Direct Photoaffinity Labeling of Juncotional Sarcoplasmic Reticulum with [\(^{14}\)C]Doxorubicin*

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Francesco Zorzato, Alfredo Margreth, and Pompeo Volpe†

From the Centro di Studio per la Biologia e la Fisiopatologia Mascotare del Consiglio Nazionale delle Ricerche, Istituto di Patologia Generale dell’Università di Padova, via Loredan 16, 35131 Padova, Italy

Doxorubicin, an anticancer drug, induces Ca\(^{2+}\) release from the terminal cisternae (TC) of skeletal muscle (Zorzato, F., Salviati, G., Facchinetti, T., and Volpe, P. (1985) J. Biol. Chem. 260, 7349–7355). Long wave ultraviolet irradiation of a TC fraction with morphologically intact feet structures (Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) J. Cell Biol. 99, 875–885) in the presence of [\(^{14}\)C]doxorubicin, led to covalent photolabeling of two proteins that exhibited apparent M, values of 350,000 and 170,000. Such proteins were found to be absent in a fraction of longitudinal sarcoplasmic reticulum but enriched in junctional face membranes obtained by Triton X-100 treatment of the TC fraction. Three additional proteins with M, values of 80,000, 60,000, and 30,000 were also faintly labeled in the junctional face membrane fraction. On a molar basis the highest level of incorporation was found in the 170,000-Da protein, probably a Ca\(^{2+}\)-binding protein (Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O. (1983) J. Biol. Chem. 258, 11267–11273). A lower level of labeling was obtained in the 350,000-Da protein, tentatively identified as a component of the feet structures (Cadwell, J. J. S., and Caswell, A. H. (1982) J. Cell Biol. 95, 543–550). Photolabeling of junctional TC proteins did not occur if (i) a 10–50-fold excess cold doxorubicin was included in the assay medium, indicating that it was displaceable and specific, and (ii) if ultraviolet irradiation was omitted. Photolabeling was inhibited by caffeine or ruthenium red, i.e. by an activator and an inhibitor of Ca\(^{2+}\) release from TC, respectively. Furthermore, photolabeling was prevented by \([\text{ethylenebis(oxyethylene-nitriilo)}]_{\text{tetracarboxylic acid}}\) suggesting that doxorubicin binding is \(\text{Ca}^{2+}\)-dependent. Doxorubicin-binding proteins are constituents of the junctional sarcoplasmic reticulum and might be involved in modulating Ca\(^{2+}\) release from TC.

SR of skeletal muscle is an intracellular membraneous network mainly involved in regulating Ca\(^{2+}\) fluxes. Morphological studies (1) have shown that the SR membrane system consists of two continuous but distinct portions, i.e. LSR and TC. Freeze-fracture studies (2) have demonstrated an identical intramembrane distribution of Ca\(^{2+}\) pump units in the LSR and in the nonjunctional area of TC. A specialized region of TC, the junctional SR, is connected by “feet” structures to sarcolemma invaginations, the transverse tubules, to form the triad (1, 2). Following transverse tubule depolarization, release of calcium from the TC (3) triggers muscle contraction. Both the mechanism of excitation-contraction coupling (4) and the molecular process underlying Ca\(^{2+}\) release (5) are not yet fully understood. Ca\(^{2+}\) release seems to occur via large conductance Ca\(^{2+}\) channels selectively localized in the TC region, as shown by single channel recording of “heavy” SR vesicles incorporated in the planar lipid bilayer (6). The components of the Ca\(^{2+}\) channel are not known but seem not to be related (7–12) to the Ca\(^{2+}\)-ATPase, the major SR membrane component (13). Moreover, it is not known whether the Ca\(^{2+}\) channels are distributed over the entire TC membrane area or are restricted to the junctional TC area.

Doxorubicin, a potent activator of Ca\(^{2+}\) release from skeletal muscle TC (15), is a potential natural photoaffinity ligand because it bears a 5,12-naphthacenedione group (Fig. 1) which is capable of being excited to a diradical triplet state intermediate by low intensity, long wave ultraviolet irradiation. This intermediate may then abstract hydrogen from a target molecule and thereby create a covalent bond (16, 17). Direct photoaffinity labeling has the advantage of covalently cross-linking a natural ligand or substrate to its protein binding site by irradiation. Using this technique proteins can be cross-linked to their ligands under the direct action of ultraviolet light, without the introduction of affinity labels on either of the reactants (14), thus reducing the probability of conformational changes at the binding site, which may occur to accommodate a modified ligand.

In this paper [\(^{14}\)C]doxorubicin is used as a probe to identify TC proteins which might be part of the Ca\(^{2+}\) release channel. We report the specific and covalent cross-linking of doxorubicin to some junctional TC proteins; on a molar basis, high \(^{14}\)C incorporation is found in two proteins with apparent M, of 350,000 and 170,000.

