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Fluorescent localization of membrane sites in glycerinated chicken skeletal muscle fibers and the relationship of these sites to the protein composition of the Z disc

(actin/α-actinin/desmin/T system/sarcoplasmic reticulum)

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ABSTRACT **Didansyl derivatives of amino acids and *N*-phenyl-1-naphthylamine were used to localize membrane hydrophobic sites in glycerol-extracted chicken skeletal muscle fibers. Epifluorescence microscopy revealed that such sites coincide with the distribution of mitochondria, the transverse tubular (T) system and the sarcoplasmic reticulum (SR). They are specifically associated with myofibril Z lines and occasionally extend from one Z plane to the next longitudinally along the muscle fiber. The hydrophobic probes interact noncovalently with the Z lines, and their induced fluorescence can be eliminated by exposure of the myofibrils to ionic detergents, nonionic detergents, or phospholipase C, before or after addition of the hydrophobic label.**

Extraction of glycerinated fibers with 0.6 M KI removes the majority of sarcomeric actin and myosin and leaves a scaffold of longitudinally interconnected Z planes. Membrane fluorescence remains tightly associated with these Z planes and with the remnant mitochondria. Shearing of such scaffolds results in the cleavage of the longitudinal connections and the production of large sheets of interconnected, close-packed Z discs in a honeycomb-like array. Comparison of the localization of two Z disc proteins, desmin and α-actinin, with that of the membrane material reveals that α-actinin is localized in the interior of each myofibril Z disc whereas both desmin and the membrane material surround each disc. Thus, glycerination and KI extraction of muscle fibers leaves remnants of T system and SR membranes tightly associated with the Z disc honeycomb lattice. Because the Z discs are connected at their peripheries through the T system to the plasma membrane, desmin and this membrane structure appear to be connected throughout the whole Z plane up to and including the plasma membrane. The congruent localization of desmin and the T system strongly suggests that this molecule mediates the adhesion of this membrane system around each Z disc.

The amplification of the electrical stimulus and its transformation to a chemical signal in the contraction of skeletal muscle fibers is mediated by two distinct, but adjoined, interfibrillar membrane systems: the transverse tubular system (T system) and the sarcoplasmic reticulum (SR). The T system is so called because it forms tubular, fingerlike invaginations that extend from the fiber's plasma membrane deep into the muscle fiber. In chicken skeletal muscle, the tubules surround every Z disc in a given Z disc plane. The lumen of the T system freely communicates with the extracellular space, and in this manner the Z discs are connected at their peripheries through the T system membrane to the outer plasma membrane. The junctional complex of the terminal cisternae of the SR with the T system (triad), is thus also found at the periphery of each Z disc (1–7). The molecular composition of the T system and its mode of association with the periphery of the Z disc have not been elucidated.

The weakest link of a muscle fiber appears to be between adjacent myofibrils. When glycerol-extracted muscle fibers are sheared in a blender, they fragment longitudinally to produce individual or, more frequently, bundles of myofibrils of random lengths. These newly generated myofibril bundles have Z lines as their terminal boundaries. If, however, the glycerinated muscle fibers are extracted with 0.6 M KI before shearing, then the path of least resistance in the fiber changes and the cleavage plane is altered. Extraction of muscle fibers with KI releases the majority of actin and myosin filaments from the myofibrils. What remains insoluble is a scaffold of Z planes longitudinally interconnected by residual fibrillar material. The weakest link of these extracted fibers now appears to be the area where the myosin filaments used to be, and shearing results in the production of large sheets of interconnected, close-packed Z discs in a honeycomb-like array (8). Such Z disc lattices provide an ideal system for investigation of the molecular basis of the association of the T system/SR membrane with the periphery of the Z disc and of the macromolecules responsible for the association of actin filaments with these membrane systems. However, because only fragments of these membrane systems are expected to remain associated with these Z disc scaffolds, we needed a convenient and rapid technique for the localization of such membrane sites in isolated myofibrils.

