3:7 Endogenous calmodulin, Ca^{2+} and phospholipase A_2 activity and their relationships to halothane sensitivity in young and adult pigs

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Introduction

Post-mortem samples of M. longissimus dorsi (LD) of halothane-sensitive pigs showed considerably higher sarcoplasmic Ca^{2+} levels than normal (Cheah et al., 1984). This excess of Ca^{2+} release in LD muscles, which was intimately linked with the formation of PSE meat (Cheah et al., 1984) was attributed to a significantly higher than normal Ca^{2+}-activated phospholipase A_2 (EC 3.1.1.4) activity (Cheah and Cheah, 1981a). The Ca^{2+}-activated phospholipase A_2 activity was demonstrated to be responsible for the enhanced mitochondrial Ca^{2+} release in halothane-sensitive pigs; both the released Ca^{2+} and the liberated long chain unsaturated fatty acids from the mitochondrial phospholipids would then induce the sarcoplasmic reticulum to release additional Ca^{2+} into the sarcoplasm (Cheah and Cheah, 1981b; Cheah, 1981).

This paper reports further investigations on mitochondrial phospholipase A_2 activity and demonstrates that a significantly higher than normal amount of endogenous calmodulin was responsible for the enhanced Ca^{2+}-activated phospholipase A_2 activity in LD muscle mitochondria of halothane-sensitive pigs. Growth studies showed that the calmodulin content and the calmodulin-Ca^{2+} dependent phospholipase A_2 activity are normal in young pigs (3-4 weeks) from a halothane-sensitive line, but these increase by 2-3 fold in the adults (23 weeks). Normal mitochondria showed all the characteristic features of halothane-sensitive mitochondria when treated with an excess of exogenous calmodulin.

Materials and Methods

Calmodulin-deficient bovine cardiac cAMP phosphodiesterase (EC 3.1.4.17), cAMP, sodium succinate, spermine tetrahydrochloride and 5'-nucleotidase (EC 3.1.3.5) were purchased from Sigma Chemical Corp.; trifluoperazine dihydrochloride from Smith Kline and French Laboratories Ltd.; calmodulin (pig brain) from Boehringer Mannheim and all other reagents were of analytical grade. Genetically-selected halothane-sensitive and halothane-insensitive British Landrace pigs were kindly supplied by Dr. A.J. Webb, Agricultural and Food Research Council Animal Breeding Research Organisation, Edinburgh.

All the pigs were killed by electrical stunning (90V, 50Hz) and exsanguination. LD muscle mitochondria were isolated immediately post-mortem by differential centrifugation with the aid of B. subtilis proteinase (Cheah and Cheah, 1981a). The mitochondria were suspended in 250 mM sucrose (final concentration) and the mitochondrial oxygen uptake was measured with a Clark oxygen electrode [Yellow Springs Oxygen Monitor (Model 53)] in a total volume of 2.50 ml. Endogenous mitochondrial phospholipase A_2 activity was determined by estimating the long chain fatty acids formed after incubation of 0.3-0.4 ml (11.3 mg protein) mitochondrial suspension at 40°C for 20 minutes in a buffer (pH 7.2) containing 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl (total volume, 0.64 ml) either in the presence or absence of trifluoperazine. Mitochondrial calmodulin was estimated on the basis of its ability to stimulate calmodulin-deficient phosphodiesterase activity by estimating the amount of phosphate released by 5'-nucleotidase (Teo et al., 1973) in boiled mitochondrial extracts. Mitochondrial Ca^{2+} was determined either 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 or by the formation of a Ca^{2+}-murexide complex. Mitochondrial protein was estimated with Folin-Phenol reagent (Lowry et al.,1951) using bovine serum albumin as standard. Muscle pH was determined in 150 mM KCl (pH 7.4) containing 5 mM sodium iodoacetate (Bendall, 1978).

