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Doxorubicin Induces Calcium Release from Terminal Cisternae of Skeletal Muscle

A STUDY ON ISOLATED SARCOPLASMIC RETICULUM AND CHEMICALLY SKINNED FIBERS*

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In this study, we investigated the effect of the anticancer drug doxorubicin on Ca^{2+} fluxes of isolated highly purified sarcoplasmic reticulum fractions (longitudinal tubules and terminal cisternae (Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) *J. Cell Biol.* 99, 875–885)) and of chemically skinned skeletal muscle fibers of the rabbit. In terminal cisternae, doxorubicin inhibits Ca^{2+} uptake (IC_{50} at 0.5 μM) and increases 2.6-fold Ca^{2+}-dependent ATPase rate (half-maximal activation at 3 μM) and unidirectional Ca^{2+} efflux (8-fold stimulation at 25 μM). On the contrary, doxorubicin is without effect on longitudinal tubules. In skinned muscle fibers, doxorubicin induces rapid and transient Ca^{2+} release, as measured by tension development (half-maximal stimulation at 6 μM), which is completely and reversibly inhibited by ruthenium red, a known inhibitor of Ca^{2+} release from isolated terminal cisternae. Doxorubicin has no effect on the sarcoplasmic reticulum Ca^{2+} pump and on the contractile apparatus of skinned muscle fibers. It is concluded that doxorubicin activates Ca^{2+} release from sarcoplasmic reticulum and opens a Ca^{2+} efflux pathway (Ca^{2+} channel) selectively localized in terminal cisternae. Doxorubicin might interact with Ca^{2+} channels involved in physiological Ca^{2+} release.

SR[1] controls myoplasmic Ca^{2+} concentration and, therefore, the contraction-relaxation cycle of the skeletal muscle fiber (1). Release of Ca^{2+} from the SR triggers contraction, and active Ca^{2+} reuptake by the SR enables relaxation. The mechanism of Ca^{2+} uptake by a membrane-bound Ca^{2+} pump is known in detail (2, 3), whereas that of Ca^{2+} release is less well understood (1, 4). Ultrastructural studies (5, 6) have shown that SR of skeletal muscle fiber is a continuous subspecialized membranous network composed of longitudinal tubules (LSR) that surround myofibrils, and terminal cisternae (TC) held in junctional association with transverse tubules responsible for storing and releasing Ca^{2+} that triggers contraction (7, 8). SR can be isolated, purified and fractionated, by density gradient centrifugation (9–12), into LSR and TC, which differ in protein composition (9–12), membrane fatty acid composition (13) and Ca^{2+} release properties (4, 14–17).

Doxorubicin (adriamycin) is a very effective anticancer drug (18) producing, among other side effects, a dose-dependent cardiotoxicity which has been related to alterations in intracellular calcium homeostasis (19–21). The putative action of doxorubicin on intracellular Ca^{2+} fluxes prompted us to investigate its interaction with *skeletal* muscle SR.

In this study we found that doxorubicin inhibits Ca^{2+} uptake and stimulates Ca^{2+}-dependent ATPase rate and unidirectional Ca^{2+} efflux of TC but is virtually without effect on LSR. In chemically skinned skeletal muscle fibers, doxorubicin evokes a transient rise of tension, *i.e.* rapid Ca^{2+} release, which is inhibited by ruthenium red, a known inhibitor of Ca^{2+} release from TC (15, 17). These results suggest that doxorubicin opens a Ca^{2+} channel that is selectively localized in TC and causes rapid Ca^{2+} release from SR.

EXPERIMENTAL PROCEDURES

Materials—Antipyrylazo III, disodium phosphocreatine, caffeine, creatine phosphokinase, ruthenium red, and phosphoenolpyruvate were obtained from Sigma. Pyruvate kinase, lactate dehydrogenase, and NADH were obtained from Boehringer Mannheim. Ionophore A23187 was obtained from Calbiochem. $^{45}CaCl_2$ (1.5 μCi/ml) was obtained from The Radiochemical Centre (Amersham, Bucks, England). The anthracycline antibiotic doxorubicin (for its structure see Ref. 18) was a gift from Farmitalia Carlo Erba (Milano, Italy), and stock solutions were prepared in bidistilled water and protected from light. All other chemicals were reagent grade.

Isolation of SR Fractions—SR was isolated from the predominantly fast-twitch skeletal muscles of the hind limbs of New Zealand White male rabbits (12). SR was fractionated by density gradient centrifugation into longitudinal tubules (LSR or R2) and terminal cisternae (TC or R4) as previously described by Saito *et al.* (12). After isolation, purified SR fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, and stored at −70 °C until used. Protein concentration was estimated according to Lowry *et al.* (22), using bovine serum albumin as a standard.

Ca^{2+} Uptake—Ca^{2+} accumulation in the absence of precipitating anions is termed Ca^{2+} uptake and was continuously monitored with a Ca^{2+}-sensitive electrode (W. Moller, Glasblaserei, Zurich, Switzerland). The assay was carried out at 37 °C in a medium containing, in a final volume of 4 ml, 20 mM histidine, pH 7.2, 0.1 M KCl, 5 mM $MgSO_4$, 2 mM ATP, 5 mM disodium phosphocreatine, and 80 μg of creatine phosphokinase. Several pulses of 10–50 nmol of $CaCl_2$ were sequentially added to calibrate the response of the Ca^{2+} electrode, and the reaction was started, when $[Ca^{2+}]$ was 10 μM, with 200–400 μg of SR protein. Doxorubicin did not chelate Ca^{2+}, as judged by the unchanged base-line of tracings (see also Ref. 18).

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; LSR, longitudinal tubules of SR; TC, terminal cisternae of SR; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; *p*Ca, negative logarithm of $[Ca^{2+}]$ in molar units; IC_{50}, concentration producing 50% inhibition.

