Interaction of Adriamycin in Vitro with Cardiac Myofibrillar Proteins

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SUMMARY. Binding of the anthracycline antibiotic doxorubicin [Adriamycin (ADR)] to selected cardiac muscle contractile proteins was determined in purified canine heart actin and α-actinin. Adriamycin binding to these proteins in solution was measured by equilibrium dialysis and gel filtration with [3H]-labeled ADR. Adriamycin did not bind when its primary amino group was blocked. Adriamycin binding did not affect actin polymerization as detected by viscometry. Viewed under the electron microscope, however, ADR promoted formation of distinct actin filaments in the presence of μM amounts of ATP without K+ and Mg++. Adriamycin-induced actin microfilaments were thicker (120 Å) than those of F-actin controls (70 Å) and stimulated myosin-ATPase to higher levels than the F-actin controls. It appears that ADR has a direct effect on the biophysical and biochemical properties of heart myofibril proteins in vitro. (Circ Res 50: 547-553, 1982)

THE ability of cardiac cells to incorporate the cardiotoxic drug, Adriamycin (ADR) (Bossa et al., 1977), makes possible the systematic study of the biochemical interaction and ultrastructure of myofibrillar proteins. This pharmacological agent induces myofibrillar alterations in myocytes (Jaenke, 1974, 1976). These morphological changes have been used clinically to evaluate the nature of ADR congestive cardiomyopathy (Billingham et al., 1978; Benjamin et al., 1978). There is considerable interest in the mechanism by which ADR produces congestive cardiomyopathy. Previous studies have shown that, immediately after intravenous infusion of ADR, nonspecific acute electrocardiographic changes occurred (Lenaz and Page, 1976; Van Hoff et al., 1977). The series of events leading to deficient myofibrillar functions and disappearance of substantial myofibrillar mass is understood poorly (Klein and Harmjanz, 1975; Balazs and Herman, 1976).

A pivotal event in ADR-heart muscle disease is disarray of the thin filaments and alterations in Z-band structure (Jaenke, 1974; Billingham et al., 1974). Parallel arrangement and organized function of the thin filaments depend upon their anchoring site(s) at the Z-band (Oakley, 1975). The Z-band is a structure in which biochemical, biophysical, and ultrastructural information also is lacking because of the absence of compounds and markers that show selectivity and specificity of action for the Z-band elements (Thomas et al., 1976). To obtain some insight into ADR cardiotoxicity, we began a study of its interaction with thin-filament and Z-band proteins.

Methods

All reagents were of analytical grade I. [γ-32P]-ATP (10 mCi/μmol) was purchased from New England Nuclear. [3H]-ADR and [14C]-ADR, specific activity = 30 μCi/mg, were generous gifts from Drs. T. Fasy, New York, and L. Zwelling, Maryland. IgG, fibronectin, and fibrinogen (each coupled to CNBr-Sepharose-4B) were used as controls, and were provided by J. Maimon, formerly of our department. Heart muscle used to isolate and purify proteins was obtained fresh from normal mongrel dogs. Sephadex and Sepharose products were from Pharmacia Fine Chemicals.

Buffer A: 2 mM Tris-HCl, 14 mM 2-mercaptoethanol (2-ME), 1 mM ethylene glycol bis(β-aminoethyl ether)N,N,N',N"-tetraacetic acid (EGTA), pH 9.
Buffer B: 20 mM Tris-HCl, 0.1 mM ethylene-diaminotetraacetic acid (EDTA), 15 mM, 2-ME, pH 7.6.
Buffer C: 0.37 M KCl, 0.05 M Tris-HCl, 14 mM 2-ME, 0.4 mM ATP, pH 6.8.
Buffer D: 2 mM Tris-HCl, 0.2 mM ATP, 14 mM 2-ME, 0.2 mM Ca++, pH 6.8.

