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# Effects of dantrolene sodium on intracellular $Ca^{2+}$-handling in normal and $Ca^{2+}$-overloaded cardiac muscle

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**Abstract**

We investigated the effects of dantrolene sodium on intracellular $Ca^{2+}$ homeostasis in normal and $Ca^{2+}$ overloaded rat cardiac muscle. In isometrically contracting rat papillary muscles loaded with the $Ca^{2+}$ indicator aequorin, dantrolene (50 μM) produced a mild negative inotropic effect (28 ± 1.8 to 21 ± 1.1 mN/mm$^2$; mean ± S.E.; $n = 6$; $P < 0.01$), which was paralleled by a decrease in peak systolic $[Ca^{2+}]_i$ (0.81 ± 0.04 to 0.67 ± 0.04 μM; $P < 0.01$). In isolated cardiac sarcoplasmic reticulum, dantrolene (50 μM) increased the initial $Ca^{2+}$ uptake rate by 23% as compared to control preparations (at pCa 6.2: 46.9 ± 1.6 to 61.1 ± 2.2 nmol/mg per min; $n = 4$; $P < 0.001$). Intracellular $Ca^{2+}$ overload was provoked in isoproterenol-pretreated (100 μM) preparations with $[Ca^{2+}]_o = 5.0$ mM at a stimulation rate of 1.0 Hz ($n = 12$). Diastolic $Ca^{2+}$ oscillations and aftercontractions increased mean diastolic $[Ca^{2+}]_i$ (0.33 ± 0.1 to 0.56 ± 0.1 μM) and tension (9.5 ± 1.8 to 15.3 ± 2.1 mN/mm$^2$), respectively. Addition of dantrolene (50 μM) reduced the amplitude of $Ca^{2+}$ oscillations and aftercontractions; mean diastolic $[Ca^{2+}]_i$ decreased to 0.44 ± 0.1 μM and diastolic tension to 13.5 ± 2.2 mN/mm$^2$. We conclude, therefore, that dantrolene sodium modifies $Ca^{2+}$ handling by the myocardial sarcoplasmic reticulum, an effect that might be useful in cardiac disorders with impaired $[Ca^{2+}]_i$ homeostasis.

*Keywords:* $Ca^{2+}$; Myocardium; Dantrolene; Sarcoplasmic reticulum; $Ca^{2+}$ indicators; Aequorin

## 1. Introduction

Dysregulation of intracellular $Ca^{2+}$-homeostasis plays an important role in the pathophysiology of heart disease. In failing myocardium contractile dysfunction and arrhythmogenecity appear to be closely associated with abnormal intracellular $Ca^{2+}$-handling (Beuckelmann et al., 1992; Morgan et al., 1990). Ischemia and reperfusion both induce intracellular $Ca^{2+}$ overload which predisposes the heart to delayed aftercontractions, triggered activity and tachyarrhythmias (January and Fozzard, 1988; Kihara et al., 1989). Several sarcolemmal mechanisms including the L-type $Ca^{2+}$-channel, the $Na^+$ $Ca^{2+}$ exchanger and the sarcolemmal $Ca^{2+}$ pump serve as integral parts of the control system for regulating the free cytosolic $Ca^{2+}$-concentration $[Ca^{2+}]_i$. In addition, a major contribution to $[Ca^{2+}]_i$-regulation is provided by the sarcoplasmic reticulum. In beating mammalian ventricular myocardium, $Ca^{2+}$ release and uptake by the sarcoplasmic reticulum essentially determine the amplitude and time course of the $Ca^{2+}$-transient that initiates myofilament interaction (Wier, 1990). A large number of biochemical and physiological studies suggest that impaired $Ca^{2+}$-homeostasis in failing as well as ischemic-reperfused myocardium is related to abnormal $Ca^{2+}$-handling involving the sarcoplasmic reticulum (Gwathmey et al., 1987; Krause et al., 1989; Meissner and Morgan, 1995). Specific pharmacological modifications of sarcoplasmic reticulum function in cardiac muscle are limited to a few experimental agents like ryanodine which has been shown to antagonize detrimental sequelae of $Ca^{2+}$ overload in ex vivo animal models (Thandroyen et al., 1988). These data suggest a pharmacological rationale for treating $Ca^{2+}$ overload conditions by way of the sarcoplasmic reticulum. However, this concept remains tentative as there is currently no clinically applicable drug with a proven and specific site of action at the level of the sarcoplasmic reticulum in cardiac muscle.

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Dantrolene sodium (1-[[[5-(4-nitrophenyl)-2-furanyl] methylene] amino]-2,4-imidazolidinedione sodium salt hydrate) was synthesized in 1967 by Snyder et al. (1967) as a skeletal muscle relaxant. A few years later the beneficial effects of dantrolene in the treatment of malignant hyperthermia became apparent. Malignant hyperthermia is a potentially fatal genetic myopathy that occurs if susceptible individuals are exposed to volatile anaesthetic agents, and primarily involves the mechanism by which $Ca^{2+}$ is released from the sarcoplasmic reticulum, i.e., $Ca^{2+}$-induced $Ca^{2+}$ release, resulting in an increase of the free ionized $[Ca^{2+}]_i$ to toxic levels (Ellis and Heffron, 1985). This results in a hypermetabolic state with rigidity and fever. In skeletal muscle, dantrolene has been shown to inhibit $Ca^{2+}$-induced $Ca^{2+}$ release from the sarcoplasmic reticulum (Ohta et al., 1990; Suarez-Isla et al., 1986) and, in this way, to ameliorate intracellular $Ca^{2+}$ overload in malignant hyperthermia (Lopez et al., 1992).

Based upon the discussion above, it is reasonable to speculate that a clinically applicable drug that specifically modifies sarcoplasmic reticulum $Ca^{2+}$ handling in cardiac muscle may have clinical utility. In cardiac muscle, dantrolene has been found to exert a mild negative inotropic effect (Honerjaeger and Alischewski, 1983). The site of action is still unclear but might involve sarcoplasmic and/or sarcolemmal $Ca^{2+}$-channels (Davidenko et al., 1986; Honerjaeger and Alischewski, 1983). In order to clarify the mode of action of dantrolene sodium in cardiac muscle, measurements of free intracellular $[Ca^{2+}]$ and isolated sarcoplasmic reticulum $Ca^{2+}$ uptake were performed in rat heart preparations. The study further attempted to test the drug in a model of intracellular $Ca^{2+}$ overload in cardiac muscle.

