CALCIUM MOVEMENTS IN SKELETAL MUSCLE MITOCHONDRIA OF MALIGNANT HYPERTHERMIC PIGS

K. S. CHEAH and A. M. CHEAH
Agricultural Research Council, Meat Research Institute, Langford, Bristol BS18 7DY, England

Received 12 September 1978

1. Introduction

Porcine malignant hyperthermia can easily be induced by anaesthesia with halothane [1–6] in certain breeds of pigs particularly those bred for lean carcasses. The predominant clinical symptoms of this syndrome are gross muscular rigidity, rapid rise in body temperature, tachycardia, hyperventilation, severe metabolic acidosis and elevated levels of serum metabolites [7,8]. The manifestations of porcine malignant hyperthermia are similar to those described for human malignant hyperpyrexia, but it is not clear to what extent the porcine data are applicable to humans. The frequency of occurrence of anaesthetic deaths in apparently healthy patients can be as high as 1 in 15 000 [9].

The etiology of porcine malignant hyperthermia is fairly well documented [7,8], but the underlying mechanism responsible for the series of biochemical events leading to the syndrome is unknown. The increase of serum Ca^{2+} in porcine malignant hyperthermia [3,10] suggests that the Ca^{2+}-accumulating organelles might be defective in halothane-sensitive pigs, but up to date no significant malfunction in these organelles has been convincingly demonstrated. The report of a difference in the sarcoplasmic reticulum in stress-susceptible pigs [11] is most likely to be due to acid denaturation of these organelles as they were isolated from muscles having pH 5.4–5.7.

Mitochondria of longissimus dorsi (LD) muscle of halothane-sensitive (i.e., malignant hyperthermic) pigs were shown [12,13] to have a much higher rate of Ca^{2+} efflux than halothane-insensitive (i.e., normal) pigs, and that the rates of Ca^{2+} efflux correlate very closely with parameters associated with porcine malignant hyperthermia. This paper provides evidence showing the possible existence of a difference either in the structural or functional integrity of the mitochondrial membranes between malignant hyperthermic and normal pigs.

2. Materials and methods

Bovine serum albumin (fatty acid-free), rotenone, sodium succinate and murexide were obtained from Sigma Chemical Corp.; crystalline *Bacillus subtilis* (Nagarse) from Teikoku Chemical Co.; all other reagents were of analytical grade.

The halothane-sensitive and -insensitive Pietrain/Hampshire pigs were killed at 75–100 kg and the mitochondria were isolated from LD muscle immediately postmortem using *B. subtilis* proteinase [14]. The Ca^{2+}-stimulated respiration for succinate oxidation was measured polarographically with a Clark oxygen electrode (Yellow Spring Oxygen Monitor (Model 53)) in total vol. 2.50 ml. The reaction medium (pH 7.20) contained 220 mM mannitol, 50 mM sucrose and 15 mM Tris–HCl in the presence of 5 mM P_i. Ca^{2+} efflux was measured with murexide [15] at 20°C using the Aminco-Chance dual-wavelength/split-beam spectrophotometer operating in the dual-wavelength mode at 540 510 nm in the same reaction medium as that described for the oxygen uptake experiments except that 2.50 mM P_i was used. Protein was determined with Folin–phenol reagent [16] using bovine serum albumin as standard.

3. Results

Mitochondria of both halothane-sensitive and -insensitive 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+} during succinate oxidation at 25°C. At 40°C, however, a marked difference in these parameters was observed when Ca^{2+} was used instead of ADP. Figure 1 represents typical polarographic experiments showing the effect of Ca^{2+} addition to mitochondria from halothane-insensitive (trace A) and halothane-sensitive (trace B) pigs, and the effect of bovine serum albumin (BSA) in counteracting the Ca^{2+}-induced uncoupling in mitochondria from halothane-sensitive pigs (trace C) during succinate oxidation at 40°C. Figure 1A represents a typical experiment of mitochondria from halothane-insensitive pig showing that these mitochondria were not easily uncoupled by Ca^{2+} at 40°C. No uncoupling was observed even after a total addition of 2297 ± 288 nmol Ca^{2+}/mg protein (mean value of 3 experiments from 3 pigs). In contrast, mitochondria of halothane-sensitive pigs showed a much lower capacity to accumulate Ca^{2+} under the same experimental conditions, and were uncoupled by the second addition of Ca^{2+} (trace B). Uncoupling was observed after a total addition of 1297 ± 163 nmol Ca^{2+}/mg protein (mean value of 4 experiments from 4 pigs). Addition of BSA restored the mitochondrial coupling integrity (trace C), and under these conditions these mitochondria from halothane-sensitive pigs could accumulate almost the same amount of Ca^{2+}/mg protein as those of halothane-insensitive pigs without showing any sign of being uncoupled. The mean value of 3 experiments from 3 halothane-sensitive pigs showed that these mitochondria could accumulate 1964 ± 224 nmol Ca^{2+}/mg protein in the presence of BSA without being uncoupled.

