Dantrolene – *In vitro* studies in malignant hyperthermia susceptible (MHS) and normal skeletal muscle

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Dantrolene sodium, a hydantoin analogue, is efficacious in the therapy of malignant hyperthermia (MH). In order to improve our knowledge of the mode of action of dantrolene, we have examined the influence of dantrolene sodium on:

(1) twitch and resting tensions, in the absence and the presence of caffeine, of intact skeletal muscle fascicles; and

(2) caffeine induced tension rises of single chemically skinned skeletal muscle fascicles.

We have found that dantrolene appears to exert its beneficial action on malignant hyperthermia susceptible (MHS) skeletal muscle by an indirect action on the sarcoplasmic reticulum (SR). Thus dantrolene inhibits twitch tensions of skeletal muscle fascicles, probably by indirectly preventing the release of calcium from the SR. To a lesser extent dantrolene inhibits caffeine induced contractures of skeletal muscle fascicles, probably by indirectly accelerating the uptake of calcium into the SR. Because the former effect is greater than the latter in vivo *dantrolene sodium is effective only when given prior to total loss of calcium from the SR. Vigilant temperature and EKG monitoring of all patients during anaesthesia is, therefore, essential.*

Key words

HYPERTHERMIA: malignant, dantrolene;
MUSCLE SKELETAL: caffeine, twitch tension, contracture, resting tension.

Dantrolene sodium, a drug efficacious in the treatment of acute malignant hyperthermic (MH) reactions, prevents release of calcium from the sarcoplasmic reticulum (SR).[1–18] The ensuing reduction in the concentration of myoplasmic calcium relaxes the myofibrils[19–31] and inhibits heat production.[32–43] These actions of dantrolene have been described in detail in our recent review.[44] Perusal of this article reveals that much of the published data regarding the action of dantrolene on the SR is at least partially contradictory. This may be because of lack of uniform experimental techniques and small sample sizes and variability among species examined. Thus it is not surprising that the results of experiments measuring the influence of dantrolene on twitch and resting tensions of skeletal muscle fascicles isolated from humans, pigs and other species have been often contradictory. We have, therefore, examined the effect of dantrolene on caffeine-augmented twitch tensions and caffeine-induced contractures of both humans and pigs, using many concentrations of both dantrolene and caffeine, and uniform experimental conditions, as nearly as species differences have permitted. In addition, we have investigated the effect of dantrolene on calcium uptake

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into and calcium release from the SR of single chemically skinned skeletal muscle fibres in order to more closely define the exact site and mode of action of dantrolene.

Methods

A *Anaesthetic and surgical techniques*

1 *Rationale*

The anaesthetic agents were chosen because they had all been demonstrated not to induce MH reactions, because none significantly altered the experimental results and finally because of species suitability.

2 *Humans*

As described previously[30,31,45] normal and MHS humans were anaesthetized with nitrous oxide, Innovar* and a sleep dose of diazepam. The trachea was intubated after the vocal cords were sprayed with 5.0 per cent cocaine. The lungs were hyperventilated by an Air Shields ventilator with nitrous oxide:oxygen = 6L:4L per minute through a semiclosed circle incorporating a soda lime cannister. Repeated doses of fentanyl were given as required for analgesia.

3 *Pigs*

Anaesthesia of both normal and MHS pigs was induced with a sleep dose of 2.5 per cent thiopentone through the tubing of an intravenous infusion of Ringers lactate in 5 per cent glucose flowing into an ear vein. The vocal cords were sprayed with 2.0 per cent lidocaine and the trachea was intubated with a red rubber tracheal tube. The lungs were hyperventilated with a Harvard Animal Ventilator at approximately 10.0 $ml \cdot kg^{-1} \cdot min^{-1}$. Anaesthesia was supplemented with increments of 2.5 per cent thiopentone, as necessary.

4 Monitoring of both human and pigs included electrocardiogram, central venous pressure, blood pressure, heart rate, nasal, skin and rectal temperatures, skin colour, moisture and circulation, skeletal muscle tone, blood gases, serum electrolytes and enzymes.

*Innovar® = Droperidol 2.5 $mg \cdot ml^{-1}$ Fentanyl 50 $\mu g \cdot ml^{-1}$.

B *Dissection, preparation and mounting of whole muscle fascicles*

As described,[31] whole muscle fascicles 1.5 to 2.0 cm long were isometrically mounted in baths of Krebs Ringer solution at 37°C and bubbled with 95 per cent O_2 and 5 per cent CO_2. Each fascicle was stimulated electrically once every five seconds at slightly less than supramaximal twitch tension for two milliseconds.

C *Determination of MH status by measurement of caffeine-induced contracture of whole muscle fascicles*

The caffeine contracture test (206) was done on each human and pig specimen to determine its MH status. The millimoles of caffeine required to raise the resting tension of a skeletal muscle fascicle by 1.0 Gm was termed the caffeine-specific concentration, or CSC.

Those individuals exhibiting a CSC of more than 4.1 were considered normal while those with a CSC equal to or less than 4.1 classified as MHS (malignant hyperthermia susceptible).

D *Addition of dantrolene and/or caffeine to solutions bathing whole skeletal muscle fascicles*

After determining the MH status of an individual, further fascicles were mounted as described above and exposed to dantrolene and/or caffeine as follows:

fascicle 1 – graded doses of caffeine (0.25–32.0 mM)

fascicle 2 – graded doses of dantrolene sodium (5, 15 and 25 $\times$ 10^{-6}M)

fascicle 3 – graded doses of dantrolene sodium (5, 15 and 25 $\times$ 10^{-6}M) plus caffeine CSC $\times$ 1 (i.e., the caffeine specific concentration)

fascicle 4 – graded doses of dantrolene sodium (5, 15 and 25 $\times$ 10^{-6}M) plus caffeine CSC $\times$ 2 (i.e. twice the caffeine specific concentration)

fascicle 5 – graded doses of caffeine (0.25–32.0 mM) plus dantrolene (5 $\times$ 10^{-6}M)

fascicle 6 – graded doses of caffeine (0.25–32.0 mM) plus dantrolene (15 $\times$ 10^{-6}M)

fascicle 7 – graded doses of caffeine (0.25–32.0 mM) plus dantrolene (25 $\times$ 10^{-6}M)

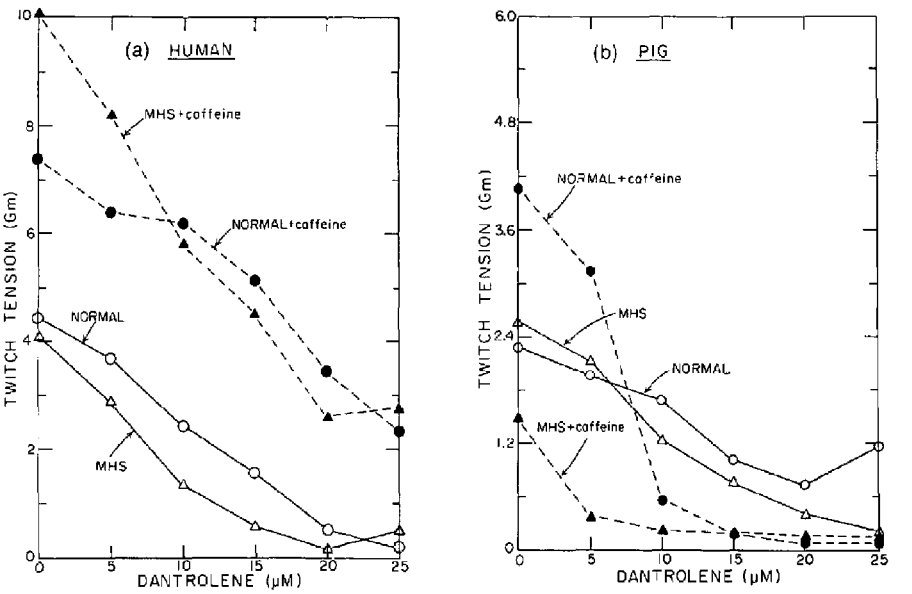

FIGURE 1 Effect of dantrolene on twitch tension.

Studies on (a) human, and (b) pig skeletal muscles.

Horizontal axis shows cumulative doses of dantrolene (in μM) added to each skeletal muscle fascicle. Thus comparisons among these dantrolene doses are within fascicles.

Vertical axis shows mean twitch tension (in Gms) of the fascicles in the presence of various dose combinations of dantrolene and caffeine.

Four plots are shown: – normal without caffeine
– normal with caffeine CSC × 2
– MHS without caffeine
– MHS with caffeine CSC × 2.

Thus comparisons between plots are between fascicles.

For each drug addition the parameters measured were twitch tension (contraction) defined as the vertical distance from the top of the contracture to the top of the twitch tension, and the resting tension (contracture) defined as the vertical distance from the baseline to the bottom of the twitch tension.

E *Addition of dantrolene to solutions bathing single skinned fibres*

The technique of preparing and measuring caffeine and calcium induced tension changes of single chemically skinned fibres has been described in detail.[31] In the present study, three different experiments were done with dantrolene. They were designed to assess initial uptake of exogenous calcium into the SR; release of calcium from the SR; and re-uptake of endogenous calcium into the SR following its release from the SR.

In the first experiment, the influence of dantrolene on initial calcium uptake into the SR was determined by adding graded concentrations of dantrolene (5, 15 and 25 × 10^{-6}M) sequentially to the preparation at the same time as the subcontractile loading calcium (p6.8) was added. Each dantrolene-calcium addition was followed 30 seconds later by caffeine which induced release of calcium from the SR. The dose of caffeine was the lowest which had, in the earlier diagnostic part of the study, been determined to initiate a rise in tension of the fibre. The greater the uptake of Ca^{++} into the SR in the presence of dantrolene sodium, the more calcium was, therefore, available for release when

the caffeine was subsequently added and so the larger was the ensuing tension rise of the fibre.

In the second experiment, the influence of dantrolene on calcium release from the SR was measured. This was done by first loading the SR with subcontractile concentrations of calcium (pCa = 6.8). Then 30 seconds later dantrolene (5, 15 and 25 $\times 10^{-6}$M) was added with the releasing caffeine. The dose of caffeine chosen in the first experiment was the minimum dose required to raise the tension of the fibre. The smaller the release of Ca^{++} from the SR in the presence of dantrolene, the smaller was the ensuing tension increase of the fibre.

