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SKELETAL MUSCLE MITOCHONDRIAL PHOSPHOLIPASE A_2 AND THE INTERACTION OF MITOCHONDRIA AND SARCOPLASMIC RETICULUM IN PORCINE MALIGNANT HYPERTHERMIA

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Comparative studies were carried out on the Ca^{2+}-transport systems of mitochondria and sarcoplasmic reticulum from longissimus dorsi muscle of genetically selected malignant hyperthermia-prone and normal pigs in order to identify the biochemical lesion responsible for the enhanced release of Ca^{2+} in the sarcoplasm occurring in porcine malignant hyperthermia. Mitochondria isolated from longissimus dorsi muscle of malignant hyperthermia-prone pigs contained a significantly ($P < 0.001$) higher amount of endogenous long-chain fatty acids. Similar amounts of endogenous mitochondrial phospholipase A_2 were observed in both types of pigs, but the total activity in malignant hyperthermia-prone pigs was at least twice that of normal. Spermine, a phospholipase A_2 inhibitor, lowered the activity in both types of mitochondria to a similar final level. Mitochondria of malignant hyperthermia-prone pigs showed a significantly ($P < 0.001$) higher oligomycin-insensitive ($Ca^{2+} + Mg^{2+}$)-ATPase activity, but the Mg^{2+}-ATPase and the ($Ca^{2+} + Mg^{2+}$)-ATPase activities were similar in both types of pigs. Sarcoplasmic reticulum isolated from longissimus dorsi muscle of malignant hyperthermia-prone pigs showed a significantly higher ($Ca^{2+} + Mg^{2+}$)-ATPase activity and a lower rate of Ca^{2+} uptake; the maximal amount and the rate of Ca^{2+} uptake by sarcoplasmic reticulum of malignant hyperthermia-prone pigs were half that of normal. Mitochondria from longissimus dorsi muscle of malignant hyperthermia-prone pigs inhibited the Ca^{2+}-transport system of the sarcoplasmic reticulum of longissimus dorsi from both normal and malignant hyperthermia-prone pigs, but mitochondria from normal pigs had no influence on the sarcoplasmic reticulum from either type. Experimental evidence favours the concept that long-chain fatty acids released from skeletal muscle mitochondria by endogenous mitochondrial phospholipase A_2 are responsible for the enhanced release of Ca^{2+} from mitochondria (Cheah, K.S. and Cheah, A.M. (1981) Biochim. Biophys. Acta 634, 70–84), and also additional release of Ca^{2+} from sarcoplasmic reticulum into the sarcoplasm during porcine malignant hyperthermia syndrome.

Introduction

Two well characterized stress syndromes exist in certain breeds of pigs, particularly those developing leaner carcasses. The first is malignant hyperthermia which can easily be induced in stress-susceptible pigs by volatile agents such as halothane [1–6], a fluorinated hydrocarbon anaesthetic, and hot climatic conditions [7]. Once initiated, the response cascades into a fulminant and vicious spiral during which the body temperature may increase at a rate of 1°C per 5 min [8], accompanied by severe metabolic acidosis and elevated levels of serum metabolites [9,10] and if uncontrolled, death occurs. The manifestations of porcine malignant hyperthermia are similar to those described for human malignant hyperthermia, which is also responsible for anaesthetic deaths in apparently healthy patients [8,11]. The second manifestation of porcine stress syndrome is a post-mortem phenomenon and is associated with the formation of pale, soft and exudative meat. The latter condition is linked with rapid post-mortem glycolysis [12,13] and denaturation of myofibrillar and sarcoplasmic proteins [12].

It is generally accepted that malignant hyper-

thermia is a primary disorder of skeletal muscle [8, 14,15], with the sympathetic nervous system being implicated only as a secondary response in the syndrome [8,16]. The increase in the level of sarcoplasmic Ca^{2+} in malignant hyperthermia [8,17] is generally believed to be responsible for the series of biochemical events which ultimately lead to severe acidosis through an increase in lactic acid formation. In spite of the well documented etiology of the porcine malignant hyperthermia syndrome, the biochemical lesion responsible for the enhanced release of Ca^{2+} in the sarcoplasm still remains to be identified.

We recently suggested [18] that phospholipase A_2 (EC 3.1.1.4, phosphatide 2-acylhydrolase) in mitochondria of skeletal muscle (longissimus dorsi) is responsible for the difference in the mitochondrial transition temperature between normal and malignant hyperthermia-prone pigs and the large-amplitude swelling in mitochondria of malignant hyperthermia-prone pigs. We also postulated that the fatty acids liberated from mitochondria of malignant hyperthermia-prone pigs by phospholipase A_2 would induce the sarcoplasmic reticulum to release more Ca^{2+} to account for the enhanced level of Ca^{2+} in the sarcoplasm, which ultimately results in the loss of control of glycolysis. This paper provides data to support this hypothesis, demonstrating that isolated mitochondria of malignant hyperthermia-prone pigs contain significantly higher endogenous phospholipase A_2 activity than normal, and that only mitochondria of malignant hyperthermia-prone pigs can influence the function of sarcoplasmic reticulum.