Dyes that are not fluorescent in water but have the remarkable ability to become fluorescent when introduced into hydrophobic environments proved to be ideal for this purpose. In our choice of such probes, we had to be confident that the fluorescence of the dye would be due exclusively to the hydrophobic properties of the membrane environment, would be due almost exclusively to membrane lipids and minimally to membrane proteins, and would not depend on the surface charge of the membrane. Didansyl derivatives of amino acids and the neutral dye *N*-phenyl-1-naphthylamine (NPN) seemed to fulfill these criteria. Didansylcystine binds rapidly to the membranes of retinal rods and cones with a simultaneous severalfold increase in its fluorescence efficiency and with no detectable binding to rhodopsin (9, 10). The binding of NPN to biological membranes, unlike that of its sulfonated derivatives 1-anilino-napththalene-8-sulfonate, occurs deep in the hydrophobic interior of membranes, preferentially with the neutral hydrocarbon tails of phospholipids and minimally with membrane proteins (11–15).

We show here that didansyl derivatives of amino acids, in particular of ornithine and lysine, and NPN can be used to study

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Abbreviations: T system, transverse tubular system; SR, sarcoplasmic reticulum; NPN, *N*-phenyl-1-naphthylamine; DD, didansyl derivative of indicated amino acid; $NaDodSO_4$, sodium dodecyl sulfate; NaCl/KCl/P_i, 0.15 M NaCl/2.5 mM KCl/0.01 M sodium phosphate, pH 7.2 (phosphate-buffered saline); PlC, phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3).

the distribution of membrane sites in isolated myofibrils by using epifluorescence microscopy. Our results demonstrate that (*i*) remnants of a T system and SR membranes (triad) remain tightly associated with isolated glycerinated myofibrils; (*ii*) this membrane residue is located at the periphery of every Z disc in isolated Z disc lattices; and (*iii*) the distribution of these sites coincides with the distribution of the hydrophobic Z disc protein, desmin.

MATERIALS AND METHODS

Preparation of Myofibrils and Z Disc Sheets. Myofibrils were prepared from chicken muscle fibers extracted with 50% (vol/vol) glycerol in 0.15 M NaCl/2.5 mM KCl/0.01 M sodium phosphate, pH 7.2 (phosphate-buffered saline; NaCl/KCl/P_i) as described (8, 17). The myofibrils were washed free of glycerol with phosphate-buffered saline by sedimentation at 1000 × *g* and used immediately. Similar results were obtained when the myofibrils were stored in glycerol at −20° for up to a month. Myofibrils are very sticky and they readily adhere to glass coverslips. Extractions with KI were carried out on myofibrils attached to coverslips because the myofibrils tend to clump irreversibly if extracted in suspension and subsequently washed free of KI with NaCl/KCl/P_i. The extraction solution contained 0.6 M KI, 0.02 M sodium thiosulfate, and 0.01 M sodium pyrophosphate (pH 7.0), and extractions were carried out for 45 min at room temperature. Z disc sheets were prepared from glycerol and KI-extracted muscle fibers as described (8).