Statistical Analysis

By plotting the logarithm of tension against the concentration of dantrolene (or logarithm of the concentration of caffeine) the response curves could be characterized by straight lines. (Observations of zero as well as resting tensions of less than 0.2 Gm and twitch tensions of less than 0.4 Gm were excluded from these calculations. Failure to exclude such observations would have seriously distorted the estimated slopes.) Therefore, the slope of this relationship was used to measure the influence of increasing concentrations of dantrolene (or caffeine) on muscle tension over the range of dantrolene (or caffeine) where the linear trend occurred. The slope, jointly calculated for several subjects (from analyses of covariance, which combine analysis of variance with regression) was an indication of the average intensity of the inhibition (or stimulation) of tension caused by increasing concentrations of dantrolene (or caffeine). Comparisons of slopes in normal and MHS muscle, and slopes between fixed drug levels, were expressed as t-statistics.

Normal and MHS muscle tensions were compared by analyses of variance involving repeated measures and performed at one concentration (the median of the range over which a response occurred) if their slopes were generally similar; but at two concentrations (medians of the first and second halves on the response range) if their slopes generally differed. In addition to the contrast of the two muscle types (normal vs. MHS), the analyses of variance evaluated also either the effect of the four fixed concentrations of dantrolene (0, 5, 15, and 25 μM) or of the three individually adjusted caffeine levels (CSC × 0, CSC × 1, and CSC × 2), and the interaction of these two kinds of effects. Detailed analysis for the effect of the four fixed dantrolene levels was carried out by applying Duncan's multiple range test. All these calculations were executed following modified logarithmic transformation of the observation [$\log_{10}$ (tension + 0.2)]. These transformations stabilized the variances (a requirement for the analyses) and, still, permitted including all readings.

The effect of adding low concentrations of dantrolene (5 μM) or caffeine (1 mM) was evaluated in analyses of variance involving repeated measures, again following the modified logarithmic transformation of the observations. Again contrasts were assessed between average tensions measured in normal and MHS muscles, as well as the effects of various caffeine and dantrolene concentrations, and the interaction of these effects. In some cases, the results were expressed in the form of t-statistics.

To analyze the data obtained on the chemically skinned fibres, the difference between tensions in normal and MHS fibres were compared by t-tests in the absence of dantrolene and by analysis of variance in the presence of the four doses of dantrolene. The differences between readings obtained at the four doses of dantrolene and those obtained in the absence of dantrolene were also compared by analysis of variance.

These statistical analyses have been summarized in the text of the following section. Detailed analysis may be obtained by writing to the senior author.

Results

Whole skeletal muscle fascicles

(a) *Twitch tensions – ascending concentrations of dantrolene sodium, fixed concentrations of caffeine*

Figures 1a and 1b and Table I show the relationship between twitch tensions and ascending (0–25 μM) dantrolene concentration at caffeine CSC × 0 and CSC × 2. Figures 2a and 2b gives the effect of caffeine (CSC × 0, CSC × 1 and CSC × 2) on twitch tension at 0, 5 and 20 μM of dantrolene. Statistical analysis is presented in Table II.

Human fascicles

Dantrolene causes dose-related inhibition of twitch tensions of normal and MHS human fascicles in the

TABLE 1 Twitch tensions of skeletal muscle fascicles in the presence of constant caffeine and cumulative dantrolene concentrations

Const. Caff. CSC / Cum. Dant. μM	(a) Human						(b) Pig					
	Normal			MHS			Normal			MHS		
	CSC × 0	CSC × 1	CSC × 2	CSC × 0	CSC × 1	CSC × 2	CSC × 0	CSC × 1	CSC × 2	CSC × 0	CSC × 1	CSC × 2
0	4.48* ±1.54 (5)	10.00 ±3.64 (4)	7.40 ±4.50 (5)	4.05 ±0.83 (11)	9.24 ±1.14 (9)	10.08 ±2.48 (7)	2.28 ±1.29 (5)	5.52 ±2.35 (5)	4.07 ±0.77 (3)	2.58 ±0.65 (9)	3.95 ±0.87 (9)	1.50 ±0.69 (6)
5	3.72 ±1.53 (5)	5.77 ±1.44 (7)	6.45 ±2.28 (4)	2.94 ±0.66 (11)	7.76 ±1.26 (10)	8.25 ±2.07 (8)	1.96 ±1.02 (5)	4.72 ±2.26 (5)	3.15 ±1.24 (4)	2.13 ±0.58 (9)	1.44 ±0.51 (9)	0.37 ±0.33 (6)
10	2.44 ±1.14 (5)	5.54 ±1.26 (7)	6.25 ±2.03 (4)	1.34 ±0.35 (11)	5.51 ±1.14 (11)	5.85 ±1.62 (8)	1.68 ±0.84 (5)	3.12 ±1.57 (5)	0.55 ±0.17 (4)	1.24 ±0.42 (9)	0.84 ±0.31 (9)	0.23 ±0.23 (6)
15	1.56 ±0.73 (5)	5.20 ±1.20 (7)	5.20 ±1.60 (4)	0.64 ±1.86 (11)	3.80 ±0.81 (11)	4.60 ±1.51 (8)	1.00 ±0.49 (5)	1.72 ±1.05 (5)	0.20 ±0.08 (4)	0.75 ±0.29 (9)	0.70 ±0.25 (8)	0.24 ±0.20 (5)
20	0.52 ±0.27 (5)	4.40 ±0.97 (7)	3.45 ±1.19 (4)	0.16 ±0.08 (10)	2.73 ±0.65 (11)	2.66 ±1.07 (7)	0.72 ±0.35 (5)	1.32 ±0.88 (5)	0.10 ±0.06 (4)	0.40 ±0.17 (9)	0.47 ±0.19 (9)	0.17 ±0.13 (6)
25	0.24 ±0.19 (5)	3.80 ±0.87 (7)	2.35 ±0.85 (4)	0.52 ±0.39 (10)	2.69 ±0.75 (9)	2.75 ±1.12 (8)	1.16 ±0.87 (5)	0.40 ±0.14 (5)	0.10 ±0.06 (4)	0.20 ±0.10 (9)	0.40 ±0.16 (9)	0.16 ±0.12 (5)

Cum. Dant. μM = Cumulative dose (in μM) of dantrolene sequentially added to each fascicle. Dose schedule is same for all fascicles.
Const. Caff. CSC = A constant dose of caffeine repeatedly added to each fascicle. Dose is different for each fascicle, being either CSC × 0 or CSC × 1 or CSC × 2.
*Average twitch tension (in Gm) ± its standard error. Number of observations is shown between parentheses.

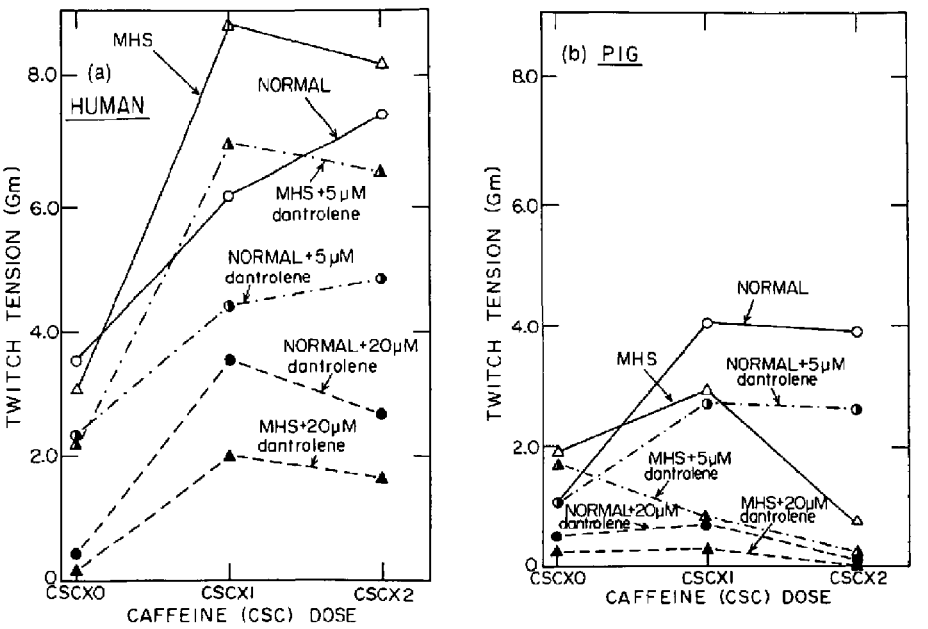

FIGURE 2 Effect of adjusted doses of caffeine on twitch tension.

Studies on (a) human, and (b) pig skeletal muscle.

Horizontal axis shows adjusted doses of caffeine (in CSC) added to skeletal muscle fascicles. Thus comparisons among these caffeine doses are comparisons among fascicles.

Vertical axis shows mean twitch tension (in Gms) of skeletal muscle fascicles.

Six plots are shown: – normal without dantrolene
– normal with 5 μM dantrolene
– normal with 20 μM dantrolene
– MHS without dantrolene
– MHS with 5 μM dantrolene
– MHS with 20 μM dantrolene

Thus contrasts between plots are between normal and MHS fascicles, but within these when different dantrolene concentrations are compared.

presence and absence of caffeine (Table I, Figure 1a).

Increasing the concentration of dantrolene has a stronger depressant influence on the logarithm of twitch tensions in the absence of caffeine than in its presence, in both normal and MHS fascicles (Table I) ($t = 5.57$, d.f. $= 10$, $p < 0.001$; and $t = 3.99$, d.f. $= 20$, $p < 0.001$; comparing the influence of dantrolene in the absence and presence of caffeine CSC × 1, for normal and MHS fascicles, respectively).

In the absence of caffeine, increasing dantrolene concentration lowers twitch tensions of MHS and normal fascicles to a similar degree ($t = 2.03$, d.f. $= 14$, $p > 0.05$). In the presence of either level of caffeine, the decrease in twitch tensions is significantly greater in MHS muscle than in normal muscle ($t = 4.74$, d.f. $= 16$, $p < 0.001$; and $t = 2.26$, d.f. $= 10$, $p < 0.05$, for caffeine levels of CSC × 1 and CSC × 2, respectively).

The above trends in the relationship between twitch tension and dantrolene concentration are maintained over the entire range of dantrolene concentrations (Table I, Figure 1a).

