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Direct Photoaffinity Labeling of Junctional 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 Muscolare del Consiglio Nazionale delle Ricerche, Istituto di Patologia Generale dell'Universita' 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_r 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_r 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 observed in the 350,000-Da protein, tentatively identified as a component of the feet strutures (Cadwell, J. J. S., and Caswell, A. H. (1982) *J. Cell Biol.* 93, 543–550). Photolabeling of junctional TC proteins did not occur (i) if 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 [ethylenebis(oxyethylenenitrilo)]tetraacetic acid suggesting that doxorubicin binding is 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[1] 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_r 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 0.3 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.[2] Briefly, TC fraction (3.3 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.

[1] 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(oxyethylenenitrilo)]tetraacetic acid.

[2] Costello, B., Saito, A., Chu, A., Maurer, A., and Fleischer, S. (1986) *J. Cell Biol.*, in press.

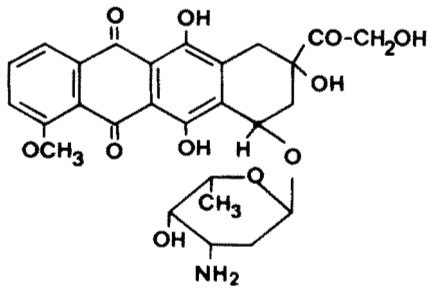

FIG. 1. **Molecular structure of doxorubicin.**

100 (0.7%). The suspension was centrifuged at 120,000 × *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 Ca^{2+}-ATPase, as shown by SDS-gel electrophoresis (see "Results"). Triton-resistant membranes were identified by thin section electron microscopy as junctional, feet-containing membranes with compartmental contents (*e.g.* calsequestrin) and morphologically preserved feet structures, unidirectionally aligned (20). 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. 21); the prominent protein components of Triton X-100-resistant TC were found to be a high molecular weight doublet, an M_r 80,000 protein and calsequestrin (Fig. 2*B* in Ref. 22).

Assays—Ca^{2+} loading, in the presence of 92 mM phosphate, and Ca^{2+}-dependent ATPase activity were measured as previously described (15).

Photolabeling of SR Fractions and JFM with [^{14}C]Doxorubicin—Membrane fractions (2 mg/ml) were equilibrated at room temperature for 5 min with specified concentrations of [^{14}C]doxorubicin in buffer A (final volume = 50 μl), in the absence or presence of 0.5 mM cold doxorubicin. Samples were kept on ice and photoactivated for 5 min with a long wave UV lamp (model UVCL-58, Ultra-Violet Products, Inc., San Gabriel, CA; wavelength >254 nm) and then solubilized by adding 25 μl of 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol, and 62.5 mM Tris-Cl, pH 6.8. Five-min exposures to ultraviolet light in the absence of doxorubicin had negligible effects on both Ca^{2+} loading and Ca^{2+}-dependent ATPase activities of TC. Photolysis, in the presence of doxorubicin, did not affect the stimulatory action of the drug on the Ca^{2+}-dependent ATPase activity (see Ref. 15 and "Results").

SDS-Polyacrylamide Gel Electrophoresis—Slab gel electrophoresis was carried out as described by Laemmli (23), using 1.5-mm thick gradient gels, with either 5–15% or 5–20% acrylamide. Slab gels were stained with Coomassie Brilliant Blue, destained in 50% methanol, 10% acetic acid, treated with Enlightning (New England Nuclear), dried onto Whatman I filter paper, and autoradiographed at −70 °C using Kodak X-Omat films. Densitometric scans of both Coomassie Blue-stained gels and autoradiographies were carried out using a 620 Bio-Rad Video Densitometer. In some experiments, [^{14}C]doxorubicin incorporation was determined by removing the Coomassie Blue-stained gel bands and digesting them with 0.4 ml of Soluene (Packard) for 16 h at room temperature before counting. Low molecular weight protein standards were obtained from Bio-Rad. High molecular weight protein standards were oligomers of phosphorylase *a* cross-linked with glutaraldehyde (24). Stains-all staining of slab gels was carried out as described by Campbell *et al.* (26).

