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CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM OF NORMAL AND DYSTROPHIC MICE

POMPEO VOLPE [a,*], ROBERT E. MRAK [b], BRIAN COSTELLO [a] and SIDNEY FLEISCHER [a]

Departments of [a] Molecular Biology and [b] Pathology, Vanderbilt University, Nashville, TN 37235, and [b] Laboratory and [b] Research Services, Veterans Administration Medical Center, Nashville, TN 37203 (U.S.A.)

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Contraction of skeletal muscle is triggered by release of calcium from the sarcoplasmic reticulum. In this study, highly purified normal and dystrophic mouse sarcoplasmic reticulum vesicles were compared with respect to calcium release characteristics. Sarcoplasmic reticulum vesicles were actively loaded with calcium in the presence of an ATP-regenerating system. Calcium fluxes were followed by dual wavelength spectrophotometry using the metallochromic indicators antipyrylazo III and arsenazo III, and by isotopic techniques. Calcium release from sarcoplasmic reticulum vesicles was elicited by (a) changing the free calcium concentration of the assay medium (calcium-induced calcium release); (b) addition of a permeant anion to the assay medium, following calcium loading in the presence of a relatively impermeant anion (depolarization-induced calcium release); (c) addition of the lipophilic anion tetraphenylboron (TPB^-) to the assay medium and (d) using specific experimental conditions, i.e. high phosphate levels and low magnesium (spontaneous calcium release). Drugs known to influence Ca^{2+} release were shown to differentially affect the various types of calcium release. Caffeine (10 mM) was found to enhance calcium-induced calcium release from isolated sarcoplasmic reticulum. Ruthenium red (20 μM) inhibited both calcium-induced calcium release and tetraphenylboron-induced calcium release, and partially inhibited spontaneous calcium release and depolarization-induced calcium release. Local anesthetics inhibited spontaneous calcium release in a time-dependent manner, and inhibited calcium-induced calcium release instantaneously, but did not inhibit depolarization-induced calcium release. Use of pharmacological agents indicates that several types of calcium release operate in vitro. No significant differences were found between normal and dystrophic sarcoplasmic reticulum in calcium release kinetics or drug sensitivities.

Introduction

Muscular dystrophy in the mouse is a genetic disease inherited as an autosomal recessive trait and is considered an important model of muscular dystrophy in man [1]. The contractile behavior of dystrophic mouse muscle is abnormal: measurements of the active state of contractility under isometric conditions have shown decreased maximal intensity, a slower rate of decay, and decreased duration of the active state plateau [2]. These changes suggest an alteration in excitation-contraction coupling and implicate the sarcoplasmic reticulum (SR) and transverse tubule sys-

* To whom correspondence should be addressed (present address): Department of Physiology, UMDNJ - New Jersey Medical School, 100 Bergen Street, Newark, NJ 07103, U.S.A.

Abbreviations: SR, sarcoplasmic reticulum; TPB^-, tetraphenylboron anion; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SKF 525-A, β-diethylaminoethyldiphenylpropylacetate chloride; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid.

tems in the pathogenesis of dystrophic muscle weakness [2].

In skeletal muscle, the sarcoplasmic reticulum membrane system lowers or raises myoplasmic calcium concentration by actively accumulating calcium or by releasing calcium, thereby allowing relaxation or contraction to occur. Calcium release from isolated sarcoplasmic reticulum can be induced by several means [3,4], which probably reflect activation of different mechanisms. Although the physiological relevance of these various types of release remains to be clarified [4], the phenomenology of calcium release in vitro can be extensively analyzed.

Recently, Mrak and Fleischer [5] have developed a procedure fór isolation of highly purified sarcoplasmic reticulum vesicles from normal and dystrophic mouse muscle. No differences were found in composition and function (calcium transport or calcium-stimulated ATPase activities) between normal and dystrophic mouse sarcoplasmic reticulum [5]. In this study, we characterize calcium release from normal and dystrophic mouse sarcoplasmic reticulum under a variety of experimental conditions, and examine the effect of several drugs which influence the calcium release process itself (e.g. caffeine, Ruthenium red and local anesthetics).

Materials and Methods

Materials. Antipyrylazo III, arsenazo III, phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, NADH, Na_2ATP (vanadate-free), disodium phosphocreatine, creatine phosphokinase, tetracaine chloride, quercetin, tetraphenylboron (sodium salt) and Ruthenium red were obtained from Sigma (St. Louis, MO). The ionophore A23187 was obtained from Calbiochem (La Jolla, CA), caffeine from Aldrich (Milwaukee, WI), and $^{45}CaCl_2$ from New England Nuclear (Boston, MA). SKF 525-A was a gift from Smith, Kline and French Laboratories (Philadelphia, PA). Quercetin and A23187 were prepared in concentrated ethanolic solutions.

Isolation of sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles from normal and dystrophic ($129B6F_1/J$-*dy*) mice, aged 6 to 8 weeks, were prepared using controlled homogenization of hindlimb and lumbar musculature and differential and density gradient centrifugations as previously described [5]. Isolated sarcoplasmic reticulum vesicles were stored in 29% sucrose (w/w)/0.1 M KCl/10 mM potassium Hepes (pH 7.1), either at −70°C or in liquid nitrogen, until used.

Assays. Protein was measured using a modification [6] of the method of Lowry et al. [7], with bovine serum albumin as a standard. ATPase activity was measured by a spectrophotometric enzyme-coupled assay [8] at 25°C, in a final volume of 1 ml, in the presence of 0.4 mM NADH, 2 mM phospho*enol*pyruvate, 7 U pyruvate kinase and 12 U lactate dehydrogenase. The absorbance change at 340 nm was monitored continuously in a Hewlett-Packard 8450A spectrophotometer. One mole of NADH oxidized corresponds to one mole of ATP hydrolized [8]. Basal ATPase activity was assayed in a medium of the following composition: 10 mM potassium Hepes (pH 7.0)/0.1 M KCl/1 mM $MgSO_4$/1 mM Na_2ATP/0.25 mM EGTA. The reaction was started by adding 10 μg of sarcoplasmic reticulum protein. Calcium-stimulated ATPase activity was measured from the incremental rate, following addition of 0.25 mM $CaCl_2$ (calculated free calcium concentration, 10 μM) in the presence of the Ca^{2+} ionophore A23187 (1.5 μg/ml). Basal ATPase activity accounted for 5–6% of total ATPase activity.