At 5 and 20 μM dantrolene, the twitch tensions in MHS and normal muscle do not differ significantly

TABLE II Analysis of variance* of twitch tensions of skeletal muscle fascicles at 5 μM and 20 μM Dantrolene

	(a) Human						*(b) Pig*					
	CSC × 0 / CSC × 1		*CSC × 0 / CSC × 2*		*CSC × 1 / CSC × 2*		*CSC × 0 / CSC × 1*		*CSC × 0 / CSC × 2*		*CSC × 1 / CSC × 2*	
Contrast	*Dant. 5*	*Dant. 20*	*Dant. 5*	*Dant. 20*	*Dant. 5*	*Dant. 20*	*Dant. 5*	*Dant. 20*	*Dant. 5*	*Dant. 20*	*Dant. 5*	*Dant. 20*
Normal / MHS	0.35† n.s.	2.13 n.s.	0.03 n.s.	1.55 n.s.	0.03 n.s.	3.34 n.s.	0.54 n.s.	1.31 n.s.	2.75 n.s.	0.13 n.s.	5.20 ‡	0.01 n.s.
Caffeine level	17.23 §	60.23 §	4.40 ‡	13.72 §	1.27 n.s.	0.32 n.s.	0.06 n.s.	0.55 n.s.	4.10 n.s.	11.02 §	2.40 n.s.	11.70 §
(Normal/MHS) × (caffeine level) interaction	0.28 n.s.	0.00 n.s.	0.88 n.s.	1.00 n.s.	0.93 n.s.	1.05 n.s.	4.36 ‡	0.08 n.s.	10.53 §	0.90 n.s.	0.26 n.s.	3.84 n.s.

*Performed on $\log_{10}$ (twitch tension + 0.2).

†F-Statistics. The numerator has always 1 degree of freedom. The degrees of freedom for the denominators are: (1) In humans, for MHS effect: 17; for caffeine effect and interaction: 22 and 21 at 5 and 20 μM dantrolene, respectively; (b) In pigs, for MHS effect: 12; for caffeine effect and interaction: 20.

n.s. not significant, $p \geq 0.05$.

‡$p < 0.05$.

§$p < 0.001$.

from each other at any level of caffeine. Although not significant, twitch tensions of MHS fascicles are higher than those of normal fascicles at both levels of caffeine with 5 μM dantrolene, and lower than those of normal fascicles at both levels of caffeine with 20 μM dantrolene (Figure 2a). The addition of caffeine CSC × 1 significantly raises twitch tensions in both MHS and normal muscle at both dantrolene levels ($F = 17.23$, d.f. = 1 and 22, $p < 0.001$; and $F = 60.23$, d.f. = 1 and 21, $p < 0.001$, for 5 and 20 μM dantrolene, respectively). Increasing the caffeine to the CSC × 2 level does not increase twitch tension further ($F = 1.27$, d.f. = 1 and 22, $p > 0.05$; and $F = 0.32$, d.f. = 1 and 21, $p > 0.05$, for dantrolene levels of 5 and 20 μM, respectively (Figure 2a, Table II).

Pig Fascicles

In the absence of caffeine, significant decreases in twitch tension occur in both normal and MHS muscle ($t = 3.64$, d.f. = 3, $p < 0.05$; and $t = 10.90$, d.f. = 8, $p < 0.001$, respectively) (Table I, Figure 1b). The decreases are significantly more marked in MHS than in normal muscle ($t = 3.36$, d.f. = 11, $p < 0.01$). In the absence of caffeine the trends in the relationship between twitch tension and dantrolene concentration are similar over the entire range of dantrolene concentration (Table I, Figure 1b). However, in the presence of caffeine, the inhibition is particularly strong at low doses of dantrolene.

Between 0 and 5 μM of dantrolene, decreases in twitch tension become significant in normal muscle with caffeine CSC × 2 ($t = 5.26$, d.f. = 2, $p < 0.05$), and in MHS muscle with CSC × 1 and CSC × 2 ($t = 3.20$, d.f. = 8, $p < 0.05$; and $t = 2.61$, d.f. = 5, $p < 0.05$, respectively) (Figure 1b, Table I). At higher concentrations (5–25 μM) of dantrolene in the presence of caffeine, decreases in twitch tension become more marked than in the absence of caffeine in normal muscle ($t = 3.92$, d.f. = 6, $p < 0.01$, for comparing effects in the absence and presence of caffeine CSC × 2), but become less marked in MHS muscle ($t = 4.83$, d.f. = 16, $p < 0.001$; and $t = 7.79$, d.f. = 13, $p < 0.001$, for comparing effects in the absence of caffeine with those in the presence of caffeine, at CSC × 1 and CSC × 2, respectively). This results in the reduction of twitch tension being significantly greater for normal muscle than for MHS muscle when caffeine is present ($t = 2.54$, d.f. = 11, $p < 0.05$; and $t = 6.68$, d.f. = 8, $p < 0.001$; for CSC × 1 and CSC × 2, respectively) (Figure 1b, Table I).

At 5 μM dantrolene, the addition of caffeine CSC × 1 increases twitch tensions substantially in normal muscle, but reduces it substantially in MHS muscle (for the interaction of the two changes: $F = 4.36$, d.f. = 1 and 20, $p < 0.05$; Table II. The addition of caffeine CSC × 2 does not result in significant further change ($F = 2.40$, d.f. = 1 and 20, $p > 0.05$; Table II). Normal muscle twitch tension is significantly higher than MHS twitch tension in the presence of caffeine, but MHS and normal twitch tensions are similar to one another in the absence of caffeine ($t = 0.72$, d.f. = 12, $p > 0.05$).

At 20 μM dantrolene, the addition of moderate amounts of caffeine (CSC × 1) does not affect twitch tension (Figure 2b) ($F = 0.55$, d.f. = 1 and 20, $p > 0.05$; Table II). Addition of the higher caffeine dose (CSC × 2), however, significantly reduces twitch tension ($F = 11.70$, d.f. = 1 and 20, $p < 0.001$; Table II). At all caffeine levels, twitch tensions of the MHS fascicles do not differ significantly from those of the normal fascicles at this dantrolene level (Table II).

(b) *Twitch tensions – ascending concentrations of caffeine, fixed concentrations of dantrolene sodium*

Figures 3a and 3b and Table III present the effect of ascending caffeine dose (0–16 mM) on twitch tensions at 0 and 25 μM dantrolene. Three phases in the dose-response relationship can be observed in these figures, although each phase is not always present for each curve. A curve containing all three phases is seen for MHS pig muscle with 25 μM dantrolene (Figure 3b). Such a curve can be described as follows: (1) and initial fall of twitch tension, followed by: (2) an increase, then followed by: (3) another fall of the twitch tension.

Figures 4a and 4b show the effect of dantrolene (5, 15 and 25 μM) on twitch tensions at 0, 1.0 and 8.0 mM of caffeine.

Statistical analysis is given in Table IV.

Human fascicles

In the absence of dantrolene, low concentrations of caffeine increase twitch tension (phase 2), but high concentrations of caffeine reduce twitch tension

TABLE III Twitch tensions of skeletal muscle fascicles: cumulative caffeine, constant dantrolene

Const. Dant. μM / Cum. Caff. mM	(a) Human								(b) Pig							
	Normal				MHS				Normal				MHS			
	0	5	15	25	0	5	15	25	0	5	15	25	0	5	15	25
0.0	2.53 ±0.87 (8)	2.07 ±0.74 (3)	1.73 ±1.15 (3)	3.28 ±1.03 (5)	3.97 ±0.98 (9)	1.46 ±0.53 (6)	2.04 ±0.65 (5)	1.76 ±0.39 (6)	3.17 ±1.14 (6)	2.08 ±1.11 (5)	1.90 ±1.21 (4)	2.68 ±0.92 (5)	2.40 ±0.69 (10)	1.28 ±0.94 (5)	1.56 ±1.46 (5)	2.04 ±1.33 (5)
1.0	4.10 ±1.18 (8)	1.74 ±0.80 (7)	0.25 ±0.15 (4)	2.25 ±0.84 (8)	4.72 ±1.40 (8)	2.10 ±0.52 (10)	2.07 ±0.61 (8)	1.31 ±0.27 (9)	3.93 ±1.39 (6)	2.12 ±1.08 (5)	1.40 ±0.72 (5)	2.00 ±0.91 (5)	3.14 ±0.78 (10)	0.74 ±0.22 (10)	1.18 ±0.49 (10)	0.89 ±0.49 (9)
2.0	4.20 ±0.51 (7)	2.69 ±1.19 (7)	0.35 ±0.20 (4)	1.31 ±0.59 (9)	6.68 ±1.54 (9)	1.88 ±0.47 (10)	1.42 ±0.40 (8)	0.75 ±0.17 (9)	5.00 ±1.63 (6)	2.36 ±1.09 (5)	1.52 ±0.74 (5)	1.56 ±0.76 (5)	2.92 ±0.82 (10)	0.64 ±0.14 (10)	0.82 ±0.31 (10)	0.68 ±0.35 (10)
4.0	7.90 ±1.63 (8)	4.11 ±1.62 (7)	0.60 ±0.34 (4)	0.96 ±0.37 (9)	6.96 ±1.55 (9)	2.50 ±0.60 (10)	1.38 ±0.36 (8)	0.78 ±0.13 (9)	6.10 ±1.84 (6)	2.96 ±1.16 (5)	1.80 ±0.71 (5)	1.80 ±0.79 (5)	2.34 ±0.62 (10)	1.02 ±0.24 (10)	1.38 ±0.57 (10)	1.09 ±0.45 (9)
8.0	9.73 ±1.61 (8)	6.23 ±2.39 (7)	1.45 ±0.85 (4)	1.64 ±0.45 (9)	6.51 ±1.31 (9)	4.48 ±1.13 (10)	3.28 ±0.95 (8)	1.62 ±0.28 (9)	6.90 ±1.91 (6)	2.70 ±0.74 (4)	2.76 ±1.09 (5)	2.84 ±1.19 (5)	1.02 ±0.39 (10)	1.12 ±0.50 (10)	1.16 ±0.34 (10)	1.68 ±0.54 (8)
16.0	9.15 ±1.77 (8)	7.60 ±2.82 (7)	3.35 ±1.68 (4)	4.04 ±0.75 (9)	5.36 ±1.11 (9)	4.40 ±1.59 (7)	4.49 ±0.97 (7)	3.13 ±0.74 (9)	7.20 ±2.23 (6)	5.52 ±1.57 (5)	3.76 ±1.35 (5)	4.44 ±1.40 (5)	0.06 ±0.03 (10)	0.18 ±0.10 (10)	0.10 ±0.05 (10)	0.55 ±0.17 (9)

Cum. Caff. mM = Cumulative dose (in mM) of caffeine sequentially added to each fascicle. Dose schedule is same for all fascicles.
Const. Dant. CSC = A constant dose (μM) of dantrolene repeatedly added to each fascicle. Dose is different for each fascicle, being either CSC × 0 or CSC × 1 or CSC × 2.