Materials and Methods

Reagents

ATP (sodium salt), bovine serum albumin, dithiothreitol, imidazole, oligomycin, phospholipase A_2 (from procine pancreas), rotenone and the sodium salts of succinic, arachidonic, linoleic, oleic and palmitic acids were obtained from Sigma Chemical Co.; crystalline *Bacillus subtilis* proteinase (Nagarse) was obtained from Teikoku Chemical Co. or Sigma Chemical Co.; all other reagents were of analytical grade.

Methods

Mitochondria were isolated from longissimus dorsi muscle using *B. subtilis* [18] proteinase and sarcoplasmic reticulum from similar muscle by modifying [19] the procedure of Martonosi and Feretos [20]. Endogenous mitochondrial long-chain free fatty acids were determined with 1,5-diphenylcarbazide [21] using 0.2 mg mitochondrial protein. The procedure of Nixon and Chan [21] for determination of long-chain fatty acids in mitochondria was modified by using 0.31 M NaOH in the copper reagent, 88% ethanol instead of absolute ethanol in the colour reagent, and 0.8 ml of 750 mM $H_2PO_4^-$ instead of 100 mM $H_2PO_4^-$ was added to the extraction solution before washing twice instead of once with 0.01 M HCl. After these modifications, a linear standard through the origin was obtained with sodium stearate. The absorbance at 550 nm of the reagent blank against chloroform was zero as compared with a value of between 0.035 and 0.120 [21]. Endogenous phospholipase A_2 activity was estimated by the formation of long-chain fatty acids after incubation of 0.80 ml (18–19 mg protein) mitochondrial suspension in 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl (pH 7.20) at 40°C for 20 min. The mitochondrial suspension was then centrifuged twice for 2 min in an Eppendorf (Model 3200) microcentrifuge, and the liberated long-chain fatty acids in the clear supernatant estimated by the above-modified method with 1,5-diphenylcarbazide [21] using sodium stearate as standard. The endogenous content of mitochondrial phospholipase A_2 was determined with Cibacron Blue F3GA [22] after the mitochondria had been treated with sodium deoxycholate. 5 mg sodium deoxycholate were added to 0.17 ml (6–7 mg protein) mitochondria in 0.23 ml of 225 mM mannitol, 75 mM sucrose, and 15 mM Tris-HCl (pH 7.20) at room temperature in a 1.5 ml Eppendorf (Model 3200) centrifuge tube. After mixing for 5–10 min at room temperature, the mitochondrial suspension was centrifuged twice for 2 min, and the phospholipase A_2 in the clear supernatant was estimated spectroscopically with Cibacron Blue F3GA. The sample cuvette (10 mm light-path) contained 0.10 ml deoxycholate supernatant in 2.50 ml of 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl (pH 7.20) and the reference cuvette contained only 2.60 ml of 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl (pH 7.20). 240 nmol Cibacron Blue F3GA were added to both the cuvettes and the spectrum was then recorded from 550 to 750 nm using the split-

beam mode with an Aminco-Chance dual-wavelength/split beam DW-2A spectrophotometer. The amount of phospholipase A_2 was estimated using the wavelength pairs at 667–645 nm, since the peak of the complex at 675 nm [22] was shifted to 666–667 nm in the presence of deoxycholate, confirmed with authentic phospholipase A_2.

The Ca^{2+}-stimulated respiration for succinate oxidation by longissimus dorsi muscle mitochondria was measured with a Clark oxygen electrode (Yellow Springs Oxygen Monitor, model 53) in a total volume of 2.50 ml at 25°C in 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl (pH 7.20). The mitocondrial ATPase activities were estimated by the P_i [23] released during 5 min incubation at 37°C in a medium (pH 7.15) consisting of 75 mM KCl, 108 mM sucrose, 50 mM Tris-HCl, 1 mM EDTA, 2 mM ATP (sodium salt) and mitochondria (0.7–0.9 mg protein) in a total volume 2.0 ml. The Mg^{2+}-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase activities were estimated in the presence of 3 mM $MgCl_3$ and 3 mM $MgCl_2$ plus 2 mM $CaCl_2$, respectively, in the presence and absence of 3 μg oligomycin. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of the sarcoplasmic reticulum was determined at 35°C by the P_i released after 1 min incubation in a medium (pH 7.20) consisting of 40 mM imidazole, 100 mM KCl, 4 mM $MgCl_2$, 0.05 mM $CaCl_2$ and sarcoplasmic reticulum (0.8–0.9 mg protein). The reaction was started by the addition of 4 mM ATP (sodium salt) and terminated by the addition of ice-cold 2 ml perchloric acid (4%). The Ca^{2+} uptake and release by sarcoplasmic reticulum were monitored continuously with a Radiometer calcium-selective electrode (F2112 Ca Selectrode) connected to a Radiometer PHM 64 research pH meter and a recorder. Protein was determined according to the method of Lowry et al. [24] using bovine serum albumin as standard.