Results and Discussion

The enhanced endogenous Ca^{2+}-activated phospholipase A_2 activity was previously shown to be responsible for the Ca^{2+}-induced uncoupling and large amplitude swelling of LD muscle mitochondria of halothane-sensitive pigs oxidising succinate at 40°C (Cheah and Cheah, 1981a). Figure 1 illustrates further studies on this enzyme at 37°C. Ca^{2+}-induced uncoupling occurred following the third addition of Ca^{2+} (Trace A), but this was prevented by a low concentration of trifluoperazine (Trace B), an inhibitor of calmodulin-dependent enzymes (Levin and Weiss, 1977; Hidaka et al., 1980). As in previous studies at 40°C (Cheah and Cheah, 1981a), spermine (not shown in Figure 1), an inhibitor of phospholipase A_2 activity (Sechi et al., 1978), also prevented the Ca^{2+}-induced uncoupling of succinate oxidation. Electron micrographs of mitochondria from experiments shown in Figure 1 revealed that the mitochondrial Ca^{2+}-activated phospholipase A_2 activity was calmodulin-dependent since the large amplitude swelling of mitochondria was prevented by trifluoperazine. As in previous studies conducted at 40°C (Cheah and Cheah, 1981a), no uncoupling of mitochondria was observed when the experiments were repeated with ADP. This result was expected since ADP is unable to stimulate phospholipase A_2 activity (Waite et al., 1969). Under these conditions no lysophosphatides and unsaturated fatty acids were produced to induce uncoupling and large amplitude swelling of skeletal muscle mitochondria as previously observed with exogenous Ca^{2+}.

Table 1 summarizes the endogenous calmodulin, Ca^{2+} and phospholipase A_2 activity in LD muscle mitochondria isolated from young (3-4 weeks) and adult (23 weeks) halothane-sensitive line pigs, and from young (4-8 weeks) and adult 23 weeks) halothane-insensitive pigs. In halothane-sensitive line pigs, the endogenous calmodulin and Ca^{2+}, and the phospholipase A_2 activity increased significantly with growth but not so in the case of halothane-insensitive pigs. No significant difference was observed in these parameters between the young halothane-sensitive line and adult halothane-insensitive pigs, and between the young pigs of both types. The endogenous phospholipase A_2 activity in the LD muscle mitochondria of both types of adult pigs was inhibited 90% with trifluoperazine (66 nmol/mg protein).

Table II summarizes the relationship between LD muscle pH at 45 minutes post-mortem and meat quality in young and adult halothane-sensitive and halothane-insensitive pigs. No significant difference was observed in the pH values between young halothane-sensitive line and young and adult halothane-insensitive pigs, but the pH values of all of these pigs were significantly ($P<0.001$) higher than those of adult halothane-insensitive pigs. Meat quality was assessed in LD muscles by a combination of pH values at 45 minutes post-mortem, colour (Hunter L) and water-holding capacity measurements. Young halothane-sensitive line pigs and young and adult halothane-insensitive pigs produced normal meat but adult halothane-sensitive pigs produced PSE meat post-mortem.

The results presented in Figure 1 and Table 1 favour the concept that a significantly higher than normal amount of endogenous calmodulin was responsible for the abnormal features of LD muscle mitochondria of halothane-sensitive pigs. This hypothesis is supported by the results obtained with normal mitochondria

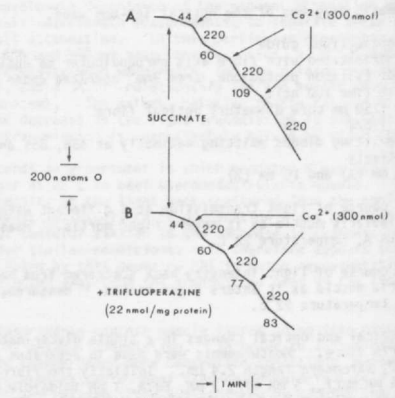

Fig. 1 Effect of trifluoperazine (B) on the Ca^{2+}-stimulated respiration of LD muscle mitochondria of halothane-sensitive pig (A) at 37°C.