Reaction with Fluorescent Probes. Didansyl amino acids—didansylcystine (DDcystine), tyrosine (DDTyr), lysine (DDLys), and ornithine (DDOrn) (Pierce)—were dissolved with continuous stirring in NaCl/KCl/P_i at a concentration of 0.1–1.0 mg/ml. Lower concentrations of these probes were used, depending on the intensity of fluorescence in different specimen preparations. NPN (Eastman) was dissolved at a concentration of 0.1 mg/ml in methanol. The specimens were incubated in suspension or on coverslips with the fluorescent probes, usually for 20–30 min at room temperature. They were then washed briefly in NaCl/KCl/P_i, mounted on slides, and sealed at their edges with Pro-texx (S.P. M7635) to prevent drying. The use of the polyvinyl alcohol compound Elvanol (PVA 51-05, E. I. DuPont de Nemours & Co.), usually employed to mount immunofluorescence specimens, completely abolished the fluorescence of the probes. Photography was carried out, immediately after preparation of the specimens, by using a Leitz Orthoplan microscope equipped with epifluorescence optics and the wide-band UV filter module A of Leitz for dansyl fluorescence without a BG 23 filter. Exposures were 5–18 sec. Indirect immunofluorescence with antibodies to desmin and α-actinin was carried out as described (8, 16) by using the wide-band blue, high-intensity-filter module H of Leitz for fluorescein isothiocyanate fluorescence. All photographs were recorded on Tri-X film with a ×100 phase-fluorescence objective lens (Ph 3, N.A. = 1.32) and developed in Diafine (Acufine Corp.).

RESULTS

Characterization of the Reaction. Fluorescence as a result of the reaction of myofibrils with the hydrophobic probes was evident within 1 min after exposure of a myofibril suspension to the dyes. The reaction was allowed to proceed usually for 20 min or more to maximize the interaction of the probes with the myofibrils. All five hydrophobic probes yielded indistinguishable fluorescent distributions. However, DDOrn and DDLys proved to be much more intensely fluorescent than NPN, DDcystine and DDTyr at the same dye concentration.

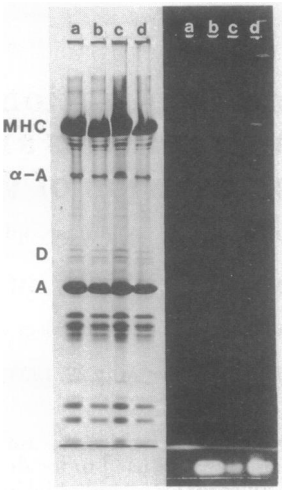

FIG. 1. One-dimensional $NaDodSO_4$ 12% polyacrylamide gel electrophoresis (17) of chicken skeletal myofibrils treated with hydrophobic fluorescent dyes. A 100-μl suspension of myofibrils was incubated with 100 μg of each of the dyes in NaCl/KCl/P_i for 2 hr at room temperature. The myofibrils were washed extensively with NaCl/KCl/P_i by sedimentation at 1000 × *g*. They were then reduced and denatured at 100° with $NaDodSO_4$ sample buffer and loaded on the gels. At the end of the run the gels were observed for fluorescence over a long-wave UV light source and photographed on Polaroid PN-55 film. The gels were then stained with Coomassie brilliant blue R-260 and destained (17). (*Left*) Stained gel. (*Right*) Corresponding fluorescence image. Lanes a, DDcystine; b, DDLys; c, DDTyr; d, DDOrn. Note that all of the dye fuorescence migrates with the buffer front. The dyes fluoresce in the gel most likely because of the presence of $NaDodSO_4$. MHC, myosin heavy chain; α-A, α-actinin; D, desmin; A, actin.

The intensity of fluorescence (in order of decreasing intensity was DDOrn ≥ DDLys ≫ NPN ≥ DDcystine > DDTyr. DDTyr fluorescence was barely detectable. Reaction of myofibrils with monodansyl cadaverine, an analogue of lysine, was uniformly negative (17). Because the specimens treated with DDOrn were the brightest, most of the photography was carried out on specimens reacted with this probe.

The interaction of the dyes with the myofibrils was stable but noncovalent. Reaction of myofibrils with an excess of DDcystine, DDLys, DDTyr, or DDOrn for more than 2 hr at room temperature and subsequent analysis of the myofibril proteins by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis under reducing conditions demonstrated that all of the fluorescent label migrated near the buffer front of the gel (Fig. 1).