EXPERIMENTAL PROCEDURES

Preparation of SR Fractions and of JFM—SR was isolated from the predominantly fast-twitch rabbit skeletal muscles and fractionated into LSR (R2; 27/32% sucrose interface) and TC (R4; 38/45% sucrose interface) as previously described by Saito et al. (18). In some experiments the protease inhibitor phenylmethylsulfonyl fluoride (2 mg/liter) was used throughout fractionation. SR fractions were suspended in 0.5 m sucrose, 5 mM imidazole, pH 7.4 (buffer A), and stored at –70 °C until used. JFM were obtained from the TC fraction following a procedure developed by Dr. Brian Costello. Briefly, TC fraction (3.5 mg/ml), kept on ice, was incubated for 10 min in buffer A supplemented by 1 mM CaCl\(_2\) and then, for 20 min with Triton X-

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† To whom correspondence should be addressed.
‡ The abbreviations used are: SR, sarcoplasmic reticulum; LSR, longitudinal SR; TC, terminal cisternae; JFM, junctional face membrane of TC; SDS, sodium dodecyl sulfate; EGTA, ethylenebis(oxyethylene-nitriilo)\(\text{tetracarboxylic acid}\).
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100 (0.7%). The suspension was centrifuged at 120,000 x g for 90 min in a Beckman 75 Ti rotor and the pellet was resuspended in buffer A. Protein concentration was estimated according to Lowry et al. (19).

Triton X-100 treatment caused almost complete solubilization of the Ca²⁺-ATPase, as shown by SDS-gel electrophoresis (see "Results"). Triton-resistant membranes were identified by thin section electron microscopy as junctional, foot-containing membranes with compartmental contents (e.g. calsequestrin) and morphologically preserved structures, unidirectionally aligned (90). Caswell and collaborators took a similar approach (21, 22) and obtained comparable results. Triton X-100 treatment of the TC-triad fraction yielded a partially purified junctional complex (Fig. 6 in Ref. 22); the prominent protein components of Triton X-100-resistant TC were found to be a high molecular weight doublet, an M, 80,000 protein and a calsequestrin/Ca²⁺-ATPase molar ratio of 0.52 (average of five preparations; see also Ref. 18). Additionally, only TC displayed a doublet of high molecular weight proteins (M, values of 350,000 and 325,000, respectively) tentatively identified as being part of the "feet" structures (22, 24, 25). The higher molecular weight protein of the doublet was more prominent, as previously reported by Seiler et al. (24). The JFM was obtained by Triton X-100 treatment of the TC fraction (20). As compared to TC, JFM (lane c) was almost devoid of Ca²⁺-ATPase but enriched in both calsequestrin and the high molecular weight doublet. An 80,000-Da poly-peptide was also present both in TC and JFM (cf. Refs. 21 and 25). The protein composition of SR fractions did not change whether phenylmethylsulfonyl fluoride, a protease inhibitor, was present or absent during SR purification (not shown).

The Stains-all staining pattern of the same fractions is shown in Fig. 2 (lanes d-f). Calsequestrin, which stains blue (26), was a very faint band in LSR (lane d); the 160,000-Da blue-staining protein was absent from JFM (lane f) whereas a 170,000-Da blue-staining protein (26) was enriched in JFM (compare lanes f and c). Both polypeptides are, however, minor components of SR fractions as indicated by either Coomassie Blue (Fig. 2, lanes b and c), Stains-all (Fig. 2, lanes e and f) or silver (not shown) staining (see also Refs. 24 and 26). As previously reported, the doublet of high molecular weight proteins stained pink (24).

Photolabeling of LSR, TC and JFM Fractions with [¹⁴C]Doxorubicin—Doxorubicin, at micromolar concentrations, elicits Ca²⁺ release from skeletal muscle TC (15). Photocitation, i.e. 5-min exposure to ultraviolet light in the presence of doxorubicin, caused irreversible activation of Ca²⁺ release from TC: 50 µM doxorubicin stimulated approximately 2-fold the Ca²⁺-dependent ATPase rate of TC even after a 1:300 dilution in the assay medium. In the absence of photocitation, instead, the Ca²⁺-dependent ATPase rate of TC reversed to control values after dilution (not shown). If doxorubicin exerted its action by binding to membrane proteins, one might be able to identify the relevant TC protein(s) using [¹⁴C]doxorubicin as a natural photoaffinity ligand.

