The effect of dantrolene on skeletal-muscle sarcoplasmic-reticulum function in malignant hyperpyrexia in pigs

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The effect of the muscle relaxant dantrolene on isolated sarcoplasmic reticulum was studied in control and malignant-hyperpyrexia-susceptible Landrace pigs. The membranes prepared from both sources showed similar Ca^{2+} -dependent ATPase activities, had comparable phospholipid/protein ratios, and their sodium dodecyl sulphate/polyacrylamide-gel patterns were indistinguishable. Membranes from both sources appeared to bind similar amounts of dantrolene. The drug did not stimulate Ca^{2+} -dependent ATPase activity in preparations from either source. The rates of Ca^{2+} exchange and Ca^{2+} efflux appeared to be similar in sarcoplasmic reticulum of control and malignant-hyperpyrexia-susceptible pigs. Dantrolene did not affect either the rates or the amount of Ca^{2+} lost from the vesicles. These results suggest that dantrolene does not directly affect the movement of Ca^{2+} across the sarcoplasmic-reticulum membrane.

Dantrolene [1-({[5-(4-nitrophenyl)-2-furanyl]methylene}amino)-2,4-imidazolidinedione] is a specific skeletal-muscle relaxant (Ellis & Carpenter, 1972; Ellis *et al.*, 1973) used clinically in the treatment of spasticity of various origins and degrees of muscular involvement. The drug has also been used successfully in the treatment of malignant hyperpyrexia in pigs (Harrison, 1975; Hall *et al.*, 1977) and humans (Friesen *et al.*, 1979). Furthermore, dantrolene has been demonstrated to be effective in both preventing and reversing contractions produced in isolated muscle by the use of various agents (Austin & Denborough, 1977) used in the identification of susceptibility to malignant hyperpyrexia (Okumura *et al.*, 1979). Dantrolene has no effect on the electrical properties of muscle (Putney & Bianchi, 1974) or on neuromuscular transmission (Ellis & Carpenter, 1972). It has been suggested that the drug depresses excitation-contraction coupling by inhibiting Ca^{2+} release from the sarcoplasmic reticulum (Ellis & Bryant, 1972; Morgan & Bryant, 1977; Desmedt & Hainaut, 1979). Van Winkle (1976) and Campbell *et al.* (1980) have further shown that dantrolene suppresses Ca^{2+} release from isolated sarcoplasmic reticulum. In an attempt to establish the possible site of action of dantrolene in pig skeletal muscle, we

have examined the effect of the drug on the Ca^{2+} -dependent ATPase (EC 3.6.1.3) activity of sarcoplasmic reticulum and the release of Ca^{2+} from fragmented sarcoplasmic reticulum isolated from control pigs. We have also prepared sarcoplasmic reticulum from pigs susceptible to malignant hyperpyrexia (MHS) and have compared the effects of dantrolene on sarcoplasmic-reticulum function. The binding of dantrolene to fragmented sarcoplasmic reticulum at concentrations of dantrolene sodium used to prevent or reverse skeletal-muscle contractions *in vitro* (Nelson & Denborough, 1977) was also examined.

Materials and methods

All chemicals used were reagent grade. [^{14}C]-Dantrolene (77.7 $\mu\text{Ci}/\text{mg}$) was a gift from Norwich-Eaton Pharmaceuticals, Norwich, NY, U.S.A. $^{45}\text{CaCl}_2$ (1.98 mCi/ml) was obtained from Amersham International, Amersham, Bucks., U.K. Proteins used as molecular-weight markers in polyacrylamide gels were obtained from Sigma Chemical Co. These proteins were myosin (205 000), β -galactosidase (116 000), phosphorylase *b* (97 000), bovine serum albumin (66 000), ovalbumin (45 000), pepsin (35 000), carbonic anhydrase (29 000), trypsinogen (24 000), β -lactoglobulin (18 000) and lysozyme (14 000).

Abbreviation used: MHS, malignant-hyperpyrexia-susceptible.

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Identification of MHS pigs and removal of muscle

Biopsies of the gracilis muscle of Landrace pigs were obtained under thiopental anaesthesia. Susceptibility or resistance to malignant hyperpyrexia was confirmed in every animal by a contracture response *in vitro* to halothane, KCl, caffeine and succinylcholine (Okumura *et al.*, 1979).