Probe Distribution in Myofibrils. Epifluorescence microscopy revealed DDOrn localized preferentially at or near the Z lines of isolated myofibril bundles (Fig. 2 *A* and *B*). Quite frequently the dye revealed the presence of fluorescent foci localized between adjacent myofibril Z lines (Fig. 2 *C* and *D*) or parallel fluorescent stripes superimposed on the Z line fluorescence (Fig. 2 *A*, *B*, *E*, and *F*). In thicker myofibril bundles, these foci and stripes were often seen to be interconnected by interfibrillar material that extended longitudinally from one Z plane to the next (Fig. 2 *G* and *H*); these structures appear to be residual mitochondria. Within a given population of glycerinated myofibrils there were a small number of myofibrils that were nonfluorescent or only partially fluorescent (not shown). In general, each myofibril appeared to exhibit its own characteristic distribution and intensity of fluorescence along the Z lines.

Reaction of myofibrils with DDOrn and subsequent exposure

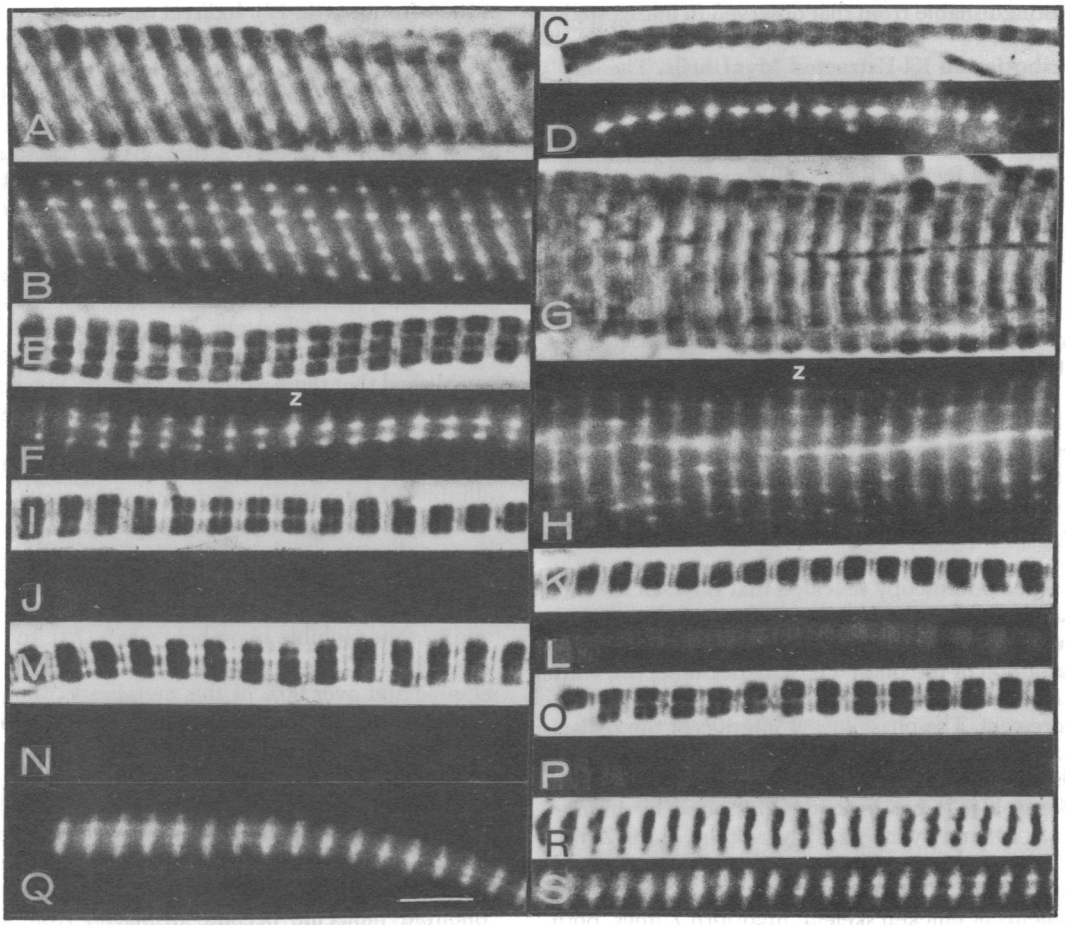

FIG. 2. For all parts, bar in Q = 5 μm. (*A–H*) Localization of DDOrn in myofibril Z lines. A 100-μl suspension of freshly prepared glycerinated myofibrils was washed free of glycerol with NaCl/KCl/P_i by repeated sedimentation at 1000 × *g*. The myofibrils were then incubated with 50 μg of DDOrn in 200 μl of NaCl/KCl/P_i for 20 min at room temperature, washed free of the dye with NaCl/KCl/P_i by repeated sedimentation at 1000 × *g*, and mounted on slides. (*A, C, E,* and *G*) Phase-contrast images. (*B, D, F,* and *H*) Corresponding fluorescence images. Z, Z line. (*I–L*) Elimination of dye fluorescence by detergents. A 100-μl glycerol-free suspension of myofibrils was suspended in 0.5 ml of 0.5% Nonidet P40 in NaCl/KCl/P_i for 15 min at room temperature either after (*I* and *J*) or before (*K* and *L*) treatment with DDOrn. The myofibrils were treated with DDOrn as above. (*M–Q*) Elimination of dye fluorescence by PlC. Digestions with PlC (*Clostridium welchii,* Sigma type I) were carried out as follows. To 100 μl of glycerol-free myofibrils in NaCl/KCl/P_i was added 100 μl of 200 mM NaCl/50 mM