## 2. Materials and methods

### 2.1. Isolated muscle performance

Male Wistar rats with a body weight of 350–400 g were used for the experiments. During deep anaesthesia with diethyl ether the heart was rapidly excised and placed in an oxygenized dissecting chamber containing a modified Krebs-Henseleit solution at room temperature. Left ventricular papillary muscles were dissected free and fixed at their muscular end with a spring clip to a muscle holder. The tendinous end was tied to a 6-0 silk suture and connected to a strain-gauge tension transducer (model MBL 341, Sensotec, Columbus, OH, USA). The preparation was placed in a tissue bath containing a modified Krebs-Henseleit solution of the following composition (mM): NaCl 120, KCl 5.9, $NaH_2PO_4$ 1.2, $CaCl_2$ 1.0, $NaHCO_3$ 25, $MgCl_2$ 1.2 and dextrose 5.5. The solution was continuously bubbled with 95% $O_2$/5% $CO_2$ at 30°C giving a pH of 7.4. Stimulation of the muscle was elicited by 5 ms square wave pulses at a rate of 0.33 Hz applied by a punctate platinum electrode at the base of the muscle. The voltage was set at approximately 10% above threshold level. The muscle contracted isometrically for an equilibration period of 30 min and was then carefully stretched to the length at which maximal tension development occurred. At the end of each experiment, the muscles were blotted and weighed. The cross-sectional area was determined from muscle weight and length by assuming a uniform cross section and a specific gravity of 1.05.

### 2.2. Aequorin signal measurements

Aequorin was loaded by the interstitial macroinjection technique into the papillary muscle (Kihara and Morgan, 1989). Optimal conditions for this procedure were obtained by lowering the temperature to 20°C and the $Ca^{2+}$ concentration to 0.3 mM in the tissue bath. Stimulation was stopped when developed tension had declined to 50% of baseline value. Subsequently, the preparation was raised from the bath and 1–2 μl aequorin solution (1 mg/ml) was injected under the epimysium at the base of the muscle with a short-shank low-resistance glass micropipette. The muscle was then returned into the bath which was slowly rewarmed to 30°C. In parallel, calcium was gradually increased to give a final concentration of 1.0 mM and stimulation was re-started at a rate of 0.33 Hz. Aequorin light signals were detected with a light-collecting apparatus as designed by Blinks (1982) which allowed for the simultaneous measurement of isometric tension. The output of the photomultiplier tube was connected to a photon counter (model C 10, Thorn EM I, Gencom, Fairfield, NJ, USA). Analog signals from the force transducer and photon counter were documented with a physiological recorder (Gould Instruments, Cleveland, OH, USA) and stored on videotype (JVC model 420 H, A.R. Vetter, Rebersburg, PA, USA). In addition to beat-to-beat analyses, the data from 20–40 steady state light signals were averaged (model No. 4562, Nicolet Instruments, Madison, WI, USA) for quantitative measurements.

### 2.3. Quantitation of $Ca_i^{2+}$

$[Ca^{2+}]_i$ was estimated by relation the recorded light signals ($L$) to the amount of light emitted after the lysis of the muscle membranes ($L_{max}$) during exposure to a solution containing the detergent Triton X-100 (5%) and 50 mM $[Ca^{2+}]_o$ at the end of the experiment (Kihara and Morgan, 1989). The normalized light signal was then converted to $[Ca^{2+}]_i$ using an in vitro calibration curve of the following form:

$$L/L_{max} = \left( \frac{(1 + K_R[Ca^{2+}])}{1 + K_R + K_{TR}[Ca^{2+}]} \right)^3$$

were $K_R$ and $K_{TR}$ are model constants.

### 2.4. Dantrolene and calcium dose-response determinations

Steady state conditions were observed for at least 30 min after the intracellular aequorin light signal had stabilized. Preliminary experiments confirmed the well-known, dose-independent (Honerjaeger and Alischewski, 1983) negative inotropic effect of dantrolene. Therefore, a single dose of 50 μM dantrolene was tested throughout the study. Baseline measurements for isometric force and intracellular light signal were checked to be constant for at least 15 min prior to addition of dantrolene.

Calcium dose-response curves for developed tension were obtained by the same protocol. In two separate groups, steady state conditions were observed in the absence or presence of dantrolene (50 μM; preincubation for 20 min) for at least 15 min. Subsequently, $[Ca^{2+}]_o$ was increased cumulatively from 0.5 to 8.0 mM and developed tension was determined at each $[Ca^{2+}]_o$ during steady-state conditions which occurred within 10 min.

### 2.5. $Ca^{2+}$ overload

Intracellular $Ca^{2+}$ overload was induced by a controlled, step-wise protocol starting with a gradual increase of the stimulation rate from 0.33 to 1.0 Hz at an extracellular $Ca^{2+}$ concentration of 1.0 mM. This resulted in a reduction of developed tension by approximately 30% due to the negative force-frequency relationship in rat cardiac muscle. Subsequently, isoproterenol was added cumulatively in concentrations of $1 \times 10^{-7}$, $1 \times 10^{-5}$ and finally $1 \times 10^{-4}$ M.

At this point, increasing the extracellular $Ca^{2+}$ concentration from 1.0 to 5.0 mM provoked the typical characteristics (mechanical aftercontractions, diastolic $Ca^{2+}$-oscillations) of intracellular $Ca^{2+}$ overload within 3 min which documented at 5 min. At this point dantrolene (50 μM) was added in the drug group whereas no drug was added in the control group. Aequorin light signals and mechanical parameters were recorded simultaneously throughout this protocol as described above.