Animals were killed, while still under anaesthesia, by using a captive bolt, and exsanguinated by severing the jugular vein. The biceps femoris and semitendinosus muscles were exposed, 500–800 g of muscle was removed, minced immediately and packed in ice or rapidly frozen in liquid N₂ for storage at -70°C . About 5 min usually elapsed between the death of the animal and the packing of minced muscle in ice. Homogenization of the fresh tissue was performed within 45 min of removal from the animal.

Preparation of fragmented sarcoplasmic reticulum

Minced muscle samples (50–100 g) were homogenized in 5 vol. of 0.25 M sucrose/10 mM-Tris/HCl buffer, pH 7.4. Frozen muscle samples were allowed to warm up to 0°C in the above medium before homogenization. The homogenization was performed in three 30 s bursts with 30 s rests in a Waring Blendor ('LOW' speed setting). After filtration through coarse gauze, the homogenate was centrifuged at $3000g_{\text{av}}$ for 5 min to remove myofibrillar proteins and large fragments of tissue. The supernatant was then centrifuged at $10000g_{\text{av}}$ for 20 min, and the mitochondrial pellet obtained was discarded. The supernatant was centrifuged at $100000g_{\text{av}}$ for 45 min, and the microsomal pellet obtained was washed once in 0.5 vol. of the original homogenization medium. The final pellet was resuspended in 0.5–1 ml of homogenization medium with a glass/Teflon homogenizer. This membrane suspension was called the fragmented sarcoplasmic-reticulum preparation and was used immediately for transport studies, or stored for other studies at -20°C . The entire preparative procedure was performed at $0-4^{\circ}\text{C}$.

Enzyme assays

Total ATPase activity was measured in 0.5 ml of a medium which comprised 0.5 mM-CaCl₂, 100 mM-NaCl, 10 mM-KCl, 10 mM-MgCl₂, 1 mM-ATP and 10 mM-Tris/HCl, pH 7.4. For measurement of Ca²⁺-independent activity, 1 mM-EGTA/Tris, pH 7.4, replaced the 0.5 mM-CaCl₂. The Ca²⁺-dependent ATPase activity was the difference between the total ATPase activity and the Ca²⁺-independent activity. The P_i liberated from ATP was measured by the method of Heinonen & Lathi (1981); the ATPase reaction was started by the addition of 20–50 μg of protein and terminated after incubation for 15 min at

37°C by the addition of the acetone/acid/molybdate colour reagent.

5'-Nucleotidase activity was assayed at 37°C by measuring P_i liberated in 15 min from a buffer containing 50 mM-5'-AMP and 0.5 M-glycine/NaOH, pH 9.1; 100–200 μg of protein was used. Succinate dehydrogenase activity was measured at 20°C by the method of King (1963). Acid phosphatase activity was measured at 37°C in 0.5 ml of medium containing 1 mM-*p*-nitrophenyl phosphate and 0.1 M-citrate, pH 5.0. The reaction was started by the addition of 100–200 μg of protein and terminated after 15 min by the addition of 0.2 ml of 10% (w/v) sodium dodecyl sulphate and 5 ml of 20 mM-NaOH. The amount of *p*-nitrophenol formed was measured at 400 nm.

Ca²⁺-transport studies

Ca²⁺ movement across fragmented sarcoplasmic-reticulum membranes was measured at 37°C by a Millipore-filtration method with 0.22 μm filters. Vesicles (2.25 mg of protein/ml) were loaded passively by overnight incubation at 0°C in 100 mM-KCl/5 mM-MgCl₂/10 mM-Tris/HCl, pH 7.4 (loading buffer) and 1 mM-CaCl₂ (including ⁴⁵Ca²⁺, 19.8 $\mu\text{Ci/ml}$). Loaded vesicles were preincubated for 15 min at 37°C with 20 μM -dantrolene (in dimethyl sulphoxide) or in dimethyl sulphoxide alone before 50-fold dilution into the tracer-free loading buffer containing 1 mM-CaCl₂ and 20 μM -dantrolene or dimethyl sulphoxide. Transport was terminated by mixing 100–200 μl portions of the diluted sample into 2 ml of an ice-cold solution of the loading buffer containing 1 mM-CaCl₂ and washing with 5 ml of the same cold buffer. In other transport experiments, Ca²⁺-free buffers were used after passive loading in 1 mM-CaCl₂ had been performed in the usual way. The filters were counted for ⁴⁵Ca²⁺ in a Packard Tri-Carb liquid-scintillation counter, with a scintillant composed of xylene/Triton X-114 (2:1, v/v) containing 0.5% (w/v) 2,5-diphenyloxazole.

