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MITOCHONDRIAL CALCIUM TRANSPORT AND CALCIUM-ACTIVATED PHOSPHOLIPASE IN PORCINE MALIGNANT HYPERTHERMIA

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Summary

The interaction of Ca^{2+} with mitochondria isolated from longissismus dorsi, a predominantly white skeletal muscle, of normal and malignant hyperthermia pigs was investigated using tightly-coupled preparations. Arrhenius plots of mitochondrial Ca^{2+}-stimulated respiration for succinate oxidation of malignant hyperthermia pigs showed a transition temperature (T_t) of 26.31 ± 0.80°C (n = 5), which was decreased by spermine to 15.41 ± 0.69°C (n = 3), a value very similar to that for normal pigs. No difference in either the T_t or in the activation energy (E_a) was observed between the two types of pigs when ADP was used instead of Ca^{2+}.

Mitochondria of malignant hyperthermia pigs were uncoupled at 40°C by exogenous Ca^{2+} at 1221 ± 301 (n = 9) nmol Ca^{2+} per mg protein during succinate oxidation and the uncoupled mitochondria showed large amplitude swelling. Both the Ca^{2+}-induced uncoupling and swelling were prevented by bovine serum albumin and by the phospholipase inhibitors, spermine and tetracaine. In contrast, mitochondria of normal pigs were still tightly coupled even after a total addition of 2313 ± 287 (n = 5) nmol Ca^{2+} per mg protein and retained the original condensed configuration in the presence or absence of spermine and tetracaine.

Mitochondria of malignant hyperthermia pigs contained significantly ($P <$ 0.001) higher quantities of endogenous Ca^{2+} and showed a significantly ($P <$ 0.001) faster FCCP-induced endogenous Ca^{2+} efflux rate than normal when monitored spectroscopically with murexide. No significant difference was observed in either the rate of exogenous Ca^{2+} uptake or in the extent of Ca^{2+} accumulated in the aerobic steady state during succinate oxidation between the two types of pigs. The rate of mitochondrial Ca^{2+} efflux of malignant hyperthermia pigs during anaerobiosis was about twice that of normal.

Experimental evidence suggests that mitochondria from musculi longissimus dorsi of malignant hyperthermia pigs contained a Ca^{2+}-stimulated phospholipase A_2 (EC 3.1.1.4, phosphatide 2-acylhydrolase), and that this enzyme if present in mitochondria of normal pigs is either latent or in very low concentration. The significance of the Ca^{2+}-stimulated phospholipase A_2 and its association with the enhanced rate of glycolysis in porcine malignant hyperthermia syndrome and in the post-mortem formation of the pale, soft and exudative conditions observed in white skeletal muscles of malignant hyperthermia pigs is discussed.

Introduction

Anaesthetic-induced malignant hyperthermia in stress-susceptible pigs is commonly used as an experimental model for investigating malignant hyperthermia in humans. The manifestations of both porcine and human malignant hyperthermia are very similar [1], the predominant clinical symptoms being gross muscular rigidity, rapid rise in body temperature, tachycardia, hyperventilation, severe acidosis and elevated levels of serum metabolites [1—3]. Porcine malignant hyperthermia is characterized by rapid glycolysis immediately post-mortem [4]. The ultimate pH value of about 5.4 in longissimus dorsi, a predominantly white skeletal muscle, is attained while the muscles are still warm. Under these conditions, denaturation of the sarcoplasmic and myofibrillar proteins occurs [5], and this is accompanied by a reduction in the water-holding capacity of the muscles [6] which ultimately results in the exudation of a large amount of drip and formation of pale, soft and exudative muscle [4,6].

In an earlier paper [7] we reported variations in the rate of mitochondrial Ca^{2+} efflux in different breeds of pigs, and suggested that the Ca^{2+} efflux was linked with porcine stress-susceptibility [4,7]. Furthermore, a difference in the rate of mitochondrial Ca^{2+} efflux was also observed within the same breed between malignant hyperthermia and normal pigs [8]. The higher rate of mitochondrial Ca^{2+} efflux in malignant hyperthermia pigs was postulated to be due to the presence of a Ca^{2+}-stimulated phospholipase since both bovine serum albumin and spermine, a phospholipase inhibitor [9], restored the values of the Ca^{2+} efflux of malignant hyperthermia pigs to normal [8].

This paper reports further studies on the mitochondria of malignant hyperthermia and normal pigs, and shows that the differences in the fluidity of the mitochondrial membranes is probably contributed by phospholipase A_2 (EC 3.1.1.4, phosphatide 2-acylhydrolase) liberating long chain unsaturated fatty acids from mitochondria of malignant hyperthermia pigs.

Materials and Methods

Materials. Pigs were kindly supplied by Dr. A.J. Webb of the Agricultural Research Council, Animal Breeding Research Organisation at Edinburgh. At 35 kg the pigs were transported to the Meat Research Institute, Langford where they were kept until slaughter at 75—100 kg.

Fatty acid-free bovine serum albumin, murexide, rotenone, sodium succinate, spermine tetrahydrochloride and tetracaine hydrochloride were purchased from Sigma Chemical Corp.; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) from Boehringer Mannheim; crystalline *Bacillus subtilis* proteinase (Nagarse) from Teikoku Chemical Co or from Sigma Chemical Corp.; all other reagents were of analytical grade.