### 2.6. Biochemical assays

Microsomal preparations enriched with sarcoplasmic reticulum vesicles were prepared by a slightly modified procedure as described previously (Kranias and Solaro, 1982). Briefly, during deep anaesthesia with diethyl ether and injection of 200 IU heparin into a femoral vein, the hearts were quickly removed and placed in ice-cold (0°C) 0.9% NaCl solution. All subsequent steps were performed on ice. After removal of the large vessels and atria the hearts were minced with cold scissors in 15 ml of an ice-cold solution containing 30 mM imidazole, 300 μM phenylmethylsulfonyl fluoride (PMSF), 10 μM dithiothreitol (DTT), 0.3 M sucrose at a pH of 7.0 (medium I). The tissue was homogenized with a motorized Teflon pestle at 500 rpm for 20 passes with a rest period of 1 min after the first 10 passes. The homogenate was centrifuged at $4300 \times g$ for 10 min. The resulting supernatant fraction was filtered through four layers of cheesecloth, and the filtrate was centrifuged at $143\,000 \times g$ for 30 min. The pellet was resuspended in medium II (medium I plus 0.6 M

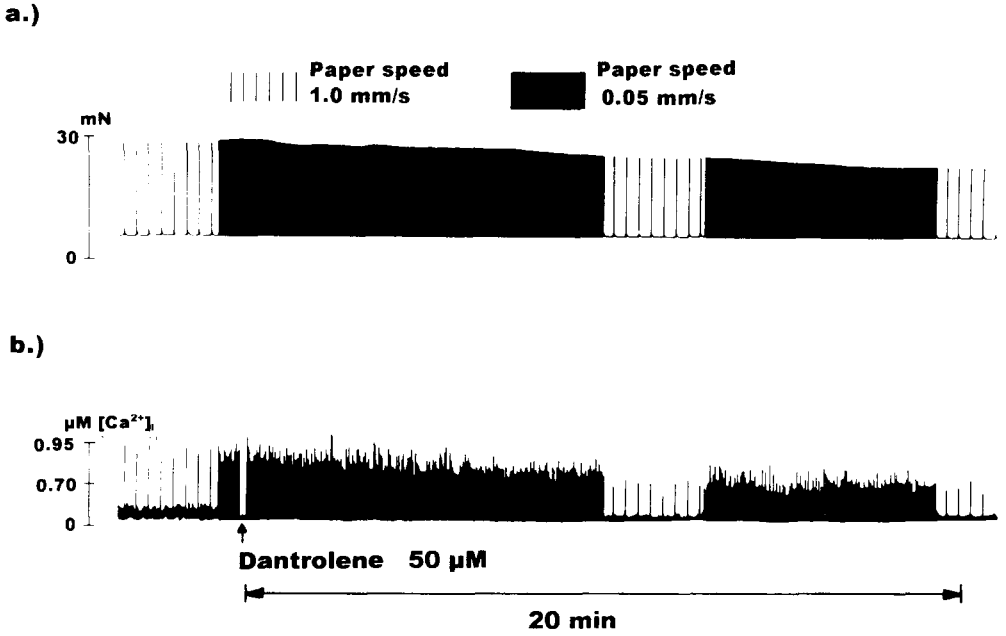


Fig. 1. Continuous chart-strip recording of (a) isometric force and (b) intracellular aequorin light signal in rat papillary muscle. Dantrolene (50 μM) was added to the tissue bath as indicated by the arrow. The negative inotropic effect was accompanied by a parallel decrease in systolic $[Ca^{2+}]_i$. $[Ca^{2+}]_o$ = 1.0 mM, 0.3 Hz, 30°C.

Table 1
Effects of dantrolene sodium on isometric force and intracellular $Ca^{2+}$ concentration in rat papillary muscle

| | Control | Dantrolene (50 μM) | |
|---|---|---|---|
| Developed tension ($mN/mm^2$) | 28 ± 1.8 | 21 ± 1.1 | $P < 0.01$ |
| Diastolic tension ($mN/mm^2$) | 9.4 ± 1.0 | 8.9 ± 0.8 | $P = 0.07$ |
| Peak sys. $[Ca^{2+}]_i$ (μM) | 0.81 ± 0.04 | 0.67 ± 0.04 | $P < 0.01$ |
| Resting $[Ca^{2+}]_i$ (μM) | 0.33 ± 0.01 | 0.28 ± 0.02 | $P = 0.012$ |

$[Ca^{2+}]_o$ = 1.0 mM, 0.3 Hz, 30°C. Values are mean ± S.E.

KCl) and centrifuged at 143 000 × *g* for 45 min. The resulting pellet was then washed in medium I for 30 min at 143 000 × *g*, and the final pellet was resuspended in a solution containing 30 mM imidazole, 300 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.3 M sucrose, 0.1 M KCl at a pH of 7.0. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

### 2.7. $Ca^{2+}$ uptake measurements

Oxalate-supported calcium uptake was measured at 37°C in a total volume of 1.5 ml using $^{45}Ca$ and a modification of the millipore filtration technique (Martonosi and Feretos, 1966). The rate of $Ca^{2+}$ uptake was measured in a medium containing 0.05–0.1 mg sarcoplasmic reticulum protein per ml of reaction mixture which included: 40 mM imidazole-HCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM $NaN_3$, 5 mM potassium oxalate, 0.5 mM EGTA, and various concentrations of $CaCl_2$ to yield 0.01–10 μM free ionized $Ca^{2+}$ at a pH of 7.0. The free $Ca^{2+}$ concentrations at pH 7.0 were calculated using a computer program (Robertson and Potter, 1984) and the apparent $Ca^{2+}$-EGTA association constant previously reported (Martell and Smith, 1974). Calcium uptake was initiated by the addition of ATP (5 mM). $Ca^{2+}$ uptake rates were linear up to 5 min at 37°C. The rate of $Ca^{2+}$ uptake was calculated from the linear regression of calcium uptake determined at 0.5, 1.0 and 1.5 min after initiation of uptake. In experiments with ruthenium red to block the sarcoplasmic reticulum release channel (Feher et al., 1988), a concentration of 20 μM of the agent was added immediately prior to starting the reaction. In contrast, dantrolene (50 μM) was preincubated with the cardiac sarcoplasmic reticulum preparation for 15 min at 37°C.