Sedimentation dialysis

The binding of [¹⁴C]dantrolene to fragmented sarcoplasmic-reticulum preparations was studied by sedimentation dialysis. The membranes (1–1.5 mg of protein/ml) were incubated for 30 min at 37°C in a buffer consisting of 100 mM-NaCl, 10 mM-KCl, 10 mM-MgCl₂ and 10 mM-Tris/HCl, pH 7.4, and 1–10 μM -dantrolene (including [¹⁴C]dantrolene, 0.24 $\mu\text{Ci/ml}$). The samples were then placed in polythene centrifuge tubes (450 μl capacity) and centrifuged at $100000g_{\text{av}}$ for 30 min in a Beckman L3-65B ultracentrifuge with a SW27 swinging-bucket rotor. Duplicate 200 μl portions of the samples were counted for radioactivity (d.p.m.) in the xylene/Triton liquid-scintillation medium before centrifuga-

tion (R_{total}) and after centrifugation (R_{free}). The concentration of bound dantrolene was obtained from the equation:

$$\text{Concn. bound } (\mu\text{M}) = \text{Total concn. } (\mu\text{M}) \times \frac{R_{\text{total}} - R_{\text{free}}}{R_{\text{total}}}$$

Other methods

For electrophoresis, 7.5–15% (w/v) continuous polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulphate were run in the discontinuous buffer system of Laemmli (1970); gels were run at a constant current (30 mA/gel), stained with Coomassie Blue R250 and scanned in a Beckman DU-8 spectrophotometer. Protein was determined by the method of Petersen (1977); phospholipid content of membranes was measured after HClO_4 hydrolysis of the phospholipid to P_i (Dittmer & Wells, 1969).

Results

Characterization of fragmented sarcoplasmic-reticulum preparation

Whether prepared from control or MHS-pig skeletal muscle, the Ca^{2+} -dependent ATPase activities of fragmented sarcoplasmic reticulum were in the range 0.3–0.8 μmol of P_i /min per mg of protein; preparations from frozen tissue also gave values in the same range. These values are comparable with those reported by McIntosh *et al.* (1977) for partially purified pig muscle sarcoplasmic-reticulum preparations. Mitochondrial contamination in the fragmented sarcoplasmic reticulum was low, as evidenced by the presence of some succinate dehydrogenase activity; this activity was about 5% of the succinate dehydrogenase activity of crude mitochondrial fractions. The presence of plasma membrane impurities was indicated by 5'-nucleotidase activity in the range 0.01–0.02 μmol of P_i /min per mg of protein. Acid phosphatase activity, used as a marker for contaminating lysosomes, was less than 0.01 μmol of *p*-nitrophenol formed/min per mg of protein.

No differences or trends were observed in the Ca^{2+} -dependent ATPase activity of control (0.49 ± 0.16) and MHS-pig material (0.46 ± 0.1) (both means $\pm$ S.D.; $n = 5$). The phospholipid/protein ratio of the fragmented sarcoplasmic-reticulum preparations from control pigs was 0.75 ± 0.05 and from MHS pigs it was 0.77 ± 0.05 (means $\pm$ S.D.; $n = 5$). These values are different from those reported by McIntosh *et al.* (1977), and may reflect differences in the muscle selected, the method of preparation and the type of sarcoplasmic-reticulum preparation. Sodium dodecyl sulphate/polyacrylamide-gel patterns, shown in Fig. 1, consisted of two

major bands of apparent molecular weights 105 000 and 97 000, corresponding to the ATPase and to phosphorylase respectively. Also present were a large number of other components, making it difficult to identify any differences between the fragmented sarcoplasmic-reticulum preparations from control