Methods. Mitochondria were isolated from musculi longissimus dorsi immediately post-mortem by modifying the procedure of Makinen and Lee [10]. 3 mg Nagarse was used instead of 5 mg per g wet weight of tissue, and homogenization was carried out with a Thomas teflon-pestle glass homogenizer in place of an Ultra-Turrax. In addition, the mitochondrial-containing 14 000 × *g* suspension was first centrifuged at 1000 × *g* for 10 min, and the supernatant from this was recentrifuged at 7000 × *g* for 10 min. The mitochondrial pellet (7000 × *g*) was washed three times before being suspended in 250 mM sucrose. Oxygen uptake was measured with a Clark oxygen electrode (Yellow Spring Oxygen Monitor (Model 53)) in a total volume of 2.50 ml. The ADP-stimulated respiration for succinate oxidation was determined in a reaction medium (pH 7.20) containing 1.0 mM EDTA, 30 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose and 20.0 mM KH_2PO_4, and the Ca^{2+}-stimulated respiration in a reaction medium (pH 7.20) containing 225.0 mM mannitol, 75.0 mM sucrose and 15.0 mM Tris-HCl in the presence of 5.0 mM P_i. The rates of mitochondrial Ca^{2+} uptake and efflux was measured at 21°C using the Amino-Chance dual-wavelength/split beam spectrophotometer operating in the dual-wavelength mode at 540—510 nm with murexide [11] in the same reaction medium as that used for measuring the Ca^{2+}-stimulated respiration for succinate oxidation except that 2.50 mM P_i was used instead of 5.0 mM P_i. Ca^{2+} was also determined by atomic absorption using an Instrumental Laboratory Inc. double-beam atomic absorption/emission spectrophotometer (Model 251) at 422.7 nm in the presence of 1% (w/v) lanthanum. Protein was determined according to the method of Lowry et al. [12] using bovine serum albumin as standard.

Electron microscopy was carried out as described by Allmann et al. [13] but without using acrolein. Thin sections of the various mitochondrial preparations, embedded in Epon 812 were cut with a glass knife and stained with 2.0% uranyl acetate in methanol [14] and lead citrate [15] before examination with an AEI (Model EM6-B) electron microscope. For examinations of the ultrastructural configurations of mitochondria during the Ca^{2+}-stimulated respiration the mitochondrial suspension at the end of each polarographic experiment was treated with an equal volume of 2.5% glutaraldehyde in 50 mM sodium cacodylate and 180 mM sucrose buffer (pH 7.4) for 2 min, and then centrifuged for 1.5 min with an Eppendorf (Model 3200) microcentrifuge. The mitochondrial pellet was fixed for 1 h with 2.5% glutaraldehyde in 50 mM sodium cacodylate and 180 mM sucrose buffer (pH 7.4) at 0°C, post-fixed with 1% osmium tetroxide in 50 mM sodium cacodylate and 300 mM sucrose buffer (pH 7.4) for 1 h at 0°C, and then dehydrated in ethanol before being embedded in Epon 812.

Results

Coupling integrity, endogenous Ca^{2+} and FCCP-induced release of endogenous Ca^{2+}

Mitochondria isolated from malignant hyperthermia and normal (control) pigs by the modified Nagarse method were tightly-coupled. The respiratory control index for the ADP-stimulated respiration during succinate oxidation of our routine mitochondrial preparations normally showed a minimum value of 6 at 25°C. The values of the respiratory control index for the Ca^{2+}-stimulated respiration during succinate oxidation at 25°C in the reaction medium (pH 7.20) containing 225.0 mM mannitol, 75.0 mM sucrose, 5.0 mM P_i and 15.0 mM Tris-HCl were found to be much higher than values previously reported by us [7] using the reaction medium (pH 7.20) consisting of 1.0 mM EDTA, 30.0 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose and 20.0 mM KH_2PO_4. Under the present experimental conditions, the values of the respiratory control index for succinate oxidation using Ca^{2+} to induce the State 3 to State 4 transition [16] was 4.72 ± 0.56 (n = 18) for malignant hyperthermia pigs, and 5.21 ± 0.48 (n = 16) for normal pigs, as compared with previous values of 1.67 [7]. No significant difference in respiratory control index was found between the two types of pigs, indicating that the coupling integrity of the isolated mitochondria was identical.

Mitochondria of malignant hyperthermia pigs contained significantly ($P <$ 0.001) higher endogenous Ca^{2+} than normal. Fig. 1 represents the direct tracings of typical spectroscopic experiments with murexide illustrating the discharge of endogenous mitochondrial Ca^{2+} by the uncoupling reagent, FCCP [17]. The amount of endogenous Ca^{2+} estimated by this method was 82.90 ± 11.27 (n = 9) nmol Ca^{2+} per mg protein for malignant hyperthermia pigs compared with 45.44 ± 9.25 (n = 9) nmol Ca^{2+} per mg protein for normal pigs. The amount of endogenous mitochondrial Ca^{2+} was also determined by atomic

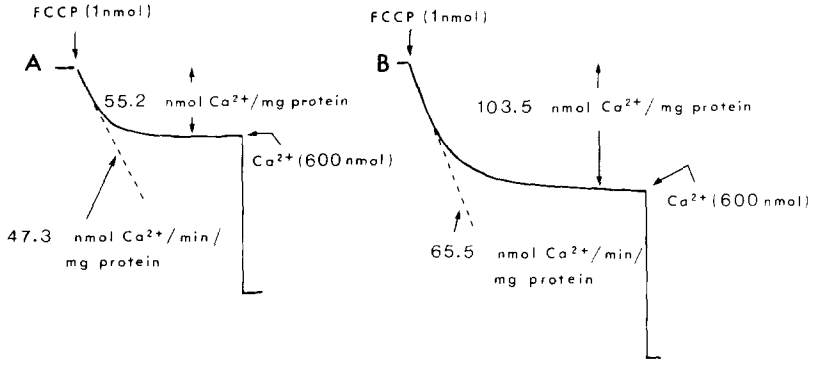

Fig. 1. Measurement of endogenous Ca^{2+} in mitochondria from musculi longissimus dorsi of normal and malignant hyperthermia pigs. The data are from typical spectroscopic experiments showing the discharge of endogenous Ca^{2+} from mitochondria of normal (A) and malignant hyperthermia (B) pigs by FCCP (1 nmol). The reaction medium (pH 7.20) contained 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl, and murexide (92 μM). Total protein, 4.98 mg (A) and 4.33 mg (B); total volume, 2.70 ml; temperature 21°C.

absorption, and the values agreed within 4—15% with the spectrophotometric values. Mitochondria of malignant hyperthermia pigs also showed significantly ($P < 0.001$) faster FCCP-induced endogenous Ca^{2+} efflux rates than normal, the FCCP-induced Ca^{2+} efflux rates were 61.77 ± 10.37 ($n = 9$) nmol Ca^{2+} per min per mg protein for malignant hyperthermia pigs as compared with 41.23 ± 5.83 ($n = 9$) nmol Ca^{2+} per min per mg protein for the control.