Results

ATPase activities of longissimus dorsi muscle mitochondria

Comparative studies of skeletal muscle mitochondrial Mg^{2+}-ATPase and the $(Ca^{2+} + Mg^{2+})$-ATPase activities [25], and their sensitivity towards oligomycin were investigated using tightly coupled preparations [18] from longissimus dorsi of malignant hyperthermia-prone and normal pigs. No significant difference was observed either in the Mg^{2+}-ATPase (Table I) or in the $(Ca^{2+} + Mg^{2+})$-ATPase (Table II) activities between the two types of pigs. 42% of the Mg^{2+}-ATPase activity of normal mitochondria was found to be insensitive to oligomycin compared with 48% in the malignant hyperthermia-prone pigs. The findings are consistent with the report of 50% oligomycin-insensitive Mg^{2+}-ATPase activity for skeletal muscle mitochondria isolated from the hind leg of rat [25]. Mitochondria of malignant hyperthermia-prone pigs, however, contained a significantly ($P < 0.001$) higher oligomycin-insensitive $(Ca^{2+} + Mg^{2+})$-ATPase activity than normal (Table II). The greater loss of oligomycin sensitivity in the $(Ca^{2+} + Mg^{2+})$-ATPase activity of malignant hyperthermia-prone pigs could be contributed by the higher Ca^{2+}-stimulated endogenous phospholipase A_2 activity in these mitochondria (see later evidence), since the activity of this

TABLE I

Mg^{2+}-ATPase ACTIVITIES OF LONGISSIMUS DORSI MUSCLE MITOCHONDRIA OF MALIGNANT HYPERTHERMIA-PRONE AND NORMAL PIGS

Experimental details as described in Materials and Methods. *n*, number of pigs used. n.s., not significant.

Longissimus dorsi muscle mitochondria	ATPase activities (μmol P_i/mg protein per 5 min) at 37°C		
	Mg^{2+}-ATPase	Oligomycin-insensitive Mg^{2+}-ATPase	Oligomycin-sensitive Mg^{2+}-ATPase
Malignant hyperthermia	0.84 ± 0.06 (n = 8)	0.40 ± 0.14 (n = 6)	0.49 ± 0.12 (n = 6)
Normal	0.81 ± 0.11 (n = 8)	0.34 ± 0.05 (n = 6)	0.52 ± 0.06 (n = 6)
Student's t-test	n.s. ($P > 0.05$)	n.s. ($P > 0.05$)	n.s. ($P > 0$ 05)

TABLE II

$(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITIES OF LONGISSIMUS DORSI MUSCLE MITOCHONDRIA OF MALIGNANT HYPERTHERMIA-PRONE AND NORMAL PIGS

Experimental details are given in Materials and Methods. *n*, number of pigs used. n.s., not significant; s, significant.

Longissimus dorsi muscle mitochondria	ATPase activities (μmol P_i/mg protein per 5 min) at 37°C		
	$(Ca^{2+} + Mg^{2+})$-ATPase	Oligomycin-insensitive $(Ca^{2+} + Mg^{2+})$-ATPase	Oligomycin-sensitive $(Ca^{2+} + Mg^{2+})$-ATPase
Malignant hyperthermia	1.35 ± 0.13 (n = 7)	0.32 ± 0.06 (n = 7)	1.00 ± 0.16 (n = 7)
Normal	1.27 ± 0.21 (n = 8)	0.17 ± 0.04 (n = 8)	1.04 ± 0.26 (n = 8)
Student's t-test	n.s. ($P > 0.05$)	s. ($P < 0.001$)	n.s. ($P > 0.05$)

enzyme is known to destroy the oligomycin sensitivity of the mitochondrial ATPase [26].

Endogenous fatty acids, phospholipase A_2 and phospholipase A_2 activity

Table III shows the amount of endogenous long-chain fatty acids and phospholipase A_2 in longissimus dorsi muscle mitochondria of malignant hyperthermia-prone and normal pigs, and the effect of spermine, a phospholipase A_2 inhibitor [27], on the endogenous phospholipase A_2 activity. Mitochondria of malignant hyperthermia-prone pigs contained a significantly ($P < 0.001$) higher amount of free long-chain fatty acids than those from normal pigs. A similar amount of endogenous phospholipase A_2 was observed in the two types of skeletal muscle mitochondria but the endogenous phospholipase A_2 activity in malignant hyperthermia-prone pigs was more than twice that of normal. Spermine inhibited the endogenous phospholipase A_2 activity in both types of mitochondria, and the difference in the endogenous phospholipase A_2 activity was abolished in the presence of 1.0 mM spermine.