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of sarcoplasmic-reticulum proteins. Approx. 100 μg of fragmented sarcoplasmic-reticulum protein of control (lower trace) and MHS pigs (upper trace) was applied to adjacent sample wells of a 7.5–15% (w/v) polyacrylamide gradient slab gel containing 0.1% (w/v) sodium dodecyl sulphate. After electrophoresis at a constant current (30 mA/slab) for approx. 6 h, the gels were stained with Coomassie Blue R250 and scanned at 520 nm in a Beckman DU-8 spectrophotometer. The apparent molecular weights ($\times 10^{-3}$) of various protein bands (arrows) were obtained by calibration of the gel with proteins of known molecular weights (see the Materials and methods section).

and MHS pigs. However, we have observed that, during preparation, the MHS-pig fragmented sarcoplasmic reticulum did not form a tightly packed pellet after the centrifugation at $100\,000g_{av.}$ for 45 min. Preparations from frozen MHS-pig tissue, however, behaved normally.

Dantrolene binding to fragmented sarcoplasmic reticulum

In preliminary studies designed to examine the binding of [^{14}C]dantrolene to fragmented sarcoplasmic-reticulum membranes, we observed that a significant portion of the label was bound to cellulose nitrate-based materials such as centrifuge tubes and Millipore filters, precluding the use of these materials in the study. Dialysis membranes also bound 10–20% of the radioactive dantrolene, amounts that were significant, especially at low dantrolene concentrations. Polythene tubes were found to bind the least amount of the radioactive label, usually less than 5%, and were used in this study. Corrections were made for this artifactual binding in every experiment. The presence of 0.5 mM- $CaCl_2$ and/or 1 mM-ATP in the buffer used did not appear to affect the amount of dantrolene bound to the fragmented sarcoplasmic reticulum.

Fig. 2. Binding of dantrolene to fragmented sarcoplasmic reticulum

The binding of dantrolene to control membranes (●; average of two determinations) and to MHS-pig membranes (○; average of four determinations) was measured by sedimentation dialysis. The incubation was done at 37°C for 30 min in 100 mM-NaCl/10 mM-KCl/10 mM-MgCl₂/10 mM-Tris/HCl, pH 7.4. Dantrolene was used in the concentration range 1–10 μM. The protein concentration was 1–1.5 mg/ml. Duplicate 200 μl samples were counted for radioactivity before and after centrifugation.

Fig. 2 shows that the amount of dantrolene bound per mg of protein increased linearly with the concentration of the drug. Fragmented sarcoplasmic reticulum from control and MHS-pig skeletal muscle appeared to bind similar amounts of dantrolene. Analysis of these data by a Scatchard plot failed to reveal any high-affinity binding sites for dantrolene in the concentration range 1–10 μM in either membrane preparation. These results are in contrast with those of Sengupta *et al.* (1980), who obtained, under different buffer conditions and using an equilibrium-dialysis system, evidence of high-affinity binding sites in pig skeletal-muscle sarcoplasmic reticulum.

Effect of dantrolene on Ca^{2+} -dependent ATPase activity

Since dantrolene has an absorbance maximum at 390 nm, it can interfere with the determination of P_i by the method of Heinonen & Lathi (1981), which uses readings at 355 nm. Therefore, in these experiments, the Ca^{2+} -dependent ATPase activity was determined by the inclusion of the appropriate amount of dantrolene in the respective EGTA-con-

Fig. 3. Effect of dantrolene on Ca^{2+} -dependent ATPase activity

Total ATPase activity was determined in a medium containing 0.5 mM- $CaCl_2$, 100 mM-NaCl, 10 mM-KCl, 10 mM-MgCl₂, 10 mM-Tris/HCl, pH 7.4, and 20 μM-dantrolene. The incubation was for 15 min at 37°C in the presence of 20–50 μg of protein. The Ca^{2+} -dependent ATPase activity was the difference between the total ATPase activity and the ATPase activity in the presence of 1 mM-EGTA. The effect of dantrolene on Ca^{2+} -dependent ATPase activity was tested in six MHS-pig and four control preparations. In the representative experiment shown, the closed symbols represent the results obtained with control sarcoplasmic reticulum and the open symbols the results with MHS-pig sarcoplasmic reticulum.