TC and JFM fractions were photolysed for 5 min under long wave ultraviolet light in the presence of a saturating concentration of [¹⁴C]doxorubicin (50 µM). Fig. 3 (lanes a and c) shows the photolabeling pattern of TC and JFM fractions: after SDS-polyacrylamide gel electrophoresis, incorporation was mainly observed in two polypeptides having apparent Mr, of 350,000 and 170,000 and was 3-fold higher in the JFM fraction (lane c); see also first column in Table I and Fig. 4, b and c). Polypeptides of Mr, 80,000, 60,000, and 30,000 were also labeled (lane c). Compared to the Coomassie Blue- and Stains-all-stained lanes (Fig. 2, lanes c and f), it is clear that though a minor component, the 170,000-Da protein is heavily labeled by doxorubicin (see below Table I). Addition of 0.5 mM cold doxorubicin to the incubation buffer before ultraviolet irradiation abolished the labeling of such proteins (lanes b and d). In the absence of ultraviolet irradiation no labeling was detected (not shown). A large and broad band of radioactivity was observed at the level of the tracking dye, even in the presence of 0.5 mM cold doxorubicin (lanes a-d), and might be either unbound [¹⁴C]doxorubicin or nonspecific la-
Fig. 2. SDS-polyacrylamide gel electrophoresis of SR fractions. Slab gel electrophoresis was carried out as described by Laemmli (23) on a linear 5-15% acrylamide gradient and stained with Coomassie Blue (lanes a-c) or Stains-all (lanes d-f). Approximately 30 μg of protein was applied per lane. Lanes a and d, LSR; lanes b and e, TC; lanes c and f, JFM. Relative molecular weights are indicated by lines (×10^{-2}) on the left side of the figure. HMW, high molecular weight proteins; 170, 170,000-Da protein; G', 160,000-Da glycoprotein; ATPase, Ca^{2+}-dependent ATPase; CS, calsequestrin.

Fig. 3. Photoaffinity labeling of TC and JFM with [3H]doxorubicin (autoradiography). TC and JFM were photolabeled for 5 min with 50 μM [3H]doxorubicin as described under “Experimental Procedures,” and then subjected to SDS-polyacrylamide gel electrophoresis (5-20% linear gradient gel). About 100 μg of protein were applied per lane. Following Coomassie Blue staining and destaining, the gels were washed, dried, and autoradiographed at -70 °C using Kodak X-Omat film (1-month exposure). Lane a, TC; lane b, TC photoactivated in the presence of 0.5 mM cold doxorubicin; lane c, JFM; lane d, JFM photoactivated in the presence of 0.5 mM cold doxorubicin. Numbers represent M, × 10^{-2}.

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Labeled protein</th>
<th>Relative dpmA</th>
<th>Relative dpmmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>TC</td>
<td>350,000</td>
<td>3.5 (3)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>170,000</td>
<td>2.9 (3)</td>
<td>7.3</td>
</tr>
<tr>
<td>JFM</td>
<td>350,000</td>
<td>10.9 (3)</td>
<td>3.8 (1)</td>
</tr>
<tr>
<td></td>
<td>170,000</td>
<td>9.0 (3)</td>
<td>4.1 (1)</td>
</tr>
</tbody>
</table>

Two specific radioactive peaks (350,000 and 170,000 Da) and JFM at least three peaks (circles in Fig. 4, b and c, respectively). Polypeptides of low M, were not easily identifiable due to a drifting base line. Labeling in the presence of excess cold doxorubicin abolished 3H incorporation (triangles in Fig. 4, b and c).

The concentration dependence of JFM photolabeling is shown in Fig. 5. After SDS-polyacrylamide gel electrophoresis of JFM, Coomassie Blue-stained gel bands were sliced, digested, and counted for radioactivity. dpm counts obtained for the polypeptides of 350,000 (a) and 170,000 (b) are plotted against [3H]doxorubicin concentration. Covalent binding was detected at 1 μM and sharply increased above 5 μM. Half-maximal binding was attained at about 15 μM. The shape of the curves resembles that previously reported for doxorubicin activation of Ca^{2+} release from the SR (cf. Fig. 5 in Ref. 15).

When radioactivity incorporated per band was normalized against Coomassie Blue staining, i.e., the relative protein content, and against apparent Mn, the highest level of label was found in the 170,000-Da protein (Table 1).
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FIG. 4. Photoaffinity labeling of SR fractions with $[{}^{14}C]$ doxorubicin (electrophoretic profile). LSR, TC, and JFM fractions were photolabeled for 5 min with $50 \mu M$ $[{}^{14}C]$doxorubicin in the absence (○) or in the presence (△) of 0.5 mM cold doxorubicin and separated on SDS-polyacrylamide gel electrophoresis (5–20% linear gradient gel). About 100 μg of protein were applied per lane. Each gel lane was then sliced, and each slice was digested with Soluene and counted for radioactivity. a, LSR; b, TC; c, JFM. Numbers represent $M_r \times 10^3$ of labeled polypeptides. In one experiment, photoactivation of TC was carried out in the presence of 0.7% Triton X-100; the labeling pattern was not affected (not shown).