$(Ca^{2+} + Mg^{2+})$-ATPase activity, Ca^{2+} uptake and Ca^{2+} binding in sarcoplasmic reticulum

Table IV summarizes the data for the $(Ca^{2+} + Mg^{2+})$-ATPase activity, Ca^{2+} uptake and Ca^{2+} binding of sarcoplasmic reticulum isolated from longissimus dorsi muscle of malignant hyperthermia-prone and normal pigs. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of malignant hyperthermia-prone pigs is higher than

TABLE III

ENDOGENOUS FATTY ACIDS, PHOSPHOLIPASE A_2 AND PHOSPHOLIPASE A_2 ACTIVITY IN LONGISSIMUS DORSI MUSCLE MITOCHONDRIA OF MALIGNANT HYPERTHERMIA-PRONE AND NORMAL PIGS

The amount of endogenous phospholipase, expressed in absorbance (A) units per mg protein, was estimated with Cibacron Blue F3GA [22] and the complex was confirmed with authentic phospholipase A_2 from porcine pancreas. Endogenous phospholipase A_2 activity was determined by the formation of long-chain fatty acids after incubation for 20 min at 4°C with 1,5-diphenylcarbazide [21]. Other experimental details are given in Materials and Methods. *n*, number of pigs used. n.s., not significant; s., significant.

Longissimus dorsi muscle mitochondria	Endogenous fatty acids (nmol/mg protein)	Endogenous phospholipase A_2 ($A_{667-645nm}$/mg protein)	Endogenous phospholipase A_2 activity (nmol fatty acid/mg protein per 20 min)	
			− spermine	+ spermine (1.0 mM)
Malignant hyperthermia	211.0 ± 21.8 (n = 10)	0.023 ± 0.003 (n = 3)	11.13 ± 1.55 (n = 3)	2.75 ± 0.80 (n = 3)
Normal	155.9 ± 23.6 (n = 9)	0.026 ± 0.001 (n = 3)	4.87 ± 0.74 (n = 3)	2.32 ± 0.59 (n = 3)
Student's t-test	s. ($P < 0.001$)	n.s.	s. ($P < 0.001$)	n.s.

TABLE IV

$(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY, Ca^{2+} UPTAKE AND Ca^{2+} BINDING BY SARCOPLASMIC RETICULUM FROM LONGISSIMUS DORSI MUCLE OF MALIGNANT HYPERTHERMIA-PRONE AND NORMAL PIGS

The $(Ca^{2+} + Mg^{2+})$-ATPase activity was estimated by the formation of P_i at 35°C, and Ca^{2+} uptake and Ca^{2+} binding with a Radiometer Ca^{2+}-selective electrode at 35°C in a total volume of 5.0 ml of reaction medium (pH 6.8) consisting of 40 mM imidazole hydrochloride, 100 mM KCl, 4 mM $MgSO_4$ and 11.4 μM $CaCl_2$. In the Ca^{2+}-uptake (+ 5.0 mM oxalate) and Ca^{2+}-binding (– oxalate) experiments, the reaction was initiated by addition of 42 μM $CaCl_2$ followed by 1.15 mM ATP. Other experimental details are given in Materials and Methods. *n*, number of pigs used in experiments. The Ca^{2+}-uptake and -binding studies were carried out in collaboration with Mr. B.C. Carvalho. n.s., not significant; s., significant.

Sarcoplasmic reticulum	$(Ca^{2+} + Mg^{2+})$-ATPase (μmol P_i/min per mg protein)	Ca^{2+} uptake (μmol/min per mg protein)	Ca^{2+} binding (μmol/mg protein)
Malignant hyperthermia	1.14 ± 0.10 (n = 3)	2.38 ± 0.46 (n = 3)	0.10 ± 0.01 (n = 3)
Normal	0.84 ± 0.13 (n = 3)	4.68 ± 1.10 (n = 3)	0.10 ± 0.02 (n = 3)
Student's t-test	s. ($P < 0.05$)	s. ($P < 0.05$)	n.s.

normal but the rate of Ca^{2+} uptake in the presence of oxalate was significantly lower. This difference observed in probably due to modifications on the sarcoplasmic reticulum of malignant hyperthermia-prone pigs by mitochondria during isolation, and is not an intrinsic difference (see evidence in Figs. 1–3). No significant difference was observed in the Ca^{2+} binding between the sarcoplasmic reticulum of the two types of pigs.