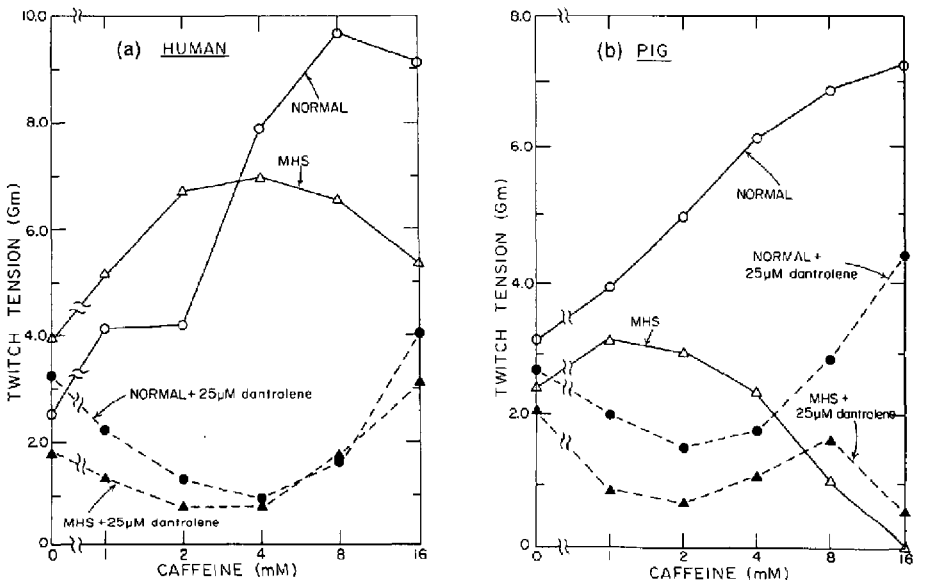

FIGURE 3 Effect of fixed concentrations of caffeine on twitch tension.

Studies on (a) human, and (b) pig skeletal muscle.

Horizontal axis shows cumulative doses of caffeine (in mM) added to each skeletal muscle fascicle. Thus comparisons among these caffeine doses are within fascicles.

Vertical axis shows mean twitch tension (in Gms) of the fascicles in the presence of various dose combinations of dantrolene and caffeine.

Four plots are shown: – normal without caffeine
– normal + 25 μM dantrolene
– MHS without caffeine
– MHS + 25 μM dantrolene.

Thus comparisons between plots are between fascicles.

(phase 3) (Figure 3a). Caffeine at low concentrations has a greater influence on the increase of twitch tension in normal muscles than in MHS muscles ($t = 2.51$, d.f. $= 15$, $p < 0.05$), however, the reduction of twitch tension begins at a lower concentration and is more marked in MHS muscle (Figure 3a).

As the dantrolene dose is increased, the decreasing phase 3 disappears; in normal muscle fascicles first with 5 μM dantrolene, and in MHS fascicles first with 15 μM dantrolene (Table III). The initial, decreasing phase 1, becomes more evident as the dantrolene dose is elevated. Thus with 25 μM dantrolene, concentrations of up to 4 mM caffeine depress twitch tension (Figure 3a). Therefore, in the absence of dantrolene the increasing phase of the curve (phase 2) occurs over a range of 0 to 4 mM caffeine, but over a higher range of at least 4 to 8 mM caffeine in the presence of dantrolene. Thus, at the middle caffeine concentration of 4 mM, the twitch tension is rising or begins to rise (phase 2) at all investigated conditions (with the exception of MHS muscle in the absence of dantrolene).

In the increasing phase of the curve (phase 2), the stimulatory effect of increasing caffeine concentration becomes more marked as the dantrolene concentration is increased in both normal and MHS muscle ($t = 5.66$, d.f. $= 8$, $p < 0.001$; and $t = 5.21$, d.f. $= 9$, $p < 0.01$, respectively).

In the presence of 1 or 8 mM caffeine, the addition of 5 μM dantrolene significantly lowers the twitch tension of normal muscle (Table IV), but

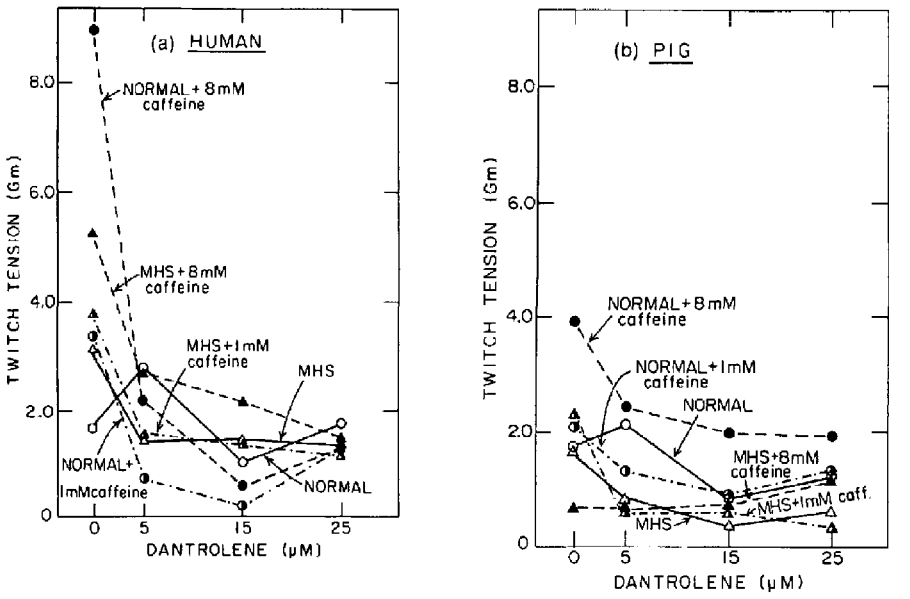

FIGURE 4 Effect of dantrolene and fixed doses of caffeine on twitch tension.

Studies on (a) human, and (b) pig skeletal muscle.

Horizontal axis shows fixed or constant doses of dantrolene (in μM) repeatedly added to each skeletal muscle fascicle. Thus comparisons among these dantrolene doses are comparisons among fascicles.

Vertical axis shows mean twitch tension (in Gms) of skeletal muscle fascicles.

Six plots are shown: – normal without caffeine
– normal + 1 mM caffeine
– normal + 8 mM caffeine
– MHS without caffeine
– MHS + 1 mM caffeine
– MHS + 8 mM caffeine.

Thus contrasts between plots are between normal and MHS fascicles, but within these when different caffeine concentrations are compared.

further increases in the dantrolene concentration do not result in substantial additional change ($p > 0.05$, with Duncan's multiple comparison). The trend is similar but not significant in MHS muscle ($p > 0.05$, with Duncan's multiple comparison).

No significant differences exist between tensions of normal and MHS fascicles at 1 mM caffeine ($F = 0.41$, d.f. = 1 and 17, $p > 0.05$; Table IV), nor at 8 mM caffeine ($F = 0.06$, d.f. = 1 and 17, $p > 0.05$; Table IV).

Pig fascicles

The dependence of twitch tension on caffeine concentration is qualitatively similar to that noted in humans (Figure 3b). In the absence of dantrolene, the addition of caffeine at any dose raises twitch tension in normal muscle. However, in MHS muscle twitch tension rises only with the addition of 1 mM caffeine and then falls at all higher doses. By contrast, in the presence of dantrolene, in both normal and MHS muscle, low levels (1 and 2 mM) of caffeine generally reduce twitch tension and levels of 4 and 8 mM caffeine raise twitch tension (phase 2). In MHS muscle, in the presence of dantrolene, increasing the caffeine concentration from 8 to 16 mM caffeine again reduces twitch tension (phase 3) whereas in normal muscle twitch tension continues to rise (Figure 3b).

In normal muscle the stimulating effect on twitch tension of increasing the concentration of caffeine (from 4 to 16 mM) is greater but not significantly greater, at the high dantrolene concentration of 25 μM than in the absence of dantrolene ($t = 2.16$, d.f. $= 5$, $p > 0.05$).

In the absence of dantrolene twitch tensions of MHS muscle decrease over the range of 1 to 16 mM caffeine (Table III, Figure 3b) whereas in the presence of dantrolene twitch tensions increase generally over the range of 2 to 8 mM caffeine. Therefore, a significant difference exists between the influence of caffeine in the presence and absence of dantrolene in MHS muscle ($t = 10.03$, d.f. $= 8$, $p < 0.001$).

The addition of 5 μM dantrolene reduces twitch tensions at low (1 mM) and at high (8 mM) concentrations of caffeine (Figure 4b, Table IV). Statistical significance for the decline can be shown only for MHS muscle at 1 mM caffeine ($F = 4.21$, d.f. $= 3$ and 37, $p < 0.05$, Table IV). Increasing dantrolene concentration to 15 or 25 μM does not significantly further change the twitch tensions from those observed at 5 μM dantrolene (by Duncan's multiple comparison, $p > 0.05$). At low concentrations of caffeine (1 mM) twitch tensions of normal and MHS muscle do not differ significantly ($F = 0.50$, d.f. $= 1$ and 15, $p > 0.05$; Table IV). At the higher concentration of caffeine (8 mM) twitch tensions of normal muscle are greater than those of MHS muscle in the absence of dantrolene ($F = 4.74$, d.f. $= 1$ and 15, $p < 0.05$; Table IV).

(c) *Resting tensions – ascending concentrations of dantrolene sodium, fixed concentrations of caffeine*

Figures 5a and 5b and Table V show the relationship between resting tension and ascending dantrolene doses (0–25 μM) at caffeine CSC × 0, CSC × 1 and CSC × 2. Since the dantrolene dependence of resting tension is parallel in normal and MHS muscle ($t < 1.22$, d.f. $= 10$, $p > 0.05$ and $t = 2.11$, d.f. $= 8$, $p > 0.05$ for humans and pigs, respectively), a dantrolene concentration of 10 μM was chosen to evaluate the caffeine effect. Figures 6a and 6b demonstrates this effect of caffeine (CSC × 0, CSC × 1 and CSC × 2). Statistical analysis is summarized in Table VI.

Human fascicles

In the absence of caffeine, in both normal and MHS muscle, dantrolene has no effect on resting tension and resting tensions remain negligible (Figure 5a).

In the presence of caffeine, at low dantrolene concentrations (between 0 and 5 M) the resting tension declines with increasing dantrolene dose (Table VI). The declines observed in normal and MHS muscles are not significantly different ($F = 0.50$, d.f. $= 1$ and 18, $p > 0.05$).