Materials—Doxorubicin and [^{14}C]doxorubicin (63.1 mCi/mmol; batch GZ 6342/40) were kindly provided by Farmitalia Carlo Erba (Nerviano, Italy). Caffeine, phenylmethylsulfonyl fluoride, and ruthenium red were obtained from Sigma, phosphorylase *a* from Boehringer, the cationic carbocyanine dye Stains-all from Eastman Organic Chemicals, and Triton X-100 from British Drug House.

RESULTS

The protein profiles of highly purified SR fractions are different (13, 18, 24). Fig. 2 (*lanes a–c*) shows the Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis of LSR, TC and JFM fractions. The predominant protein band in LSR (*lane a*), also referred to as the Ca^{2+} pump membrane (18), was the Ca^{2+}-ATPase (*cf.* Ref. 13), whereas calsequestrin, a Ca^{2+} binding protein selectively localized in the lumen of TC (13), was virtually absent. TC (*lane b*), which consists of two different types of membrane, the junctional face membrane (15–20%) and the Ca^{2+} pump membrane (18), was characterized by a high content of calsequestrin (13, 18, 24) and a calsequestrin/Ca^{2+}-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_r 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 Ca^{2+}-ATPase but enriched in both calsequestrin and the high molecular weight doublet. An 80,000-Da polypeptide 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 glycoprotein was absent from JFM (*lane f*) whereas a 170,000-Da blue-staining protein (26) was enriched in JFM (compare *lanes f* and *e*). 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 stains pink (24).

Photolabeling of LSR, TC and JFM Fractions with [^{14}C] Doxorubicin—Doxorubicin, at micromolar concentrations, elicits Ca^{2+} release from skeletal muscle TC (15). Photoactivation, *i.e.* 5-min exposure to ultraviolet light in the presence of doxorubicin, caused irreversible activation of Ca^{2+} release from TC: 50 μM doxorubicin stimulated approximately 2-fold the Ca^{2+}-dependent ATPase rate of TC even after a 1:300 dilution in the assay medium. In the absence of photoactivation, instead, the Ca^{2+}-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 [^{14}C] 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 [^{14}C]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 M_r 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 M_r 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 [^{14}C]doxorubicin or nonspecific la-

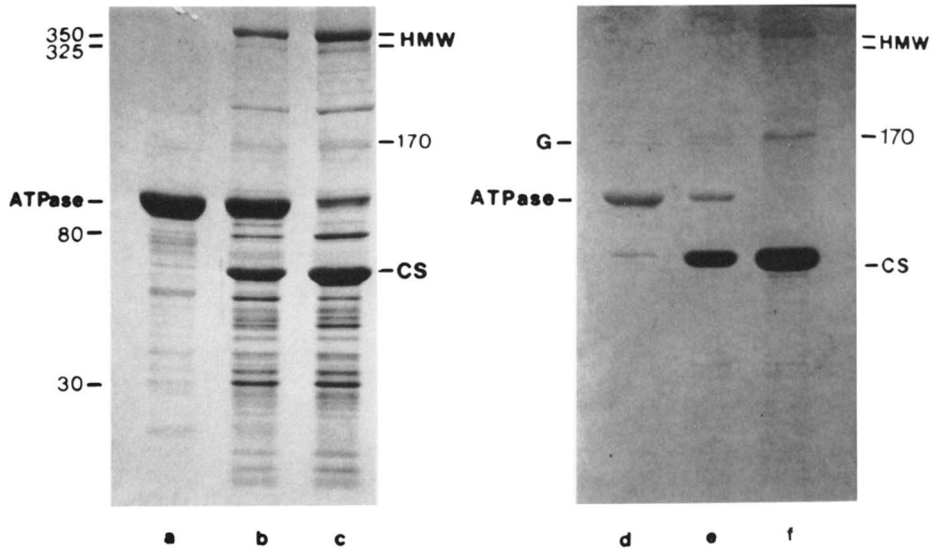

FIG. 2. **SDS-polyacrylamide gel electrophoresis of SR fractions.** Slab gel electrophoresis was carried out as described by Laemmli (23) on a linear 5–15% acrylamide gradient and stained with Coomassie Blue (*lanes a–c*) or Stains-all (*lanes d–f*). Approximately 30 μg of protein was applied per lane. *Lanes a* and *d*, LSR; *lanes b* and *e*, TC; *lanes c* and *f*, JFM. Relative molecular weights are indicated by lines ($\times 10^{-3}$) 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.