Stability of sarcoplasmic reticulum

In all the Ca^{2+}-electrode experiments (Fig. 1), Ca^{2+} uptake was initiated by addition of ATP to sarcoplasmic reticulum of normal (Traces A and C) and malignant hyperthermia-prone (Traces B and D) pigs. The second and subsequent additions of a similar amount of ATP were introduced only after the release of Ca^{2+} from the sarcoplasmic reticulum was complete. The comparative data on the cycling of Ca^{2+} uptake and release induced by successive additions of ATP (2.5 μmol) to sarcoplasmic reticulum (Fig. 2) of normal and malignant hyperthermia-prone pigs demonstrate that sarcoplasmic reticulum of malignant hyperthermia-prone pigs could be less stable. No Ca^{2+} uptake was observed at the 12th addition of 2.5 μmol ATP (30 μmol added) to the sarcoplasmic reticulum of malignant hyperthermia-prone pigs. In contrast, normal sarcoplasmic reticulum was still able to take up exogenous Ca^{2+} at the 12th addition of ATP, and the maximal amount of Ca^{2+} taken up at the 6th addition of ATP (15 μmol added) was

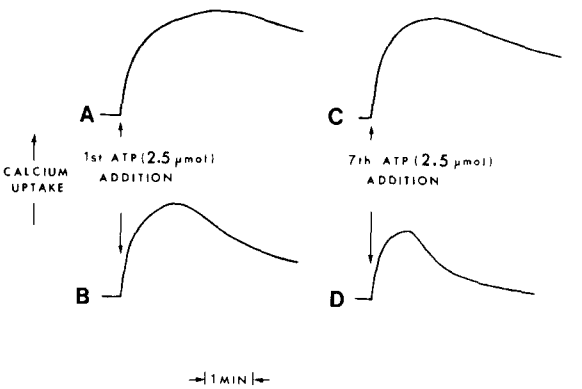

Fig. 1. Comparative studies of the Ca^{2+}-accumulating ability of sarcoplasmic reticulum isolated from longissimus dorsi of malignant hyperthermia-prone and normal pigs. The figure shows typical Ca^{2+}-electrode traces obtained at 31°C with sarcoplasmic reticulum of normal (traces A and C) and of malignant hyperthermia-prone (traces B and D) pigs showing the Ca^{2+} accumulation induced by ATP addition and subsequent release. Unlike the sarcoplasmic reticulum preparation described in Materials and Methods, the preparation used was isolated by centrifuging the first 11 000 × *g* supernatant [18] at 15 000 × *g* for 30 min at 4°C to remove mitochondria, followed by 40 000 × *g* for 45 min to sediment the sarcoplasmic reticulum. The experiments were conducted in a thermostatically controlled water-jacketed perspex cell in a reaction medium (pH 7.20) containing 225.0 mM mannitol, 75.0 mM sucrose, 15.0 mM Tris-HCl and 10 mM $MgCl_2$. Total volume, 5.0 ml. The sarcoplasmic reticulum concentration was 5.2 mg protein/ml for normal (traces A and C) and 10.0 mg/ml for malignant hyperthermia-prone (traces B and D) pigs.

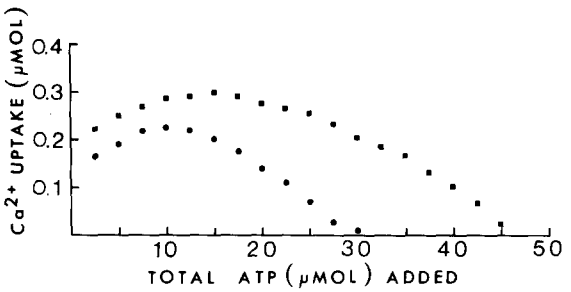

Fig. 2. Comparative studies of the Ca^{2+}-accumulating ability and stability of sarcoplasmic reticulum isolated from longissimus dorsi of normal (▪) and malignant hyperthermia-prone (•) pigs. Experimental details as described in the legend to Fig. 1. Ca^{2+} uptake was calculated by replotting calibration curves of changes in millivolts following additions of Ca^{2+}, and replotting the data on a linear scale.

at least twice that of malignant hyperthermia-prone pigs. (Note: the amount of protein used in the experiment for the sarcoplasmic reticulum preparation of malignant hyperthermia-prone pigs was twice that of normal). Normal sarcoplasmic reticulum also retained exogenous Ca^{2+} much longer than that of malignant hyperthermia-prone pigs (see Figs. 1 (cf. traces A and B, and traces C and D) and 2) before Ca^{2+} release occurred.