Uptake, accumulation and release of exogenous Ca^{2+}

In all experiments 120—150 nmol Ca^{2+} per mg protein were added to the mitochondrial suspensions. This was about 10% of the total exogenous Ca^{2+} added to uncouple mitochondria of malignant hyperthermia pigs at 40°C [8]. Thus, in all our experiments the kinetic uptake, accumulation and release of exogenous Ca^{2+} were estimated with coupled mitochondria.

Table I summarizes the rate of Ca^{2+} uptake and the percentage of Ca^{2+} accumulated by mitochondria during aerobiosis, and the rate of Ca^{2+} released at the onset of anaerobiosis between mitochondria from musculi longissimus dorsi of malignant hyperthermia and control pigs. No significant difference was observed in either the rate of Ca^{2+} uptake or in the extent of Ca^{2+} accumulated between the mitochondria from both types of pigs. A significant ($P < 0.001$) difference in the anaerobic rate of mitochondrial Ca^{2+} efflux was however observed between the two types of pigs when the estimations were carried out either in the absence [18] or presence of 2.50 mM P_i (Table I). P_i stimulated both the rate of Ca^{2+} uptake and the initial fast phase of Ca^{2+} efflux but inhibited the slow phase of the biphasic Ca^{2+} efflux. Maximum Ca^{2+} efflux was obtained with 2.50 mM P_i, but a further increase in P_i concentration only resulted in an inhibition of the Ca^{2+} efflux in the fast phase.

Effect of exogeneous Ca^{2+} on succinate oxidation

Mitochondria isolated from musculi longissimus dorsi of both malignant hyperthermia and control pigs showed no significant difference in their coupling integrity, and in their State 3 and State 4 respiratory rates induced by either ADP or Ca^{2+} [16] during succinate oxidation at 25°C. At 40°C, however,

TABLE I

Ca^{2+} UPTAKE, ACCUMULATION AND Ca^{2+} EFFLUX IN MITOCHONDRIA FROM MUSCULI LONGISSIMUS DORSI OF MALIGNANT HYPERTHERMIA AND NORMAL PIGS

The data were obtained with mitochondria oxidizing succinate (+rotenone) in the presence of 2.50 mM P_i using murexide to monitor the kinetic uptake, accumulation and release of exogenous Ca^{2+}. The sequence of additions to the mitochondrial suspension was 1 μM rotenone, 92 μM murexide and Ca^{2+} (120—150 nmol per mg protein), and then followed by 10 mM succinate to initiate the uptake of exogenous Ca^{2+}. Temperature, 21°C; total volume, 2.70 ml; 10 mm light path cuvette. The values are means ± S.D. for the numbers of pigs in parentheses. n.s., non significant; s., significant.

Muscle mitochondria	Ca^{2+} uptake (nmol/min/mg protein)	Ca^{2+} accumulation (%)	Ca^{2+} efflux (nmol/min/mg protein)
Malignant hyperthermia	275.5 ± 62.9 ($n = 12$)	92.3 ± 4.0 ($n = 12$)	214.5 ± 31.5 ($n = 31$)
Control	273.4 ± 56.7 ($n = 14$)	92.3 ± 3.6 ($n = 14$)	119.4 ± 19.1 ($n = 27$)
t-Test	n.s. ($P > 0.05$)	n.s. ($P > 0.05$)	s. ($P < 0.001$)