### 2.8. Chemicals

Ryanodine (Calbiochem, San Diego, CA, USA) was dissolved in deionized water at a concentration of 0.5 mM. The concentration of the stock ruthenium red (Sigma, St. Louis, MO, USA) was determined by the absorbance at 533 nm with an extinction coefficient of $6.16 \times 10^{-4}$ $M^{-1}$ (Feher et al., 1988). For biochemical studies, dantrolene sodium (Sigma) was dissolved immediately before use in propylene glycol and diluted in the test solution. The final concentration of propylene glycol did not exceed 0.5% and did not affect the $Ca^{2+}$ release and $Ca^{2+}$ uptake mechanisms of the cardiac sarcoplasmic reticulum. In the papillary muscle preparations, dantrolene sodium was added in aqueous solution (deionized water) to give a concentration of 50 μM in the tissue bath. Aequorin was purchased from the laboratory of Dr. John R. Blinks (Friday Harbor Laboratories, Friday Harbor, WA, USA).

### 2.9. Statistical analysis

Results are reported as means ± S.E. Data were compared using unpaired and paired (when appropriate) Student's *t*-tests.

Statistical significance was set at $P < 0.05$.

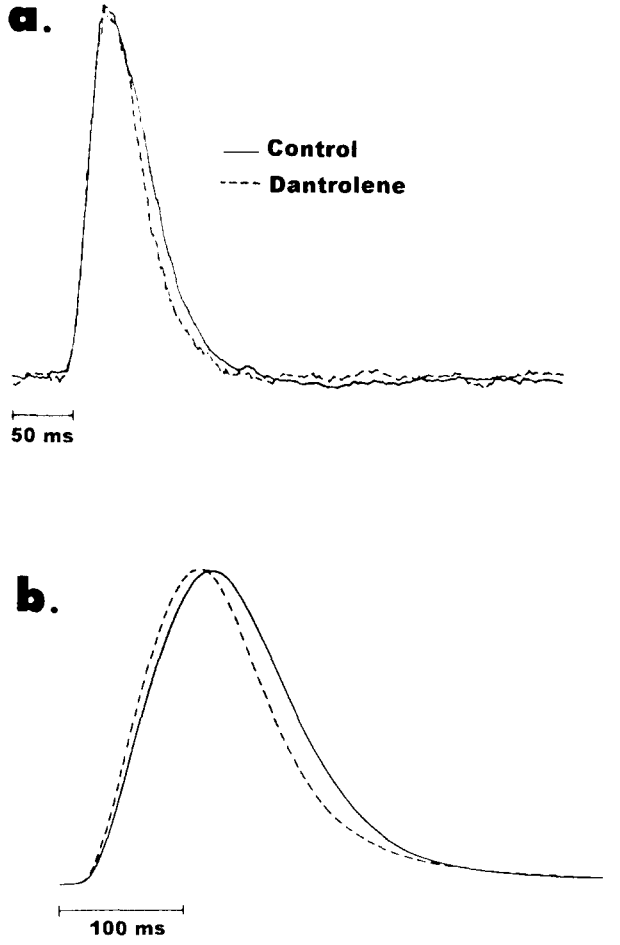


Fig. 2. Time-course of the (a) intracellular aequorin light transient and (b) isometric twitch before and after dantrolene (50 μM) administration. $[Ca^{2+}]_o$ = 1.0 mM, 0.3 Hz, 30°C.

## 3. Results

### 3.1. Dantrolene effects on isometric tension and $[Ca^{2+}]_i$

Dantrolene (50 μM) induced a mild negative inotropic effect which resulted in a reduction of developed tension from 28 ± 1.8 to 21 ± 1.1 mN/mm$^2$ ($n = 6$; $P < 0.01$). In parallel, peak intracellular systolic $Ca^{2+}$ concentration declined from 0.81 ± 0.04 to 0.67 ± 0.04 μM ($P < 0.01$). Resting diastolic tension was reduced by a small amount and was accompanied by a slight decrease in diastolic $Ca^{2+}$ concentration (Table 1). All of these effects were slow in onset and reached a steady-state only 20 min after addition of dantrolene (Fig. 1).

The time courses of the isometric twitch and intracellular light transient demonstrated minor changes after dantrolene administration (Fig. 2). Except for the time to peak tension (control: 114 ± 4.6; dantrolene: 105 ± 5.2 ms; $P < 0.05$), all other parameters including the time to 90% decline from peak tension (control: 152 ± 3.3; dantrolene: 137 ± 7.9 ms; $P = 0.10$), time to peak light (control: 40 ± 1.7; dantrolene: 37 ± 1.1 ms; $P = 0.14$) and time to 90% decline from peak light (control: 75 ± 1.7; dantrolene: 74 ± 3.1 ms; $P = 0.26$) did not change significantly.

### 3.2. Calcium dose-response curves

$Ca^{2+}$ dose-response curves were determined in two separate groups without ($n = 5$) and with ($n = 5$) addition of dantrolene (50 μM; preincubation for 20 min). At baseline (1.0 mM $Ca^{2+}$, no drug), both groups were comparable in terms of the absolute amount of developed tension (29 ± 2.8 and 31 ± 2.6 mN/mm$^2$, respectively; $P = 0.36$).

In the presence of dantrolene, there was a marked shift in the steep part of the $Ca^{2+}$ dose-response curve to the right (Fig. 3). However, the maximal inotropic responses to $Ca^{2+}$ which were reached at 4.0 mM $Ca^{2+}$ in the control group (43 ± 1.7 mN/mm$^2$) and at 6.0 mM $Ca^{2+}$ in the dantrolene group (47 ± 2.7 mN/mm$^2$) were not different ($P = 0.11$). Thus, the negative inotropic effect of dantrolene could be completely antagonized by elevating the extracellular $Ca^{2+}$ concentration.