Induction of calcium release and efflux. In this paper, 'calcium release' refers to the net loss of calcium by sarcoplasmic reticulum vesicles, and 'calcium efflux' to the unidirectional outward flow of calcium across the sarcoplasmic reticulum membrane, measured in the presence of 100 μM quercetin. A variety of experimental conditions were used to induce calcium release from sarcoplasmic reticulum.

(*A*) *Spontaneous calcium release.* Calcium phosphate loading was carried out at 25°C using sarcoplasmic reticulum vesicles (30 μg in 1 ml) in 112.5 mM potassium phosphate (pH 7.0)/1 mM $MgSO_4$/1 mM Na_2ATP/5 mM disodium phosphocreatine/20 μg/ml creatine phosphokinase/200 μM antipyrylazo III [9]. Loading was performed by the addition of several consecutive pulses of $CaCl_2$ (25–50 nmol per pulse) to a 1 ml assay medium and was followed by monitoring differential absorbance changes (710–790 nm) of

the metallochromic indicator antipyrylazo III [10] in a Hewlett-Packard 8450A spectrophotometer. Spontaneous calcium release occurred after the completion of calcium loading with an intervening lag period of approx. 100 s. At the onset of release, free calcium concentration is in the submicromolar range [9]. Maximal rates of calcium release are obtained at free $[Ca]_0$ between 10^{-8} and 10^{-7} M [9]. Release rates were calibrated using the final $CaCl_2$ addition prior to release.

(*B*) *Spontaneous calcium efflux.* Unidirectional, spontaneous calcium efflux was measured spectrophotometrically in the presence of quercetin [9,11,12]. Quercetin inhibits both the forward and backward reactions of the calcium pump [13,14] and does not enhance unidirectional calcium efflux [9,12]. The rate of calcium efflux measured by means of isotope techniques, after quenching the calcium pump with 1 mM EGTA [12], agrees closely with the calcium efflux rate measured in the presence of quercetin [12]. This technique, therefore, allowed measurement of unidirectional calcium efflux from preloaded sarcoplasmic reticulum vesicles without significant reuptake of calcium. Sarcoplasmic reticulum vesicles were preloaded with calcium phosphate as described above, and 100 μM quercetin was added at the completion of loading, at the beginning of the lag phase which precedes spontaneous calcium release [9,12]. Efflux rates were calibrated by adding a $CaCl_2$ pulse at the end of each experiment (see Fig. 1B).

(*C*) *Calcium-induced calcium efflux.* Calcium efflux at fixed free calcium concentrations was measured isotopically. The assay medium was the same as that described in B, but included a small quantity of $^{45}CaCl_2$ of high specific activity. Sarcoplasmic reticulum vesicles (60 μg in 2 ml) were preloaded with 60 nmol of non-radioactive calcium and the calcium loading was monitored spectrophotometrically. At the completion of loading, 100 μM quercetin was added followed immediately by 40 μl of a concentrated calcium-EGTA buffer (250 mM EGTA, pH 7.0) to hold the free external calcium concentration at 10^{-5} M during calcium efflux. Sequential aliquots of 0.1 ml were then withdrawn and rapidly filtered using a microfilter kit with 0.2 μm nitrocellulose filters (Schleicher and Schuell, Keene, NH), and the $^{45}Ca^{2+}$ content of the filtrates determined by liquid scintillation counting. The calcium-EGTA buffer solution was prepared with the aid of a computer program (Table I in Ref. 9) using the association constants given by Fabiato and Fabiato [15].

(*D*) *Tetraphenylboron-induced calcium release.* Tetraphenylboron was used to induce calcium release essentially as described by Shoshan et al. [16]. Calcium preloading and calcium release were measured spectrophotometrically at 25°C. Sarcoplasmic reticulum vesicles (90 μg in 1 ml) were preloaded in 4.4 mM sodium phosphate/88 mM NaCl/5 mM disodium phosphocreatine/20 μg/ml creatine phosphokinase/200 μM antipyrylazo III/1 mM $MgSO_4$/1 mM Na_2ATP/17.6 mM sodium Hepes (pH 7.0). Sodium salts were used in these experiments because tetraphenylboron is not soluble in the presence of potassium [16]. Following calcium preloading, 100 μM tetraphenylboron was added.

(*E*) *Depolarization-induced calcium release.* The protocol used was similar to that of Caswell and Brandt [17]. Calcium loading and calcium release were measured spectrophotometrically following the differential absorbance changes (660–740 nm) of the metallochromic indicator arsenazo III [10]. Sarcoplasmic reticulum vesicles (150 μg in 1 ml) were preincubated for 9 min at 0°C in 100 mM potassium gluconate/125 mM sucrose/3.5 mM disodium phosphocreatine/40 μg/ml creatine phosphokinase/20 μM arsenazo III/5 mM NaCl/3 mM $MgSO_4$/15 mM imidazole (pH 6.8). The assay medium was warmed to 37°C and 3.25 mM Na_2ATP was added. Calcium loading was performed by addition of two consecutive 50 nmol $CaCl_2$ pulses. When loading was complete (approx. 350 s), 50 μl of 2 M KSCN (or potassium gluconate, as control) were added and rapidly mixed to achieve a final concentration of 98 mM (or 198 mM potassium gluconate). The rate of the ensuing release was calculated from the average slope of the absorbance traces during the 45 seconds following KSCN addition.