At higher dantrolene concentrations (10 through 25 μM), in the presence of caffeine, the resting tensions of normal and MHS muscle increase very slightly (Table VI, Figure 5a).

In the presence and absence of caffeine, the resting tensions of normal and MHS muscle do not differ significantly (Table VI, Figure 5a).

In normal and MHS muscle, caffeine CSC × 1 raises resting tension slightly ($F = 1.28$, d.f. $= 1$ and 23, $p > 0.05$, Table VI). However, resting tension at CSC × 2 are substantially and significantly higher than those at CSC + 1 ($F = 15.81$, d.f. $= 1$ and 23, $p < 0.001$; Figure 6a, Table VI).

Pig fascicles

In the absence of caffeine, in both normal and MHS muscle, dantrolene has no effect on twitch tension which remains negligible (Figure 5b).

In the presence of caffeine, the addition of low

TABLE IV Analysis of variance* of twitch tensions of skeletal muscle fascicles at 1 and 8 mM caffeine

	(a) Human		*(b) Pig*	
Contrast	*1 mM Caffeine*	*8 mM Caffeine*	*1 mM Caffeine*	*8 mM Caffeine*
Normal MHS	0.41† n.s.	0.06 n.s.	0.50 n.s.	4.74 ‡
Dantrolene level	7.44 §	10.23 §	4.21 ‡	0.61 n.s.
(Normal/MHS) × (dantrolene level)	0.75 n.s.	1.29 n.s.	0.84 n.s.	2.04 n.s.

*Performed on $\log_{10}$ (twitch tension + 0.2). The "Dantrolene Level" effect compares twitch tensions at 0, 5, 15 and 25 μM dantrolene.

†The F-statistics assessing MHS effects have 1 and 17 degrees of freedom (d.f.) in humans and d.f. = 1 and 15 in pigs. In humans, d.f. = 3 and 39 for the analysis of the dantrolene effect and the interaction, and in pigs df = 3 and 37.

n.s. = not significant, $p \geq 0.05$.

‡$p < 0.05$.

§$p < 0.001$.

TABLE V Resting tensions of skeletal muscle fascicles: constant caffeine, cumulative dantrolene

Const. Caff. CSC / Cum. Dant. μM	(a) Human						(b) Pig					
	Normal			MHS			Normal			MHS		
	CSC × 0	CSC × 1	CSC × 2	CSC × 0	CSC × 1	CSC × 2	CSC × 0	CSC × 1	CSC × 2	CSC × 0	CSC × 1	CSC × 2
0	0.00 ±0.00 (5)	0.73 ±0.53 (3)	2.54 ±0.58 (5)	0.00 ±0.00 (11)	1.34 ±0.46 (11)	2.31 ±0.40 (8)	0.00 ±0.00 (5)	0.82 ±0.32 (5)	2.18 ±0.51 (4)	0.00 ±0.00 (9)	2.00 ±0.44 (9)	4.55 ±2.29 (6)
5	0.00 ±0.00 (5)	0.03 ±0.03 (7)	1.47 ±0.89 (4)	0.01 ±0.01 (11)	0.10 ±0.06 (11)	0.96 ±0.54 (8)	0.00 ±0.00 (5)	0.68 ±0.35 (5)	1.48 ±0.34 (4)	0.00 ±0.00 (9)	0.88 ±0.63 (9)	1.57 ±0.87 (6)
10	0.00 ±0.00 (5)	0.10 ±0.07 (7)	0.90 ±0.42 (4)	0.02 ±0.01 (11)	0.09 ±0.07 (11)	0.56 ±0.38 (8)	0.00 ±0.00 (5)	0.60 ±0.35 (5)	1.20 ±0.30 (4)	0.00 ±0.00 (9)	0.93 ±0.72 (9)	1.48 ±0.91 (6)
15	0.00 ±0.00 (5)	0.11 ±0.07 (7)	1.77 ±1.28 (4)	0.02 ±0.01 (11)	0.08 ±0.07 (11)	0.46 ±0.29 (8)	0.00 ±0.00 (5)	0.52 ±0.34 (5)	1.03 ±0.24 (4)	0.04 ±0.03 (9)	0.90 ±0.72 (9)	1.30 ±0.83 (6)
20	0.00 ±0.00 (5)	0.14 ±0.07 (7)	1.22 ±0.68 (4)	0.02 ±0.01 (11)	0.10 ±0.07 (11)	0.41 ±0.29 (8)	0.00 ±0.00 (5)	0.48 ±0.34 (5)	1.03 ±0.32 (4)	0.04 ±0.03 (9)	0.91 ±0.69 (9)	1.13 ±0.79 (6)
25	0.00 ±0.00 (5)	0.17 ±0.09 (7)	1.37 ±0.75 (4)	0.02 ±0.02 (10)	0.13 ±0.10 (10)	0.80 ±0.62 (7)	0.00 ±0.00 (5)	0.42 ±0.30 (5)	1.13 ±0.47 (4)	0.06 ±0.04 (9)	0.89 ±0.65 (9)	1.02 ±1.72 (6)

Cum. Dant. μM = Cumulative dose (in μM) of dantrolene sequentially added to each fascicle. Dose schedule is same for all fascicles.
Const. Caff. CSC = A constant dose of caffeine repeatedly added to each fascicle. Dose is different for each fascicle, being either CSC × 0 or CSC × 1 or CSC × 2.

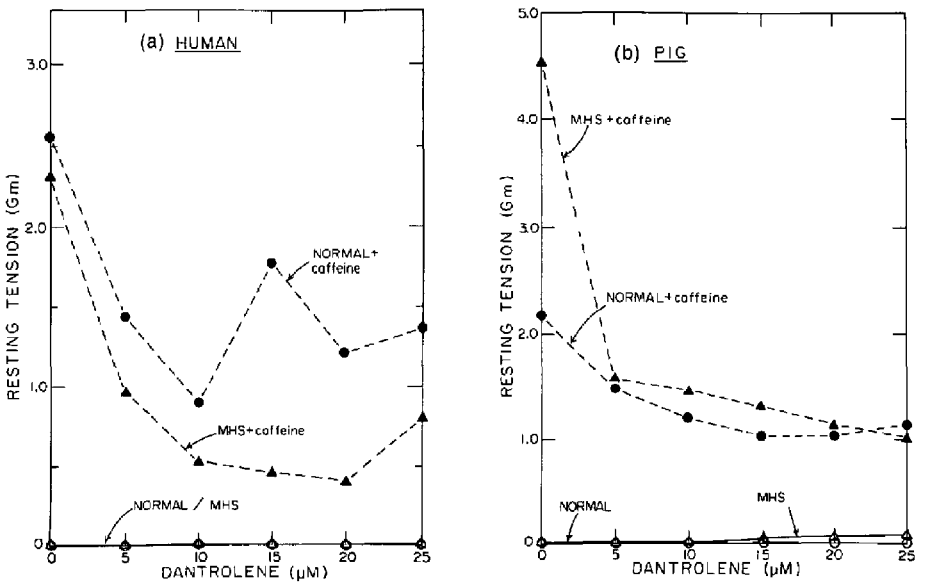

FIGURE 5 Effect of dantrolene on resting tension.
Same as Figure 1 except vertical axis measures resting tension.

concentrations of dantrolene (5 μM) lowers the resting tension (Figure 5b, Table V). Reductions in resting tension are significant in normal muscle with caffeine CSC × 2 ($t = 3.89$, d.f. = 3, $p < 0.05$), and in MHS muscle with CSC × 1 ($t = 2.29$, d.f. = 8, $p < 0.05$). No significant contrasts have been found between normal and MHS muscle at this low dantrolene concentration. At higher dantrolene concentrations resting tension is generally little affected by increasing dantrolene concentration: the resting tension response curve (Figure 5b) flattens over high dantrolene concentrations (15–25 μM).

Resting tensions of normal and MHS muscle in the presence and absence of dantrolene are not significantly different from each other (Table VI).

Caffeine significantly raises resting tensions in both normal and MHS muscle (Figure 6b). Resting tensions with CSC × 1 are higher than those in the absence of caffeine ($F = 11.25$, d.f. = 1 and 20, $p < 0.001$, Table VI). Resting tensions with CSC × 2 are even higher than those with CSC × 1 ($F = 4.45$, d.f. = 1 and 20, $p < 0.05$; Table VI).

(d) *Resting tensions – ascending concentrations of caffeine, fixed concentrations of dantrolene sodium*

The influence of ascending caffeine (0–16 mM) on resting tension at 0 and 25 μM dantrolene is given in Figures 7a and 7b and at all dantrolene doses in Table VII. Figures 8a and 8b present the effect of dantrolene (0, 5, 15 and 25 μM) on resting tension at 0 and 8.0 mM of caffeine. Under most conditions, substantial resting tension responses were not obtained until beyond 4 mM caffeine (Figures 7a and 7b, Table VII). Therefore, a concentration of 8 mM caffeine was chosen to perform the statistical analysis (Table VIII).

Figures 9a and 9b illustrates caffeine concentrations which yield a resting tension of 0.2 gm. A tension of 0.1 gm was the minimally detectable reading but exhibited substantial uncertainly. Consequently, a level of 0.2 gm was chosen to evaluate threshold caffeine concentrations.

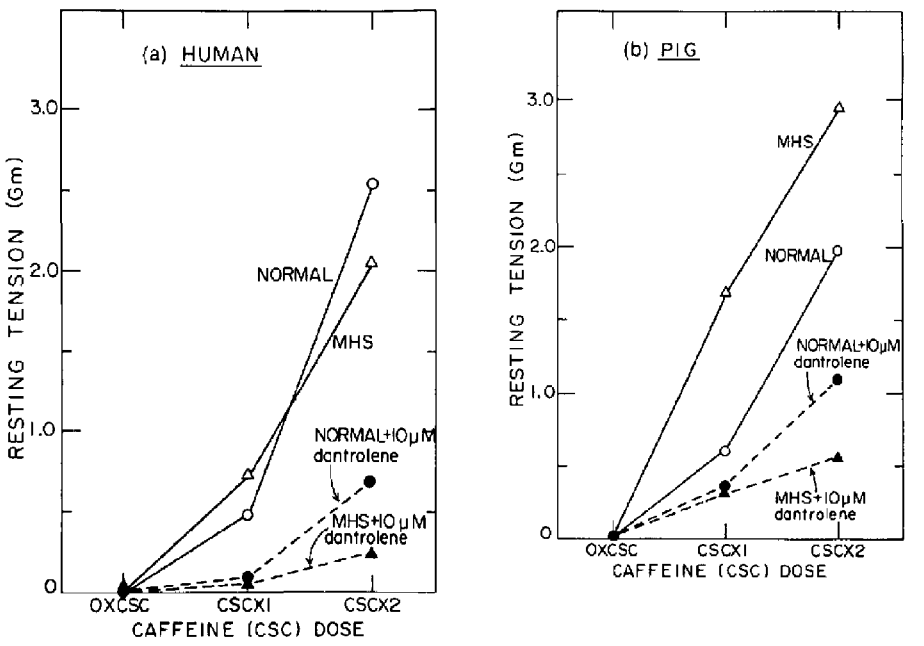

FIGURE 6 Effect of adjusted doses of caffeine on resting tension.
Same as Figure 2 except vertical axis measures resting tension.