Figure 2 illustrates typical experiments showing the effect of BSA (traces B and D) on the rate of mitochondrial Ca^{2+} efflux of halothane-sensitive

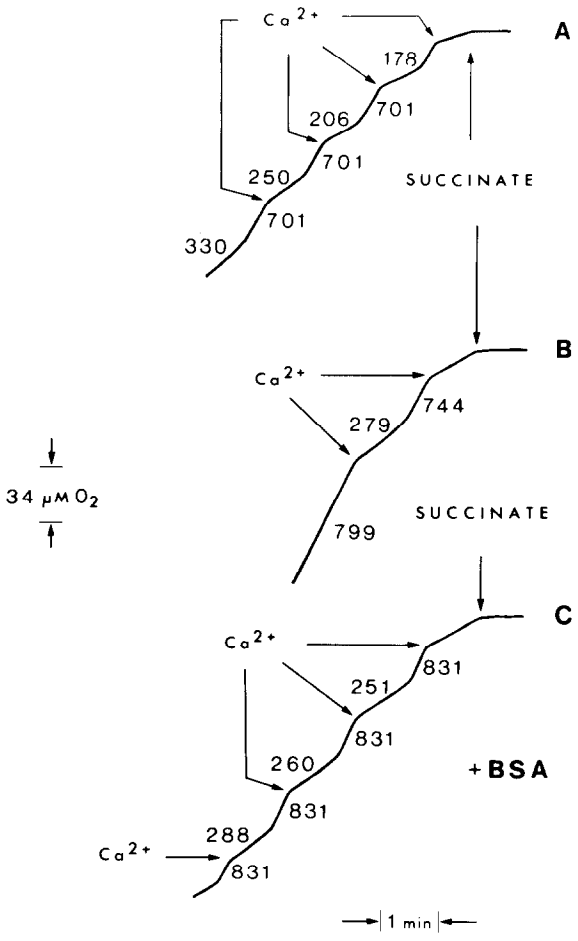

Fig.1. Effect of Ca^{2+} on succinate oxidation by mitochondria of halothane-insensitive and -sensitive pigs at 40°C. Trace A illustrates a typical experiment showing the state 3–state 4 transition induced by Ca^{2+} during succinate oxidation by mitochondria of halothane-insensitive pig. Four additions of 300 nmol Ca^{2+} were added without causing uncoupling of mitochondria. Total protein, 0.50 mg; total Ca^{2+} added, 2400 nmol/mg protein. Trace B represents a typical experiment showing the effect of Ca^{2+} on mitochondria of halothane-sensitive pig during succinate oxidation. The second addition of 300 nmol Ca^{2+} completely uncoupled the mitochondria. Total protein, 0.48 mg; total Ca^{2+} added, 1250 nmol/mg protein. Trace C illustrates a typical experiment showing bovine serum albumin (BSA) completely counteracted the Ca^{2+}-induced uncoupling in mitochondria of halothane-sensitive pig represented by trace B. No uncoupling was observed even after a total addition of 1050 nmol Ca^{2+} (3 additions of 300 nmol and 1 addition of 150 nmol). Total protein, 0.48 mg; total Ca^{2+} added, 2188 nmol/mg protein. Rotenone (2 μM) and succinate (10 mM) were added prior to Ca^{2+} addition in all the experiments represented by traces A–C. BSA (1.0 mg) 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/min/mg protein.