Ca^{2+} Loading—Ca^{2+} accumulation in the presence of precipitating anions, *i.e.* phosphate, is termed Ca^{2+} loading and was continuously monitored by following differential absorbance change of the Ca^{2+} indicator antipyrylazo III (23) at 710–790 nm in a Perkin-Elmer 356 spectrophotometer. The assay was carried out at room temperature (22–24 °C) in a medium containing, in a final volume of 1 ml, 92 mM K phosphate, pH 7.0, 1 mM $MgSO_4$, 1 mM Na_2ATP, 0.15 mM NADH, 0.5 mM phosphoenolpyruvate, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, 200 μM antipyrylazo III, and 50 μM $CaCl_2$ (24). The reaction was started by adding 30 μg of SR protein. Ca^{2+} loading and Ca^{2+}-dependent ATPase rates were measured in the same medium in order to determine coupling ratio of Ca^{2+} transport (see "Results").

Ca^{2+}-dependent ATPase—ATPase rate was measured by a spectrophotometric enzyme-coupled assay (25). The absorbance change at 340 nm (*i.e.* NADH oxidation) was continuously monitored in a Perkin-Elmer 551S spectrophotometer. The assay was carried out at 37 °C in a medium containing, in a final volume of 3 ml, 20 mM histidine, pH 7.2, 0.1 M KCl, 5 mM $MgSO_4$, 2 mM ATP, 0.15 mM NADH, 0.5 mM phosphoenolpyruvate, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, and 5–15 μg of SR protein. Basal ATPase rate was measured in the presence of 0.2 mM EGTA and total ATPase rate after addition of 0.2 mM $CaCl_2$ (estimated $[Ca^{2+}]$ = 10 μM). Basal ATPase rate accounted for 7% of the total ATPase rate. The difference between total and basal ATPase is termed Ca^{2+}-dependent ATPase. Maximal Ca^{2+}-dependent ATPase rate was obtained in the presence of the Ca^{2+} ionophore A23187 (1.5 μg/ml).

Unidirectional Ca^{2+} Efflux after Passive Ca^{2+} Accumulation—Ca^{2+} efflux was measured as previously described (26) with slight modifications. SR fractions (7–10 mg/ml) were incubated overnight at 0–4 °C in a medium containing 0.1 M KCl, 20 mM histidine, pH 7.2, and 2 mM $^{45}CaCl_2$ (~8000 cpm/nmol). Aliquots were then diluted 100-fold at room temperature (22–24 °C) in a medium containing 20 mM histidine, pH 7.2, 0.1 M KCl, 0.1 mM free $[Mg^{2+}]$, 5 mM K_2-EGTA, and 0.01 μM free $[Ca^{2+}]$ in the presence and absence of 25 μM doxorubicin. After dilution, several aliquots were withdrawn within 40 s and rapidly filtered under suction. The filters (0.22 μm pore size, GS filters, Millipore Co., New Bedford, MA) were immediately washed with 2 ml of an ice-cold solution containing 10 mM $LaCl_3$ and 20 mM $MgSO_4$ and counted for radioactivity. Free $[Mg^{2+}]$ and $[Ca^{2+}]$ were determined using the association constants given by Fabiato and Fabiato (27).

Preparation of Chemically Skinned Fibers—Fiber preparation has been described in detail previously (28, 29). Briefly, the adductor muscle of New Zealand White rabbits was isolated, bundles were excised from the bulk of the muscle, and each bundle was chemically skinned by exposure to a "skinning" solution containing 5 mM K_2-EGTA, 0.17 M K propionate, 2.5 mM K_2-Na_2ATP, 2.5 mM Mg propionate, and 10 mM imidazole propionate, pH 7.0. After 24 h at 0 °C, the bundles were transferred to skinning solution made up in 50% glycerol and stored at −20 °C until used. SR of chemically skinned fibers is functionally and morphologically intact (28, 29).

Isometric Force Measurements—Single fibers (average mean diameter, 50 μm) were positioned between two clamps, one of them attached to a strain gauge (800 series, Akers Electronics, Horton, Norway), and stretched to 130% of slack length (29). After 30 s in solution R (5 mM K_2-EGTA, 0.17 M K propionate, 2.5 mM Mg propionate, 5 mM Na_2-K_2ATP, and 10 mM imidazole propionate, pH 7.0), fibers were allowed to accumulate Ca^{2+} for 60 s in a *p*Ca 6.4 solution (0.17 M K propionate, 2.5 mM Mg propionate, 5 mM Na_2-K_2ATP, 2.15 mM $CaCl_2$, 5 mM K_2-EGTA, and 10 mM imidazole propionate, pH 7.0), rinsed ~30 s in solution W, which is a modified solution R without EGTA, and then challenged with doxorubicin. At the end of each experiment, fibers were placed in solution W and then exposed to a *p*Ca 5 solution (0.17 M K propionate, 2.4 mM Mg propionate, 5 mM Na_2-K_2ATP, 4.78 mM $CaCl_2$, 5 mM K_2-EGTA, and 10 mM imidazole propionate).

Steady-state isometric force/*p*Ca curves were obtained by exposing fibers sequentially to solutions of different $[Ca^{2+}]$ (from *p*Ca 6.4 to *p*Ca 4.8). Isometric tension generated in each solution was continuously monitored, and base-line tension was established as the steady-state voltage output recorded with a fiber in solution R. At the end of each experiment the fiber was again placed in solution R, and the cycle was repeated in the presence of 100 μM doxorubicin. All *p*Ca solutions were modified solution R containing different total Ca concentrations (from 2.15 to 4.89 mM), different total Mg concentrations (from 2.44 to 2.40 mM), and fixed EGTA concentration (5 mM). Free $[Mg^{2+}]$, kept constant at 0.09 mM, and free $[Ca^{2+}]$ were determined using association constants given in Orentlicher *et al.* (30). All experiments were carried out at room-temperature (22–24 °C) and 0.2 M ionic strength.