Results

Spontaneous calcium release and efflux

Spontaneous calcium release occurs from vesicles of mouse sarcoplasmic reticulum preloaded with calcium in the presence of high con-

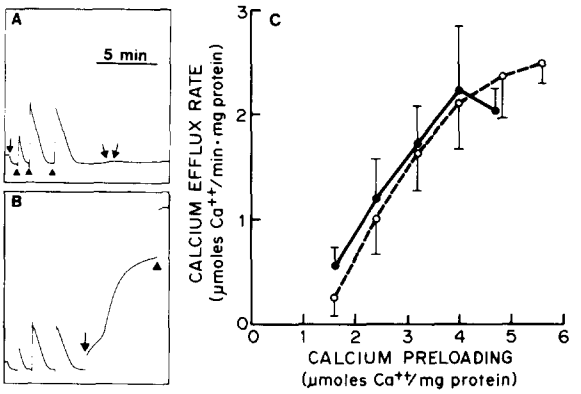

Fig. 1. Spontaneous calcium release and calcium efflux from normal and dystrophic mouse sarcoplasmic reticulum. Calcium loading, calcium release, and calcium efflux were measured spectrophotometrically using antipyrylazo III as calcium indicator as described in Materials and Methods. (A) Typical calcium release experiment: Normal mouse sarcoplasmic reticulum (30 μg protein, added at arrow) was loaded with $CaCl_2$ (one 25 nmol and two 50 nmol pulses, added at arrowheads). At the completion of calcium loading, after depletion of extravesicular calcium, and following a lag period of 100 s, a miniscule calcium release (double arrow) occurred, followed by reuptake. (B) Typical calcium efflux experiment: Normal mouse sarcoplasmic reticulum was loaded with $CaCl_2$ as in (A). At the completion of calcium loading quercetin (100 μM) was added (arrow). Following a lag period of 100 s, calcium efflux occurred without subsequent reuptake. Arrowhead denotes recalibration of antipyrylazo III by addition of 50 nmol $CaCl_2$. (C) Dependence of calcium efflux on calcium preloading. Experiments were carried out as in (B), with 30 μg sarcoplasmic reticulum protein. O------O, Normal mouse sarcoplasmic reticulum; ●———●, dystrophic mouse sarcoplasmic reticulum. Three different sarcoplasmic reticulum preparations were assayed; bars represent one S.D.

centrations of inorganic phosphate and low magnesium, after depletion of extravesicular calcium [9] and after a lag period of approx. 100 s (Fig. 1A). This release, however, is considerably slower than that seen using rabbit skeletal muscle sarcoplasmic reticulum under identical experimental conditions [9,12]; release rates of 0.1–0.2 μmol Ca^{2+}/min per mg protein obtained for normal mouse sarcoplasmic reticulum are approximately one tenth the values obtained for rabbit sarcoplasmic reticulum [9]. Normal and dystrophic mouse sarcoplasmic reticulum did not differ significantly in the rate of spontaneous calcium release (data not shown).

Unidirectional calcium efflux from preloaded sarcoplasmic reticulum vesicles was measured by adding quercetin at the completion of calcium loading (Fig. 1B and Refs. 9 and 12). The concentration of quercetin employed (100 μM) was sufficient to completely inhibit both calcium transport and calcium-stimulated ATPase activities (Table I). Spontaneous calcium efflux rates were dependent upon the level of calcium preloading [9], and maximal values of 2.0–2.5 μmol Ca^{2+}/min per mg protein were obtained, approx. 10–20-times the measured rate of spontaneous calcium release (Fig. 1C). There was no significant difference in spontaneous calcium efflux between normal and dystrophic mouse sarcoplasmic reticulum.

The effect of various pharmacological agents on spontaneous calcium efflux from normal and dystrophic mouse sarcoplasmic reticulum is shown in Table II. SKF 525-A (100 μM) and tetracaine (300 μM), two tertiary amine local anesthetics [12], inhibited spontaneous calcium efflux. The inhibition by these local anesthetics was time-dependent, as reported for rabbit light sarcoplasmic reticulum [12]. Local anesthetics administered at the completion of calcium loading were only slightly inhibitory, but much more effective when added just prior to calcium loading, thereby allowing 5–6-min incubation before calcium efflux took place (Table II). Ruthenium red (20 μM) showed partial inhibition (30–40%). Higher concentrations of Ruthenium red were found to have no greater effect (data not shown). Caffeine (10 mM) was found to inhibit spontaneous calcium efflux *. Normal and dystrophic sarcoplasmic reticulum did not differ in the sensitivity of spontaneous calcium efflux to these pharmacological agents.

* The effect of caffeine on spontaneous calcium efflux from mouse sarcoplasmic reticulum depends upon the extent of calcium preloading. There is no inhibition when the calcium preloading is 0.8 μmol Ca^{2+}/mg protein, i.e. lower than that used in the experiments of Table II. A similar pattern has been observed for rabbit light sarcoplasmic reticulum. Caffeine enhanced spontaneous calcium release rate with low calcium preloading (1.5 μmol Ca^{2+}/mg protein). but markedly inhibited spontaneous calcium release with higher preloading (4.2 μmol Ca^{2+}/mg protein) (Volpe, P., Mitchell, R.D., Palade, P. and Fleischer, S., unpublished observations).

TABLE I

EFFECT OF DRUGS ON CALCIUM LOADING AND CALCIUM-STIMULATED ATPase ACTIVITIES OF NORMAL AND DYSTROPHIC MOUSE SARCOPLASMIC RETICULUM (SR)

Calcium loading activity was measured spectrophotometrically as described in Materials and Methods under Spontaneous calcium release. Calcium-stimulated ATPase activity was measured by a spectrophotometric enzyme-coupled assay as described in Materials and Methods except where noted otherwise. The average control activities were: Calcium loading, 1.51 and 1.14 μmol Ca^{2+}/min per mg protein for normal and dystrophic mouse sarcoplasmic reticulum, respectively; calcium-stimulated ATPase, 3.13 and 2.56 μmol ATP hydrolized/min per mg protein for normal and dystrophic mouse sarcoplasmic reticulum, respectively. (These values indicate a 20–25% difference in purity for these preparations of normal and dystrophic sarcoplasmic reticulum [5].) Sarcoplasmic reticulum vesicles and drug were added at the same time. For drugs added as ethanolic solutions, controls were run with additions of ethanol alone. The effect of the ethanol was negligible in these experiments and those reported in subsequent tables. Values are given as mean ± S.D. for determinations on three different sarcoplasmic reticulum preparations.