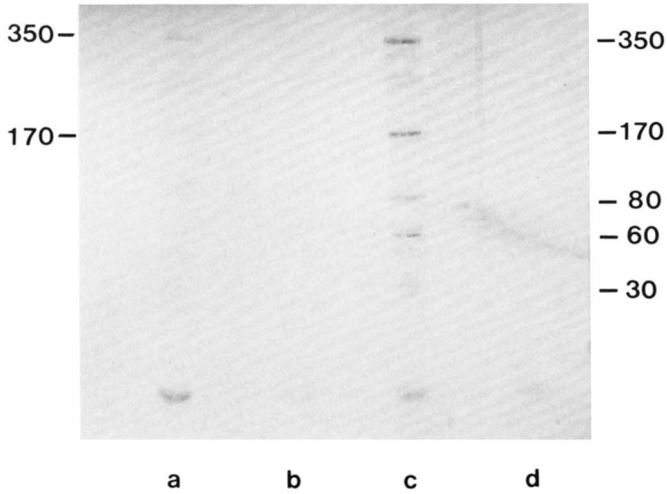

FIG. 3. **Photoaffinity labeling of TC and JFM with [^{14}C] doxorubicin (autoradiography).** TC and JFM were photolabeled for 5 min with 50 μM [^{14}C]doxorubicin as described under "Experimental Procedures," and then subjected to SDS-polyacrylamide gel electrophoresis (5–20% linear gradient gel). About 100 μg of protein were applied per lane. Following Coomassie Blue staining and destaining, slab gels were treated with Enlightning, 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 $M_r \times 10^{-3}$.

beling of membrane phospholipids and proteolipid. Photolabeling of JFM with a subsaturating concentration of [^{14}C] doxorubicin (10 μM) showed two major bands (M_r 350,000 and 170,000) which disappeared if photoactivation was carried out in the presence of 0.5 mM cold doxorubicin (not shown). Doxorubicin has no effect on Ca^{2+} fluxes in LSR (15); photoactivation of LSR with 50 μM [^{14}C]doxorubicin did not yield any of the above labeled polypeptides but only a faint band corresponding to the Ca^{2+}-ATPase (not shown). These results indicate that the relevant doxorubicin-binding proteins belong to the junctional SR.

Similar results were obtained by a complementary approach, *i.e.* by slicing and counting each gel lane after electrophoresis separation of photoactivated SR fractions (Fig. 4). LSR fraction displayed no peak (Fig. 4*a*). TC fraction showed

TABLE I

Radioactivity covalently incorporated into the 350,000- and 170,000-Da proteins of TC and JFM fractions photolabeled with [^{14}C] doxorubicin

TC and JFM (100 μg of protein) were photolabeled for 5 min with either 50 or 25 μM [^{14}C]doxorubicin (columns A and B, respectively) and electrophoresed as described in the legend to Fig. 4. From densitometric measurements of Coomassie Blue-stained gels, a 9:1 ratio was found between the 350,000- and 170,000-Da proteins (see also Fig. 2, b and c). Coomassie Blue-stained bands of M_r 350,000 and 170,000 were sliced, digested with Soluene and counted for radioactivity. Background counts have been subtracted (see legend to Fig. 5). dpm/A was obtained dividing dpm of each band by the densitometric area of the corresponding Coomassie Blue-stained band. Each of these ratios was normalized to that of the 350,000-Da protein. The numbers in parentheses indicate the number of experiments.

Labeled protein	dpm × 10^{-2}		Relative dpm/A		Relative dpm/mol	
	A	B	A	B	A	B
TC						
350,000	3.5 (3)		1		1	
170,000	2.9 (3)		7.3		3.8	
JFM						
350,000	10.9 (3)	3.8 (1)	1	1	1	1
170,000	9.0 (3)	4.1 (1)	7.4	9.7	3.8	4.9

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_r were not easily identifiable due to a drifting base line. Labeling in the presence of excess cold doxorubicin abolished ^{14}C 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 M_r 350,000 (*a*) and 170,000 (*b*) are plotted against [^{14}C]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 M_r, the highest level of label was found in the 170,000-Da protein (Table I). On a molar