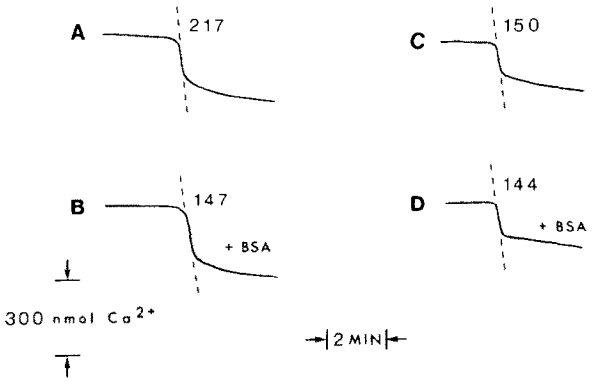

Fig.2. Effect of bovine serum albumin (BSA) on mitochondrial Ca^{2+} efflux of halothane-sensitive and -insensitive pigs. The rate of mitochondrial Ca^{2+} efflux was estimated with murexide at 540–510 nm with the Aminco-Chance spectrophotometer. Murexide (92 μM) was added to the mitochondrial suspension in a 10 mm lightpath cuvette containing rotenone (2 μM) and P_i (2.50 mM). Reaction was initiated by addition of Ca^{2+} (600 nmol) and succinate (10 mM). Figure 2 shows the final kinetic traces of Ca^{2+} efflux where differences were observed between the halothane-sensitive and -insensitive pigs. The numbers alongside the traces represent the rates of Ca^{2+} efflux expressed in nmol/min/mg protein at 20°C. Traces A and C represent typical kinetic traces of Ca^{2+} efflux of halothane-sensitive and -insensitive pigs. Traces B and D show the effect of BSA (0.3 mg) on the rate of mitochondrial efflux of halothane-sensitive and -insensitive pigs, respectively.

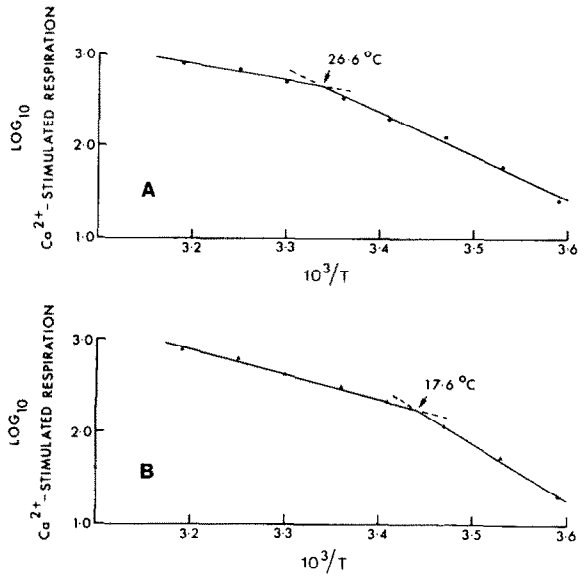

Fig.3. Arrhenius plots of Ca^{2+}-stimulated respiration of mitochondria from halothane-sensitive and -insensitive pigs. The Ca^{2+}-stimulated respiration was estimated with a Clark oxygen electrode from 40° to 5°C. Figure 3 represents typical results obtained with mitochondria from halothane-sensitive (A) and -insensitive (B) pigs.

(tracc A) and -insensitive (trace C) pigs. BSA reduced the high efflux rate of halothane-sensitive pigs (trace B) to the low rate characteristic of halothane-insensitive pigs (trace D). The mean value of Ca^{2+} efflux rates from 4 halothane-sensitive pigs were reduced from 227 ± 24 nmol/min/mg protein to 114 ± 18 nmol/min/mg protein by 0.3 mg BSA. Under the same experimental conditions, BSA (0.3 mg) had no effect on the rate of Ca^{2+} efflux from halothane-insensitive pigs. Like BSA, spermine also reduced the mitochondrial Ca^{2+} efflux rate of halothane-sensitive pigs to that observed for halothane-insensitive pigs. The mean value of Ca^{2+} efflux rates from 4 halothane-sensitive pigs were reduced from 227 ± 24 nmol/min/mg protein to 149 ± 8 nmol/min/mg protein by 1.0 mM spermine. The same concentration of spermine had no effect on the efflux rate of mitochondria of halothane-insensitive pigs.