RESULTS

Effect of Doxorubicin on Ca^{2+} Uptake by SR Fractions—Fig. 1 shows that doxorubicin was a potent inhibitor of Ca^{2+} uptake by TC with an IC_{50} of 0.5 μM. Such effect was selective for TC since Ca^{2+} uptake by LSR was only slightly influenced at 10 μM doxorubicin, a concentration which completely inhibited Ca^{2+} uptake by TC.

Ca^{2+} uptake is the net difference of Ca^{2+} influx, mediated by the Ca^{2+} pump (2), and Ca^{2+} efflux via Ca^{2+} channels (1, 4) and/or other pathways (passive diffusion, carrier-mediated facilitated diffusion, Ca^{2+} pump-mediated efflux; for review see Ref. 31). Thus, the action of doxorubicin on Ca^{2+} uptake of TC might be due to inhibition of Ca^{2+} influx, activation of Ca^{2+} efflux, or both. These possibilities were tested as described below.

Effect of Doxorubicin on Ca^{2+}-dependent ATPase Rate—Ca^{2+} uptake into the SR lumen is coupled to Ca^{2+}-dependent ATP hydrolysis (2). Fig. 2*A* shows that doxorubicin stimulated Ca^{2+}-dependent ATPase rate of TC. Above 1 μM there was a sharp increase of doxorubicin effectiveness. Half-maximal stimulation was attained at about 3 μM and maximal stimulation (2.6-fold) at 25 μM. Higher concentrations of doxorubicin were found to have no greater effect (Fig. 2*A*). This result indicates, together with inhibition of Ca^{2+} uptake measured under similar conditions, that doxorubicin may activate Ca^{2+} efflux from TC, dissipate transmembrane Ca^{2+} gradient, and, therefore, stimulate Ca^{2+}-dependent ATPase rate by removing Ca^{2+} back inhibition on the Ca^{2+} pump (32). This interpretation is also supported by the observation that the Ca^{2+} ionophore A23187 was as effective as doxorubicin in stimulating Ca^{2+}-dependent ATPase rate (2.8-fold *versus* 2.6-fold, respectively; see legend to Fig. 2).

The selectivity of action of doxorubicin is further illustrated in Fig. 2*B*. Ca^{2+}-dependent ATPase of LSR increased by only 20% at a concentration of doxorubicin higher than 10 μM.

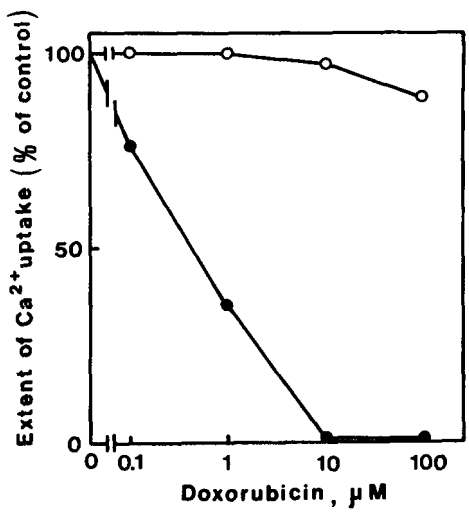

FIG. 1. **Effect of doxorubicin on Ca^{2+} uptake by SR fractions.** Ca^{2+} uptake was measured at 37 °C with a Ca^{2+}-sensitive electrode, as described under "Experimental Procedures," on 400 and 240 μg of protein of TC (●) and LSR (○), respectively. Two SR preparations were used and data were averaged. The extent of Ca^{2+} uptake, in the absence of doxorubicin, was 92.6 and 54.8 nmol of Ca^{2+}/mg of protein for LSR and TC, respectively. Peak Ca^{2+} accumulation was attained within 15–20 s.