Purification of Dog Heart α-Actinin

The procedure for isolation of canine cardiac α-actinin was adapted, with some modification, from a method used for smooth muscle (Feramisco and Burridge, 1980). Dog heart muscle obtained immediately after sacrifice was defatted with scissors and minced into approximately 1-cm cubes. The muscle was homogenized with 10 volumes of deionized, distilled water containing 14 mM 2-ME in a Waring Blender, with three 10-second bursts. The frothy mixture was centrifuged at 7,000 g for 10 minutes; its supernatant was discarded. The pellet was resuspended in water and recentrifuged. The resulting pellet was resuspended in buffer A, stirred gently for 10 minutes, recentrifuged at 7,000 g, and the supernatant saved. The supernatant's pH was adjusted to 7-7.2 with 0.5 mM acetic acid, and 1 mM MgCl2 was added to obtain a final concentration of 10 mM. Within minutes, a white precipitate formed. The material was collected by a 10-minute centrifugation at 7,000 g and discarded. The supernatant was chilled to 4°C and ammonium sulfate fractionation was performed by addition of the finely powdered solid. Added in the first step were 14.9 g of (NH4)2SO4/100 ml of supernatant. Little precipitation was detected and the supernatant cleared by filtration.
through Whatman no. 4 filter paper. Addition of 5.6 g (NH₄)₂SO₄/100 ml formed a precipitate. It was centrifuged and the pellet dissolved and dialyzed exhaustively against buffer B. The dialyzed protein solution was ultracentrifuged at 100,000 g for 20 minutes and applied to a DEAE-cellulose column (2.5 x 30 cm) equilibrated with buffer B. The column was washed with buffer B until absorbance at 280 nm returned to baseline values and the proteins eluted with a gradient of 0-0.4 M NaCl in buffer B. Three large peaks were detected. The third peak was pooled and ultracentrifuged at 100,000 g for 60 minutes. To the supernatant, 26 g/100 ml (NH₄)₂SO₄ were added and the precipitate pelleted, resuspended, and dialyzed against buffer B. The resulting protein solution was stored at 4°C for immediate use and the remainder frozen at -20°C with 10% sucrose. Total proteins were determined by the method of Lowry et al. (1951), and SDS-polyacrylamide slab-gel electrophoresis (Laemmli, 1970) was performed on individual or pooled fractions to establish polypeptide purity.

Preparation of Dog Heart Muscle Actin

The technique used was a modification of one previously described (Spudich and Watt, 1971). Fresh dog heart (150 g wet weight), trimmed, minced, and washed in saline, was blended in a Waring Blender, using two 10-second bursts in 200 ml of buffer C. The frothy suspension was stirred for 10 minutes, centrifuged at 10,000 g for 5 minutes, and the supernatant saved for myosin purification. The residue was washed consecutively with three volumes of 0.05 M bicarbonate buffer, pH 8.0, for 15 minutes, followed by a wash in 20 volumes of 1 mM EDTA, pH 7.0, and with 10 volumes of deionized water to remove EDTA. The residue was extracted with two volumes of ice-cold acetone for 5 minutes, filtered through gauze, air-dried overnight, and extracted at 4°C for 16 hours with 300 ml of buffer D. The extract was filtered and the supernatant's pH adjusted to 7.6. The suspension was clarified by ultracentrifugation at 100,000 g for 20 minutes, and solid KCl and 1 mM MgCl₂ were added to 100 mM and 1 mM, respectively. After 2 hours of standing at room temperature, solid KCl was added to make it 0.6 M and promote further polymerization. After 1.5 hours at room temperature, the solution was centrifuged at 100,000 g for 1.5 hours; the clear gelatinous pellet was suspended in and dialyzed against buffer D. The dialyzed mixture was clarified, polymerized, ultracentrifuged, and depolymerized twice. The final supernatant was stored frozen with 10% sucrose at -20°C.

Myosin Preparation

The salvaged supernatant material from the actin preparation in buffer C was diluted with 10 volumes of cold deionized, distilled water, and the translucent white fibrillar precipitate was allowed to sediment at 1 g. This material was pelleted at 7,000 g for 10 minutes; the pellet was resolubilized in buffer C and clarified by centrifugation at 37,000 g for 10 minutes. This procedure was repeated twice and the final solution stored in 50% glycerol at -20°C.