Trace A illustrates the control experiment. Trace B is the same as Trace A except that trifluoperazine was added to the mitochondrial suspension. Rotenone (2 μM) was added prior to succinate. Total protein, 1.57 mg (Trace A and Trace B). The numbers alongside the Traces (A-B) represent the rates of oxygen uptake expressed in natoms O/min/mg protein.

treated with calmodulin to bring it to a level equivalent to or in excess of that of LD muscle mitochondria of halothane-sensitive pigs. Figure 2 illustrates a typical experiment showing the Ca^{2+}-stimulated respiration of succinate oxidation by halothane-insensitive LD muscle mitochondria (Trace A), the Ca^{2+}-induced uncoupling observed when the mitochondria were treated with an excess of exogenous calmodulin (Trace B) and the prevention of the Ca^{2+}-induced uncoupling by a low concentration of trifluoperazine (Trace C). In these experiments the LD muscle mitochondria were aged overnight in ice in order to increase their permeability towards exogenous calmodulin. The control experiment (Fig. 2, Trace A) showed that the aged mitochondria retained nearly all their original coupling integrity, assessed on the values of the respiratory control index and Ca^{2+}/O ratio, of the freshly isolated mitochondria during succinate oxidation. In contrast to the aged preparations, 600 ng calmodulin was required to cause the Ca^{2+}-induced uncoupling of fresh LD muscle mitochondria (not shown in Figure 2) instead of 25 ng calmodulin for the aged preparations (Fig. 2, Trace B). The Ca^{2+}-induced uncoupling observed with 600 ng calmodulin in freshly isolated mitochondria was prevented by the same low concentration of trifluoperazine used for aged mitochondria.

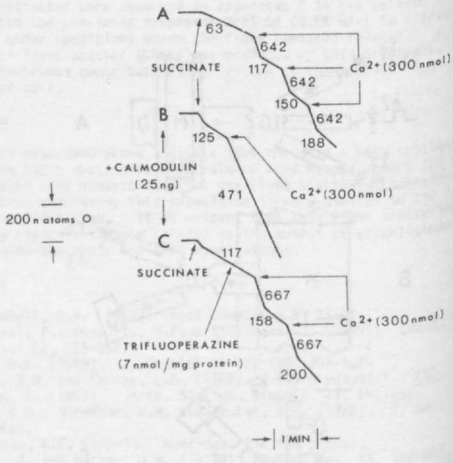

Fig. 2 Effect of exogenous calmodulin on the Ca^{2+}-stimulated respiration of succinate oxidation by halothane-insensitive LD muscle mitochondria aged overnight in ice at 1°C.

Trace A (control) illustrating no Ca^{2+}-induced uncoupling was observed in aged mitochondria. The preparation retained 85% of the respiratory control index and 93% of the Ca^{2+}/O ratio of freshly isolated mitochondria.

Trace B shows that Ca^{2+}-induced uncoupling occurred after the mitochondria were pretreated with 25 ng calmodulin.

Trace C illustrates that the Ca^{2+}-induced uncoupling in the presence of 25 ng calmodulin was prevented by a low concentration of trifluoperazine. Under these conditions, the mitochondria retained 86% of the control respiratory control index and 72% of the control Ca^{2+}/O ratio. Total protein (Traces A-C) 1.02 mg protein.

Table 1 Endogenous calmodulin, Ca^{2+} and phospholipase A_2 activity in LD muscle mitochondria of halothane-sensitive and halothane-insensitive pigs.

Calmodulin was estimated using boiled mitochondrial extracts by measuring the cAMP phosphodieterase activity in the presence of 1.0 mM Ca^{2+} using calmodulin-deficient phosphodiesterase. The results are means ± SD for the number of pigs in parentheses. Phospholipase A_2 activity is expressed in nmol fatty acids per mg protein per 20 minutes at 40°C. Other experimental details are described in materials and methods.