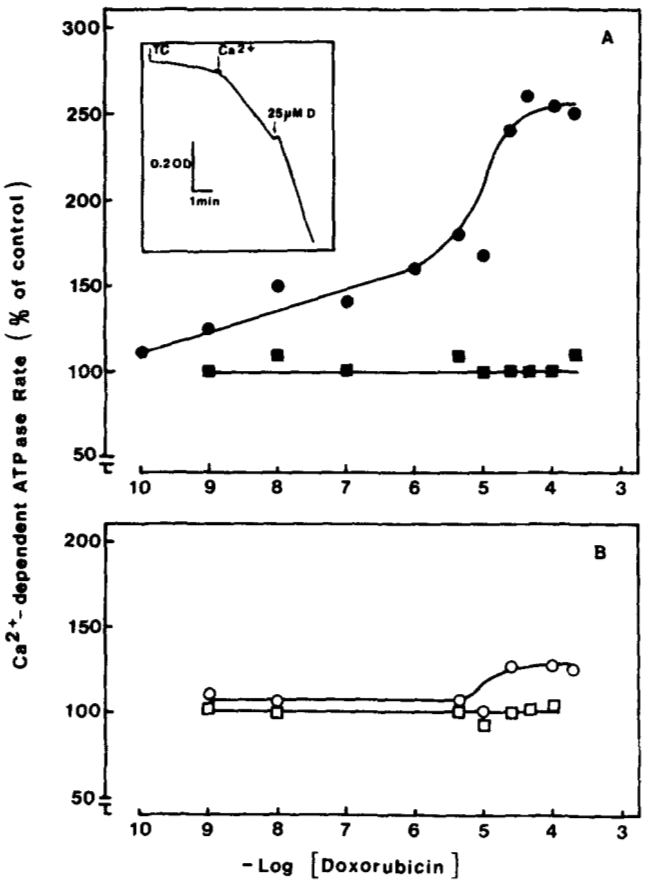

FIG. 2. **Effect of doxorubicin on Ca^{2+}-dependent ATPase rate of SR fractions.** ATPase rate was measured at 37 °C by a spectrophotometric enzyme-coupled assay, as described under "Experimental Procedures." Ca^{2+}-dependent ATPase rates were obtained by subtracting basal ATPase rates from total ATPase rates and are expressed as percentages of control rates. Three SR preparations were analyzed and data from a representative experiment are shown (*panel A*, TC; *panel B*, LSR). In the absence of A23187, the control rate was 7.36 and 2.74 μmol of P_i/mg of protein/min for LSR (○, 5 μg/ml) and TC (●, 5 μg/ml), respectively. In the presence of 1.5 μg/ml A23187, the control rate was 18.07 and 7.69 μmol of P_i/mg of protein/min for LSR (□, 2 μg/ml) and TC (■, 2 μg/ml), respectively. Note that A23187 stimulated the Ca^{2+}-dependent ATPase rate by a factor of 2.6 and 2.8 in LSR and TC, respectively. There is no convincing explanation for the 2-step curve of Fig. 2*A*. The *inset* in Fig. 2*A* shows a typical trace; 25 μM doxorubicin (*D*) increased the Ca^{2+}-dependent ATPase rate of TC (5 μg/ml).

This finding can be explained by the small degree of cross-contamination of this fraction by TC (see Fig. 3 in Ref. 12).

Maximal turnover rate of the Ca^{2+} pump, as judged by measuring Ca^{2+}-dependent ATPase rate with A23187 (Fig. 2), was not affected by doxorubicin. This result indicates that doxorubicin had no effect on the Ca^{2+} pump of both TC and LSR.

Effect of Doxorubicin on Coupling Ratio of TC—Ca^{2+} loading and Ca^{2+}-dependent ATPase rates of TC were measured under identical experimental conditions (*cf.* Ref. 33). 25 μM doxorubicin decreased the coupling ratio by a factor of 2. Ruthenium red stimulated the Ca^{2+}-loading rate, increased the coupling ratio, and antagonized the doxorubicin action (Table I). Taken together these results indicate that doxorubicin reduced the coupling ratio by activating Ca^{2+} efflux because ruthenium red, a known inhibitor of Ca^{2+} release from TC (17), blocked the doxorubicin effect.

Effect of Doxorubicin on Unidirectional Ca^{2+} Efflux from TC after Passive Ca^{2+} Accumulation—The experiment depicted in Fig. 3 was carried out to unambiguously assess the effect of doxorubicin on unidirectional Ca^{2+} efflux from TC. TC vesicles were equilibrated overnight in 2 mM $^{45}CaCl_2$ (passive Ca^{2+} accumulation) and then diluted 100-fold in a Ca-EGTA buffer (*p*Ca 8). Under the specific experimental conditions, *i.e.* lack of ATP, Ca^{2+} influx mediated by the Ca^{2+} pump is

TABLE I

Effect of doxorubicin on coupling ratio of TC

The Ca^{2+}-loading rate, in the presence of 92 mM K phosphate, was measured at room temperature, using antipyrylazo III as Ca^{2+} indicator, as described under "Experimental Procedures." Ca^{2+}-dependent ATPase rate was measured *in the same medium* by a spectrophotometric coupled-enzyme assay, following the rate of NADH reduction. The numbers in parentheses indicate the number of determinations carried out on the same TC preparation. Note that 25 μM doxorubicin decreased the Ca^{2+} loading rate only by 43% and stimulated 1.2-fold the Ca^{2+}-dependent ATPase rate. This was probably due to the presence of phosphate that greatly enhances Ca^{2+} influx by lowering intraluminal free [Ca^{2+}]. Also note that A23187 stimulated 1.3-fold the Ca^{2+}-dependent ATPase rate.

Addition	Ca^{2+}-loading rate	Ca^{2+}-dependent ATPase rate	Ca^{2+}/ATP
	μmol/mg protein/min		
None	0.35 (3)	1.14 (2)	0.31
25 μM doxorubicin	0.20 (2)	1.34 (1)	0.15
20 μM ruthenium red	1.55 (2)	0.88 (1)	1.76
25 μM doxorubicin + 20 μM ruthenium red	1.48 (2)	0.94 (1)	1.57
1.5 μg/ml A23187		1.41 (2)	