Interaction between mitochondria and sarcoplasmic reticulum

In the experiments (Figs. 1 and 2), the lower amount of Ca^{2+} uptake and its retention time, and the lower stability of the sarcoplasmic reticulum of malignant hyperthermia-prone pigs were demonstrated using preparations known to contain approx. 15% contamination with mitochondrial proteins. The experiments illustrated in Figs. 1 and 2 for malignant hyperthermia-prone pigs were designed to demonstrate that the differences observed between the two types of sarcoplasmic reticulum could be due to the influence of mitochondria on the Ca^{2+}-transport system of the sarcoplasmic reticulum of malignant hyperthermia-prone pigs. Further experiments were thus carried out to demonstrate the effect of mitochondria on the Ca^{2+} uptake of sarcoplasmic reticulum and to show that the differences previously observed in the sarcoplasmic reticulum preparations of malignant hyperthermia-prone pigs could be accounted for by the presence of contaminating mitochondria. Fig. 3 illustrates typical Ca^{2+}-electrode experiments demonstrating that mitochondria of malignant hyperthermia-prone pigs could depress the Ca^{2+} uptake of sarcoplasmic reticulum both from malignant hyperthermia-prone (trace A) and from normal (trace B) pigs, whereas normal mitochondria had no influence on normal sarcoplasmic reticulum (trace C).

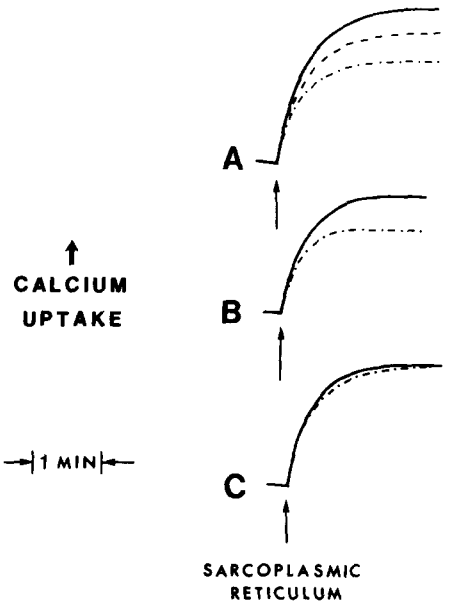

Fig. 3. Effect of mitochondria on Ca^{2+} accumulation by sarcoplasmic reticulum isolated from longissimus dorsi of malignant hyperthermia-prone and normal pigs. The figure shows typical Ca^{2+}-electrode traces showing that mitochondria from longissimus dorsi of malignant hyperthermia-prone pigs depress the Ca^{2+} accumulation by sarcoplasmic reticulum isolated from malignant hyperthermia-prone (trace A) and normal (trace B) pigs, and the lack of effect of normal mitochondria on normal sarcoplasmic reticulum (trace C). The experiments were conducted as described in the legend to Table IV, except that azide (1.0 mM), mitochondria and oligomycin (2 μg/mg mitochondrial protein) were added to the medium prior to the sarcoplasmic reticulum. The sarcoplasmic reticulum was only added after a steady base-line was obtained. Trace A: 1.0 mg sarcoplasmic reticulum of malignant hyperthermia-prone pig alone (———), with 0.5 mg (- - - - -) and 0.9 mg (– • – • –) mitochondria of malignant hyperthermia-prone pig. Trace B: 1.0 mg sarcoplasmic reticulum of normal pig (———) reacting with 0.9 mg mitochondria of malignant hyperthermia-prone pig (– • – • –). Trace C: 1.0 mg sarcoplasmic reticulum of normal pig (———) reacting with 0.9 mg mitochondria of normal pig (– • – • –).

TABLE V

INFLUENCE OF MITOCHONDRIA ON Ca^{2+} ACCUMULATION BY SARCOPLASMIC RETICULUM

Both mitochondria and sarcoplasmic reticulum were isolated from longissimus dorsi muscle of malignant hyperthermia-prone and normal pigs. Other experimental details are given in the legend to Fig. 3. *n*, number of pigs used in experiments.

Mitochondria	Sarcoplasmic reticulum	Mitochondria/ sarcoplasmic reticulum	Inhibition (%) of Ca^{2+} uptake (sarcoplasmic reticulum)
Malignant hyperthermia	Malignant hyperthermia	0.57 : 1.00	12 (n = 4)
		0.93 : 1.00	22 (n = 4)
		1.40 : 1.00	37 (n = 2)
Malignant hyperthermia	Normal	0.56 : 1.00	16 (n = 3)
		0.93 : 1.00	22 (n = 2)
Normal	Normal	0.64 : 1.00	0 (n = 2)
		1.54 : 1.00	0 (n = 2)
		1.70 : 1.00	10 (n = 2)