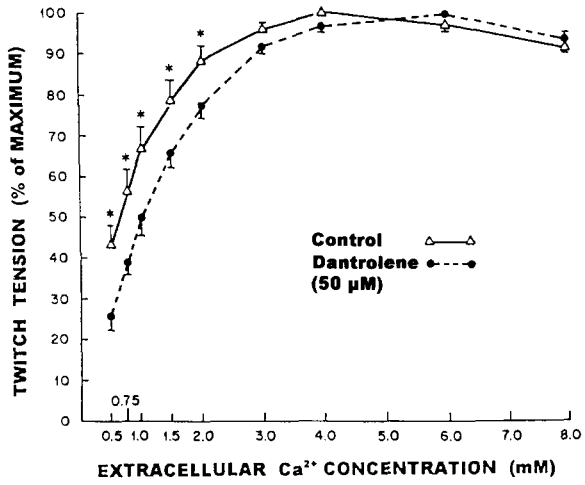


Fig. 3. $Ca^{2+}$ dose–response curves in rat papillary muscle with and without addition of dantrolene (50 μM). 0.3 Hz, 30°C, * $P < 0.05$, $n = 5$.

### 3.3. Dantrolene effects on intracellular $Ca^{2+}$ overload

The increase of stimulation rate from 0.3 to 1.0 Hz led to a fall of developed tension from 26 ± 1.4 to 19.1 ± 1.1 mN/mm$^2$ and of systolic $[Ca^{2+}]_i$ from 0.79 to 0.67 μM due to the negative force-frequency relationship in rat cardiac muscle. Diastolic $[Ca^{2+}]_i$ was unchanged at 0.32 μM. Addition of isoproterenol ($10^{-7}$, $10^{-5}$, $10^{-4}$ M) increased developed tension to 21.5, 25.2 and 28.1 mN/mm$^2$. In parallel, systolic $[Ca^{2+}]_i$ rose to 0.72, 0.92, and 1.00 μM. Diastolic $[Ca^{2+}]_i$ was unaltered at 0.31 μM.

Initiation of $Ca^{2+}$ overload following elevation of $[Ca^{2+}]_o$ to 5.0 mM in these isoproterenol pretreated preparations resulted in a further rise of systolic $[Ca^{2+}]_i$ from 1.00 ± 0.10 to 1.21 ± 0.10 μM ($P < 0.05$). In contrast, developed tension declined from 28 ± 2.7 to 22 ± 1.8 mN/mm$^2$ ($P < 0.05$; Fig. 4). The diastolic light signal was transformed from a straight to an oscillatory course which corresponded to the appearance of mechanical after-contractions (Fig. 5). Therefore, the *mean* diastolic $[Ca^{2+}]_i$ had to be determined which rose from 0.31 ± 0.10 to 0.54 ± 0.20 μM ($P < 0.01$) and was paralleled by an increase of diastolic tension from 9.3 ± 1.4 to 14.9 ± 2.6 mN/mm$^2$ ($P < 0.01$). All measurements were taken 5 min

Table 2
Isometric force and intracellular $Ca^{2+}$ concentration in $Ca^{2+}$ overloaded rat papillary muscle

| | Control 0 min | $Ca^{2+}$ overload 5 min | $Ca^{2+}$ overload + dantrolene 20 min |
|---|---|---|---|
| Developed tension (mN/mm$^2$) | 27 ± 4.2 | 21 ± 4.7 | 20 ± 4.5 |
| Diastolic tension (mN/mm$^2$) | 9.5 ± 1.8 | 15.2 ± 2.1 | 13.5 ± 2.2 [a] |
| Systolic $[Ca^{2+}]_i$ (μM) | 1.07 ± 0.1 | 1.28 ± 0.2 | 0.96 ± 0.1 [a] |
| Diastolic $[Ca^{2+}]_i$ (μM) | 0.33 ± 0.1 | 0.56 ± 0.1 | 0.44 ± 0.1 [a] |

The preparations were pretreated with 100 μM isoproterenol and stimulated at 1.0 Hz. $Ca^{2+}$ overload was induced by elevation of $[Ca^{2+}]_o$ from 1.0 to 5.0 mM and was documented at 5 min. Dantrolene (50 μM) was added at 5 min after induction of $Ca^{2+}$ overload and the effect was documented at 20 min.
[a] $P < 0.05$ refers to 20 min versus 5 min values, $n = 6$, mean value ± S.E.

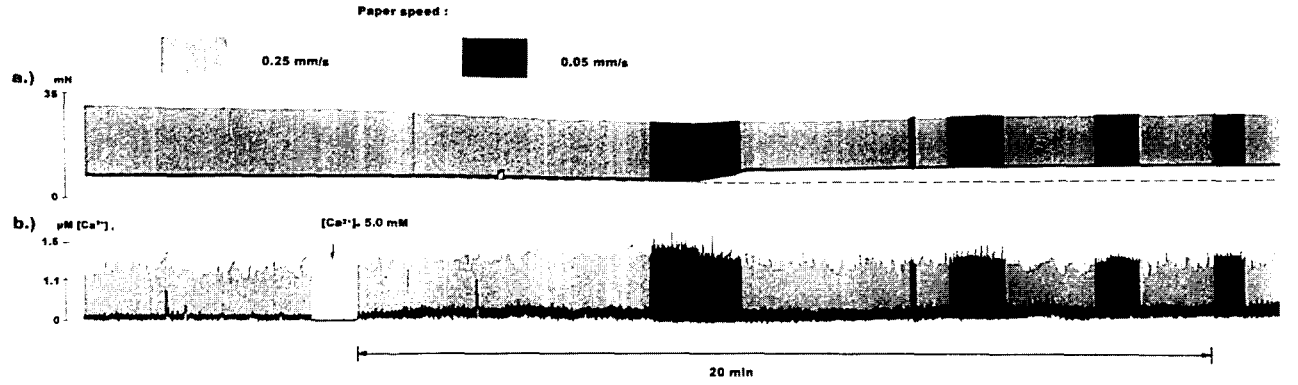


Fig. 4. Continuous chart-strip recording of (a) isometric force and (b) intracellular aequorin light signal in $Ca^{2+}$ overloaded rat papillary muscle. The preparation was pretreated with 100 μM isoproterenol and stimulated at 1.0 Hz. $Ca^{2+}$ overload was induced by elevating $[Ca^{2+}]_o$ from 1.0 to 5.0 mM (arrow).

after initiation of $Ca^{2+}$ overload and did not change significantly over a 15 min period in the control group. In a second group, comparable changes were observed after 5 min of $Ca^{2+}$ overload (Table 2). Addition of dantrolene (50 μM) reduced systolic $[Ca^{2+}]_i$ from $1.28 \pm 0.20$ to $0.96 \pm 0.10$ μM after 15 min in this group ($P < 0.05$; $n = 6$; Fig. 6) while developed tension did not change ($21 \pm 4.7$ to $20 \pm 4.5$ mN/mm$^2$; $P = 0.24$). Diastolic light oscillations and mean diastolic $[Ca^{2+}]_i$ ($0.56 \pm 0.10$ to $0.44 \pm 0.10$ μM; $P < 0.05$) were also reduced. In parallel, mechanical aftercontractions declined in amplitude (Fig. 7) and diastolic tension decreased from $15.2 \pm 2.1$ to $13.5 \pm 2.2$ mN/mm$^2$ ($P < 0.05$).