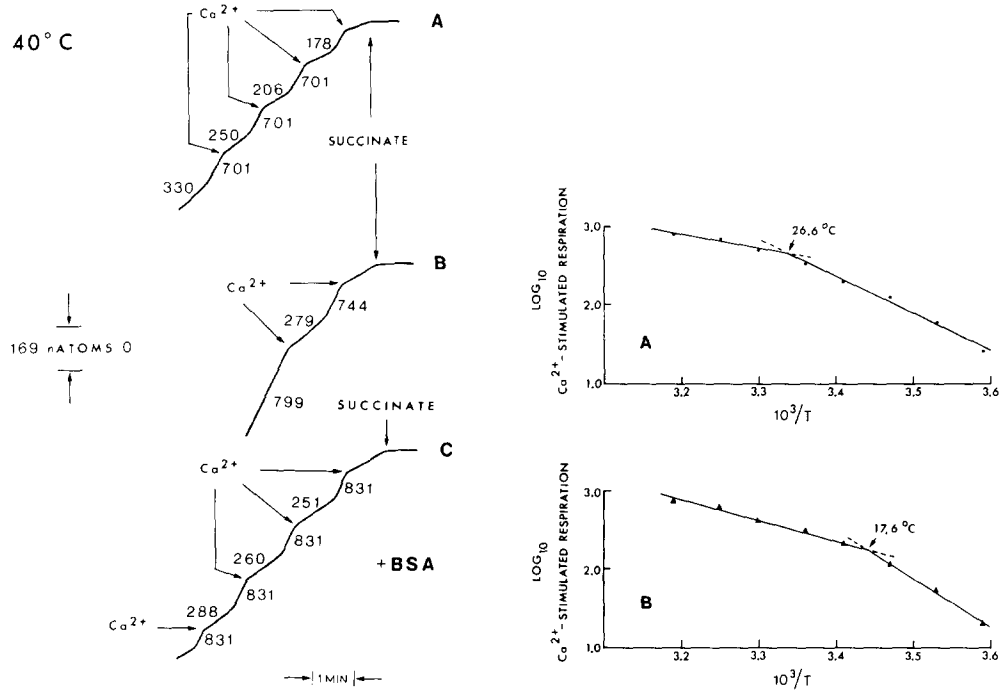

Fig. 2. Effect of exogenous Ca^{2+} on succinate oxidation by mitochondria from musculi longissimus dorsi of normal and malignant hyperthermia pigs at 40°C. Trace A illustrates a typical polarographic experiment showing the State 3-State 4 transition induced by Ca^{2+} during succinate oxidation by mitochondria of normal pig. Four additions of 600 nmol Ca^{2+} per mg protein were introduced without causing uncoupling of mitochondria. Total protein, 0.50 mg; total Ca^{2+} added, 2400 nmol Ca^{2+} per mg protein. Trace B represents a typical experiment showing the effect of Ca^{2+} on mitochondria of malignant hyperthermia pig during succinate oxidation. The second addition of 625 nmol Ca^{2+} per mg protein completely uncoupled the mitochondria. Total protein, 0.48 mg; total Ca^{2+} added, 1250 nmol per mg protein. Trace C illustrates a typical experiment showing bovine serum albumin (BSA) completely counteracted the uncoupling in mitochondria of malignant hyperthermia pig represented by Trace B. No uncoupling was observed even after a total addition of 2188 nmol Ca^{2+} per mg protein (three additions of 625 nmol and one addition of 313 nmol). Total protein, 0.48 mg. Rotenone (2 μM) and succinate (10 mM) were added prior to Ca^{2+} addition in all the experiments represented by Traces A—C. 1 mg bovine serum albumin (BSA) was added before rotenone and succinate in Trace C. The numbers alongside the Traces (A-C) represent the rates of oxygen uptake expressed in natoms O per min per mg protein, and experimental traces run from right to left.

Fig. 3. Arrhenius plots of Ca^{2+}-stimulated respiration of mitochondria from musculi longissimus dorsi of malignant hyperthermia and normal pigs. The Ca^{2+}-stimulated respiration was estimated with a Clark oxygen electrode. The data represent typical results obtained with mitochondria from malignant hyperthermia (A) and normal (B) pigs. Changes in the slope of the Arrhenius plots were determined by least mean square analysis. By a computer programme the residual sum of squares was calculated for combinations of points fitted to two straight lines from the upper to the lower temperature extremes. A change in slope was considered to occur at the first minimum for the sum of the residual sum of squares for the two straight lines.

a marked difference in the mitochondrial coupling was observed following the addition of Ca^{2+} in that mitochondria from malignant hyperthermia pigs were more easily uncoupled by Ca^{2+}. Fig. 2 illustrates typical polarographic experiments showing the effect of Ca^{2+} addition to mitochondria of control (Trace A) and of malignant hyperthermia (Trace B) pigs, and the effect of bovine serum albumin in counteracting the Ca^{2+}-induced uncoupling (Trace C)

in mitochondria from malignant hyperthermia pigs. No uncoupling was observed with the control pigs even after a total addition of 2313 ± 287 (n = 5) nmol Ca^{2+} per mg protein. In contrast, mitochondria of malignant hyperthermia pigs were uncoupled under the same experimental conditions, by the second addition of Ca^{2+} (Trace B). Uncoupling was observed after a total addition of 1221 ± 301 (n = 9) nmol Ca^{2+} per mg protein. Addition of bovine serum albumin maintained the coupling integrity (Trace C) of mitochondria from malignant hyperthermia pigs even after 2038 ± 236 (n = 4) nmol Ca^{2+} per mg protein were added. No significant difference was observed in the mitochondrial coupling integrity between the two types of pigs when ADP was used instead of Ca^{2+} at 40°C.

Arrhenius plots of Ca^{2+}- and ADP-stimulated respiration of succinate oxidation

Fig. 3 represents a typical Arrhenius plot of the Ca^{2+}-stimulated respiration of mitochondria isolated from musculi longissimus dorsi of malignant hyperthermia (A) and control (B) pigs. In both cases, discontinuities in the Arrhenius plots were observed, showing a distinct difference in the transition temperature (T_t) in succinate oxidation. Mitochondria of malignant hyperthermia pigs showed a T_t of 26.31 ± 0.80°C (n = 5), and this was 9°C higher than the T_t of 17.29 ± 0.77°C (n = 4) for the control pigs. The activation energy (E_a) above the T_t was 40.38 ± 15.69 kJ/mol (n = 5) for malignant hyperthermia pigs as compared with a value of 50.72 ± 2.89 kJ/mol (n = 4) for the control. Below the T_t, the values of E_a were 101.97 ± 24.81 kJ/mol (n = 5) for malignant hyperthermia pigs, and 127 ± 26.47 kJ/mol (n = 4) for the control. Thus, the Arrhenius plots of the Ca^{2+}-stimulated respiration for succinate oxidation showed that there was a significant difference ($P < 0.001$) in the T_t between the two types of pigs, but with only small and non-significant changes in the E_a either above or below the T_t. When the experiments were repeated using ADP instead of Ca^{2+} with the same mitochondrial preparations, no difference was observed in either the T_t (Fig. 4) or E_a between the two types of pigs. The average value of T_t for two pigs of each type was 16.74°C and 17.68°C for malignant hyperthermia and normal pigs, respectively. The E_a above the T_t was 58.74 kJ/mol for malignant hyperthermia pigs as compared with a value of E_a of 51.65 kJ/mol for the control, their corresponding E_a values below the T_t were 141.89 kJ/mol and 132.47 kJ/mol, respectively.