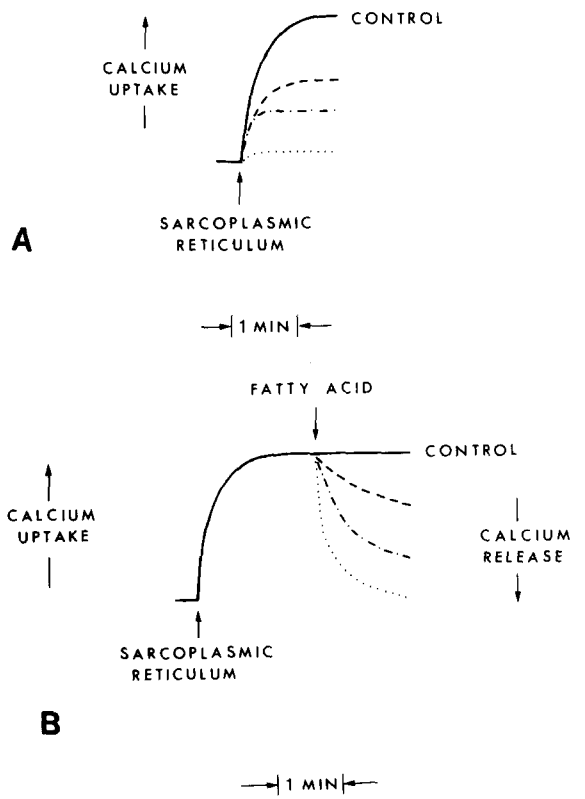

Fig. 4. Effect of unsaturated fatty acids on Ca^{2+} accumulation (A) and release (B) by sarcoplasmic reticulum from longissimus dorsi muscle. The data represent typical Ca^{2+}-electrode experiments showing the inhibition by arachidonic (·····), oleic (– · – · –) and linoleic (————) acids on Ca^{2+} uptake (A) and induction of Ca^{2+} release from sarcoplasmic reticulum preloaded with exogenous Ca^{2+} by fatty acids (B). Temperature, 35°C; total volume, 5.0 ml; sarcoplasmic reticulum, 1.0 mg protein; fatty acid, 0.5 μg/mg sarcoplasmic reticulum. Ca^{2+} uptake was calculated by adding Ca^{2+} at the end of the experiment to bring the concentration of Ca^{2+} back to the original level.

22% inhibition of Ca^{2+} uptake by sarcoplasmic reticulum of malignant hyperthermia-prone and normal pigs was observed when an equal amount of mitochondria from malignant hyperthermia-prone pigs was allowed to interact with the sarcoplasmic reticulum preparations (Table V). No inhibition of Ca^{2+} uptake by normal sarcoplasmic reticulum was detected even when normal mitochondria at a ratio of 1.54 : 1.00 were added. However, when the ratio of normal mitochondria to normal sarcoplasmic reticulum was increased to 1.70 : 1.00 only 10% inhibition of Ca^{2+} uptake by normal sarcoplasmic reticulum was observed. This situation could not be equated with in situ conditions in longissimus dorsi muscle, which contains more sarcoplasmic reticulum than mitochondria.

Effect of long-chain fatty acids on Ca^{2+} uptake and release by sarcoplasmic reticulum

The endogenous phospholipase A_2 activity in longissimus dorsi muscle mitochondria of malignant hyperthermia-prone pigs is over twice that of normal (Table III). The long-chain fatty acids liberated from

the mitochondrial membranes by the phospholipase activity could affect the function of the sarcoplasmic reticulum by increasing the $(Ca^{2+} + Mg^{2+})$-ATPase activity and decreasing the rate of Ca^{2+} uptake (Table IV). In order to substantiate this hypothesis, arachidonic ($C_{20:4}$), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids were selected for investigation, since these are the major long-chain fatty acids of the mitochondrial phospholipids [28] known to be released by phospholipase A_2 [29]. All the three long-chain unsaturated fatty acids affected the Ca^{2+} uptake by sarcoplasmic reticulum (Fig. 4A), and similar concentrations of these fatty acids also induced the release of Ca^{2+} (Fig. 4B). The relative effectiveness (arachidonic > oleic > linoleic) of the fatty acids on Ca^{2+} uptake and release by sarcoplasmic reticulum (Fig. 4) is consistent with the suggestion that fatty acids released from the mitochondrial phospholipids by the action of phospholipase A_2 could depress the function of sarcoplasmic reticulum. The effects produced by the fatty acid could be counteracted by bovine serum albumin, a binder of fatty acids [30], and under these conditions the sarcoplasmic reticulum regained its original function.