Tris·HCl, pH 7.5/5 mM $CaCl_2$/2 mM phenylmethylsulfonyl fluoride (Sigma)/2 mM glutathione. To this suspension, 20 μg of enzyme was added and the mixture was incubated for 60 min at room temperature. The reactions were terminated by the addition of 2 mM ethylene glycol-bis(β-aminoethyl ether)*N,N'*-tetraacetate. (*M* and *N*) Exposure of myofibrils to DDOrn prior to PlC treatment. (*O* and *P*) Exposure of myofibrils to DDOrn and subsequent treatment with PlC in the presence of 20 μg of soybean trypsin inhibitor (Sigma type I-S). (*Q*) Exposure of myofibrils to DDOrn and subsequent treatment with PlC in the presence of 1 mM *o*-phenanthroline (Sigma; the *o*-phenanthroline was dissolved in methanol; fluorescence image only). (*M* and *O*) Phase-contrast images. (*N* and *P*) Corresponding fluorescence images. (*R* and *S*) Localization of DDOrn in KI-extracted myofibrils. Glycerol-free myofibrils were attached to coverslips and extracted with a buffer containing 0.6 M KI. The myofibrils were then treated with DDOrn as above. During the KI extraction, myofibrils shorten (8, 17). (*R*) Phase contrast image. (*S*) Fluorescence image.

of the labeled myofibrils to the nonionic detergent Nonidet P40 (0.5%, 15 min) completely eliminated the dye fluorescence (Fig. 2 *I* and *J*). Exposure of the labeled myofibrils to the ionic detergent sodium deoxycholate (0.1%, 15 min) similarly eliminated the dye fluorescence (not shown). Extraction of myofibrils with 0.5% Nonidet P40 (or 0.1% deoxycholate) prior to labeling and subsequent reaction with DDOrn revealed the presence of a weak and hazy fluorescence distributed uniformly throughout the myofibril (Fig. 2 *K* and *L*). This fluorescence is probably due to the generation of a hydrophobic environment along the myofibril by the binding of the detergents to the myofibril proteins. Such a nonselective interaction of ionic and nonionic detergents with the hydrophobic sites of proteins is well documented (18–21). Analysis of the myofibril extracts by $NaDodSO_4$ electrophoresis indicated that small quantities of a considerable number of proteins were released from the myofibrils by these detergents (not shown).