[¹⁴C]-ADR Binding to Proteins Determined by Gel Filtration

Preincubation of 10⁻⁴ to 10⁻⁵ M mixtures of ADR with radiolabeled [¹⁴C]-ADR at room temperature with 1.0-2.0 mg of purified α-actinin or actin was carried out for 1 hour. The unbound, unlabeled, and [¹⁴C]-ADR was separated from the protein-bound ADR by gel filtration column chromatography using a 1 x 20 cm column, packed with Seph-

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ax G-50 and equilibrated with buffer B. Fractions of 1.0 ml were collected and 100-ml aliquots were counted in a Packard scintillation spectrometer. For G-actin, an identical column was used, but with Sephadex G-25 equilibrated with buffer D to separate free ADR from actin-bound ADR. Protein elution was monitored at 280 nm with a Beckman 25 kinetic recording spectrophotometer.

Negative Staining Electron Microscope Studies

Purified G-actin aliquots, incubated with and without ADR, were examined by negative staining electron microscopy. Ten microliters of approximately 1 mg/ml G-actin were added to 90 μl of buffer D containing ADR in varied concentrations ranging from 10⁻⁴ to 10⁻⁵ M. The molar ratio of ADR:actin ranged from 4.0 to 0.4. The mixture was incubated at room temperature for 60 minutes and an aliquot removed for electron microscopy. Samples were deposited on Formvar copper-coated grids and allowed to stand for 20 seconds. The excess solution was removed, and negative staining was performed for 2 minutes with 1% uranyl acetate. Grids were dried and observed with a JEOL 100-B electron microscope at 80 kV.

Viscometry Studies

Measurement of ADR, incubated with various concentrations of ADR, was performed with an Ostwald viscometer with a capillary flow time of 250 seconds for 2 ml of buffer B in a water bath at 20°C. Various concentrations of ADR incubated with G-actin were examined. Measurements were performed repeatedly until a constant flow time was achieved. F-actin was polymerized with 0.1 M K⁺ and 0.1 mM Mg²⁺ in the presence and absence of ADR.

Coupling of G-Actin to CNBr-Sepharose-4B

Before conjugation, 2 ml of 6 mg/ml G-actin were dialyzed overnight in buffer D, centrifuged at 100,000 g for 20 minutes, and the supernatant added slowly to 5 ml of swollen CNBr-Sepharose 4B gel beads with constant gentle agitation. The material was mixed in an orbital shaker slowly for 2 hours at room temperature, the beads washed alternately with 250 ml of 10 mM HCl, 400 ml distilled water, 300 ml of 0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl and left in a 1:1 (vol/vol) slurry. An equal volume of 0.5 M ethanolamine was added, allowed to shake in an orbital shaker for 2 hours at room temperature and the beads washed with 50 ml of 10 mM HCl, 500 ml 0.1 M NaHCO₃, pH 8.3, with 0.5 M NaCl using 100-ml volumes alternately. The Sepharose beads were stored at 4°C in 0.05 M bicarbonate buffer, pH 8.

Approximately 3 ml of the gel beads were applied to a small column to yield a gel pack of 1 x 2.5 cm. The gel was equilibrated with buffer D containing 0.1 mM ATP, and 0.5 ml ADR (10⁻⁵ M) was applied to the column. One-milliliter fractions were collected and, after the 4th ml, the buffer was changed to buffer D with 0.6 M KCl. Absorbance was monitored at 500 nm. IgG, fibrinogen, and fibronectin, each coupled to CNBr-Sepharose 4B, were used as controls and failed to retain ADR on affinity chromatography determinations.

ADR Conjugation to CNBr-Sepharose 4B

The method essentially was similar to the procedure used for G-actin conjugation to Sepharose 4B, except that 25 mg ADR in 2 ml of physiological saline were substituted for G-actin. The column bed was built with approximately 3 ml of the gel applied to a small column to yield a gel pack of
Myosin Preparation

Myosin was obtained as a by-product of the procedure used for the separation of α-actinin. The yield of myosin was low. The major polypeptides of myosin were detected. Small amounts of contaminating actin and α-actinin were seen. Screened for ATPase activity, the intrinsic myosin-Mg\(^{++}\)-ATPase was low, indicating that the small amount of actin contamination could not generate ATPase activity in the presence of Mg\(^{++}\).