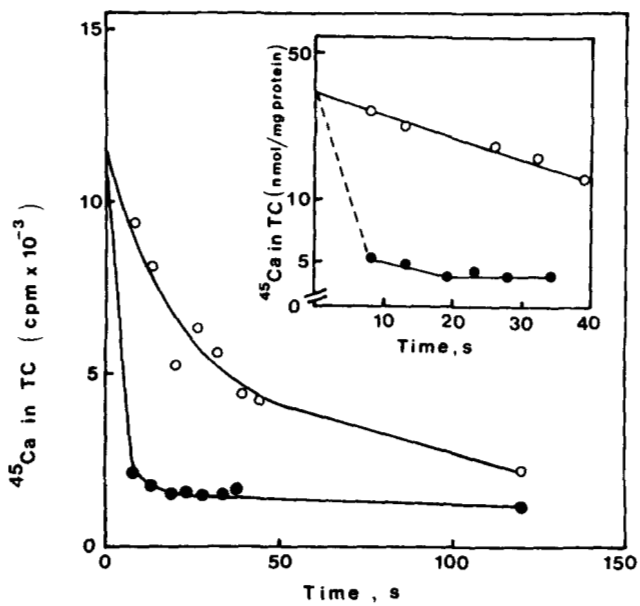

FIG. 3. **Effect of doxorubicin on unidirectional Ca^{2+} efflux from TC after passive Ca^{2+} accumulation.** TC vesicles (7 mg/ml) were passively loaded with $^{45}CaCl_2$, and efflux was initiated by 100-fold dilution into a Ca-EGTA buffer (*p*Ca 8), as described under "Experimental Procedures." $^{45}Ca^{2+}$ counts remaining in TC vesicles (*i.e.* trapped on filters) are plotted *versus* time after initiation of Ca^{2+} efflux by dilution, in the presence (●) and absence (○) of 25 μM doxorubicin. This concentration was chosen because it completely inhibited Ca^{2+} uptake (see Fig. 1) and maximally stimulated Ca^{2+}-dependent ATPase rate (Fig. 2*A*). In the *inset*, data are presented as semilog plot. The total amount of $^{45}Ca^{2+}$ trapped by TC vesicles (32.3 nmol/mg of protein) was determined by back extrapolation of the control efflux curve (○) to the time of dilution. The first-order rate constant was determined from linear regression analysis in the absence of doxorubicin (○) and graphically in the presence of doxorubicin (●); $t_{1/2}$ of Ca^{2+} efflux was 67 and 7.5 s, respectively.

suppressed, and unidirectional Ca^{2+} efflux can be measured (14–16, 26). Because the rate of Ca^{2+} efflux depends on the composition of the dilution buffer, being high at *p*Ca 5–6 (14–16) and low above *p*Ca 7 and below *p*Ca 5, we chose a buffer of *p*Ca 8 that elicits *per se* minimal Ca^{2+} efflux.

As shown in Fig. 3, Ca^{2+} efflux declined exponentially in the control experiment. Doxorubicin (25 μM) greatly increased Ca^{2+} efflux from TC. Approximately 85% of accumulated Ca^{2+} was released within 8 s, this being an upper limit. From a semilog plot of $^{45}Ca^{2+}$ retained in TC *versus* time (*inset*), it was calculated that the first-order rate constant (k) of the initial efflux component (up to 35 s after dilution) was increased 8-fold by doxorubicin (from 0.024 to 0.21 s^{-1}). As a lower limit we estimated a Ca^{2+} efflux rate of 3.3 nmol of Ca^{2+}/mg of protein/s in the presence of doxorubicin. It must be noted, however, that values obtained for k and Ca^{2+} efflux rate are still 3 orders of magnitude lower than those estimated for Ca^{2+} release *in vivo* (4).

Doxorubicin (25 μM) did not affect Ca^{2+} efflux from LSR ($k = 0.013$ s^{-1}).

Effect of Doxorubicin on Ca^{2+} Release from SR of Skinned Fibers—Experiments carried out with SR fractions suggest that doxorubicin induces Ca^{2+} efflux selectively from TC. If doxorubicin opens Ca^{2+} channels localized in TC, it might be able to induce contraction of either intact or skinned single fiber, *i.e.* induce rapid and massive Ca^{2+} release to activate the contractile machinery. A chemically skinned fiber preparation was used to test this hypothesis.

Ca^{2+} release from SR of rabbit adductor skinned fibers was indirectly measured by following tension development (28). Single fibers were exposed to Ca^{2+} uptake solution (*p*Ca 6.4) for 60 s, so that SR could accumulate Ca^{2+}, rinsed in solution W, and then challenged with the drug. At very low drug concentrations, the time course of force development was biphasic (Fig. 4*A*). Doxorubicin, below 1 μM down to 1 nM, induced immediate but slowly developing tension which turned into a faster one after 20–60 s (Fig. 4*A*). An S-shaped time course suggests that $\Delta[Ca^{2+}]$ after activation was small (34). Doxorubicin, above 1 μM, induced immediate fast developing tension (Fig. 4, *B* and *C*). The rate of tension rise increased up to 100 μM doxorubicin, the half-maximal rate being attained at 6 μM (Fig. 5). Since the rate of tension rise is related to the *p*Ca of bathing solution (35), it would appear that there is a dose-dependent increase of intermyofibrillar Ca^{2+} concentration. Doxorubicin effect occurred in two steps (Fig. 5) that probably reflect the nonlinear relationship between tension and Ca^{2+} saturation of troponin C binding sites (36).

At all doxorubicin concentrations tested, tension reached a peak and then began to drop (Fig. 4). This seems to indicate that Ca^{2+} release triggered by doxorubicin was transient and that part of released Ca^{2+} was reaccumulated by longitudinal tubules of SR (see the legend to Fig. 4).

Fig. 6 shows the effect of ruthenium red on doxorubicin-induced tension. Ruthenium red (10–20 μM) completely inhibited doxorubicin at 100 μM. After rinsing the fiber in solution W, 100 μM doxorubicin induced again tension development.

Effect of Caffeine and Doxorubicin on Ca^{2+} Release from SR of Skinned Fibers—Caffeine is a drug thought to act directly on SR and to cause Ca^{2+} release from TC of isolated preparations (17) and of skinned fibers (28).

Fig. 7 (*upper tracing*) shows that 10 mM caffeine induced tension development of single skinned fiber. At peak tension 5 mM EGTA was added to block Ca^{2+} reaccumulation by SR. After being rinsed in solution W, fiber was exposed to 100 μM doxorubicin that evoked miniscule force development. Fig. 7 (*lower tracing*) shows an experiment in which the order of additions was reversed. 100 μM doxorubicin induced tension development, whereas subsequent addition of 10 mM caffeine elicited a minute contraction. These results indicate that doxorubicin and caffeine mobilized the same pool of Ca^{2+} from TC.