Reaction of myofibrils with DDOrn and subsequent treatment with phospholipase C (PlC; phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) also resulted in the complete removal of the dye fluorescence (Fig. 2 *M* and *N*). Myofibrils that were treated first with PlC and then with DDOrn exhibited no detectable fluorescence in smaller myofibril bundles and very faint Z line fluorescence in thicker bundles. All the PlC reactions were carried out in the presence of phenylmethylsulfonyl fluoride to inhibit any serine proteases contaminating the enzyme preparation. The presence of soybean trypsin inhibitor in addition during the reaction also did not inhibit the removal of the dye fluorescence (Fig. 2 *O* and *P*). Thus, the removal of the dye fluorescence does not appear to be due to the proteolytic activity of a serine protease or a trypsin-like activity. The inclusion of 1 mM *o*-phenanthroline, a specific inhibitor of PlC, during the reaction completely inhibited the removal of the dye fluorescence (Fig. 2*Q*). Similarly, PlC treatment in the presence of *o*-phenanthroline and subsequent reaction of the myofibrils with DDOrn resulted in a distribution

of the dye indistinguishable from that observed in undigested myofibrils (not shown).

Probe Distribution in KI-Extracted Myofibrils. The distribution of the dye fluorescence remained essentially the same when glycerinated myofibrils were first extracted with 0.6 M KI and then reacted with DDOrn. The Z lines remained fluorescent, the fluorescent foci and stripes still remained associated with the Z lines (Fig. 2 *R* and *S*), and the mitochondrial remnants were still prevalent.

Probe Distribution in Z Disc Sheets. Z disc sheets are composed of a phase-dense lattice that surrounds each phase lucent myofibril Z disc (8) (see Fig. 3*A*). The geometrical shape of each Z disc varied but, most frequently, hexagonal, pentagonal, and tetragonal shapes were observed, giving an overall impression of a honeycomb-like structure. At the periphery of each Z disc, the points of intersection of three or more Z discs were occupied frequently by phase-dense foci. Occasional foci were large and extremely phase-dense and appeared as the most phase-dense objects in the whole scaffold. Electron micrographs revealed that these large densities were in fact residual mitochondria (8).

Epifluorescence microscopy revealed that the DDOrn fluorescence was localized at the periphery of each Z disc (Fig. 3 *A* and *B*). The distribution of this fluorescent lattice coincided with the distribution of the phase-dense lattice observed with phase-contrast optics. The small and the large phase-dense foci also were fluorescent. The phase-lucent interiors of the Z discs were nonfluorescent.

Immunofluorescent Localization of Desmin and α-Actinin in Z Disc Scaffolds. It has been demonstrated by immunofluorescence (22) and by labeling the proteins of the Z disc covalently with dansyl cadaverine as catalyzed by guinea pig liver transglutaminase (17) that desmin and α-actinin are two major components of chicken skeletal myofibril Z lines. Both proteins remain associated with the Z lines after KI extraction of the myofibrils (8, 17). Indirect immunofluorescence carried out on Z disc scaffolds with antibodies specific for α-actinin and desmin revealed that α-actinin is localized in the interior of the Z disc (Fig. 3*D*). The localization of desmin, on the other hand, is complementary to that of α-actinin. Desmin was localized at the periphery of each Z disc, giving rise to an image of a fluorescent lattice (Fig. 3*C*). Thus, the localization of desmin coincides with the distribution of the dye fluorescence.