Effect of Drugs, Cations, and EGTA on Photolabeling of JFM Fraction—We investigated the effect of several agents reported to enhance or inhibit Ca$^{2+}$ release from isolated TC and from the SR of skinned fibers of skeletal muscle; it was postulated that doxorubicin acted on a Ca$^{2+}$ efflux pathway, i.e. a Ca$^{2+}$ channel, selectively localized in TC. Our previous results (15), however, did not clarify the mode of action of doxorubicin which could conceivably interact with the channel itself, with the gate or with a putative drug binding site(s).

In the present study we used $[{}^{14}C]$doxorubicin as a natural photoaffinity ligand to identify doxorubicin-binding TC proteins which might play a role in the Ca$^{2+}$ release process from TC. The effect of doxorubicin on Ca$^{2+}$ release became irreversible following photoactivation. A few proteins having apparent $M_r$ of 350,000, 170,000, 80,000, 60,000 and 30,000 were specifically photolabeled with saturating concentrations of doxorubicin (Fig. 3, lane c). On a molar basis, a high level of incorporation was found in the 350,000- and 170,000-Da proteins; a much lower labeling was detected for other proteins.

DISCUSSION

In a recent report (15), it was shown that doxorubicin induced Ca$^{2+}$ release from isolated TC and from the SR of skinned fibers of skeletal muscle; it was postulated that doxorubicin acted on a Ca$^{2+}$ efflux pathway, i.e. a Ca$^{2+}$ channel, selectively localized in TC. Our previous results (15), however, did not clarify the mode of action of doxorubicin which could conceivably interact with the channel itself, with the gate or with a putative drug binding site(s).

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Electrophoresis and densitometric scanning. Autoradiographic lanes were carried out in a 620 Bio-Rad Video reader as described in the legend to Fig. 3. Both ruthenium red, a Ca²⁺ channel blocker (6, 28, 29) that inhibits the effect of doxorubicin on skeletal muscle SR (15), and caffeine, a Ca²⁺ release activator (28) that competes with doxorubicin (15), prevented [³⁵S]doxorubicin incorporation into the 350,000-, 170,000-, and 80,000-Da proteins. Silver ions, which also activate Ca²⁺ release (11), partially prevented photolabeling. Thus, the 350,000-, 170,000-, and 80,000-Da proteins are common targets for several pharmacological agents and are probable drug-binding sites of the Ca²⁺ release channel. This implication is strengthened by the finding that EGTA prevents photolabeling. Since Ca²⁺-induced Ca²⁺ release displayed by skeletal SR is inhibited by very low levels of free Ca²⁺ (7, 29-31), the Ca²⁺ dependence of doxorubicin binding may reflect critical conformational changes of the Ca²⁺ channel complex.

The protein profile of JFM (Fig. 2) was very similar to that reported by Caswell and Brunschwig (Fig. 2B in Ref. 22) for comparable membrane fractions. Tentative identification of major doxorubicin-binding proteins is based on electrophoretic mobility as compared to that reported by Caswell and collaborators (21, 22, 25) and on the Stains-all staining properties (26). As previously indicated, the 350,000-Da polypeptide is a component ("spanning protein") of the foot structures (22, 25; see also Ref. 24). The 170,000-Da protein, which stains blue with Stains-all, is a Ca²⁺ binding protein (26) (see also Fig. 4 in Ref. 32) and might share some primary sequence and structural properties with calsequestrin. The 80,000-Da protein may be the "anchoring protein" (22), an integral membrane protein present in both transverse-tubule and junctional SR membranes. At present, however, the functional role of these proteins is not clear.

Is there a causal link between the effect of doxorubicin on Ca²⁺ release (15) and its binding to junctional SR proteins (present paper)? The 350,000-Da spanning protein connecting adjacent transverse tubule and TC membranes is known to be a calmodulin-binding protein (24) and a substrate for an endogenous Ca²⁺-calmodulin protein kinase and also for an exogenous aCAMP-dependent protein kinase (24). On the basis of these findings, Seiler et al. (24) have suggested that phosphorylation may be involved in mediating Ca²⁺ fluxes at the SR junction. Doxorubicin may thus interfere with selective phosphorylations and consequently induce Ca²⁺ efflux from TC. As to the 170,000-Da protein, it is tempting to speculate that it bears the Ca²⁺ binding sites of the Ca²⁺ channel. Such a protein is a minor constituent of the junctional SR membrane, is a Ca²⁺ binding protein (26), and is labeled by doxorubicin in a Ca²⁺-dependent manner. However, isolation and further characterization of the doxorubicin-binding proteins are required to identify such proteins as components of the TC Ca²⁺ channel and to understand their role in the Ca²⁺ release process.

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