Effect of spermine and Mg^{2+} on Arrhenius plots of Ca^{2+}-stimulated respiration of succinate oxidation

Fig. 5 illustrates typical Arrhenius plots of a set of experiments showing the effect of various temperatures on the Ca^{2+} stimulated respiration of mitochondria from musculi longissimus dorsi of malignant hyperthermia pigs (A), and the effect of spermine on the T_t (B). The T_t of malignant hyperthermia pigs was lowered by spermine from 26.31 ± 0.80°C (n = 5) to 15.41 ± 0.69°C (n = 3), and by Mg^{2+} (4.0 mM) to 17.27°C (n = 2) and this new T_t in the presence of spermine or Mg^{2+} was very similar to the T_t of 17.29 ± 0.77°C (n = 4) for normal pigs. The E_a of 59.26 ± 3.88 kJ/mol (n = 3) and 143.00 ± 27.10 kJ/mol (n = 3) above and below the T_t in the presence of spermine was also practically identical to the values of 50.72 ± 2.89 kJ/mol (n = 4) and 127.02 ± 26.47 kJ/

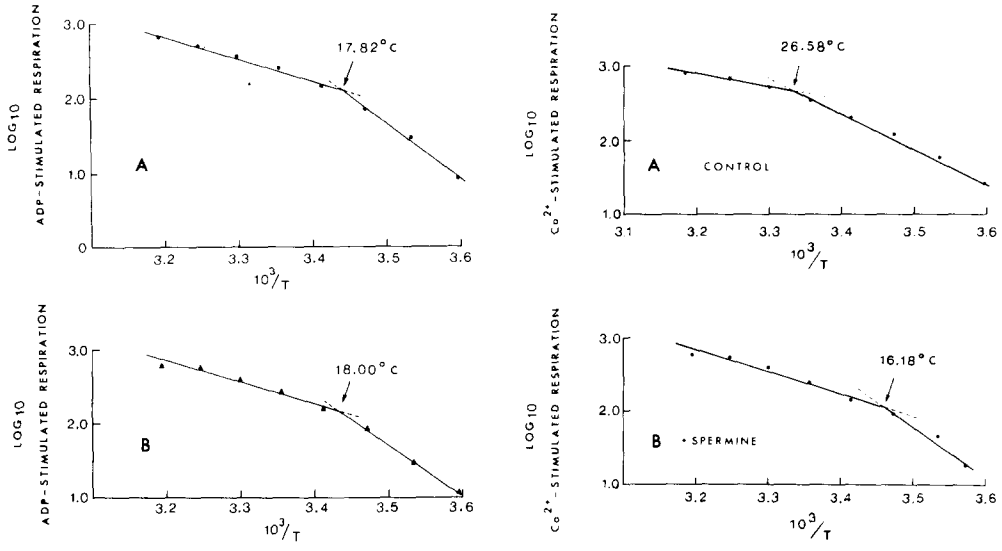

Fig. 4. Arrhenius plots of ADP-stimulated respiration of mitochondria from musculi longissimus dorsi of malignant hyperthermia and normal pigs. The ADP-stimulated respiration was estimated with a Clark oxygen electrode. The data represent typical results obtained with mitochondria from malignant hyperthermia (A) and normal (B) pigs. The straight lines were drawn as described in the legend to Fig. 3.

Fig. 5. Arrhenius plots of Ca^{2+}-stimulated respiration of mitochondria from musculi longissimus dorsi of malignant hyperthermia pigs determined in the absence (A) and presence of spermine (B).

mol (n = 4) observed for normal pigs. With mitochondria of normal pigs however, spermine, as expected, was ineffective in altering the T_t and the E_a (Fig. 6). The average T_t was 15.93°C (n = 2) and the E_a (n = 2) above and below the T_t was 43.04 and 145.17 kJ/mol, respectively in the presence of spermine.

Effect of spermine, tetracaine and Mg^{2+} on Ca^{2+}-stimulated respiration of succinate oxidation

The ability of bovine serum albumin to prevent the Ca^{2+}-induced uncoupling in mitochondria of malignant hyperthermia pigs (Fig. 2, C) implied that free fatty acids were most likely to be responsible for the mitochondrial uncoupling. It is known that bovine serum albumin could easily counteract the un-

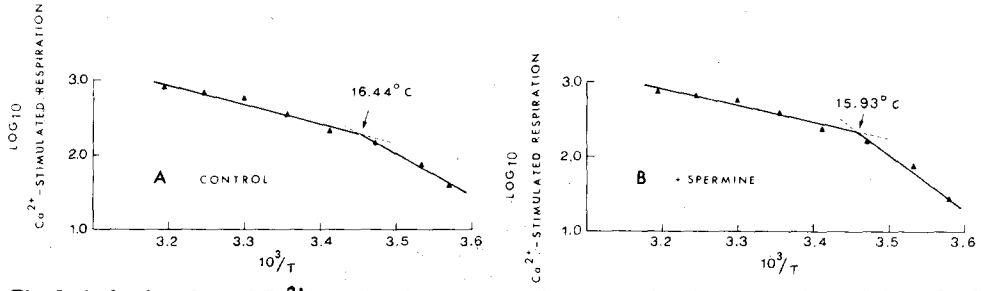

Fig. 6. Arrhenius plots of Ca^{2+}-stimulated respiration of mitochondria from musculi longissimus dorsi of normal pigs estimated in the absence (A) and presence of spermine (B).

coupling of mitochondria caused by free fatty acids [19—21], and restore oxidative phosphorylation by its ability to bind with free fatty acids [19—23]. As only the mitochondria of malignant hyperthermia pigs were observed to be uncoupled by exogenous Ca^{2+} and not by exogenous ADP at high temperatures, it seems very likely that one of the sources of free fatty acids was the action of the Ca^{2+}-activated phospholipase A_2 in mitochondria from musculi longissimus dorsi of malignant hyperthermia pigs. This explanation was supported by data