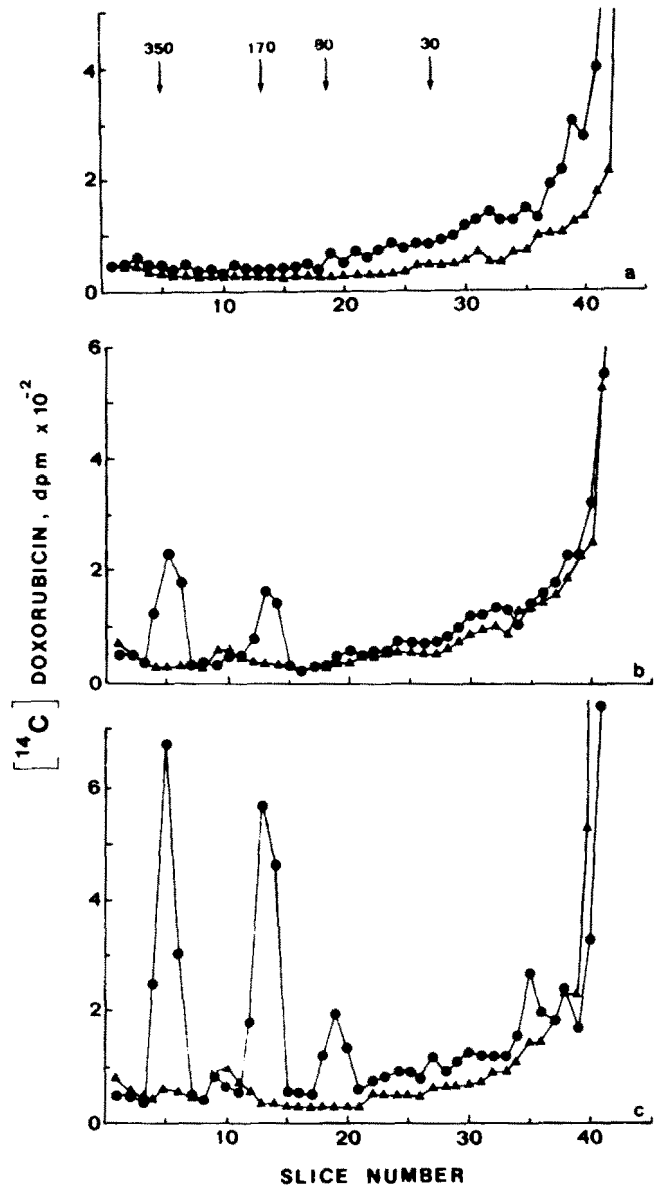

FIG. 4. **Photoaffinity labeling of SR fractions with [^{14}C] doxorubicin (electrophoretic profile).** LSR, TC, and JFM fractions were photolabeled for 5 min with 50 μM [^{14}C]doxorubicin in the absence (●) or in the presence (▲) of 0.5 mM cold doxorubicin and separated on SDS-polyacrylamide gel electrophoresis (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).

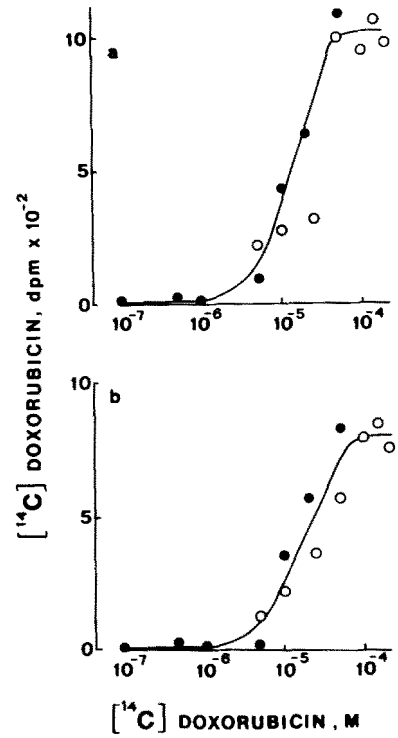

FIG. 5. **[^{14}C]Doxorubicin incorporation into JFM proteins.** Photolabeling of JFM (100 μg of protein) was carried out for 5 min with [^{14}C]doxorubicin as described under "Experimental Procedures." [^{14}C]Doxorubicin concentration ranged from 0.1–200 μM, as indicated on the abscissa. After photoactivation, JFM samples were subjected to SDS-polyacrylamide gel electrophoresis (5–20% gradient gel). Coomassie Blue-stained bands of M_r 350,000 (*a*) and 170,000 (*b*) were sliced, digested with Soluene, and counted for radioactivity (see "Experimental Procedures" for details). Background counts were obtained by counting and averaging several unstained gel slices, and were subtracted from total dpm. *Open* and *closed symbols* represent data from two separate experiments on different JFM preparations.