Additions	% of control activity			
	Calcium loading		Calcium-stimulated ATPase (+A23187)	
	Normal SR	Dystrophic SR	Normal SR	Dystrophic SR
Quercetin (100 μM)	1.0	3.9	1.8	1.0
Tetraphenylboron (100 μM) [a]	2.5	2.3	79.8 ± 4.8 [b]	88.4 ± 9.0 [b]
Caffeine (10 mM)	95.3	92.4	99.2	93.1
SKF 525-A (100 μM)	94.7 ± 7.0	85.8 ± 6.0	79.0 ± 8.2	85.1 ± 9.2
Tetracaine (300 μM)	115.4	106.3	90.2	93.7
Ruthenium red (20 μM)	116.0 ± 11.5	103.7 ± 6.8	99.3	106.3

[a] All salts in the assay medium were sodium salts.

[b] These ATPase activities were determined by measuring the inorganic phosphate produced [38] as described by Ottolenghi [39]. The enzyme-coupled assay could not be used since pyruvate kinase requires potassium which precipitates tetraphenylboron.

Effect of salt additions on spontaneous calcium release and efflux

The lag period preceding spontaneous calcium release, or spontaneous calcium efflux, and the rates of release or efflux, were altered by the addition of concentrated salt solutions at the beginning of the lag phase (Fig. 2, Table III and Ref. 11). Increasing the sodium concentration of the medium 4.8-fold, either by addition of NaCl or Na_2SO_4, resulted in shortening of the lag period and enhancement of both calcium release and calcium efflux from normal and dystrophic mouse sarcoplasmic reticulum (Figs. 2 C and G, and Table III). Calcium release showed greater stimulation than efflux (Table III), suggesting that these results are at least partially attributable to decreased calcium transport activity. Vesicle rupture does not seem to be the cause of the increased calcium release since the lag period is not eliminated, albeit shortened.

In contrast to the effect of added sodium salts, addition of equivalent amounts of Tris chloride or of Tris sulfate resulted in lengthening of the lag period and nearly eliminated calcium efflux (Figs. 2 D and H, and Table III). Considering the relative permeabilities of $Cl^- > Na^+ \gg SO_4^{2-} > Tris^+$ [18], it would appear that conditions which might induce a sustained and large negative-inside sarcoplasmic reticulum membrane potential (e.g. TrisCl, $Tris_2SO_4$) inhibit spontaneous calcium release, whereas conditions which might induce a less negative-inside (or more positive-inside) sarcoplasmic reticulum membrane potential (such as Na_2SO_4) enhance spontaneous calcium release.

Calcium-induced calcium efflux

Ohnishi [19] and Miyamoto and Racker [22] have recently shown that calcium release from rabbit sarcoplasmic reticulum can be elicited when free $[Ca]_0$ is around 10–20 μM. In this study, calcium-induced calcium efflux was measured in the presence of quercetin to block calcium reaccumulation, and calcium-EGTA buffer to fix the free external calcium concentration at 10^{-5} M (see Methods). The rate of calcium efflux was about 80 nmol Ca^{2+}/min per mg protein for both normal

TABLE II

EFFECT OF DRUGS ON SPONTANEOUS CALCIUM EFFLUX FROM NORMAL AND DYSTROPHIC MOUSE SARCOPLASMIC RETICULUM (SR)

Spontaneous calcium efflux was measured by adding quercetin (100 μM) at the completion of calcium loading as described in Materials and Methods and in the legend to Fig. 1B. Normal and dystrophic mouse sarcoplasmic reticulum was preloaded to 3.3 μmol Ca^{2+}/mg protein. This preloading level was chosen because it gave good efflux rates. The average control rates for calcium efflux were 1.63 and 1.70 μmol Ca^{2+}/min per mg protein for normal and dystrophic mouse sarcoplasmic reticulum, respectively. Drugs tested were added just prior to calcium loading, thereby allowing a 5–6-min interval before calcium release took place. Caffeine, instead, was added at the completion of calcium loading. Values are given as mean ± S.D. for determinations on three different sarcoplasmic reticulum preparations.

Additions	Calcium efflux rate (% of control activity)	
	Normal SR	Dystrophic SR
SKF 525-A (100 μM) [a]	18.0 ± 7.2	22.4 ± 3.7
Tetracaine (300 μM) [a]	35.9	33.1
Ruthenium red (20 μM)	63.8 ± 8.5	73.7 ± 7.9
Caffeine (10 mM)	28.5 ± 6.6	23.2 ± 7.1

[a] Spontaneous calcium efflux was only slightly inhibited (10–15%) when SKF 525-A and tetracaine were added at the completion of calcium loading (see also Ref. 12).

and dystrophic mouse sarcoplasmic reticulum (Table IV).

The effect of several drugs on calcium-induced calcium efflux was examined and the results are shown in Table IV. Caffeine (10 mM) stimulated calcium-induced calcium efflux rate more than 2-fold at 10^{-5} M $[Ca]_0$, with approx. 50% of the preloaded calcium released within 4 min (data not shown). Ruthenium red (20 μM) and SKF 525-A (100 μM) added at the completion of calcium loading, markedly inhibited calcium-induced calcium efflux and caffeine stimulation (Table IV). SKF 525-A exerted its action on this calcium efflux instantaneously, that is without the incubation required for the inhibition of spontaneous calcium efflux (cf. Table II). Caffeine, SKF 525-A and Ruthenium red had similar effects on calcium-induced calcium efflux from normal and dystrophic mouse SR (Table IV).