Effect of Doxorubicin on Steady-state Isometric Force/pCa Curve—Doxorubicin could stimulate tension development in skinned fibers by acting, at least in part, on the contractile apparatus rather than by releasing Ca^{2+} from SR. This possibility was checked by studying isometric force/*p*Ca relations. Skinned fibers were sequentially exposed to solutions of increasing $[Ca^{2+}]$, in the absence and, then, in the presence of 100 μM doxorubicin, and steady force generated at each subsaturating *p*Ca was measured (Fig. 8). Doxorubicin did not change: (*a*) maximum force generation at saturating *p*Ca (4.8); (*b*) *p*Ca required for half-maximal force (*p*Ca 6.1); and (*c*) steepness of the curve. These findings show that doxorubicin did not affect the contractile apparatus.

DISCUSSION

Skeletal muscle contraction occurs when Ca^{2+} is released from TC in response to transverse-tubule depolarization. Terminal cisternae seem to be the unique site of storage and release of Ca^{2+} (7, 8, 37), as opposed to SR longitudinal tubules primarily devoted to reuptake of released Ca^{2+} (4). Addition-

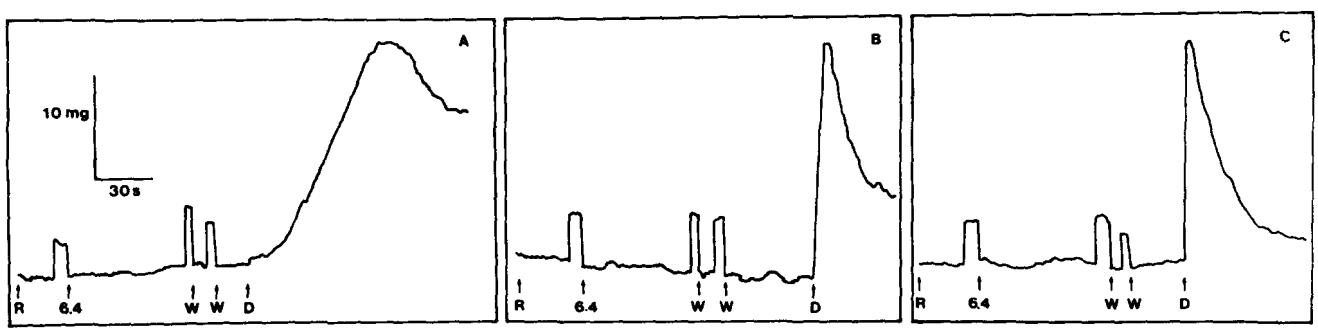

FIG. 4. **Doxorubicin induces tension development in skinned fibers.** Rabbit adductor skinned fibers were prepared and isolated as described under "Experimental Procedures." Isometric force measurements were carried out as described under "Experimental Procedures." Single fibers were placed in solution R, then in Ca^{2+} uptake solution (*p*Ca 6.4), and finally in solution W. Doxorubicin (*D*) administration induced tension development. 1 μM doxorubicin (*panel A*) evoked immediate, slowly developing tension that, after 20 s, became faster. 10 and 100 μM doxorubicin (*panels B* and *C*, respectively) elicited immediate fast developing tension. Note that tension after reaching a peak value dropped and that part of released Ca^{2+} was reaccumulated by SR (52).

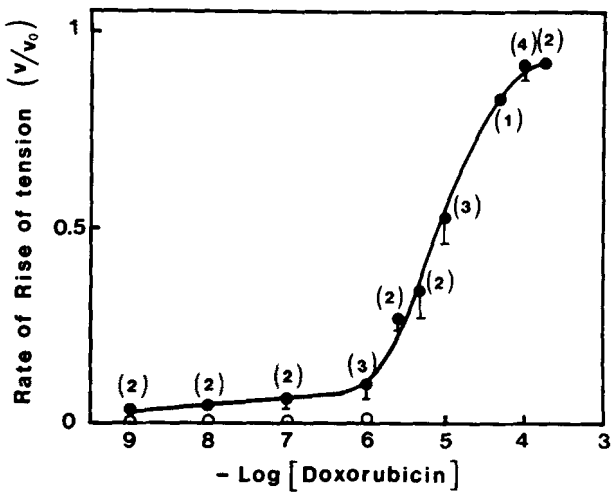

FIG. 5. **Effect of doxorubicin on rate of rise of tension in skinned fibers.** Data were obtained from several experiments as shown in Fig. 4 and are expressed as means ± 1 S.D. (*n*). At the end of each experiment, fibers were exposed to a *p*Ca 5 solution. In this case rise of tension was rate limited only by diffusion of Ca^{2+} from the bathing medium to the axis of the fiber (53). The rate of tension rise in the presence of doxorubicin (v) was normalized against that obtained in *p*Ca 5 solution (v_0). Up to 1 μM, doxorubicin evoked a biphasic response; an initial slow rate turned after 20–60 s into a faster one (*cf.* Fig. 4*A*). *Empty circles* represent such slower rates. The length of slow phase was inversely related to drug concentration (not shown).

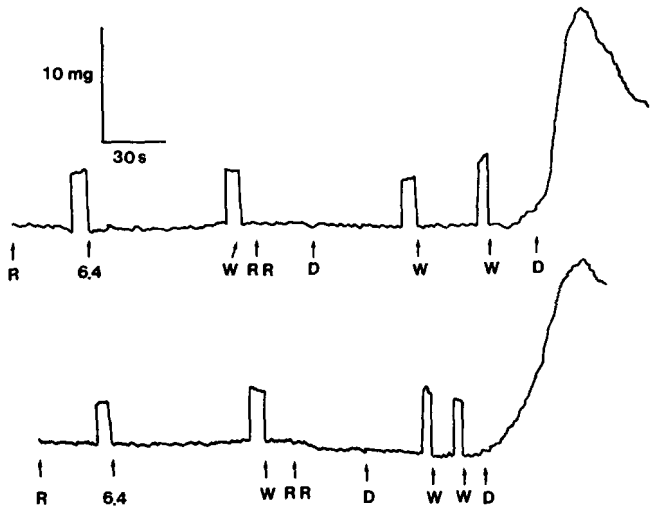

FIG. 6. **Ruthenium red inhibits doxorubicin-induced tension development in skinned fibers.** A single fiber was placed in solution R, then in Ca^{2+} uptake solution (*p*Ca 6.4), and finally in solution W supplemented with either 10 μM (*upper tracing*) or 20 μM (*lower tracing*) ruthenium red (*RR*). Administration of 100 μM doxorubicin (*D*) was without effect. Afterwards fiber was rinsed twice in soltuion W, and doxorubicin was again applied. Tension developed, although the rate of the rise of tension was slower than expected (*cf.* Fig. 4*C*), probably because ruthenium red had not been removed completely.