DISCUSSION

The mechanism by which NPN and the didansyl derivatives of ornithine, lysine, and cystine bind to glycerinated myofibrils is unknown, but it does not appear to be due to a covalent interaction of these dyes with membrane proteins. It is clearly established that these probes fluoresce only when they enter a hydrophobic environment (9–15). In the glycerol-extracted myofibrils this environment must be generated, at least in part, by membrane phospholipids because the dye fluorescence is eliminated by nonionic and ionic detergents and PlC, all of which solubilize membrane phospholipids. The action of PlC on membranes causes the conversion of the relatively polar phospholipids and sphingomyelin of the membranes to the relatively nonpolar 1,2-diglyceride and ceramide, respectively, and to water-soluble phosphate esters (23). In skeletal muscle microsomes, 70% of the total phospholipid is phosphatidylcholine and over 90% of this membrane phospholipid is hydrolyzed by PlC, whereas phosphatidyl serine, phosphatidylethanolamine, and sphingomyelin are only minimally hydrolyzed. Phosphorylcholine, being water soluble, is rapidly released into the microsome-free solution whereas the diglyceride product of the hydrolysis separates from the microsomal membrane but remains associated with it in the form of small, dense, lipid droplets. Thus all of the components of the phospholipid molecule become displaced from the membrane during enzymic degradation (23–25).

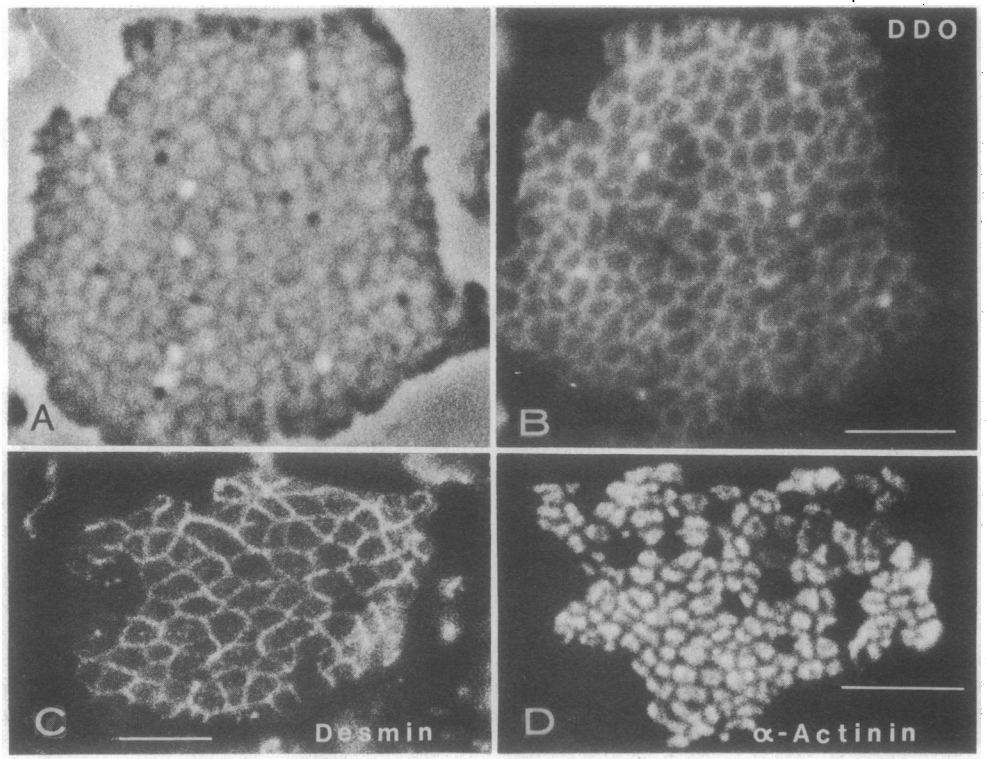

FIG. 3. Distribution of DDOrn, desmin, and α-actinin in Z disc lattices. (*A* and *B*) Lattices treated with DDOrn. (*A*) Phase-contrast image; (*B*) fluorescence image. (*C*) Indirect immunofluorescence using antibodies to desmin. (*D*) Indirect immunofluorescence using antibodies to α-actinin. Bars = 5 μm.

