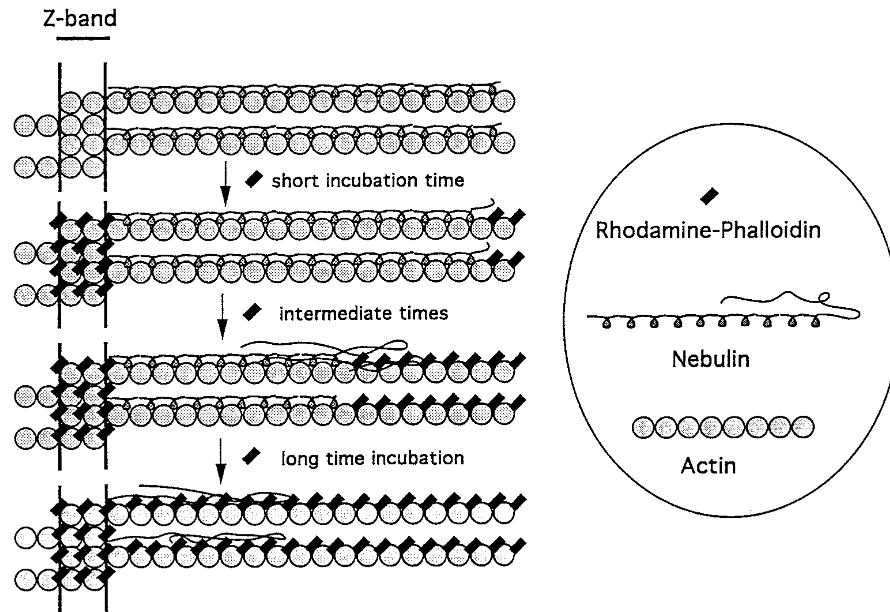


Fig. 7 Schematic diagram of a tentative model for the competitive binding between phalloidin and nebulin to thin filaments in rabbit skeletal myofibrils.

1994) could stabilize the nebulin-actin interaction whereas at the pointed end, the capping protein tropomodulin, only appears to interact with actin and tropomyosin (Weber et al., 1994). On initial phalloidin binding to the pointed end, a new nebulin unattached 'end domain' is produced with weaker binding to actin, allowing for a directional 'unzipping' of the nebulin from actin, finally resulting in uniform phalloidin binding. However, it has been noted that nebulin modules of similar size from different regions of the sequence have different binding properties (Jin and Wang, 1991), so it is possible that non-uniformity of module strength may also contribute to the directional de-inhibition by phalloidin.

A recent report has shown that certain nebulin fragments which bind to both actin and myosin S1 inhibit actin velocity in an in vitro motility assay and Ca^{2+}-calmodulin reverses the inhibition (Root and Wang, 1994). Our studies which show that calmodulin reverses the inhibition by nebulin of phalloidin binding and by dissociating nebulin from actin, indicate that calmodulin may function similarly to dissociate nebulin from actin. It is not clear, however, if there is a functional involvement for nebulin-calmodulin in view of the ability of nebulin-containing myofibrils to contract and the high concentrations of calmodulin (5-10 μM) necessary to observe the effect. In the in vitro motility studies (Root and Wang, 1994), the fluorescence of Rh-Ph labeled actin could be visualized after addition of the nebulin modules. This suggests that their modules do not compete with Rh-Ph binding in apparent disagreement with our results involving intact nebulin in thin filaments. This discrepancy could be reconciled if nebulin modules bind to actin at sites other than at the Rh-Ph site. Indeed, as the schematic model of nebulin-actin interaction indicates (Fig. 7), in addition to strong nebulin binding sites between actin subunits, additional sites would appear to be necessary to align the nebulin strand along the thin filament.

Our observation that phalloidin binds readily to actin in the Z-band, suggests that if nebulin is present in that region, it is not bound to actin in the same manner as in the I-band. This is consistent with studies of the Z-disc, whose basic structure seems to be composed mainly of actin and α-actinin (Luther, 1991; Morris et al., 1990). The anti-nebulin staining at the Z-band seen in the immuno-fluorescence study (Fig. 5a) could be due to an optical artifact considering that there is a nebulin epitope 0.1 μm away from the Z-band by immuno-electron microscopy (Wang and Wright, 1988) and the 0.2 μm resolution of the objective. Thus, the apparent Z-band staining can be explained by the overlapping of the fluorescent anti-nebulin close to each side of the Z-band. The apparent decrease of the Z-band staining after nebulin is displaced by Rh-Ph (Fig. 5b) is probably due to the relative increase of fluorescence of the prominent band upon collapse of nebulin which dominates the image. However, recent analysis of the sequence of nebulin suggests that its C-terminal SH3 domain does partially extend into the Z-disc (Labeit and Kolmere, 1995). However, in view of its unique sequence it would not be expected to interact with actin in the same manner as the repeating modules along its sequence and could therefore not inhibit Rh-Ph binding in the Z-disc. Clearly, further studies are needed to clarify the different modes of nebulin interaction with actin along the length of the thin filament.

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