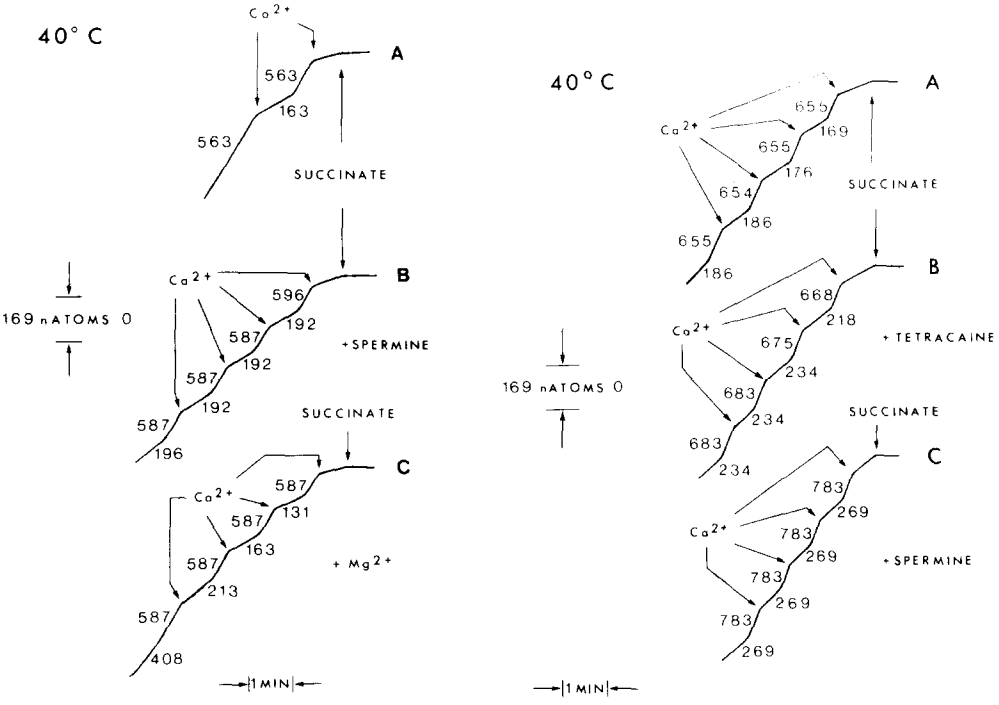

Fig. 7. Effect of spermine and Mg^{2+} on Ca^{2+}-stimulated respiration by mitochondria from musculi longissimus dorsi of malignant hyperthermia pigs at 40°C. Trace A illustrates a typical experiment showing the effect of Ca^{2+} on mitochondria of malignant hyperthermia pig during succinate oxidation. The second addition of 727 nmol Ca^{2+} per mg protein completely uncoupled the mitochondria. Total protein, 0.55 mg; total Ca^{2+} added, 1454 nmol per mg protein. Trace B shows a typical experiment of spermine completely counteracting the uncoupling in mitochondria of malignant hyperthermia pig illustrated in Trace A. No uncoupling was observed after a total addition of 2908 nmol Ca^{2+} per mg protein (four additions of 727 nmol). Total protein, 0.55 mg; spermine, 1.0 mM. Trace C illustrates a typical experiment showing Mg^{2+} preventing the uncoupling induced by Ca^{2+} in mitochondria of malignant hyperthermia pig during succinate oxidation. No uncoupling was observed after a total addition of 2908 nmol Ca^{2+} per mg protein, but the last addition of 727 nmol Ca^{2+} per mg protein resulted in the mitochondria showing signs of becoming uncoupled. Total protein, 0.55 mg; Mg^{2+}, 4.0 mM. Spermine in Trace B and Mg^{2+} in Trace C were added into the mitochondrial suspension prior to the additions of rotenone (2 μM) and succinate (10 mM). All other experimental details as described in the legend to Fig. 2.

Fig. 8. Effect of tetracaine and spermine on Ca^{2+}-stimulated respiration by mitochondria from musculi longissimus dorsi of normal pigs at 40°C. Trace A illustrates a typical polarographic experiment showing the State 3 to State 4 transition induced by Ca^{2+} during succinate oxidation by mitochondria of normal pigs, and Trace B and Trace C when the experiment described in Trace A was repeated in the presence of tetracaine and spermine, respectively. No Ca^{2+}-induced uncoupling was observed in the presence of tetracaine (B) and spermine (C) even after a total addition of 2624 nmol Ca^{2+} per mg protein. Total protein in each experiment, 0.61 mg; tetracaine, 0.5 mM; spermine, 1.0 mM. All other experimental details as described in the legend to Fig. 2 and Fig. 7.

obtained using two phospholipase inhibitors, spermine [9] and tetracaine [24,25], and Mg^{2+}, the cation specific for the Ca^{2+}-binding sites [26—32] and for protecting the structural integrity and stabilization of mitochondrial membranes [33—35].

Fig. 7 illustrates direct polarographic tracings of typical experiments showing the effect of spermine (B) and Mg^{2+} (C) in preventing the mitochondrial uncoupling of malignant hyperthermia pigs (A) caused by the addition of exogenous Ca^{2+} during succinate oxidation at 40°C. The first addition of 727 nmol Ca^{2+} per mg protein gave a respiratory control index value of 3.45 (A). The second addition of a similar amount of Ca^{2+}, however resulted in uncoupling of the mitochondria. Uncoupling was observed after a total addition of 1221 ± 301 (n = 9) nmol Ca^{2+} per mg protein. When spermine (B) was incubated with the mitochondrial suspension in the reaction vessel for 10 min prior to the subsequent addition of exogenous Ca^{2+}, no uncoupling was observed even after a total addition of 2489 ± 315 (n = 5) nmol Ca^{2+} per mg protein. The average value of the respiratory control index was 3.05 (B) as compared with a value of 3.45 (A) obtained following the first addition of 727 nmol Ca^{2+} per mg protein in the absence of spermine, before the mitochondria were uncoupled by the second addition of 727 nmol Ca^{2+} per mg protein. Uncoupling of mitochondria by exogenous Ca^{2+} was also prevented by tetracaine. Under these conditions (not shown), the average value of the respiratory control index obtained by three additions of 727 nmol Ca^{2+} per mg protein was 2.57, which was slightly lower than the value of 3.45 observed in the presence of spermine (B). When the experiments were repeated in the presence of Mg^{2+} (C), no uncoupling was observed after a total addition of 2279 ± 399 (n = 3) nmol Ca^{2+} per mg protein, but a progressive decline in the value of the respiratory control index was observed. The first addition of 727 nmol Ca^{2+} per mg protein gave a respiratory control index of 4.48; the subsequent separate additions of similar amounts of Ca^{2+} resulted in values of the respiratory control index of 3.60, 2.76 and 1.44, respectively. Even though the average value of respiratory control index of 3.08 in the presence of Mg^{2+} (C) was almost identical to the value of 3.05 observed in the presence of spermine (B), the mitochondria were showing signs of becoming slightly uncoupled following the last addition of 727 nmol Ca^{2+} per mg protein, i.e. after a total addition of 2909 nmol Ca^{2+} per mg protein in this particular set of experiments. This was not the case with the last addition of 727 nmol Ca^{2+} per mg protein when the experiments were carried out in the presence of spermine (B).