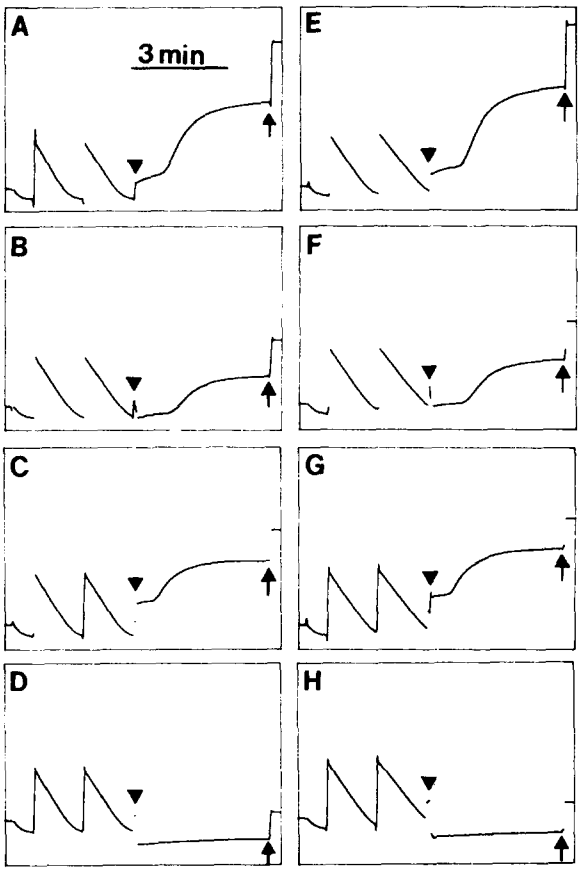

Fig. 2. Effect of salt additions on spontaneous calcium efflux from normal and dystrophic mouse sarcoplasmic reticulum. Spontaneous calcium efflux was measured spectrophotometrically in the presence of quercetin (100 μM), added at the completion of calcium loading, as described in Materials and Methods and in the legend to Fig. 1B, except that sodium salts were used instead of potassium salts. (A), (B), (C) and (D): Normal sarcoplasmic reticulum. (E), (F), (G) and (H): dystrophic sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles were preloaded to 3.3 μmol Ca^{2+}/mg protein. Arrowhead denotes the addition: of quercetin in (A) and (E); of quercetin and 125 mM sodium phosphate (pH 7.0) in (B) and (F); of quercetin and 0.8 M NaCl in (C) and (G); and of quercetin and 0.8 M Tris-HCl in (D) and (H). Aliquots (250 μl) of concentrated salt solutions were added, and final concentrations are given above. At the end of each experiment 50 nmol of $CaCl_2$ were added (arrow) to recalibrate the antipyrylazo III response to calcium.

Tetraphenylboron-induced calcium release

The lipophilic anion tetraphenylboron (100 μM), added at the completion of calcium loading, induced immediate calcium release from normal and dystrophic mouse sarcoplasmic reticulum

TABLE III

EFFECT OF SALT ADDITIONS ON SPONTANEOUS CALCIUM RELEASE AND EFFLUX FROM NORMAL AND DYSTROPHIC MOUSE SARCOPLASMIC RETICULUM (SR)

Spontaneous calcium release and calcium efflux were measured spectrophotometrically as described in Materials and Methods, except that sodium salts were used instead of potassium salts. Sarcoplasmic reticulum vesicles were preloaded to 3.3 μmol Ca^{2+}/mg protein. At the completion of calcium loading and at the beginning of the lag period, either a concentrated salt solution or isotonic sodium phosphate buffer (control) was added as a 250 μl aliquot. In calcium efflux experiments, quercetin (100 μM) was also added, just prior to the salt addition. Release and efflux rates were calibrated by adding a $CaCl_2$ pulse at the end of each experiment.

Additions	μmol Ca^{2+}/min per mg protein				Lag period [a] (s)	
	Calcium release rate		Calcium efflux rate			
	Normal SR	Dystrophic SR	Normal SR	Dystrophic SR	Normal SR	Dystrophic SR
None	0.10	0.13	2.05	2.19	87	77
Sodium phosphate, pH 7.0 (125 mM) (control/dilution)	0.11	0.13	1.64	1.75	92	83
NaCl (0.8 M)	2.09	2.27	2.86	3.05	44	42
Na_2SO_4 (0.4 M)	1.95	2.01	2.53	2.89	59	57
TrisCl (0.8 M)	0.29	0.25	0.14	0.10	182	187
$Tris_2SO_4$ (0.4 M)	0.19	0.17	0.13	0.13	174	182

[a] The lag period is defined as the time elapsing between completion of calcium loading and beginning of either calcium release or calcium efflux. Quercetin did not change the length of the lag period [9,12].

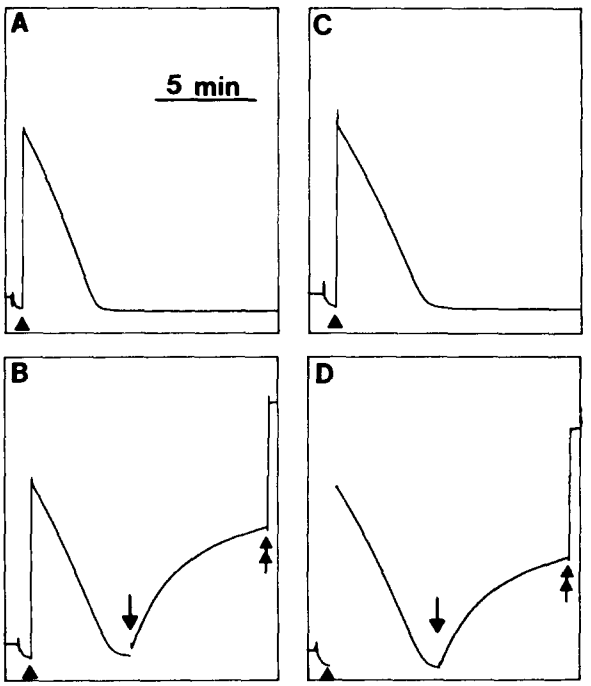

Fig. 3. Tetraphenylboron-induced calcium release from normal and dystrophic mouse sarcoplasmic reticulum. Calcium loading and calcium release were followed spectrophotometrically using antipyrylazo III as calcium indicator, as described in Materials and Methods. Sarcoplasmic reticulum vesicles (90 μg protein in 1 ml) were preloaded with 50 nmol $CaCl_2$ (arrowhead). (A) and (B): Normal sarcoplasmic reticulum. (C) and (D): Dystrophic sarcoplasmic reticulum. In the absence of tetraphenylboron, (A) and (C), no calcium release was detected. In (B) and (D) 100 μM tetraphenylboron was added at the completion of calcium loading (arrow). Double arrow indicates recalibration of antipyrylazo III with 50 nmol $CaCl_2$.