### 3.4. Dantrolene effects on $Ca^{2+}$ uptake in isolated sarcoplasmic reticulum vesicles

In vitro experiments with isolated sarcoplasmic reticulum vesicles demonstrated the effect of dantrolene on cardiac sarcoplasmic reticulum $Ca^{2+}$ handling. Dantrolene (50 μM) significantly increased the initial rate of $Ca^{2+}$ uptake by approximately 23% as compared to $Ca^{2+}$ uptake in the absence of the drug (at pCa 6.0: $48.6 \pm 1.6$ to $61.1 \pm 2.2$ nmol/mg per min; $P < 0.05$; Table 3). Parallel experiments with ruthenium red (20 μM), which is known to provide complete closure of the sarcoplasmic reticulum release channel (Feher et al., 1988), showed a 70% increase of the initial rate of sarcoplasmic reticulum $Ca^{2+}$ uptake. The effect of dantrolene on sarcoplasmic reticulum $Ca^{2+}$ activity was not $Ca^{2+}$ dependent as assessed by $Ca^{2+}$ uptake measurements performed at different free $Ca^{2+}$ concentrations (Table 3).

## 4. Discussion

The present study provides for the first time immediate evidence for the intracellular $Ca^{2+}$-modulating effect of dantrolene sodium in cardiac muscle. Systolic force generation and peak systolic $[Ca^{2+}]_i$ declined gradually and in parallel before reaching a steady state after approximately 20 min. Previous investigators reported a similar time course and extent of the negative inotropic effect in several species which appears to be dose-independent (Honerjaeger and Alischewski, 1983; Salata et al., 1983). However, only

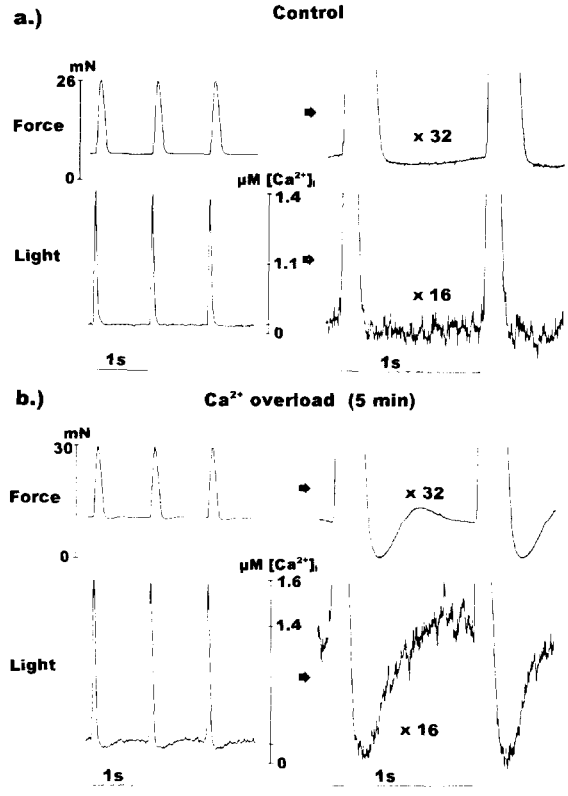


Fig. 5. Isometric force and intracellular aequorin light signal during (a) control conditions and (b) 5 min after induction of intracellular $Ca^{2+}$ overload. The preparation was pretreated with 100 μM isoproterenol and stimulated at 1.0 Hz. $Ca^{2+}$ overload was induced by elevating $[Ca^{2+}]_o$ from 1.0 to 5.0 mM. Averaged signals of 20 consecutive beats. Arrows indicate magnification of tension (×32) and light (×16).

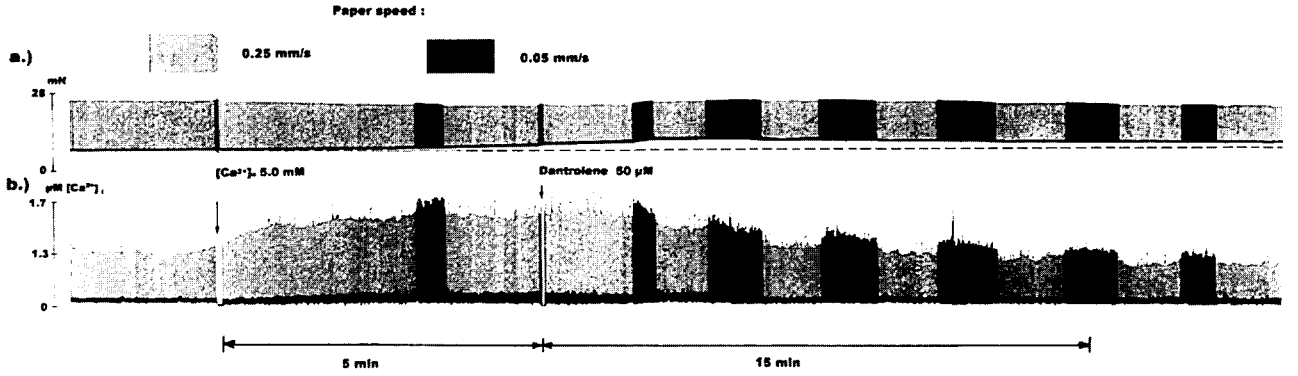


Fig. 6. Continuous chart-strip recording of (a) isometric force and (b) intracellular aequorin light signal in $Ca^{2+}$ overloaded rat papillary muscle. The preparation was pretreated with 100 μM isoproterenol and stimulated at 1.0 Hz. $Ca^{2+}$ overload was induced by elevating $[Ca^{2+}]_o$ from 1.0 to 5.0 mM (arrow). Dantrolene was added as indicated 5 min after induction of $Ca^{2+}$ overload.

indirect evidence about the underlying mechanism of action in cardiac muscle has been available.