The effect of inhibitors of phospholipase A_2 was repeated using mitochondrial preparations from control pigs (Fig. 8). In the absence of a phospholipase inhibitor, no uncoupling by exogenous Ca^{2+} was observed after a total addition of 2313 ± 287 (n = 5) nmol Ca^{2+} per mg protein, and the average value of the respiratory control index was 3.66 (A). The mitochondria were still coupled when the same total amount of exogenous Ca^{2+} was added during succinate oxidation either in the presence of tetracaine (B) or spermine (C); the average value for the respiratory control index under these conditions being 2.95 (B) and 2.91 (C), respectively. The decrease in the values of the respiratory control index in the presence of the phospholipase inhibitors was principally due to an increase in the State 4 respiratory rate.

Effect of phospholipase inhibitors on mitochondrial configuration

The mitochondrial configuration of both malignant hyperthermia and control pigs were examined (Fig. 9) under various conditions at the end of the polarographic experiments during succinate oxidation at 40°C in the presence of exogenous Ca^{2+}. Thin sections of mitochondria prepared either in the presence of spermine (Fig. 9B) or tetracaine (Fig. 9C) showed that the mitochondria were intact and were either in the orthodox or condensed configuration [36] but not drastically swollen as observed in the absence of a phospholipase inhibitor (Fig. 9A). A few swollen mitochondria were however observed

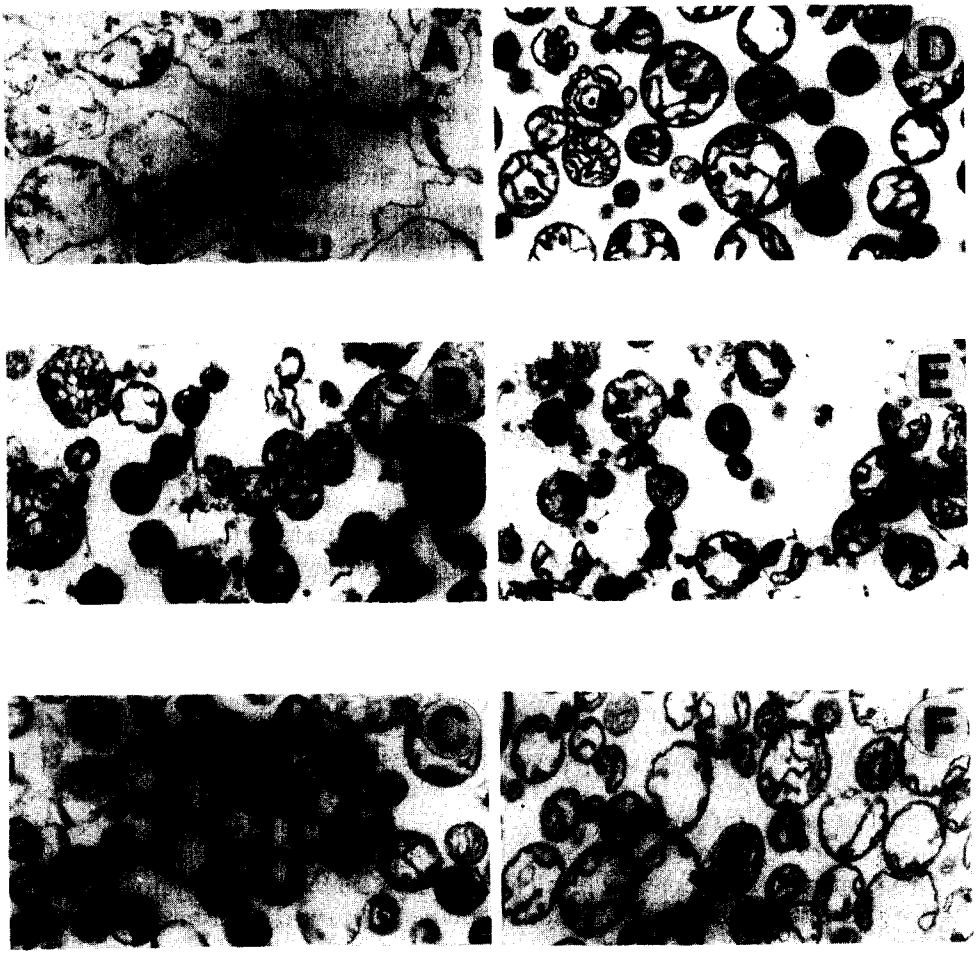

Fig. 9. Thin sections of mitochondria from musculi longissimus dorsi showing their ultrastructural configuration during succinate oxidation at 40°C. The electron micrographs A—C were prepared from mitochondria of malignant hyperthermia pigs when uncoupled by exogenous Ca^{2+} during succinate oxidation (A), and when the Ca^{2+}-induced uncoupling was prevented by either spermine (B) or tetracaine (C). The electron micrographs D—F were prepared from mitochondria of normal pigs as illustrated in Fig. 8. D, mitochondria of normal pig after the State 3 to State 4 transitions induced by Ca^{2+} (see Fig. 8A). E, as described in D except that the experiment was repeated in the presence of spermine (see Fig. 8C). F, as described in D except that the experiment was repeated in the presence of tetracaine (see Fig. 8B). Final magnification for all electron micrographs, 8500X.

in the presence of tetracaine (Fig. 9C) but the extent of swelling was not as drastic as that in the absence of any phospholipase inhibitor (Fig. 9A).

Mitochondria of normal pigs, on the other hand assumed a condensed configuration, and were found to be intact and not swollen (Fig. 9D) during succinate oxidation in the presence of 2313 ± 287 (n = 5) nmol Ca^{2+} per mg protein at 40°C, a condition where the mitochondria still fully retained their coupling integrity (see Fig. 8A). In the presence of either tetracaine or spermine, the mitochondria were still coupled (Fig. 8, B and C), and thin sections prepared under these conditions showed that the mitochondria were intact and were also in a condensed configuration without showing any sign of becoming swollen (Fig. 9, E and F).