taining tubes. Dimethyl sulphoxide alone failed to affect the Ca^{2+} -dependent ATPase activity. The effect of dantrolene was tested in six preparations from MHS pigs and four from controls. In a representative experiment shown in Fig. 3, up to $20\text{ }\mu\text{M}$ -dantrolene did not appear to increase or decrease to any significant extent the Ca^{2+} -dependent ATPase activity of control or MHS-pig membrane preparations. It was also observed that dantrolene did not appear to affect the $\text{Na}^{+} + \text{K}^{+}$ -dependent ATPase activity.

Ca^{2+} movement in the presence of dantrolene

All transport experiments were performed on

freshly prepared sarcoplasmic-reticulum material. Initial experiments established that over 90% of the membrane protein was retained by $0.22\text{ }\mu\text{m}$ Millipore filters. Further, in every experiment, the amount of protein filtered was chosen such that filtration and washing was completed in 30–40 s. As shown in Fig. 4(a), the efflux of Ca^{2+} from the vesicles was slow at 0°C , so that losses of Ca^{2+} during the brief filtration and washing procedure were minimal.

Passively loaded fragmented sarcoplasmic-reticulum vesicles showed a very rapid movement of Ca^{2+} across the membrane when diluted into $1\text{ mM-}\text{Ca}^{2+}$ solutions maintained at 37°C (Fig. 4a).

Fig. 4. *Equilibrium exchange of Ca^{2+} in sarcoplasmic reticulum*

The exchange of Ca^{2+} across control fragmented sarcoplasmic-reticulum membranes (a) and MHS-pig membranes (b) was monitored at 37°C by a Millipore-filtration method. Passively loaded vesicles (see the Materials and methods section) were diluted 50-fold into a medium containing $1\text{ mM-}\text{Ca}^{2+}$. The radioactivity trapped on the filter at the times shown was expressed as a percentage of the 0 min sample. The rate of exchange of Ca^{2+} at 0°C is shown as (■); the results obtained in the presence of $20\text{ }\mu\text{M}$ -dantrolene are indicated (▼). These experiments were done with five different preparations each of control (●) and MHS-pig membranes (○); representative experiments are shown.

Fig. 5. *Ca^{2+} efflux from sarcoplasmic reticulum*

The efflux of Ca^{2+} from five different preparations of control membranes (a) and MHS-pig membranes (b) was measured by dilution of passively loaded vesicles into a Ca^{2+} -free medium (see Fig. 4 and the Materials and methods section for details). Efflux rates in the presence of $20\text{ }\mu\text{M}$ -dantrolene are shown (▼). The results of a representative experiment are shown for control (●) and MHS-pig (○) membranes.

A similar result was obtained when MHS-pig fragmented sarcoplasmic-reticulum vesicles were studied in the same way (Fig. 4*b*). Approx. 50% of the internal $^{45}\text{Ca}^{2+}$ was exchanged within 2 min, a rate similar to that reported by Campbell *et al.* (1980). The exchange of label was usually completed in about 15 min. When tested, 20 μM -dantrolene sodium did not appear to affect either the rate of exchange of label or the total amount of label lost from the vesicles.

Fig. 5(*a*) shows the efflux of Ca^{2+} from passively loaded vesicles into a Ca^{2+} -free medium; the carry-over of Ca^{2+} from the original loading buffer resulted in the final Ca^{2+} concentration during efflux being 0.02 mM. The rate of efflux of Ca^{2+} appeared to be the same as the equilibrium exchange process, and the amount of Ca^{2+} lost appeared to be the same. Here again, 20 μM -dantrolene did not appear to decrease the rate or amount of Ca^{2+} lost from the vesicles. Further, MHS-pig membranes released Ca^{2+} at the same rate as control membranes (Fig. 5*b*).