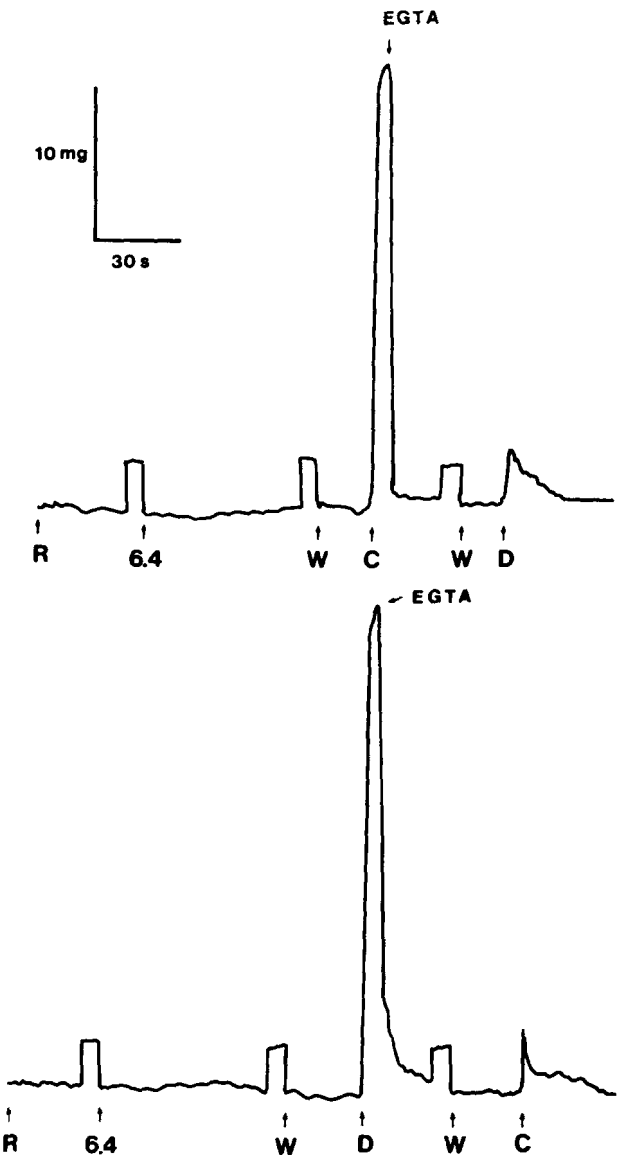

FIG. 7. **Effect of caffeine and doxorubicin on tension development in skinned fibers.** Single fibers were placed in solution R for 30 s, in Ca^{2+} uptake soluton (*p*Ca 6.4) for 60 s, and then in solution W. *Upper tracing*, 10 mM caffeine (*C*) induced force development. At peak tension, fiber was relaxed in 5 mM EGTA, rinsed in solution W, and then exposed to 100 μM doxorubicin (*D*) which elicited very small tension. In the *lower tracing* the order of additions was reversed; 100 μM doxorubicin induced tension development, whereas subsequent addition of 10 mM caffeine was virtually ineffective.

ally, the rate of Ca^{2+} release *in vivo* is 3 orders of magnitude higher than the Ca^{2+} uptake rate (8), indicating that these two processes are mediated by two entirely different mechanisms, *i.e.* the opening of a channel (1, 15, 16, 38) and active transport (2), respectively.

Accordingly, as it was first reported by Weber and Herz (39), isolated TC and LSR fractions differ in their Ca^{2+} release properties. For instance, only TC fractions exhibit Ca^{2+}-induced Ca^{2+} release which, in turn, reflects the existence of Ca^{2+}-gated Ca^{2+} channels. Although Ca^{2+} release from both LSR and TC fractions can be obtained by several experimental manipulations (for review see Ref. 4), Ca^{2+} channels, at least those involved in physiological Ca^{2+} release, are expected to be selectively found in TC fractions.

This study provides the first evidence that doxorubicin interacts with a Ca^{2+} channel localized in purified TC fractions. It is shown that doxorubicin evokes rapid Ca^{2+} release from SR of skinned fibers and stimulates Ca^{2+} efflux from isolated TC fractions but is without effect on isolated LSR fractions.