Human fascicles

Figure 7a and Table VII present dose–response relationships depicting the effect of caffeine concentration on resting tension. Resting tensions increase steeply as the concentration of caffeine increases.

Figure 9a shows the caffeine concentration required to evoke a resting tension response of 0.2 gm. At each dose of dantrolene, the required caffeine concentration was lower in MHS than in normal muscle. The threshold concentration increased with each succeeding dose of dantrolene.

In the absence of dantrolene, the effect of caffeine is stronger in normal than in MHS muscle ($t = 2.69$, d.f. $= 15$, $p < 0.05$) (Figure 5a). In normal muscle, however, the stimulating effect of increasing concentrations of caffeine decreases as the dose of dantrolene increases ($t = 4.29$, d.f. $= 9$, $p < 0.01$), whereas in MHS muscle, the stimulating effect increases slightly ($t = 1.53$, d.f. $= 9$, $p > 0.05$; Figure 10a). Thus, with 25 μM dantrolene, the effect of caffeine is much stronger in MHS than in normal muscle ($t = 3.04$, d.f. $= 17$, $p < 0.01$).

At 8 mM caffeine the resting tension is higher in MHS than in normal muscle ($F = 6.82$, d.f. $= 1$ and 17, $p < 0.05$; Table VIII), and dantrolene significantly reduces resting tension of both normal and MHS muscle ($F = 25.74$, d.f. $= 3$ and 42, $p < 0.001$; Figure 8a, Table VIII).

Pig fascicles

As in human muscle, resting tension increases as the concentration of caffeine increases (Figure 7b, Table VIII).

Figure 9b shows the caffeine concentration required to evoke a resting tension response of 0.2 gm. As in human muscle, at each dose of dantrolene the required caffeine concentration was lower in MHS than in normal muscle. Dantrolene increased the required caffeine concentration in both normal and MHS muscle, especially between 0 and 5 μM dantrolene.

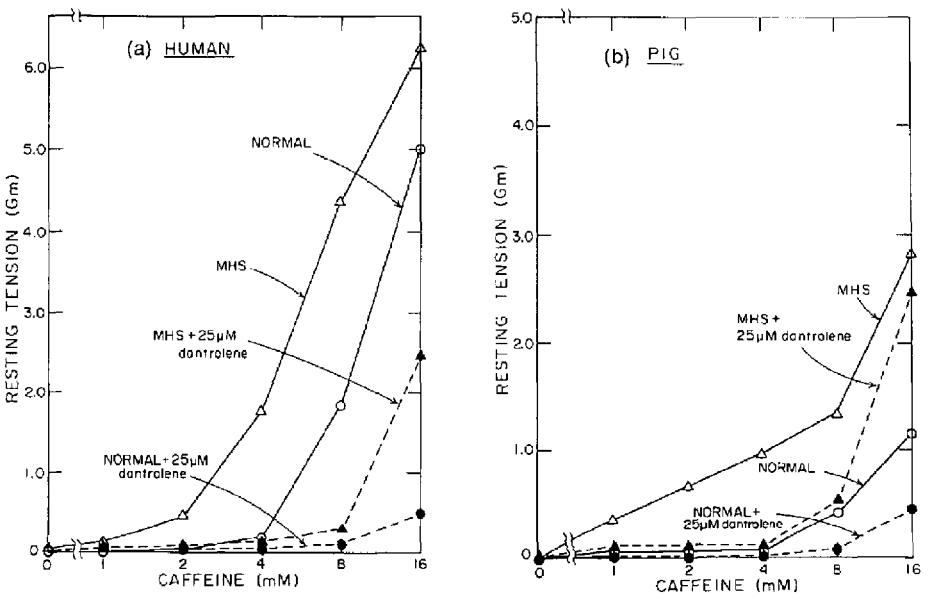

FIGURE 7 Effect of fixed concentrations of caffeine on resting tension.
Same as Figure 3 except vertical axis measures resting tension.

TABLE VI Analysis of variance* of resting tensions of skeletal muscle facicles at 10 μM Dantrolene

	(a) Human			*(b) Pig*		
Contrast	$\frac{CSC \times 0}{CSC \times 1}$	$\frac{CSC \times 0}{CSC \times 2}$	$\frac{CSC \times 1}{CSC \times 2}$	$\frac{CSC \times 0}{CSC \times 1}$	$\frac{CSC \times 0}{CSC \times 2}$	$\frac{CSC \times 1}{CSC \times 2}$
Normal	0.02†	2.52	3.21	0.00	0.31	1.38
MHS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Caffeine	1.28	10.63	15.81	11.25	23.03	4.45
level	n.s.	§	§	§	§	‡
(Normal/MHS) ×	0.37	0.94	0.69	0.01	0.64	2.36
(caffeine level)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

*The analyses were performed following the transformation of $\log_{10}$ (resting tension + 0.2). The listed F-statistics have the following degrees of freedom: For the MHS effect, 1 and 17 in humans, and 1 and 12 in pigs; for the caffeine effect and the interaction, 1 and 23 in humans, and 1 and 20 in pigs.
†Values represent F-statistics (df 1, df 2) performed on $\log_{10}$ (resting tension + 0.2).
n.s. = not significant, $p \geq 0.05$.
‡$P < 0.05$.
§$p < 0.001$.

TABLE VII Resting tensions of skeletal muscle fascicles: cumulative caffeine, constant dantrolene

Cum. Caff. mM \ Const. Dant. μM	(a) Human								(b) Pig							
	Normal				MHS				Normal				MHS			
	0	5	15	25	0	5	15	25	0	5	15	25	0	5	15	25
0.0	0.00 ±0.00 (9)	0.00 ±0.00 (7)	0.00 ±0.00 (4)	0.00 ±0.00 (8)	0.00 ±0.00 (9)	0.00 ±0.00 (10)	0.00 ±0.00 (9)	0.00 ±0.00 (10)	0.00 ±0.00 (6)	0.00 ±0.00 (5)	0.00 ±0.00 (4)	0.00 ±0.00 (5)	0.00 ±0.00 (10)	0.00 ±0.00 (5)	0.00 ±0.00 (5)	0.00 ±0.00 (5)
1.0	0.01 ±0.01 (9)	0.07 ±0.04 (7)	0.05 ±0.05 (4)	0.00 ±0.00 (8)	0.10 ±0.06 (9)	0.04 ±0.03 (10)	0.06 ±0.03 (9)	0.03 ±0.02 (10)	0.00 ±0.00 (6)	0.04 ±0.04 (5)	0.00 ±0.00 (5)	0.00 ±0.00 (5)	0.33 ±0.11 (10)	0.07 ±0.05 (10)	0.08 ±0.06 (10)	0.01 ±0.01 (9)
2.0	0.01 ±0.01 (9)	0.07 ±0.04 (7)	0.05 ±0.03 (4)	0.02 ±0.02 (9)	0.42 ±0.20 (9)	0.04 ±0.02 (10)	0.08 ±0.03 (9)	0.05 ±0.03 (10)	0.00 ±0.00 (6)	0.04 ±0.04 (5)	0.02 ±0.02 (5)	0.02 ±0.02 (5)	0.65 ±0.20 (10)	0.04 ±0.02 (10)	0.08 ±0.05 (10)	0.02 ±0.01 (10)
4.0	0.21 ±0.11 (9)	0.10 ±0.06 (7)	0.08 ±0.05 (4)	0.03 ±0.02 (9)	1.26 ±0.38 (9)	0.12 ±0.07 (10)	0.13 ±0.05 (9)	0.13 ±0.05 (10)	0.02 ±0.02 (6)	0.04 ±0.04 (5)	0.02 ±0.02 (5)	0.04 ±0.02 (5)	0.95 ±0.22 (10)	0.41 ±0.17 (10)	0.09 ±0.05 (10)	0.08 ±0.04 (9)
8.0	2.07 ±0.80 (9)	0.34 ±0.19 (7)	0.08 ±0.05 (4)	0.10 ±0.05 (9)	3.92 ±1.03 (9)	0.86 ±0.39 (10)	0.28 ±0.09 (9)	0.32 ±0.12 (10)	0.42 ±0.10 (6)	0.06 ±0.06 (5)	0.12 ±0.10 (5)	0.08 ±0.06 (5)	1.33 ±0.20 (10)	1.70 ±0.39 (10)	0.95 ±0.22 (10)	0.55 ±0.19 (8)
16.0	5.63 ±1.01 (8)	2.27 ±1.02 (7)	0.75 ±0.30 (4)	0.54 ±0.22 (9)	5.57 ±1.00 (9)	4.59 ±1.03 (10)	2.44 ±0.62 (9)	2.52 ±0.98 (10)	1.15 ±0.34 (6)	0.24 ±0.19 (5)	0.32 ±0.27 (5)	0.46 ±0.32 (5)	2.83 ±0.42 (10)	3.83 ±0.67 (10)	3.11 ±0.62 (10)	2.48 ±0.68 (9)

Cum. Caff. mM = Cumulative dose (in mM) of caffeine sequentially added to each fascicle. Dose schedule is same for all fascicles.
Const. Dant. CSC = A constant dose (μM) of dantrolene repeatedly added to each fascicle. Dose is different for each fascicle, being either CSC × 0 or CSC × 1 or CSC × 2.