basis, 4 mol of doxorubicin are incorporated into the 170,000-Da protein for every mol incorporated into the 350,000-Da protein. The 4:1 relationship holds for different concentrations of [^{14}C]doxorubicin and for identical proteins of the TC fraction (Table I). On molar basis, the relative ^{14}C incorporation into the 80,000- and 30,000-Da proteins was 0.11 and 0.05, respectively, when normalized to that of the 350,000-Da protein.

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 SR on the [^{14}C]doxorubicin labeling pattern (Fig. 6). Ultraviolet irradiation of JFM was carried out with 25 μM [^{14}C]doxorubicin (*a*). Concentrations of 5 mM EGTA (*b*), 5 mM caffeine (*c*), and 20 μM ruthenium red (*e*) almost abolished labeling of the 350,000- and 170,000-Da proteins as well as the 80,000-Da protein, as seen by densitometric scans of autoradiographic lanes of SDS-polyacrylamide gel electrophoresis. A concentration of 10 μM Ag^{2+} (*g*) reduced the ^{14}C incorporation, whereas 100 μM Ca^{2+} (*f*) and 10 mM Mg^{2+} (*d*) did not change the labeling profile.

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).

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.

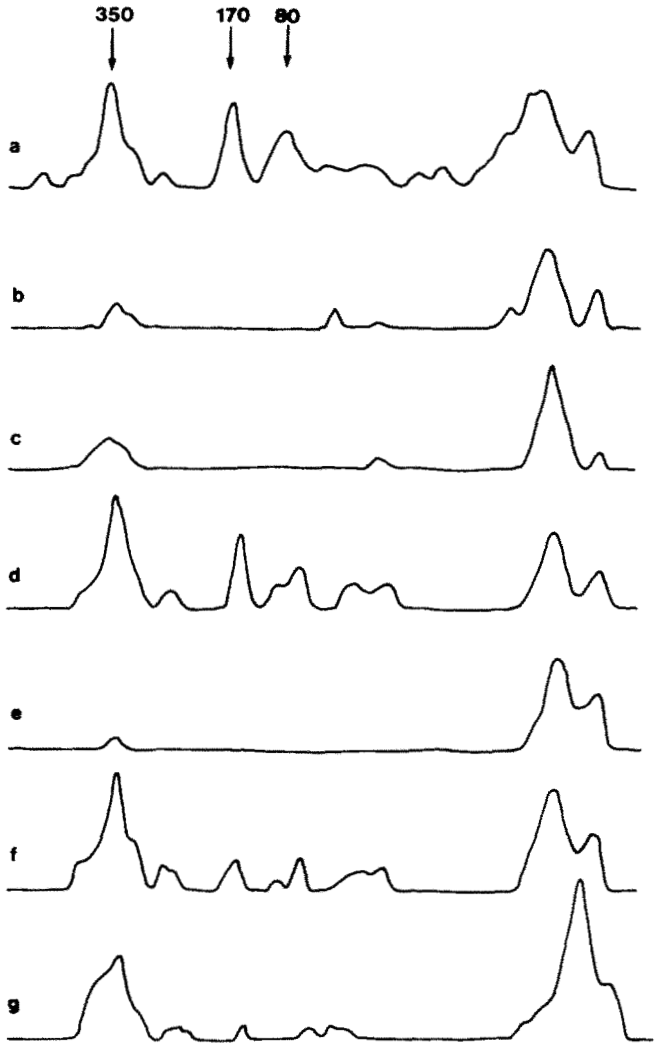

FIG. 6. **Effect of drugs, cations, and EGTA on [^{14}C]doxorubicin photolabeling of JFM (densitometric scans).** Photolabeling of JFM (150 μg of protein) was carried out for 5 min with 25 μM [^{14}C] doxorubicin as described under "Experimental Procedures," in the absence and presence of several agents. SDS-polyacrylamide gel electrophoresis (5–20% gradient gel) and autoradiography were carried out as described in the legend to Fig. 3. Densitometric scan of autoradiographic lanes was carried out in a 620 Bio-Rad Video Densitometer. *a*, JFM photoactivated with 25 μM [^{14}C]doxorubicin (control); *b*, plus 5 mM EGTA; *c*, plus 5 mM caffeine; *d*, plus 10 mM Mg^{2+}; *e*, plus 20 μM ruthenium red; *f*, plus 100 μM Ca^{2+}; *g*, plus 10 μM Ag^{2+}. *Numbers* represent $M_r \times 10^{-3}$ of labeled polypeptides.

Labeling is deemed specific because (i) it was abolished by excess cold ligand (Fig. 3, *lanes b* and *d*) and (ii) was not observed if ultraviolet irradiation was omitted. In principle, nonspecific labeling was reduced because experiments were carried out in the presence of 5 mM imidazole, a "scavenger" that can react with those excited species of the photolabel formed in solution (16) and because of the relative inertness of the ketone triplet state to reaction with water (27). Labeling was affected by EGTA and ruthenium red (Fig. 5) and this provides indirect evidence against a hydrophobic labeling mechanism. Since ^{14}C incorporation was higher in the 170,000-Da protein, labeling of other proteins might be due to contiguity, provided that labeled polypeptides are spatially adjacent. Alternatively, independent binding sites may exist.