It appears then that the hydrophobic fatty acid tails of phosphatidylcholine may be the sites of binding of the didansyl amino acids (in particular DDOrn and DDLys) and NPN. Removal of these sites by PlC hydrolysis or by detergents eliminates the hydrophobic dye fluorescence. It is difficult to eliminate the possibility that the observed fluorescence is due not only to the hydrophobic environment of phospholipids but also, to some extent, to a hydrophobic phospholipid/protein interface in the membrane. In the cases examined so far it has been demonstrated that both NPN and DDcystine do not interact appreciably with membrane proteins (9–11). It seems likely that the observed dye fluorescence in myofibrils is predominantly due to the hydrophobic fatty acid environment of membrane phospholipids. The fluorescence of myofibrils is intense when exposed to DDOrn or DDLys, but exposure to DDcystine or DDTyr yields a much weaker fluorescence. On the other hand, when DDcystine binds to the cell membranes of vertebrate rods and cones, the membranes of these cells become brilliantly fluorescent (10). This difference in the fluorescence intensity of DDcystine may reflect differences in phospholipid composition between the membranes of rods and cones and those of muscle fibers.

The distribution of the fluorescent dyes in glycerol-extracted chicken skeletal muscle fibers and isolated myofibril bundles corresponds closely to the distribution of mitochondria, the T system and SR membranes in intact muscle as revealed by electron microscopy (1–3, 26). The remarkably specific localization of the dye fluorescence at the periphery of Z discs in isolated Z disc lattices further attests to the fact that these dyes reveal the localization of the T system–SR membrane. The exact effect that glycerol has on these membrane systems is unknown. From published electron micrographs of glycerol-extracted muscle fibers, it appears that this molecule fragments, but does not solubilize extensively, these membranous organelles (27). The fluorescence data presented here clearly demonstrate that a T system/SR membrane complex (triad) remains associated with glycerol-extracted myofibrils. This membrane complex must be very tightly adherent to the Z discs because extensive extraction of glycerol-extracted muscle fibers with 0.6 M KI does not remove it from the periphery of the Z discs. At this site the membrane material occupies the area where two neighboring Z discs are joined. The localization of the hydrophobic Z disc protein desmin coincides with the distribution of the T-system membrane at the periphery of the Z disc. Shearing of Z disc lattices results in cleavage of the inter-Z disc area on one side or the other of the desmin- or membrane-containing structures. Thus, a number of the Z discs at the edge of a fragmented Z disc lattice are only partally surrounded by desmin, or membrane material, such that the outermost side of these discs is not desmin- or membrane-bound. It has recently been demonstrated (28) that desmin forms a tenacious hydrophobic complex with actin that is insoluble in high KI concentrations but is soluble at low pH or in the strong anionic detergent Sarkosyl. The desmin-actin complex can polymerize *in vitro* into twisting and intertwining filaments, each with an average diameter of 120 Å (28). From these observations we postulated that desmin filaments form a ring around the Z disc which functions (*i*) to integrate all the actin filaments that terminate in the Z disc and (*ii*) to link one Z disc laterally to the next (8, 16). The coincident distribution of desmin and the T-system membrane suggests that desmin has yet a third function: to mediate the adhesion of the T-system membrane to the periphery of the Z disc. Thus, actin filaments can interact with this membranous system by virtue of their ability to interact with desmin.

The distribution of α-actinin is complementary to that of desmin and the T-system membrane; it is localized exclusively in the interior of the Z disc. Thus, α-actinin is most likely not involved in the adhesion of the T-system membrane with the periphery of the Z disc. However, a considerable amount of evidence demonstrates that α-actinin does interact with actin filaments and may be responsible for linking actin filaments within the Z disc (29–31). The type of molecular bonding that holds the Z disc proteins together is not well understood. The recent demonstration that Z disc actin, α-actinin, and desmin serve as substrates for the crosslinking enzyme guinea pig liver transglutaminase suggests that some of these proteins may be held together by ϵ-(γ-glutamyl)lysine covalent bonds (17).

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