Parameters	Halothane-sensitive line		Halothane-insensitive line	
	Young (3-4 weeks)	Adult (23 weeks)	Young (4-8 weeks)	Adult (23 weeks)
Calmodulin (ng/mg protein)	31.1 ± 7.8 (n = 3)	43.8 ± 2.2 (n = 4)	22.0 ± 1.7 (n = 4)	23.7 ± 5.0 (n = 4)
Ca^{2+} (nmol/mg protein)	35.5 ± 12.0 (n = 7)	79.1 ± 13.4 (n = 16)	33.6 ± 11.5 (n = 4)	43.6 ± 11.8 (n = 14)
Phospholipase A_2 activity	3.7 ± 0.5 (n = 4)	11.2 ± 1.3 (n = 4)	3.3 ± 0.6 (n = 4)	4.3 ± 1.3 (n = 4)

The results support the hypothesis that LD muscle mitochondrial Ca^{2+}-activated phospholipase A_2 activity is calmodulin-dependent, and that a significantly higher than normal amount of endogenous calmodulin is responsible for the enhanced Ca^{2+}-activated phospholipase A_2 activity in adult halothane-sensitive pigs. The calmodulin-Ca^{2+} dependent phospholipase A_2 activity is normal in young halothane-sensitive pigs but its activity increases by 3-fold in the adults. No significant increase in the endogenous calmodulin, Ca^{2+} and phospholipase A_2 activity is observed in the LD muscle mitochondria with age in halothane-insensitive pigs. Our present studies also suggest that PSE is a developmental disorder and is not expressed in young halothane-sensitive line pigs.

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References

Bendall, J.R. (1978). Meat Sci. 2, 91.
Cheah, A.M. (1981). Biochim. Biophys. Acta 648, 113.
Cheah, K.S. and Cheah, A.M. (1981a). Biochim. Biophys. Acta 634, 70.
Cheah, K.S. and Cheah, A.M. (1981b). Biochim. Biophys. Acta 638, 40.
Meat Sci. 10, 117.
Hikita, H., Yamaki, T., Naka, M., Tanaka, T., Hayashi, H. and Kobayashi, R. (1980). Mol. Pharmacol. 17, 66.
Lawry, R.M. and Weiss, B. (1977). Mol. Pharmacol. 13, 690.
Lehn, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265.
Sechi, A.M., Cabrini, L., Landi, L., Pasquali, P. and Lenaz, G. (1978). Arch. Biochem. Biophys. 186, 248.
Tee, T.S.
Waite, M., Wang, T.H. and Wang, J.H. (1973). J. Biol. Chem. 248, 588.
Scherphof, G.L., Boshouwers, F.M.G. and van Deenen, L.L.M. (1969). J. Lipid Res. 10, 411.

Table II Muscle pH and meat quality of LD muscles of halothane-sensitive and halothane-insensitive young and adult British Landrace pigs.

The pH of LD muscles were determined at 45 minutes post-mortem and the parameters (colour, water-holding capacity) of meat quality as previously described (Cheah et al., 1984). The values for the water-holding capacity of LD muscles, determined by the Grau-Hamm type press, refer to the ratio value of muscle area/fluid area; for PSE meat the ratio is less than 0.60 and the Hunter L value greater than 53. (Cheah et al., 1984). The results are means ± SD for the number of pigs in parentheses.

Parameters	Halothane-sensitive line		Halothane-insensitive line	
	Young (3-4 weeks)	Adult (23 weeks)	Young (3-4 weeks)	Adult (23 weeks)
pH (45 min post-mortem)	6.33 ± 0.04 (n = 4)	5.49 ± 0.06 (n = 9)	6.46 ± 0.19 (n = 4)	6.30 ± 0.18 (n = 5)
Colour (Hunter L)	44.7 ± 1.3 (n = 3)	57.0 ± 2.2 (n = 6)	46.9 ± 3.0 (n = 4)	47.7 ± 3.2 (n = 3)
Water-holding capacity	0.62 ± 0.16 (n = 7)	0.48 ± 0.04 (n = 9)	0.80 ± 0.14 (n = 4)	0.74 ± 0.08 (n = 9)
Meat quality	Normal	PSE	Normal	Normal