Affinity Binding of ADR to CNBr-Sepharose 4B

The covalent binding was performed as described for ADR-CNBr-Sepharose 4B, and the column was built under conditions similar to those described for ADR-CNBr-Sepharose 4B.

Determination of ATPase Activity

ATPase activities of proteins were determined by the release of inorganic phosphorus (P\(_i\)) from ATP. The released P\(_i\) was extracted into a N-butanol layer. Mixtures of radioactive [\(^{32}\)P]-ATP (1 μCi in 0.1 nmol) and non-radioactive ATP (0.1 mmol) were employed. One-half-milliliter aliquots of the N-butanol layer were counted in a scintillation spectrometer. ATPase assays were performed in volumes of 1.0 ml. Proteins and reagents initially were mixed in a volume of 0.9 ml with 50 mm Tris-HCl, pH 7.5, and incubated at room temperature for 10 minutes. For assay purposes, the quantity of protein was 0.01 mg of muscle myosin/ml. Cardiac actin (0.02 mg/ml), with or without α-actinin, in the presence or absence of 10\(^{-4}\) M ADR, was used for activation of myosin-Mg\(^{++}\)-ATPase. ADR-actin mixtures contained no K\(^+\) in the medium. Reaction was initiated by addition of 0.1 ml of 10\(^{-4}\) M unlabeled and [\(^{32}\)P]-ATP and allowed to proceed at 37°C for 10 minutes. Aliquots of 1 ml were removed at various time intervals. Reactions were stopped by addition of 0.4 ml of 20% trichloroacetic acid. ATPase was estimated as the difference between P\(_i\) at zero time and P\(_i\) at the end of the reaction (Fuszk Basin et al., 1977).

Results

Protein Purification

Dog heart α-actinin was purified successfully, based on a procedure described for smooth muscle tissue (Feramisco and Burridge, 1980). After ion exchange chromatography, three protein peaks were detected, the third containing α-actinin. Total protein content in the peak was 150 mg. Protein composition, analyzed by polyacrylamide slab-gel electrophoresis, demonstrated a prominent band of homogeneous density in the region of 100,000 daltons, which corresponds to α-actinin subunits. The protein was approximately 90% homogeneous.

Dog Heart Muscle G-Actin

From approximately 150 g of heart muscle, the yield of actin was about 225 mg. Sodium dodecyl sulfate slab-gel electrophoresis of the purified product demonstrated a discrete band at 43,000 MW, with approximately 90% purity. The small amount of contamination present appears to be related to troponin-myosin and troponin.

Myosin Preparation

Myosin was obtained as a by-product of the procedure used for the separation of α-actinin. The yield of myosin was low. The major polypeptides of myosin were detected. Small amounts of contaminating actin
TABLE 1
Binding of [%C]-ADR to Cardiac Muscle G-actin and α-Actinin

<table>
<thead>
<tr>
<th>Protein</th>
<th>ADR added (μmol/mg protein)</th>
<th>Mol ADR bound/mol protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-Actin</td>
<td>0.02-0.33</td>
<td>2.1-3.0</td>
</tr>
<tr>
<td>α-Actinin</td>
<td>0.07-0.17</td>
<td>0.66-1.0</td>
</tr>
</tbody>
</table>

Binding was performed at 4°C for 24 hours, using equilibrium dialysis cuvettes. The volume of each side of the cuvette was 0.8 ml and the amount of protein loaded varied between 1 and 2 mg. Mixtures containing constant amounts of [%C]-ADR and varied concentrations of unlabeled ADR (0.02-0.4 μmol) were inserted in the opposite compartment to the one containing the proteins. The data show that approximately 1-3 mol of ADR bound to the proteins studied.

formed with an average diameter of 120 Å at all concentrations of ADR tested. Potassium-induced F-actin filaments produced long, typically thin filaments with an average diameter of 60-70 Å.

Viscometry Studies on G- and F-Acts in the Presence of ADR

As shown in Figure 3, ADR in concentrations of 10^-6 to 10^-3 M did not alter the specific viscosity of G-actin. Polymerized F-actin did not demonstrate altered viscosity in the presence of ADR. The addition of millimolar concentrations of K^+ and Mg^{++} to ADR-G-actin caused polymerization of G-actin with a noticeable increase in viscosity. Viscosity did not change afterwards, and the level of viscosity increase was identical to that obtained with F-actin control.