Dantrolene did not diminish slow response action potentials in cat (Salata et al., 1983) or guinea pig ventricular muscle (Honerjaeger and Alischewski, 1983), arguing against an inhibitory effect on sarcolemmal L-type $Ca^{2+}$ channels. In patients with AV nodal reentry tachycardia dantrolene was also shown to exert no calcium channel blocking action on the cardiac conduction system (Kentsch et al., 1991). In contrast, electrophysiological parameters in canine Purkinje fibers and rabbit sinoatrial as well as atrioventricular nodes were modified by dantrolene in a way similar to that of typical calcium channel blocking agents (Salata and Jalife, 1982; Davidenko et al., 1986). The $Ca^{2+}$ dose-response curves in our study suggest a purely $Ca^{2+}$ antagonistic effect which could involve both sarcolemmal and/or sarcoplasmic mechanisms. Biochemical and single channel studies in cardiac sarcoplasmic reticulum preparations regarding possible effects of dantrolene are not available from the literature. Therefore, our finding that dantrolene increased the initial $Ca^{2+}$ uptake rate of isolated cardiac sarcoplasmic reticulum by 23% provides unique support for the concept of a sarcoplasmic site of action in cardiac muscle. As dantrolene acts as a negative inotropic agent in cardiac muscle and has a negligible effect on sarcoplasmic reticulum $Ca^{2+}$ ATPase activity in skeletal muscle (White et al., 1983), the most probable mechanism involved is sarcoplasmic reticulum $Ca^{2+}$ release. This hypothesis suggests an as yet unspecified modification of the sarcoplasmic reticulum $Ca^{2+}$ release channel which translates into reduced $Ca^{2+}$ release with a subsequent decrease of the intracellular $Ca^{2+}$ transient and systolic force generation. In quantitative terms, the observed changes in sarcoplasmic reticulum $Ca^{2+}$ uptake, amplitude of the $Ca^{2+}$ transient and twitch tension in response to dantrolene were very similar in magnitude in this study indicating a functional cause–effect relationship. Nevertheless, as experimental studies looking for dantrolene effects on isolated sarcolemmal preparations are still missing, an additional $Ca^{2+}$ channel antagonist mode of action cannot be excluded from a theoretical point of view. Modifications of myofilament interaction, however, seems to be not involved as the $Ca^{2+}$ sensitivity of the contractile proteins in skeletal muscle has been found to be unchanged in the presence of dantrolene (Brocklehurst, 1975).

In comparison, the mechanism of action for dantrolene seems to be strikingly similar in skeletal and cardiac muscle. Dantrolene has been shown to be an inhibitor of $Ca^{2+}$ release from isolated skeletal sarcoplasmic reticulum vesicles (Danko et al., 1985; Ohta et al., 1990). In addi-

Table 3
Effects of ruthenium red and dantrolene sodium on the initial rate of $Ca^{2+}$ uptake by isolated cardiac sarcoplasmic reticulum

| pCa | Calcium uptake (nmol/mg per min) in the absence (-) versus presence (+) of 20 μM ruthenium red | | | Calcium uptake (nmol/mg per min) in the absence (-) versus presence (+) of 50 μM dantrolene | | |
|---|---|---|---|---|---|---|
| | 20 μM ruthenium red (nmol/mg per min) | | -fold increase in rate of calcium uptake in presence of drug | 50 μM dantrolene (nmol/mg per min) | | -fold increase in rate of calcium uptake in presence of drug |
| | − | + | | − | + | |
| 6.2 | 26.49 ± 0.43 | 43.07 ± 0.75 [b] | 1.63 ± 0.05 | 24.64 ± 0.52 | 30.42 ± 0.96 [a] | 1.23 ± 0.02 |
| 6.0 | 51.74 ± 0.50 | 97.42 ± 2.57 [b] | 1.88 ± 0.04 | 46.86 ± 1.65 | 61.14 ± 2.19 [a] | 1.22 ± 0.06 |
| 5.5 | 110.30 ± 0.75 | 186.09 ± 2.47 [b] | 1.69 ± 0.01 | 99.24 ± 3.02 | 123.06 ± 5.12 [a] | 1.23 ± 0.03 |

[a] $P < 0.01$, $n = 4$, mean value ± S.E. [b] $P < 0.001$, $n = 4$, mean value ± S.E.

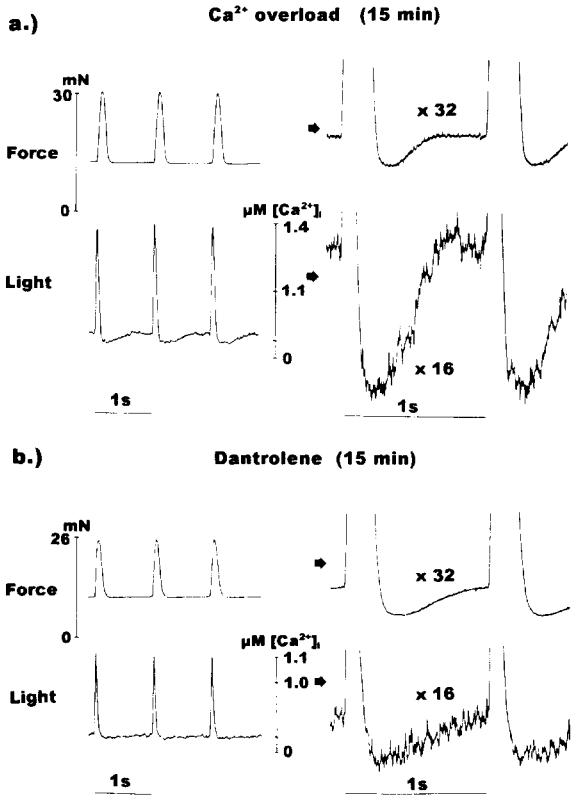


Fig. 7. Isometric force and intracellular aequorin light signal during $Ca^{2+}$ overload (a) in an isochronic control preparation and (b) 15 min after dantrolene administration (50 μM). The preparations were pretreated with 100 μM isoproterenol and stimulated at 1.0 Hz. $Ca^{2+}$ was induced by elevating $[Ca^{2+}]_o$ from 1.0 to 5.0 mM. Averaged signals of 20 consecutive beats. Arrows indicate magnification of tension (×32) and light (×16).