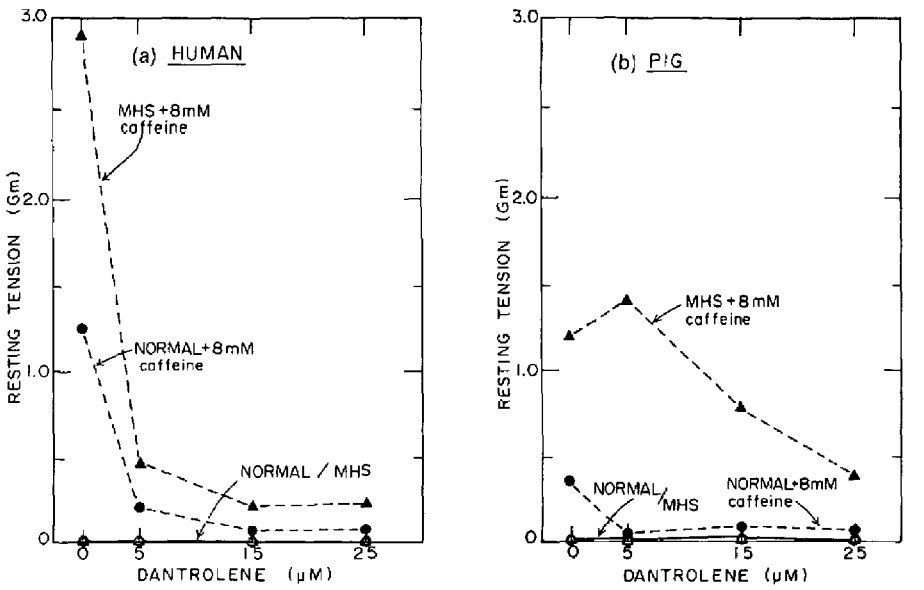

FIGURE 8 Effect of dantrolene and fixed doses of caffeine on resting tension. Same as Figure 4 except vertical axis measures resting tension.

TABLE VIII Analysis of variance of resting tensions of skeletal muscle fascicles

	(a) Human			*(b) Pig*		
Contrast	*F*	*df*	*Sig*	*F*	*df*	*Sig*
Normal/MHS	6.82	1, 17	*	48.22	1, 15	‡
Dantrolene level	25.74	3, 42	‡	5.52	3, 36	†
(Normal/MHS) × (Dantrolene level) *interaction*	0.25	3, 42	n.s.	1.75	3, 36	n.s.

Performed on $\log_{10}$ (resting tension + 0.2) at caffeine = 8 mM. All data were used. The "dantrolene level" effect consists of resting tension at no dantrolene, 5 μM, 15 μM and 25 μM dantrolene.
*$p < 0.05$.
†$p < 0.01$.
‡$p < 0.001$
n.s. = not significant, $p \geq 0.05$.

As in humans, in the absence of dantrolene, the effect of caffeine is stronger in normal than in MHS muscle ($t = 5.51$, d.f. = 14, $p < 0.001$; Figure 10b). In MHS muscle, caffeine has a significant, stimulating effect on resting tension which is more marked in the presence than in the absence of dantrolene ($t = 4.45$, d.f. = 13, $p < 0.001$). In normal muscle, caffeine also enhances resting tension significantly, but does so to a lesser degree in the presence than in the absence of dantrolene ($t = 3.31$, d.f. = 6, $p < 0.05$). Thus, with 25 μM dantrolene, the effect of caffeine is much stronger in MHS than in normal muscle ($t = 2.99$, d.f. = 12, $p < 0.05$), as in humans.

Evaluated at a caffeine concentration of 8 mM, the resting tension is very significantly higher in MHS than in normal muscle ($F = 48.22$, d.f. = 1 and 15, $p \ll 0.001$; Table VIII).

Resting tensions are significantly reduced by increasing concentrations of dantrolene ($F = 5.52$, d.f. = 3 and 36, $p < 0.01$; Figure 7b, Table VIII).

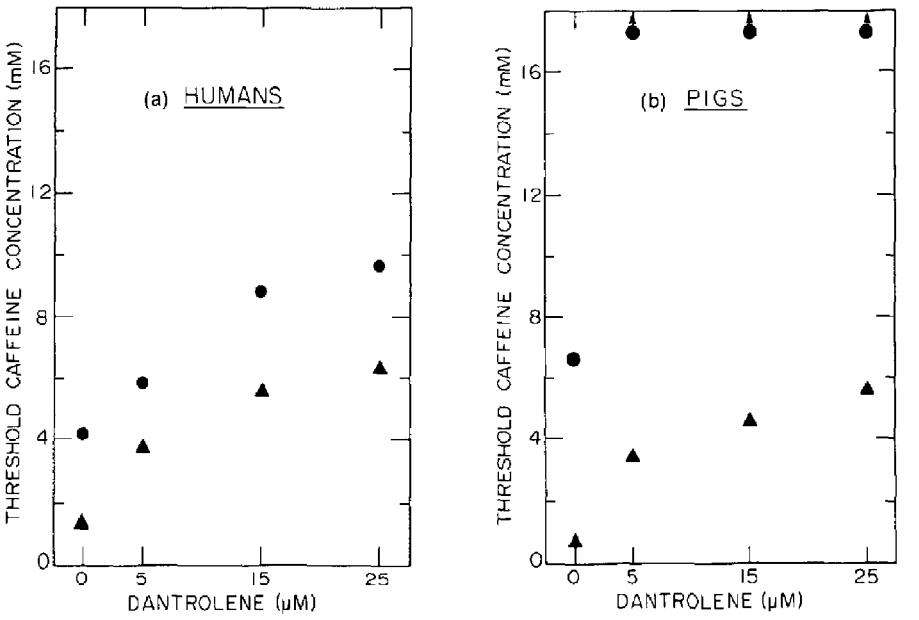

FIGURE 9 Threshold caffeine concentrations for resting tension of 0.2 gm.
▲ MHS muscle. ● Normal muscle. ↑ Threshold ≫ 16 mM caffeine.

Human chemically skinned fibres – influence of dantrolene sodium on calcium release from and calcium uptake into the sarcoplasmic reticulum

Figures 11a and 11b and Table IX show the influence of dantrolene on calcium release from the uptake into the SR of single, chemically skinned, skeletal muscle fascicles.

(a) *Caffeine induced calcium release from the SR* (proportional to tension observed after addition of dantrolene with releasing caffeine (Figure 11a, Table IX)

As the dantrolene concentration increases from zero to the lowest dantrolene dose (10^{-7} M), tensions rise slightly but not significantly in normal fibres ($t = 0.60$, d.f. = 6, $p > 0.05$), and fall slightly but not significantly in MHS fibres ($t = 1.21$, d.f. = 6, $p > 0.05$).

In the absence of dantrolene, tension is higher in MHS than in normal fibres, although this difference is not significant ($t = 0.65$, d.f. = 12, $p > 0.05$). In the presence of dantrolene, tension is lower in MHS than in normal muscle, but this difference, again, is not significant ($F = 0.19$, d.f. = 1 and 12, $p > 0.05$). Thus, although calcium release from the SR in the presence of dantrolene appears to be slightly less in MHS than in normal fibres, the difference is not large enough to be of importance.

The differences between readings obtained at the four dantrolene concentrations and those obtained in the absence of dantrolene are not statistically significantly different in normal and MHS fibres ($F = 2.58$, d.f. = 1 and 12, $p > 0.05$).

(b) *Uptake of calcium into the SR* (proportional to tension observed after addition of dantrolene with loading calcium) (Figure 11b, Table IX)

As the dantrolene concentration increases from zero to 10^{-7} M, no significant changes in tension occur in normal fibres ($t = 1.18$, d.f. = 6, $p > 0.05$), but a modest, significant, fall develops in MHS fibres ($t = 2.59$, d.f. = 6, $p < 0.05$).

In the absence of dantrolene, the tension is higher in MHS fibres than in normal fibres, but the difference is statistically not significant ($t = 0.65$, d.f. = 12, $p > 0.05$). In the presence of dantrolene

TABLE IX Calcium release from and uptake into the SR of single chemically skinned skeletal muscle fibres in the presence of cumulative concentrations of dantrolene

	*Calcium release**		*Calcium uptake†*	
Concentration	*Normal*	*MHS*	*Normal*	*MHS*
Control	6.14 ±0.61 (7)	7.03 ±1.23 (7)	6.14 ±0.61 (7)	7.03 ±1.23 (7)
10^{-7}	6.69 ±0.98 (7)	6.26 ±1.25 (7)	5.34 ±1.37 (7)	4.66 ±0.52 (7)
10^{-6}	6.74 ±0.89 (7)	6.09 ±0.99 (7)	6.09 ±0.72 (7)	4.64 ±0.97 (7)
10^{-5}	6.37 ±1.19 (7)	5.80 ±0.82 (7)	5.47 ±0.87 (7)	3.89 ±1.14 (7)
5.7×10^{-5}	6.40 ±1.00 (6)	4.65 ±0.79 (4)	6.33 ±0.66 (6)	3.95 ±0.53 (4)

*Calcium release as determined by increase in tension observed after the addition of dantrolene with releasing caffeine.
†Calcium uptake as determined by increase in tension observed after the addition of dantrolene with releasing caffeine.

the tension in MHS fibres is lower than in normal fibres but this difference, again, is not statistically significant ($F = 2.32$, d.f. = 1 and 12, $p > 0.05$).

The differences between readings obtained at the four dantrolene concentrations and those obtained in the absence of the drug were statistically significantly different in normal and MHS muscle ($F = 5.51$, d.f. = 1 and 12, $p < 0.05$).

Discussion

In both normal and MHS human and porcine skeletal muscle fascicles, we have found that dantrolene inhibits twitch tensions and contractures. These values are in substantial agreement with those of Ellis and Carpenter (who used normal rat diaphragms),[6,13] Moulds (who used normal mouse soleus[8] and of Nelson (who used vastus lateralis from two MHS humans).[33]

Inhibition of twitch tensions would indicate that dantrolene might have reduced calcium release from the SR.[46-50] Inhibition of contractures would suggest that dantrolene sodium might have increased uptake of calcium into the SR.[46-50] It might, however, only mean that at the time of measurement the rate (increased) of calcium release from the SR is exceeding the rate (normal) of calcium reuptake into the SR.[51]

Our examination of the influence of dantrolene on twitch and resting tensions of whole skeletal muscle fascicles does not reveal whether the dantrolene is acting directly on the SR,[10,14,24,26] or whether its action on the SR is an indirect one. Thus it might have been that the primary effect of dantrolene is not to directly alter calcium uptake into, binding to or release from the SR. Rather these changes might be secondary to primary dantrolene influences occurring elsewhere in the muscle cell. For instance, dantrolene might, by a direct action on the sarcolemma[28] inhibit influx of extracellular fluid calcium into the myoplasm.[15,52-54] Release of "regenerative" or "trigger" calcium from the sarcolemma might be inhibited by dantrolene.[7,10] This "trigger" calcium in turn precipitates the release of much more calcium from the SR.[55-58] Recent exciting work by Nelson[59] suggests that indeed in MHS SR calcium induced calcium release occurs at much lower doses of calcium than in normal SR. Alternatively, dantrolene might exert its inhibitory effect on the transverse tubules or at the E–C coupling step at the gap junctions between the transverse tubules and the SR.[4,8,10,13,16,24,28]

On the other hand, it might be that dantrolene does act directly on the SR. Thus rather than inducing release of calcium through non voltage-dependent channels, dantrolene may inhibit release of calcium through the voltage dependent "chloride" channel of the SR.[60-62]

Alternatively, dantrolene might alter some calcium related function of the mitochondria, for example, it might increase calcium uptake into or inhibit calcium release from the mitochondria,[26,63,64] Cheah has suggested that the primary MHS muscle defect lies not in the SR, but rather in the mitochondria.[65] He has reported that endogenous phospholipase a_2 from MHS mitochondria, is greater than normal. He postulates that by liberating unsaturated fatty acids this phospholipase a_2 causes a greater than normal calcium release from the SR.[65] Finally, the functions of various cytoplasmic proteins which play a role in intracellular calcium metabolism such as calmodulin[66] might be altered by dantrolene.