Discussion

In our previous paper [18] we reported a transition temperature 9°C above normal for the Ca^{2+}-stimulated respiration during succinate oxidation of longissimus dorsi muscle mitochondria from malignant hyperthermia-prone pigs, and that this difference was abolished by spermine, a phospholipase A_2 inhibitor [28]. We also suggested that mitochondria of malignant hyperthermia-prone pigs contained a Ca^{2+}-activated phospholipase A_2, and that this enzyme is either latent or in a very low concentration in normal pigs. The present data show that mitochondria from longissimus dorsi muscle of both malignant hyperthermia-prone and normal pigs contain the same amount of endogenous phospholipase A_2, but its endogenous activity in malignant hyperthermia-prone pigs is at least twice that of normal. The higher endogenous phospholipase A_2 activity is probably due to increased amounts of endogenous free long-chain fatty acids (Table III) and Ca^{2+} [18], both known to activate mitochondrial phospholipase A_2 activity [29].

The fatty acids released from the mitochondrial phospholipids by the action of mitochondrial phospholipase A_2 are most likely to be responsible for the difference in the Ca^{2+} permeability (Table IV) and stability (Figs. 1 and 2) of isolated sarcoplasmic reticulum from longissimus dorsi muscle between malignant hyperthermia-prone and normal pigs. This suggestion is consistent with the finding that mitochondria isolated from longissimus dorsi muscle of malignant hyperthermia-prone pigs were able to inhibit the Ca^{2+} uptake of sarcoplasmic reticulum isolated from similar muscle of malignant hyperthermia-prone and normal pigs (Fig. 3 and Table V). Mitochondria from normal pigs, however, did not have any influence on the function of the sarcoplasmic reticulum unless added greatly in excess of in situ conditions. Long-chain unsaturated fatty acids (arachidonic, oleic and linoleic), known to be released from the mitochondrial phospholipids by phospholipase A_2 [31], also inactivated the Ca^{2+}-transport system of sarcoplasmic reticulum by inhibiting Ca^{2+} uptake and inducing Ca^{2+} release (Fig. 4), and by stimulating the $(Ca^{2+} + Mg^{2+})$-ATPase activity [19]. An alternative explanation of the mitochondrial inhibition of the Ca^{2+} uptake by sarcoplasmic reticulum could be either the direct effect of phospholipase A_2 alone [34] or in combination with fatty acids liberated from mitochondria, acting on the sarcoplasmic reticulum.

To understand the main lesion in malignant hyperthermia syndrome, a satisfactory explanation for the enhanced release of Ca^{2+} into the sarcoplasm is required. The present and previous data [18] are consistent in identifying the endogenous mitochondrial phospholipase A_2 as one of the main factors responsible for porcine malignant hyperthermia syndrome. The enzyme activity is at least twice as great as normal. Furthermore, longissimus dorsi muscle of stress-susceptible, malignant hyperthermia-prone pigs are more sensitive to anoxia [32], a condition favouring the activation of mitochondrial phospholipase A_2 [33]. The sequence of biochemical events leading to an increase in sarcoplasmic Ca^{2+} level in malignant hyperthermia-prone pigs can perhaps be visualized as follows. Firstly, activation of mitochondrial phospholipase A_2 brought about through a combination of any of the above-mentioned factors resulting in the formation of lyso derivatives from the

mitochondrial phospholipids and liberation of unsaturated fatty acids. Secondly, the fatty acids and lyso derivatives then destabilize the mitochondrial membranes causing the mitochondria to swell. This is accompanied simultaneously by a faster than normal Ca^{2+} release [18]. (Large-amplitude swelling in longissimus dorsi muscle mitochondria of malignant hyperthermia-prone pigs was indeed observed both in in vitro [18] and in situ (unpublished data) experiments, but not with mitochondria of similar muscle in normal pigs.) Thirdly, the long-chain fatty acids alone, or in combination with phospholipase A_2, inactivate the Ca^{2+}-transport system of sarcoplasmic reticulum, causing the release of additional Ca^{2+} into the sarcoplasm. Fourthly, the enhanced level of Ca^{2+} stimulates glycolysis by activating the phosphorylase kinase [35] and the myofibrillar ATPase so that more glycogen is degraded to lactate than under normal conditions.

If our explanation for the increase in Ca^{2+} permeability is correct, one would expect the post-mortem glycolysis in muscle of malignant hyperthermia-prone pigs always to operate at maximum rate. This was confirmed by a series of experiments employing electrical stimulation, a technique used to increase the rate of post-mortem glycolysis [36]. Electrical stimulation had no effect on the rate of glycolysis of longissimus dorsi muscle from malignant hyperthermia-prone pigs, indicating that glycolysis is already operating at maximal rate. With normal pigs, the rate of glycolysis was increased significantly by electrical stimulation, but the enhanced rate was not as high as that observed with malignant hyperthermia-prone pigs.

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