Labeled polypeptides were absent in LSR (Fig. 4*a*) and enriched in a fraction derived from the junctional face of TC (Figs. 3*c* and 4*c*), clearly suggesting that doxorubicin-binding proteins are junctional in origin. If doxorubicin activates Ca^{2+} release from isolated TC and from the SR of skinned fibers (15) by interacting with such proteins, an important conclusion might be that the Ca^{2+} release channels are localized in the junctional membrane of the SR.

Evidence that doxorubicin-binding proteins are related to the Ca^{2+} release process is based on the observation that several Ca^{2+} release modulators influenced the photolabeling pattern (Fig. 6). Both ruthenium red, a Ca^{2+} channel blocker (6, 28, 29) that inhibits the effect of doxorubicin on skeletal muscle SR (15), and caffeine, a Ca^{2+} release activator (28) that competes with doxorubicin (15), prevented [^{14}C]doxorubicin incorporation into the 350,000-, 170,000-, and 80,000-Da proteins. Silver ions, which also activate Ca^{2+} 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 Ca^{2+} release channel. This implication is strengthened by the finding that EGTA prevented photolabeling. Since Ca^{2+}-induced Ca^{2+} release displayed by skeletal SR is inhibited by very low levels of free Ca^{2+} (7, 29–31), the Ca^{2+} dependence of doxorubicin binding may reflect critical conformational changes of the Ca^{2+} channel complex.

The protein profile of JFM (Fig. 2) was very similar to that reported by Caswell and Brunschwig (Fig. 2*B* 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 feet structures (22, 25; see also Ref. 24). The 170,000-Da protein, which stains blue with Stains-all, is a Ca^{2+} binding protein (26) (see also Fig. 4 in Ref. 32) and might share some primary sequence and structural properties with calsequestrin.[3] 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 Ca^{2+} 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 Ca^{2+}-calmodulin protein kinase and also for an exogenous cAMP-dependent protein kinase (24). On the basis of these findings, Seiler *et al.* (24) have suggested that phosphorylation may be involved in mediating Ca^{2+} fluxes at the SR junction. Doxorubicin may thus interfere with selective phosphorylations and consequently induce Ca^{2+} efflux from TC. As to the 170,000-Da protein, it is tempting to speculate that it bears the Ca^{2+} binding sites of the Ca^{2+} channel. Such a protein is a minor constituent of the junctional SR membrane, is a Ca^{2+} binding protein (26), and is labeled by doxorubicin in a Ca^{2+}-dependent manner. However, isolation and further characterization of the doxorubicin-binding proteins are required to identify such proteins as components of the TC Ca^{2+} channel and to understand their role in the Ca^{2+} release process.

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[3] K. P. Campbell, personal communication.

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