Effect of ADR on Myosin-ATPase Activity

Measurement of myosin-ATPase activity in the presence of millimolar concentrations of Mg^{++} is an expression of activation of myosin by actin. The basic myosin-ATPase activity stimulated by Mg^{++} in the absence of actin was low. The effect found upon addition of F-actin and preincubation for 10 minutes before addition of [γ^32P]-ATP is illustrated in Figure 4. ATPase activity was increased with time until approximately 10 minutes of incubation. G-actin previously incubated with ADR for 1 hour and added to myosin produced an increase in the level of ATP hydrolysis. The increase in ATPase activity was approximately 40% over the duration of the experimental period.

Affinity Chromatography

In Figure 5, we illustrate the binding of ADR to actin-conjugated CNBr-Sepharose 4B column. Bound ADR did not elute from the column utilizing buffer B. Upon addition of KCl to bring K^+ concentration to 0.6 M, ADR eluted in a discrete peak as measured by absorbance at 500 nm. Binding specificity of ADR to cardiac myofibrillar proteins was tested in a control experiment using covalently coupled IgG to CNBr-Sepharose 4B (Fig. 6). Adriamycin emerged retarded by the gel beads. Similar negative results were
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FIGURE 3. Viscometric determinations of actin incubated with various concentrations of ADR. ADR added to G-actin did not alter actin's viscosity after incubation at 25°C for 1 hour. Similar results were obtained using F-actin. The native state of actin was unaffected by ADR since additions of K⁺ and Mg²⁺ produced a rapid increase in actin specific viscosity to similar levels of those of F-actin.

Affinity Chromatography with ADR-Coupled Sepharose 4B

Adriamycin-bound CNBr-Sepharose 4B did not affect elution of G-actin, F-actin, or α-actinin when applied to the column (Fig. 7). All proteins eluted in the void volume as discrete peaks detected by their absorbance at 280 nm.

Discussion

Although the morphological changes seen at the Z-band in ADR cardiotoxicity have been documented (Jaenke, 1974, 1976), biochemical details of the nature of these alterations are scant (Someya et al., 1978). In this work, we have explored the interaction of ADR with cardiac actin and α-actinin. Successful purification of α-actinin from cardiac muscle was achieved during a modification of the method for smooth muscle. α-Actinin separated from other proteins as a discrete peak using ion-exchange chromatography. Biochemical and biophysical properties of cardiac α-actinin were similar to those of its counterpart from striated muscle (Goll et al., 1972; Puszkin et al., 1977; Ullrick et al., 1977). Cardiac actin was purified by well-established methods and demonstrated biochemical properties similar to actin from other muscle sources.

The molecular nature of ADR binding to these cardiac muscle proteins may relate to ionic interactions between charged groups on the protein and the ligand (Trouet, 1979). Bound ADR dissociated from the proteins in the presence of high ionic strength.

FIGURE 4. ADR-actin-stimulated ATPase activity. Cardiac muscle G-actin was preincubated with 10⁻⁵ M ADR for 1 hour at room temperature in the absence of K⁺ and Mg²⁺. F-actin was formed using cardiac G-actin in the presence of 0.1 M K⁺ and 0.1 mM Mg²⁺ and incubation at room temperature. Other ATPase assay conditions are described in text under Methods. ADR-actin showed 2.5 minutes 27.5%; 5.0 minutes 41%; 7.5 minutes 48%; and at 10 minutes 31% stimulation of ATP hydrolysis compared to F-actin controls. Values represent the average of triplicate determinations.

FIGURE 5. Affinity chromatography showing absence of ADR binding to CNBr-Sepharose 4B coupled with IgG. IgG molecules were bound covalently to Sepharose-4B beads, as described in the legend for Figure 5. This was carried out to determine the specificity of binding of ADR. IgG molecules did not show binding. ADR emerged unretarded in the void volume.
buffers. Adriamycin binding to cardiac actin and α-actinin appears to be selective and is being examined kinetically in our laboratory. Manifestation of this binding to G-actin also is implied by formation of unusually thick filaments of actin in the absence of K⁺ and Mg²⁺ as observed by negative-staining electron microscopy. In solution, these ADR-formed filaments did not exhibit the increased viscosity found for F-actin when it was polymerized by K⁺ and Mg²⁺; rather, the filaments formed by ADR with G-actin showed specific viscosity similar to control values for untreated G-actin. These filaments were thick (10–15 nm in diameter) as compared to F-actin (5–7 nm) as visualized by negative staining electron microscopy. This ultrastructural observation may account for the lack of increased viscosity since long, delicate F-actin filaments are responsible for increased specific viscosity.