tion, the drug has been demonstrated to block the conductance of $Ca^{2+}$ channels in purified skeletal sarcoplasmic reticulum membranes studied with patch-clamp in artificial bilayers (Suarez-Isla et al., 1986). Thus, the skeletal muscle relaxant effect could be attributed to interaction with the sarcoplasmic reticulum $Ca^{2+}$ release channel complex which comprises the ryanodine receptor as an integral part (Takeshima et al., 1989). Malfunction of the sarcoplasmic reticulum $Ca^{2+}$ release mechanism with ensuing elevation of resting myoplasmic free $Ca^{2+}$ concentration in skeletal muscle has been identified as the pathophysiological basis of malignant hyperthermia in swine and man which can be treated successfully by dantrolene sodium (Lopez et al., 1992). It is, therefore, tempting to speculate that dantrolene might have beneficial effects in cardiac $Ca^{2+}$ overload as well.

Intracellular $Ca^{2+}$ overload in cardiac muscle has been defined as an abnormal rise in intracellular free calcium concentration during diastole (Clusin et al., 1983; Kihara and Morgan, 1991). In experimental models, this condition is provoked by lowering or removing $[K^+]_o$, lowering $[Na^+]_o$, raising $[Ca^{2+}]_o$ and exposing the tissue to catecholamines or cardiotonic steroids (January and Fozzard, 1988). Recently, dysregulation of diastolic $[Ca^{2+}]$ homeostasis has been implicated in the pathophysiology of heart failure (Gwathmey et al., 1987) and ischemia-reperfusion (Meissner and Morgan, 1995). Thus, increased diastolic tonus, reduced systolic performance and triggered activity have been proposed to be manifestations of impaired diastolic $[Ca^{2+}]_i$ regulation (Lakatta, 1989). According to this hypothesis, spontaneous $Ca^{2+}$ release from the sarcoplasmic reticulum during diastole is the basic characteristic of $Ca^{2+}$ overload and secondarily induces contractile and electrophysiological dysfunction (Wier and Hess, 1984; Allen et al., 1985).

In our model, $Ca^{2+}$ overload was induced by a combination of rapid pacing, high $[Ca^{2+}]_o$ and catecholamine. In close agreement with other reports (Allen et al., 1985) the onset of $Ca^{2+}$ overload was characterized by an additional increase in peak systolic $[Ca^{2+}]_i$ while systolic force generation paradoxically declined. At the same time, diastolic $Ca^{2+}$-oscillations appeared which correlated temporally with aftercontractions. It has been hypothesized that diastolic $Ca^{2+}$ oscillations result in inhomogeneities of diastolic sarcoplasmic reticulum $Ca^{2+}$ loading, heterogeneous myofilament interaction, different diastolic sarcomere length, and an increase in diastolic tonus (Lakatta, 1989). Subsequently, inhomogeneous systolic $[Ca^{2+}]_i$ levels and reduced systolic force generation will result. Moreover, diastolic $Ca^{2+}$ oscillations due to spontaneous sarcoplasmic reticulum $Ca^{2+}$ release are considered as the cause of a transient inward current ($i_{TI}$) which induces delayed afterdepolarizations and triggered activity (January and Fozzard, 1988). Dantrolene was quite effective in reducing the magnitude of diastolic $Ca^{2+}$ oscillations and aftercontractions and, by this way, decreased diastolic $[Ca^{2+}]_i$ and diastolic tension. In contrast, systolic force generation remained nearly constant despite a significant fall in peak systolic $[Ca^{2+}]_i$. This discrepancy might be due to a still near-maximal activation of the myofilaments at elevated $[Ca^{2+}]_i$ (approx. 1 μM) and/or reduced diastolic inhomogeneities secondary to diminished diastolic oscillations. It should be noted that in the presence of spatiotemporal inhomogeneities estimates of $[Ca^{2+}]_i$ by means of the aequorin method can be erroneously high (Yue and Wier, 1985). Nevertheless, the gradual development and immediate temporal relation to aftercontractions clearly demonstrate that diastolic $Ca^{2+}$ oscillations and an associated increase in diastolic $[Ca^{2+}]_i$ characterized the $Ca^{2+}$ overload state. The converse holds true after dantrolene administration.

Reports about dantrolene effects in cardiac disease with potentially impaired $Ca^{2+}$ homeostasis are limited to a few studies. In an ischemia model in rats, dantrolene significantly reduced the incidence of extrasystoles, ventricular tachyarrhythmias and ventricular fibrillation (Brooks et al., 1989). Furthermore, the drug has been shown to effectively antagonize reperfusion injury in an experimental rat heart model (Mitchell et al., 1993). In humans with malig-

nant hyperthermia, supraventricular and ventricular arrhythmias usually precede the onset of fever and rigidity. If given early in the course of the disease, cardiovascular symptoms including arrhythmias are ameliorated by dantrolene administration (Ward et al., 1986). However, it is unknown whether this effect is related to a specific effect on myocardial $Ca^{2+}$ homeostasis or general relief of malignant hyperthermia.

In summary, this study confirms the negative inotropic effect of dantrolene sodium in cardiac muscle which could be immediately attributed to altered intracellular $Ca^{2+}$ handling. $Ca^{2+}$ uptake studies in isolated cardiac sarcoplasmic reticulum vesicles indicated a modulation of sarcoplasmic reticulum $Ca^{2+}$ pumping activity as the possible mechanism of action which creates a potentially novel approach in the clinical treatment of heart disease. In a pharmacological model of $Ca^{2+}$ overload, dantrolene effectively antagonized diastolic $Ca^{2+}$ oscillations and after-contractions. Thus, this drug might become a valuable investigational and clinical tool for cardiac disorders with impaired $Ca^{2+}$ homeostasis.

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