Discussion

Our present data suggest that mitochondria from musculi longissimus dorsi of malignant hyperthermia pigs contained a Ca^{2+}-activated phospholipase A_2, and that this enzyme, if present in normal pigs is either latent or in very low concentration. Mitochondria contain phospholipase A_2 [37—43], and this enzyme can be activated by low concentrations of Ca^{2+} [44]. However, the total activity of the enzyme and its exact location is still not fully established. The enzyme might be localized in both the outer and inner mitochondrial membranes with the majority of the enzyme on the outer membrane [39], or in the intermembrane space between the inner and outer membranes [45] or in the inner membrane [41]. Mitochondrial phospholipase A_2, on stimulation by Ca^{2+} [41,44] caused the hydrolysis of the phospholipids in the mitochondrial membranes, resulting in the liberation of unsaturated fatty acids and lyso-derivatives of the mitochondrial phospholipids [41,46,47]. The fatty acids released would bring about the uncoupling of mitochondria [20,21], and the free fatty acids and lyso-derivatives of the mitochondrial membrane phospholipids would cause the destabilization of the mitochondrial membranes, resulting in swelling of the mitochondria [24,48—52].

A change in fluidity of the mitochondrial membranes has been demonstrated by the T_t of Arrhenius plots from either ADP or Ca^{2+}-stimulated respiration [53,54]. The 9°C increase in the T_t observed in mitochondria of malignant hyperthermia pigs in the Ca^{2+}-stimulated respiration could be explained by hydrolysis of phospholipids of mitochondrial membranes by Ca^{2+}-activated phospholipase A_2 releasing unsaturated fatty acids. The overall effect of phospholipase A_2 action would be a net increase in the content of saturated fatty acids in the mitochondrial membranes of malignant hyperthermia pigs which would in turn result in an increase in the T_t (Fig. 3, A and Refs. 53, 55). The involvement of a Ca^{2+}-activated phospholipase A_2 in mitochondria of malignant hyperthermia pigs was substantiated by the ability of spermine to decrease the T_t to a value (Fig. 5) which was not significantly different from that of normal (Fig. 6). This was further supported by the similar T_t obtained for the mitochondria of both types of pigs when ADP was used instead of Ca^{2+} to stimulate respiration during succinate oxidation (Fig. 4), as mitochondrial phospholipase A_2 could not be activated by ADP.

Stabilization of the mitochondrial membrane of malignant hyperthermia pigs

by Mg^{2+} could only partially overcome the fatty acid-induced uncoupling of the mitochondria through the action of the Ca^{2+}-stimulated phospholipase A_2 activity, even though the concentration of Mg^{2+} employed in our in vitro experiments was about 4-fold excess of that under physiological conditions, in which the free Mg^{2+} is probably close to 1 mM [56]. As a consequence of mitochondrial membrane stabilization, the T_t of mitochondria from malignant hyperthermia pigs was decreased by Mg^{2+} to 17.27°C ($n = 2$), a value which was similar to that observed in the presence of spermine, and also to that of normal pigs. Experimental evidence from the use of spermine, a mitochondrial phospholipase A_2 inhibitor [9] and a stabilizer of mitochondrial membranes [29,57, 58], and Mg^{2+}, a stabilizer of mitochondrial membrane [33—35] and not a phospholipase inhibitor [59] implies that the Ca^{2+}-stimulated phospholipase A_2 in mitochondria from musculi longissimus dorsi of malignant hyperthermia pigs is directly responsible for the higher T_t observed in the Ca^{2+}-stimulated respiration during succinate oxidation.

Porcine malignant hyperthermia appears to be a primary disorder of skeletal muscle [60,61], with the sympathetic nervous system being implicated only as a secondary response in the syndrome [62]. This was substantiated by the failure of curare or anaesthesia to prevent musculi longissimus dorsi of stress-susceptible pigs from producing much higher levels of lactate than normal [63]. Furthermore, musculi longissimus dorsi of anaesthetized stress-susceptible pigs was found to be more sensitive to anoxia induced by administration of pure nitrogen or by exsanguination than similar muscle of normal pigs [64]. Lactic acid levels were also found to be significantly ($P < 0.05$) higher in biopsy samples of stress-susceptible pigs breathing oxygen than similar muscle of stress-resistant pigs [64].

It is most likely that the mitochondrial Ca^{2+}-activated phospholipase A_2 plays a central role in the series of biochemical events in porcine malignant hyperthermia and in the ultimate formation of the pale, soft and exudative conditions associated with muscles of stress-susceptible animals. At high temperature, the mitochondria of malignant hyperthermia pigs are uncoupled by fatty acids released through the action of the Ca^{2+}-activated phospholipase A_2 acting on the mitochondrial phospholipids. The fatty acids and the lyso-derivatives of the mitochondrial phospholipids caused the mitochondria to undergo large amplitude swelling, and accompanied simultaneously by a faster Ca^{2+} efflux than normal. A similar situation would also occur when the mitochondria were de-energized during anoxia. It is most likely that the fatty acids released from the mitochondria of malignant hyperthermia pigs could induce the sarcoplasmic reticulum to release their endogenous Ca^{2+}. The release of additional Ca^{2+} from the sarcoplasmic reticulum could also be induced by Ca^{2+} [65] released from mitochondria of malignant hyperthermia pigs or by phospholipase A_2 modifying the lipids of the sarcoplasmic reticulum. Through a combination of a faster mitochondrial Ca^{2+} efflux rate and an inactivation of the sarcoplasmic reticulum by fatty acids [66] an overall increase in the level of Ca^{2+} occurs in the myoplasm. The excess Ca^{2+} then stimulates glycolysis by activating the phosphorylase kinase [67] and the myofibrillar ATPase resulting in more glycogen being degraded to lactate than under normal circumstances.

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