Discussion

Our interest in pig skeletal-muscle sarcoplasmic-reticulum function stems from the use of pigs as experimental animals for studies on malignant hyperpyrexia in humans, which appears to be a disorder of Ca^{2+} movement in skeletal muscle (Gronert, 1980). The ability of dantrolene to prevent or reverse the abnormal contracture response *in vitro* of MHS-pig muscle (Austin & Denborough, 1977) focused our attention on the possible effects of the drug on sarcoplasmic-reticulum function. The range of dantrolene concentrations used in the present study was 1–20 μM ; it is in this range that the drug is effective *in vitro* (Austin & Denborough, 1977). Further, all these studies were performed at 37°C, as the drug-induced contractures of MHS-pig muscle are temperature-dependent (Sullivan & Denborough, 1981).

We have prepared crude sarcoplasmic-reticulum membranes from control and MHS-pig skeletal muscle under conditions that minimized changes in the tissue *post mortem*. Further, we have used a limited number of steps in the isolation of the reticulum in order to attenuate any changes that may take place preferentially in the material from MHS pigs. In spite of these precautions, that changes *post mortem* had already taken place was evident from the fact that the MHS-pig microsomal fraction failed to pack tightly during high-speed centrifugation. We have noted, however, that sarcoplasmic reticulum prepared from frozen MHS-pig muscle pelleted normally. Apart from this aberrant sedimentation behaviour, sarcoplasmic reticulum from control and MHS pigs were very similar in

their phospholipid/protein ratios, protein composition and Ca^{2+} -dependent ATPase activity. It may be noted that McIntosh *et al.* (1977) have isolated sarcoplasmic reticulum from pig skeletal muscle selected on the basis of the degenerative changes occurring *post mortem*.

In comparing the movement of Ca^{2+} across membranes prepared from control and MHS-pig muscle, we have found the rate of Ca^{2+} loss and the amount of Ca^{2+} lost from either preparation to be indistinguishable. These results were obtained with passively loaded fragmented sarcoplasmic reticulum, and are in agreement with the results of Gronert *et al.* (1979). Since the efflux studies of McIntosh *et al.* (1977) were done in the presence of oxalate, their results cannot be compared directly with the results of the present study. Dantrolene did not appear to have any effect on Ca^{2+} movement in either control or MHS-pig membranes. Our studies indicate that control and MHS-pig sarcoplasmic reticulum bind the same amount of dantrolene; this binding appears to represent low-affinity saturation of the membrane phospholipids with the drug. No evidence was obtained for high-affinity binding of dantrolene to the membranes in the 1–10 μM concentration range.

We have chosen two different experimental approaches to examine the movement of Ca^{2+} across the sarcoplasmic-reticulum membrane. The equilibrium method allows the measurement of Ca^{2+} movement in the absence of any stimulus, whereas the efflux of Ca^{2+} down its concentration gradient may be considered representative of the situation *in vivo*. The results obtained in this study indicate that the sarcoplasmic reticulum is permeable to Ca^{2+} , a property consistent with its role in the release of Ca^{2+} during muscle contraction. However, in these experiments, it is unlikely that calsequestrin or the high-affinity Ca^{2+} -binding protein inside the isolated sarcoplasmic reticulum would have immobilized any of the exogenous Ca^{2+} . The failure of dantrolene to decrease either the rate of loss of Ca^{2+} or the amount of Ca^{2+} lost suggests that the drug may not act directly on Ca^{2+} fluxes. Our results do not support the observations by Van Winkle (1976) or Campbell *et al.* (1980), who showed that the drug decreased the rate of Ca^{2+} release from the sarcoplasmic reticulum. It may be necessary to use a 'heavy' sarcoplasmic-reticulum fraction (Campbell *et al.*, 1980) in order to demonstrate the effect of the drug in pig muscle. Although it is possible that dantrolene may exert its effect by directly stimulating Ca^{2+} -dependent ATPase activity, thereby resulting in an enhanced Ca^{2+} uptake, the results of the present study indicate that the drug does not act in this way. We suggest that the mechanism of action of dantrolene on Ca^{2+} movement across the sarcoplasmic reticulum may be less direct. The ability of the drug

to block the phosphorylation of calsequestrin (Campbell & Shamoo, 1980) provides a mechanism whereby the amount of Ca^{2+} bound by calsequestrin may be influenced by its phosphorylation state.

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