(Figs. 3 B and D) and approx. 80% of the preloaded calcium was released within 5 min. Tetraphenylboron inhibited calcium loading but did not inhibit calcium-stimulated ATPase (Table I), indicating tetraphenylboron does not act on the calcium pump [16].

Quercetin enhanced tetraphenylboron-induced calcium release (Table V) probably by inhibiting the calcium pump [13]. Ruthenium red inhibited tetraphenylboron-induced calcium release (Table V). Normal and dystrophic mouse sarcoplasmic reticulum did not differ in tetraphenylboron-induced calcium release or in the sensitivity of this release to the drugs tested.

Depolarization-induced calcium release

Calcium release can be induced from isolated sarcoplasmic reticulum and from sarcoplasmic reticulum of skinned muscle fibers by replacement

of a non permeant anion by a permeant one [4] or by addition of a permeant anion (e.g. SCN^-) following calcium loading in the presence of a relatively impermeant anion (e.g. gluconate) [17,21]. This type of calcium release (depolarization-induced calcium release) has been suggested to be due to development of a negative-inside diffusion potential across the sarcoplasmic reticulum membrane [4]. Figs. 4 A and C shows that such release can be observed in mouse sarcoplasmic reticulum. Addition of KSCN to either normal or dystrophic mouse sarcoplasmic reticulum, preloaded with 0.67 μmol Ca^{2+}/mg protein, triggered an immediate and sustained calcium release with a rate of approx. 0.1 μmol Ca^{2+}/min per mg protein. Addition of an equivalent amount of concentrated potassium gluconate (Figs. 4 B and D), instead of KSCN, did not induce calcium release.

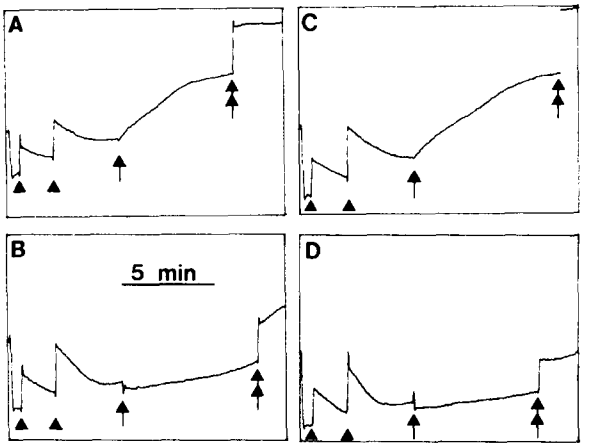

Fig. 4. Depolarization-induced calcium release from normal and dystrophic mouse sarcoplasmic reticulum. Calcium loading and calcium release were followed spectrophotometrically using arsenazo III as calcium indicator, as described in Materials and Methods. Sarcoplasmic reticulum vesicles (150 μg protein in 1 ml) were preloaded with two consecutive 50 nmol $CaCl_2$ pulses (arrowheads). (A) and (B): Normal SR. (C) and (D): Dystrophic SR. In (A) and (C) KSCN (50 μl of a 2 M solution) was added at the completion of calcium loading (arrow) and rapidly mixed, resulting in a final KSCN concentration of 98 mM. In (B) and (D) potassium gluconate (50 μl of a 2M solution) was added at the completion of calcium loading and rapidly mixed, raising the potassium gluconate concentration from 100 to 198 mM. At the end of each experiment, 50 nmol of $CaCl_2$ were added to recalibrate the arsenazo III (double arrow).

TABLE IV

EFFECT OF DRUGS ON CALCIUM-INDUCED CALCIUM EFFLUX FROM NORMAL AND DYSTROPHIC MOUSE SARCOLPLASMIC RETICULUM (SR)

Calcium loading was followed spectrophotometrically as described in Materials and Methods. Sarcoplasmic reticulum vesicles were preloaded to 0.98 μmol Ca^{2+}/mg protein. This relatively low preloading level was optimized for the experiments carried out with caffeine. It appears that only partially filled sarcoplasmic reticulum vesicles respond well to caffeine (Volpe, P. and Mrak, R.E., unpublished observations, and Ref. 31). At the completion of calcium loading, quercetin (100 μM) was administered, the external free calcium concentration was fixed at 10^{-5} M by adding 40 μl of a concentrated calcium-EGTA solution (250 mM EGTA), and calcium efflux was measured isotopically (see Materials and Methods). Caffeine, Ruthenium red, or SKF 525-A were administered immediately following addition of the calcium-EGTA solution. Results of a typical experiment are shown.

Additions	nmol Ca^{2+}/min per mg protein	
	Normal SR	Dystrophic SR
None [a]	74.5	80.1
Ruthenium red (20 μM)	22.3	18.4
SKF 525-A (100 μM)	25.1	20.2
Caffeine (10 mM)	210.4	190.2
Caffeine (10 mM) and Ruthenium red (20 μM)	30.7	16.3
Caffeine (10 mM) and SKF 525 A (100 μM)	33.1	22.7

[a] The addition of quercetin, in the absence of the Ca-EGTA solution, elicited no measurable calcium efflux.