We detected increased levels of ATPase activity using ADR-actin and ADR-myosin. This finding is in contradistinction to a previous report of depressed ATPase activity by an actin-heavy meromyosin complex formed in the presence of ADR (Someya et al., 1978). The increased ATPase activity seen with actin-ADR is indicative that these filaments mimic the biochemical properties of F-actin. Despite changes in the filament, myosin interacted with such filaments, and ATPase activation occurred.

Adriamycin was conjugated to CNBr-Sepharose 4B for use in affinity chromatography. Because of its color, ADR turned the gel matrix bright pink in neutral pH range and to blue-purple above pH 9. Passage of various buffers at neutral, acid, and alkaline pH through the matrix during its preparation demonstrated color change related to the various PK of the adriamycinone moiety of ADR. This can be used as a visual clue to monitor the adherence of the drug to the gel matrix in the preceding reaction. The utility of the ADR affinity column lies in its ability to have the free amino group of daunosamine blocked while leaving only functional groups from adriamycinone available for use in this system. In the systems we have examined, it served as a negative control, for interaction of ADR with any macromolecule that potentially interacts with the free amino groups of daunosamine. The roles of other functional groups of ADR interacting with contractile proteins in vitro are being explored.

Affinity chromatography of ADR using actin-conjugated Sepharose-4B demonstrated binding of ADR to the matrix. Elution of ADR from an affinity column using high salt demonstrates this binding to be ionic in nature. Conversely, ADR-coupled CNBr-Sepharose 4B neither held nor retarded the elution of proteins examined. Thus, the lack of binding of proteins to ADR-conjugated CNBr-Sepharose 4B was manifested by blockage of the free amino group in its sugar moiety (Fig. 8). The data suggest that blockage of this ADR amino group may be a worthwhile tool to study alterations of contractility in vitro. The therapeutic utility of DNA-bound ADR in clinical trials demonstrated its efficacy (Trouet et al., 1978).

Interaction of ADR with cardiac proteins may play a role in the development of altered cardiac contractility. This interaction relates to the binding of drug through the free amino group of daunosamine sugar to appropriate sites on the protein. Uptake, incorporation, and disposition of this substance in the myocyte may be related to its action on nuclear DNA resulting in altered transcription and translation. All of these phenomena ultimately may lead to observed sarcomeric changes. Adriamycin demonstrates some direct action on selected myofibrillar elements in vitro. These effects may prove to be the target of toxicity in the cardiac sarcomere.

In summary, ADR interacted with selected cardiac myofibrillar proteins in vitro. The nature of this interaction appears to be related to biochemical struc-

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**Figure 7.** Affinity chromatography of α-actinin, G-actin, and F-actin on ADR-CNBr-Sepharose 4B. None of the proteins used bound to ADR coupled to Sepharose 4B beads. All proteins emerged in the void volume. When the buffer was changed to contain 0.6 M K⁺ (at 6 ml), no proteins emerged.

**Figure 8.** Structure of the molecule of ADR, composed of two major parts. The daunosamine moiety appears as the site for binding of macromolecules through its primary amino group (arrow). Blockage of this group by binding to CNBr on Sepharose 4B beads completely abolishes binding to α-actinin, G-actin, and F-actin as described in the legend for Figure 7.
tures in the Z-band and thin filaments that include α-actinin and actin as demonstrated by the binding of ADR to these proteins. Adriamycin appears to induce G-actin to form unusual filamentous structures which, biophysically, are dissimilar to native F-actin. This same ADR-actin complex enhances ATPase activity of myosin. The binding of ADR by its primary amino group in the daunosamine moiety may play a role in the interaction of ADR and contractile proteins, both experimentally and clinically.

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