Doxorubicin is *selective* because it modifies Ca^{2+} permeability characteristics of TC only (Figs. 1–3). The possibility of a nonspecific detergent action can be ruled out since LSR

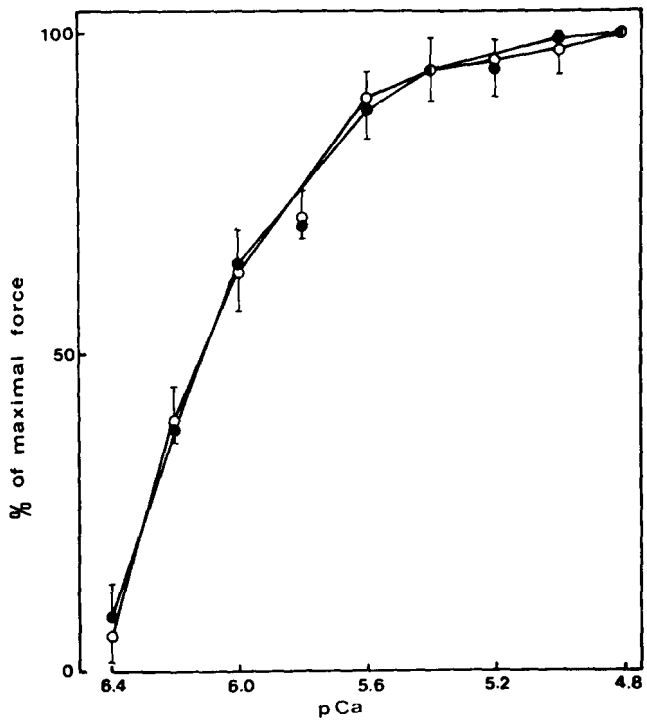

FIG. 8. **Effect of doxorubicin on steady-state isometric force/pCa curve.** Each single fiber was stretched to 130% of slack length in solution R and then sequentially exposed to a solution of increasing [Ca^{2+}] values, in the absence (O) and presence (●) of 100 μM doxorubicin. Data are expressed as mean percentages of maximal force ± 1 S.D. ($n = 3$).

was unaffected by the drug. Thus, doxorubicin could either interact with a Ca^{2+} channel (intrinsic Ca^{2+} efflux pathway) or introduce into TC membranes an extrinsic Ca^{2+} efflux pathway, *i.e.* it might behave like an ionophore. The latter hypothesis does not hold for the following reasons. 1) Ionophores, such as A23187 and X537-A, dissipate Ca^{2+} gradients across any biological or artificial membrane (40). On the contrary, doxorubicin is without effect on LSR and in phosphatidylcholine liposomes does not increase membrane permeability to cations (41), unless cardiolipin is present. It is known, however, that this phospholipid species is absent from SR membranes (42). We also found that 100 μM doxorubicin, at variance with A23187 (2.5 μg/ml), did not increase Ca^{2+} permeability of phosphatidylcholine-phosphatidylserine (95:5) liposomes.[2] 2) Even high concentrations of doxorubicin evoke transient Ca^{2+} release from SR of skinned fibers (Fig. 4*C*), at variance with A23187 that contractures fibers or other agents, *e.g.* tetraphenylboron, reported to lock SR Ca^{2+} channels in open conformation state and to induce sustained tension of chemically skinned fibers (43). Thus, doxorubicin increases Ca^{2+} permeability without disrupting lipid bilayer or permanently altering TC membranes.

In conclusion, our results are consistent with the view that doxorubicin interacts with an intrinsic Ca^{2+} efflux pathway, *i.e.* a Ca^{2+} channel of TC. The selectivity criterion does not prove that doxorubicin acts on the Ca^{2+} channel involved in physiological Ca^{2+} release. It allows, however, a plausible hypothesis strengthened by the findings that: 1) doxorubicin elicits rapid Ca^{2+} release from the SR of skinned fibers; 2) ruthenium red, an inhibitor of Ca^{2+} release from TC and blocker of Ca^{2+} channels (17), fully antagonizes doxorubicin action (Fig. 6 and Table I); and 3) caffeine and doxorubicin mobilize the same pool of Ca^{2+} from TC (Fig. 7). It remains to be clarified whether doxorubicin binds directly to the channel, interferes with its gating mechanism, or interacts with one of the putative several drug-binding sites of the channel (44), *i.e.* the molecular mechanism of doxorubicin action is not yet known.

Doxorubicin is *specific* because it does not influence the Ca^{2+} pump turnover rate of both LSR and TC (Fig. 2) and, therefore, distinguishes Ca^{2+} influx pathway from Ca^{2+} efflux pathway. A few drugs have been found to affect Ca^{2+} efflux preferentially over Ca^{2+} influx in skeletal SR: local anesthetics (45), ruthenium red (17), and ryanodine (46), which are blockers; caffeine (39) and calmodulin antagonists (47), which are activators. These data of pharmacologic nature are in agreement with other biochemical data (16, 43, 48, 49) indicating that Ca^{2+} pump and Ca^{2+} channel systems are composed of different proteins. Furthermore, doxorubicin is, on a concentration basis, the most *potent* among drugs activating Ca^{2+} efflux.

It is known that doxorubicin can be enzymatically reduced to a semiquinone species which behaves as an electron shuttle and might bring about oxidation of proteins as well as lipid peroxidation (18). However, it appears that doxorubicin *per se*, rather than its derivatives, was responsible for the effects described in this paper. In fact: 1) doxorubicin exerts its action instantaneously; 2) doxorubicin undergoes spectral changes when reduced, with maximal absorption peak shifted from 500 to 420 nm (this shift was not detected in any of the several assay media used (not shown)); 3) since drug effects are seen only with TC fractions, one should postulate that endogenous substrates (NADH or NADPH) and reductase systems (50) capable of activating doxorubicin were present in a sizable amount only in TC areas of SR. TC fractions, instead, did not change the doxorubicin spectrum even in the presence of exogenous substrates (not shown).

Recently, attempts have been made to identify SR proteins as putative components of the Ca^{2+} channel. Ikemoto (51) suggested a 32,000 intrinsic SR protein, whereas Tuana and MacLennan (47) stressed the role of two 20,000 and 60,000 proteins. In this context, doxorubicin might be a tool in identifying constituents of the TC Ca^{2+} efflux pathway, *i.e.* the channel itself, the gate, or the drug receptor, due to its potency, selectivity (TC *versus* LSR), and specificity (Ca^{2+} channel *versus* Ca^{2+} pump).

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[2] P. Volpe and F. Zorzato, unpublished results.

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