Table VI summarizes the effect of several drugs on depolarization-induced calcium release from both normal and dystrophic mouse sarcoplasmic reticulum. In each case, the drug was added just before KSCN administration. Quercetin (100 μM) enhanced calcium release (cf. Ref. 13). Ruthenium red (20 μM) partially inhibited calcium release. SKF 525-A (100 μM) was without effect in both normal and dystrophic mouse sarcoplasmic reticulum. However, when SKF 525-A was added prior to calcium loading, thereby allowing approximately 6-min incubation before addition of KSCN, it enhanced release in normal and dystrophic mouse sarcoplasmic reticulum. This effect is not due to inhibition of the calcium pump because both calcium loading and calcium-stimulated ATPase are only slightly affected by SKF 525-A (Table I and Ref. 12).

TABLE V

EFFECT OF DRUGS ON TETRAPHENYLBORON-INDUCED CALCIUM RELEASE FROM NORMAL AND DYSTROPHIC MOUSE SARCOPLASMIC RETICULUM (SR)

Calcium loading and tetraphenylboron-induced calcium release were measured spectrophotometrically as described in Materials and Methods and in the legend to Fig. 3. Sarcoplasmic reticulum vesicles were preloaded to 0.55 μmol Ca^{2+}/mg protein. Tetraphenylboron-induced calcium release rates for control preparations were 0.24 and 0.21 μmol Ca^{2+}/min per mg protein, on average, for normal and dystrophic mouse sarcoplasmic reticulum, respectively. The drugs tested were added at the completion of calcium loading, just prior to the addition of tetraphenylboron (100 μM). The effect of local anesthetics on tetraphenylboron-induced calcium release could not be determined because of interferences in dye absorption. Values are given as mean ± S.D. for determinations on three different sarcoplasmic reticulum preparations.

Additions	Calcium release rate (% of control activity)	
	Normal SR	Dystrophic SR
Ruthenium red (20 μM)	17.0 ± 3.3	15.6 ± 4.1
Quercetin (100 μM)	145.6 ± 12.9	147.5 ± 16.2

TABLE VI

EFFECT OF DRUGS ON DEPOLARIZATION-INDUCED CALCIUM RELEASE FROM NORMAL AND DYSTROPHIC MOUSE SARCOPLASMIC RETICULUM (SR)

Calcium loading and depolarization (KSCN)-induced calcium release were measured spectrophotometrically as described in Materials and Methods and in the legend to Fig. 4. Sarcoplasmic reticulum vesicles were preloaded to 0.67 μmol Ca^{2+}/mg protein. The calcium release rate for control preparations was 0.1 μmol Ca^{2+}/min per mg protein, on average, for both normal and dystrophic mouse sarcoplasmic reticulum. The drugs tested were added at the completion of calcium loading, and just prior to the addition of KSCN. Values are given as mean ± S.D. for determinations on three sarcoplasmic reticulum preparations.

Additions	Calcium release rate (% of control activity)	
	Normal SR	Dystrophic SR
Quercetin (100 μM)	162.7 ± 21.1	166.0 [b]
Ruthenium red (20 μM)	76.0 ± 11.5	61.0 ± 9.2
SKF 525-A (100 μM) [a]	100.7 ± 14.3	97.0 ± 12.5

[a] Addition of SKF 525-A, prior to calcium loading, enhanced calcium release rate by 70% ± 32 and 33% ± 16 in normal and dystrophic mouse sarcoplasmic reticulum, respectively.

[b] Only two preparations were analyzed. The results varied by less than 10%.

Discussion

This study is the first comparative characterization of calcium release processes from normal and dystrophic mouse sarcoplasmic reticulum. We have employed several experimental conditions which appear to involve different mechanisms of calcium release [3,4,9,16,17,22]. Under all of the conditions used, the calcium release properties of purified sarcoplasmic reticulum from normal and dystrophic mouse were similar.

Mechanisms of calcium release

Contraction of skeletal muscle is initiated by release of calcium from the terminal cisternae of the sarcoplasmic reticulum [4,23] following depolarization of the transverse tubule membrane. Both the coupling process between transverse tubule depolarization and calcium release from sarcoplasmic reticulum, and the calcium release mechanism itself are poorly understood [4]. In vitro, calcium release from sarcoplasmic reticulum vesicles can be induced by a number of different means: by changing the external free calcium concentration (Table IV and Ref. 22), by addition of tetraphenylboron which apparently changes the sarcoplasmic reticulum membrane surface charge (Fig. 3 and Ref. 16), by modulating the sarcoplasmic reticulum membrane potential (Fig. 4 and Ref. 21) or by imposing a pH gradient (alkaline-inside) [3,24]. Calcium release can also be made to occur spontaneously (Fig. 1 and Refs. 9,20,25 and 26). Calcium can also be released via reversal of the calcium pump, with synthesis of ATP from ADP and inorganic phosphate [27], or through the calcium channel in the calcium pump without synthesis of ATP [3,28].

Specific and different experimental conditions must be employed to evoke these calcium releases. In this respect, one could ask whether the mechanisms of calcium release are independent or interrelated. In skinned skeletal muscle fibers, calcium-induced calcium release differs from depolarization-induced calcium release in that only the first type of release is enhanced by increasing calcium preloading and free ATP concentration and inhibited by local anesthetics and increasing free magnesium concentration [4]. In skinned skeletal muscle fibers, two sarcoplasmic reticulum

calcium efflux pathways have been implied, only one being sensitive to calcium and to caffeine [29]. It has been suggested that calcium-induced calcium release and depolarization-induced calcium release act in series in isolated sarcoplasmic reticulum vesicles [22], and that spontaneous calcium release from isolated sarcoplasmic reticulum vesicles is distinct from calcium-induced, depolarization-induced and pH-induced calcium release, as well as from calcium pump reversal [9,12]. In this study, we provide further evidence, mainly of a pharmacological nature, that different mechanisms of calcium release and, possibly, different calcium efflux pathways, operate in sarcoplasmic reticulum in vitro.