Our studies favour the hypothesis that the benefi-

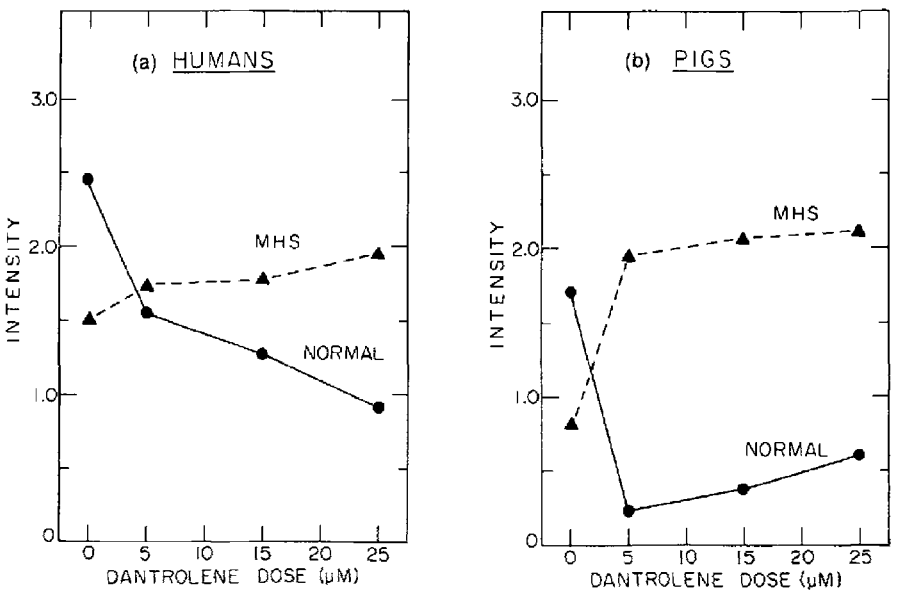

FIGURE 10 Intensity† of the effect of caffeine on resting tension.

† Intensity of the caffeine effect is measured by the slope of the straight lines fitted in plots contrasting the logarithm of resting tension with the logarithm of caffeine concentration.

cial action of dantrolene on the SR is partly, although perhaps not entirely, indirect. Thus dantrolene does suppress caffeine-potentiated twitches and caffeine-induced contractures of whole muscle fascicles. In the skinned fibre preparation, dantrolene does not improve calcium uptake into the SR or reduce calcium release from the SR. In fact, the lowest dose of dantrolene actually moderately inhibits calcium uptake into the SR. Consequently, the inability of dantrolene to inhibit caffeine potentiated twitches and contractures of whole muscle fascicles appears to be mainly due to an action at some site other than the SR. Dantrolene might well have a suppressive action on signal transmission across the E–C coupling step at the gap junction between the transverse tubules and the SR. Our studies are consistent with, but do not prove this postulation.

Since Moulds[8] has shown that dantrolene sodium reduces potassium-induced contractures of mammalian skeletal muscle, the sarcolemma must remain a candidate for a primary site of action of dantrolene. Potassium, by depolarizing the sarcolemma, induces release of "regenerative" calcium from the sarcolemma. Inhibition by dantrolene of "regenerative" calcium release from the SR would, therefore, be expected to reduce indirectly calcium release from the SR.

Reduction by dantrolene of influx of extracellular fluid calcium across the sarcolemma to the myoplasm is also a possibility in this regard. Derdemezi, working in our laboratory, is presently investigating the influence of dantrolene on the uptake of Ca^{++45} into electrically stimulated, isometrically mounted whole muscle fascicles.[67]

Suppression by dantrolene of calcium release from the mitochondria is not probable as mitochondria, although well able to accumulate considerable quantities of calcium, are not thought to release calcium in response to electrical or other stimulation. Rather, mitochondria act as long-term storage reservoirs for excess calcium, in the form of calcium phosphate appetite crystals, which is beyond the capacity of the SR to accumulate between

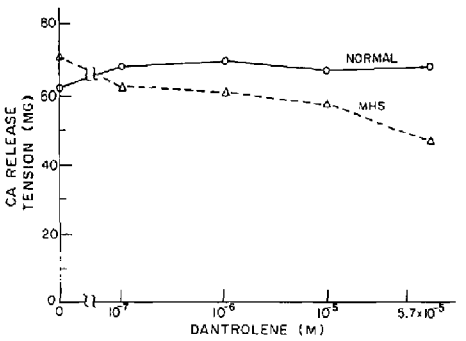

FIGURE 11a Calcium release from the SR of single chemically skinned skeletal muscle fibres.

Horizontal axis gives cumulative dose of dantrolene (in μM) added to each skinned fibre with the releasing caffeine. The greater the calcium released by the caffeine, the greater the ensuing tension increase of the fibre. Since dantrolene is added with releasing caffeine the tension developed by each fibre is a reflection of the influence of dantrolene on calcium release from the SR.

Vertical axis gives mean tension (in mg) of single chemically skinned human skeletal muscle fibre. Tension rise of skinned fibre is caused by caffeine induced calcium release from the SR.

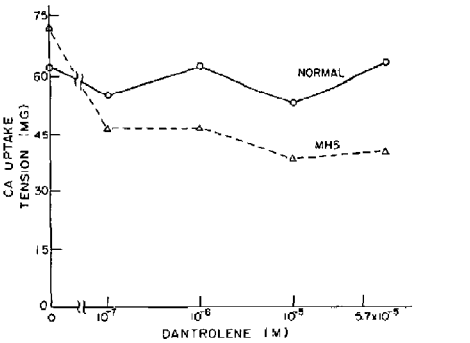

FIGURE 11b Calcium uptake into the SR of single chemically skinned skeletal muscle fibres.

Horizontal axis gives cumulative dose of dantrolene (in μM) added to each skinned fibre with the loading calcium. The greater the uptake of calcium into the Sr, the greater the calcium available for release by the caffeine and, therefore, the greater the ensuing tension increase of the fibre. Since dantrolene is added with the loading calcium, the tension developed by each fibre is a reflection of the influence of dantrolene in calcium uptake into the SR.

Vertical axis gives mean tension (in mg) of single chemically skinned human skeletal muscle fibre. Tension rise of skinned fibre is caused by caffeine induced calcium uptake into the SR.

myofibrillar contractions.[63,64,68-71] Uptake of calcium into the mitochondria is thus dependent on the myoplasmic concentration of calcium. On the other hand, if Cheah's postulations[65] are correct then dantrolene might act by lowering phospholipase a_2 from MHS mitochondria. Calcium release from the SR would thereby be suppressed because of impairment of fatty acid mobilization.

At several points in this paper we have observed that both the passage of time and the administration of a high caffeine concentration have a fatiguing effect on MHS muscle which is absent, or at least less in normal muscle. Thus, initially a smaller dose of caffeine is required to initiate contractures in MHS than in normal muscle at low caffeine concentrations the amplitude of the ensuing contractures is greater in the MHS than in the normal muscle. However, as time goes by, and as the dose of caffeine rises, the MHS muscle seems to suffer some untoward change which renders it less and less capable of sustaining tension increases. Whether this deterioration is due to the development of a greater unresponsiveness of the MHS than of the normal myofibrils to a prolonged period of an excessively high calcium-containing milieu or to a greater draining of calcium out of the MHS SR than out of the normal SR or to some other as yet unknown factor, has not so far been determined.

The effect of fatigue was noted when the concentration of either dantrolene or caffeine was left constant and the dose of the second drug was varied. Consequently, in each of the pairs of Figures 1 vs. 4, 3 vs. 2, 5 vs. 8 and 7 vs. 6, the first figure may illustrate fatigue effect along with changing dantrolene concentration, and the second figure may illustrate the effect of varying caffeine doses.

In conclusion, we find: (1) the reason for the beneficial effect of dantrolene in the prophylaxis and therapy of MH reactions remains uncertain; however, (2) the principal action of dantrolene is probably not directly on the SR; (3) the *in vivo* action of dantrolene on MHS muscle is reflected *in vitro* on its influence on the caffeine contracture

test, but not on its influence on the caffeine-skinned fibre tension test.

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Résumé

Le dantrolène sodique, un analogue de l'hydantoin, est efficace dans le traitement de l'hyperthermie maligne. Pour améliorer notre connaissance du mode d'action du dantrolène, nous avons étudié l'influence du dantrolène sodique sur:

1) la tension de contraction et de repos avec ou sans caféine sur des faisceaux de muscle squelettique intacts; et

2) l'augmentation de la tension induite par la caféine sur des faisceaux de muscle squelettique enrobés chimiquements.

Nous avons trouvé que le dantrolène semble exercer son action bénéfique sur le muscle squelettique susceptible à l'hyperthermie maligne par une action indirecte sur le réticulum sarcoplasmique (RS). Le dantrolène inhibe les tensions de contraction des faisceaux de muscle squelettique, probablement en prévenant indirectement le largage du calcium du RS. A un moindre degré, le dantrolène inhibe les contractures des faisceaux du muscle squelettique induites par la caféine probablement en accélérant indirectement la captation du calcium dans le RS.

Parce que le premier effet est plus important que le deuxième in-vivo, le dantrolène est efficace uniquement lorsqu'il est administré avant le largage total du calcium par le RS. Une surveillance vigilante de la température et de l'électrocardiogramme pendant l'anesthésie est essentielle.