The calcium release and efflux described here do not appear to be mediated by the calcium pump. This is because (A) the ATP-regenerating system employed precludes significant accumulation of ADP, which would be required for calcium pump reversal, and (B) quercetin, an inhibitor of the backward and forward mode of the calcium pump [13], did not inhibit calcium efflux. MacLennan et al. [3] have employed quercetin to identify calcium releases occurring through the calcium channel in the calcium pump, i.e. EGTA- and pH-induced calcium release. We used quercetin to block calcium pump function and calcium reaccumulation, and to measure unidirectional calcium efflux (Fig. 1B and Table IV; see also Ref. 12). The stimulation of calcium release by quercetin (Fig. 1B and Tables V and VI) can be largely accounted for by elimination of unidirectional calcium influx. However, Watras et al. [20] have recently reported that quercetin activates a calcium efflux pathway in rabbit light sarcoplasmic reticulum. Such a pathway is activated at $[Ca]_0$ ranging from 0.2 to 1 μM [20]. Thus, the contribution of this phenomenon should be negligible in our experiments (e.g. Fig. 1B and Table IV).

We find that calcium-induced calcium efflux and spontaneous calcium efflux differ in their drug sensitivities. Calcium-induced calcium efflux is enhanced by caffeine and inhibited by Ruthenium red (Table IV), while spontaneous calcium efflux may be inhibited * by caffeine and is only partially inhibited by Ruthenium red (Table II). Furthermore, inhibition of calcium-induced calcium efflux by SKF 525-A, is immediate, indicating an external site of action (Table IV and Ref. 30), whereas inhibition of spontaneous calcium efflux by SKF 525-A is time-dependent, implying an internal site of action (Table II and Ref. 12). An explanation for this finding is that these calcium effluxes occur through separate pathways, possibly localized in sarcoplasmic reticulum vesicles deriving from terminal cisternae (heavy) sarcoplasmic reticulum and longitudinal (light) sarcoplasmic reticulum, respectively. It has been reported that calcium-induced, caffeine-stimulated [31], Ruthenium red-sensitive calcium release occurs preferentially in rabbit heavy sarcoplasmic reticulum [22,30], whereas spontaneous, Ruthenium red-insensitive calcium release is preferentially displayed by rabbit light sarcoplasmic reticulum [9,12]. In this regard, our isolated mouse sarcoplasmic reticulum contains both light and heavy sarcoplasmic reticulum (Figs. 1 and 2 in Ref. 5). Thus, the partial inhibition of spontaneous calcium efflux by Ruthenium red (Table II) may be due to blockage of Ruthenium red-sensitive calcium channels selectively located in terminal cisternae sarcoplasmic reticulum vesicles [22].

Depolarization-induced calcium release is qualitatively different from spontaneous calcium efflux. Depolarization-induced calcium release is triggered by the addition of salt solutions designed to impose a transient negative-inside sarcoplasmic reticulum membrane potential (Fig. 4, and Ref. 21). Spontaneous calcium efflux, on the other hand, is enhanced by the addition of salt solutions designed to impose a more positive-inside membrane potential on the sarcoplasmic reticulum vesicles and inhibited when the sarcoplasmic reticulum membrane is made more negative inside (Fig. 2 and Table III). Should the same efflux pathway mediate, at least in part, both depolarization-induced and spontaneous calcium release, different voltage gating mechanisms might be involved [11].

Depolarization-induced calcium release differs from other forms of calcium release in sensitivity to local anesthetics (cf. Tables II, IV, VI). In fact, SKF 525-A did not inhibit depolarization-induced calcium release (Table VI), as previously reported by Ohnishi [32] for procaine, and a short incuba-

* See footnote on p. 70.

tion of sarcoplasmic reticulum with SKF 525-A actually enhanced this calcium release (Table VI and Refs. 4, 33).

The physiological role of these types of calcium release is not clear [3,4,9]. Recent studies by Miyamoto and Racker [22] and Stephenson [29] have envisaged a physiological role for the calcium-induced calcium release in skeletal muscle, whereas other investigators [3,16,34] have suggested that surface potential perturbations might modulate calcium release. Tetraphenylboron is thought to act by altering sarcoplasmic reticulum membrane surface potential [16], and tetraphenylboron-induced calcium release is blocked by Ruthenium red * (Table V). Ruthenium red, an exavelent cation, might antagonize the surface potential change brought about by tetraphenylboron [35]. It remains to be ascertained whether calcium-induced calcium release, which is similarly inhibited by Ruthenium red (Table IV), occurs through the same pathway as tetraphenylboron-induced calcium release.

Excitation-contraction coupling in dystrophic mouse muscle

Studies of muscle contractile behavior in dystrophic mouse have implicated a defect in the excitation-contraction coupling in murine muscular dystrophy [2]. Such a defect might involve (A) generation and propagation of the surface membrane action potential, (B) signal transmission through the transverse tubule-sarcoplasmic reticulum junction, (C) release and/or uptake of calcium by the sarcoplasmic reticulum, or (D) the contractile apparatus. Indeed, Kerr and Sperelakis [36] have recently reported normal surface membrane potentials, but decreased specific membrane resistivity and depressed action potential maximum rates of rise in ultrastructurally normal muscle fibers from dystrophic mice.

Isolated sarcoplasmic reticulum vesicles from dystrophic mice have been shown to have normal calcium pump function [5], and sarcoplasmic reticulum from normal and dystrophic mice are similar in lipid composition [37]. In the present study, we find essentially similar calcium release characteristics in sarcoplasmic reticulum of normal and dystrophic mice sarcoplasmic reticulum under a variety of experimental conditions. Thus, a biochemical basis for the reported alterations of muscle contractile behavior in dystrophic mouse has not been demonstrable in the sarcoplasmic reticulum under the experimental conditions used. If a membrane defect does exist in murine dystrophy, it may be localized in the surface membranes; the plasma membrane, the transverse tubule, or both [36].

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* The possibility that Ruthenium red exerts its action by direct precipitation of tetraphenylboron was ruled out for the following reasons: (a) the absorbance spectrum (200–800 nm) of Ruthenium red does not change upon addition of tetraphenylboron; (b) the light scattering (measured at 300 nm) of a solution containing 5 mM sodium phosphate/100 mM NaCl/20 mM sodium Hepes (pH 7.0) and 20 μM Ruthenium red, does not increase upon addition of 100 μM tetraphenylboron.

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