The effect of various temperatures on the Ca^{2+}-stimulated respiration of LD muscle mitochondria of both halothane-sensitive and -insensitive pigs is represented by their respective Arrhenius plots (fig.3). Halothane-sensitive pigs (A) showed a much higher transition temperature than that of -insensitive pigs, the average values for 3 pigs of each type being 26.6 ± 0.60°C and 17.6 ± 0.65°C, respectively.

4. Discussion

The existence of a difference either in the structural or functional integrity of the mitochondrial membranes of halothane-sensitive (malignant hyperthermia prone and stress-susceptible) pigs is probably responsible for the higher rate of mitochondrial Ca^{2+} efflux and lower capacity to accumulate Ca^{2+} at higher temperature in these pigs. The composition of the membrane lipids of halothane-sensitive pigs could be different from those of normal as shown by the difference of 9°C in the transition temperature of the Arrhenius plots of Ca^{2+}-stimulated respiration (fig.3) between the two types of pigs. It is well known that

the unsaturated : saturated fatty acids ratio in membranes is the determining factor for a phase transition at a particular temperature [17–22]. The higher transition temperature coupled with the relationship of unsaturated to saturated fatty acids in regulating membrane-bound enzymic systems [20,21] suggests that halothane-sensitive pigs might contain more saturated fatty acids in their mitochondrial membranes than normal pigs. The functional integrity of the Ca^{2+} transport system of halothane-sensitive pigs could also be affected by modification of the Ca^{2+}-binding sites by fatty acids released as a consequence of the Ca^{2+}-stimulated phospholipase, resulting in the marked difference in both the Ca^{2+} efflux rates ([12,13] and fig.2) and Ca^{2+} accumulation at high temperature (fig.1). This suggestion is based on the evidence that BSA (0.3 mg) and spermine (1.0 mM) restore the values of the Ca^{2+} efflux rates and Ca^{2+} accumulation of halothane-sensitive pigs to normal. Uncoupling of mitochondria by free fatty acids [23,24] could be counteracted by BSA, which restores oxidative phosphorylation by binding with free fatty acids [24,25]. Spermine, on the other hand, affects either the kinetics of Ca^{2+} transport by interacting with the Ca^{2+}-binding sites of the mitochondrial membrane [26] or inhibits the phospholipase activity [27]. No significant difference was observed in either the rate of Ca^{2+} uptake and release or the ATPase activity of sarcoplasmic reticulum isolated from halothane-sensitive and -insensitive pigs. From the evidence presented, it thus appears that the Ca^{2+} transport system in mitochondria probably plays an important role in the underlying mechanism of the porcine malignant hyperthermia syndrome and perhaps also of human hyperpyrexia.

Acknowledgement

The authors are grateful to Dr A. J. Webb, Agricultural Research Council, Animal Breeding Research Organisation, Edinburgh, for supplying the halothane-sensitive and -insensitive Pietrain/Hampshire pigs.

References

[1] Harrison, G. G., Biebuyk, J. F., Terblanche, J., Dent, D. M., Hickman, R. and Saunders, J. J. (1968) Brit. Med. J. 3, 594–595.
[2] Allen, W. M., Berrett, S., Harding, J. D. J. and Patterson, D. S. P. (1970) Vet. Rec. 87, 64–69.
[3] Berman, M. C., Harrison, G. G., Bull, A. A. and Kench, D. D. (1970) Nature 225, 653–655.
[4] Nelson, T. E., Jones, E. W., Venable, J. H. and Kerr. D. D. (1972) Anaesthesiol. 36, 52–56.
[5] Hall, L. W., Trim, C. M. and Woolf, N. (1972) Brit. Med. J. 2, 145–148.
[6] Gronert, G. A. and Theye, R. A. (1976) Anaesthesiol. 44, 36–43.
[7] Brucker, R. F., Williams, C. H., Popinigis, J., Glavez, T. L., Vail, W. J. and Taylor, C. A. (1973) in: Int. Symp. Malignant Hyperthermia (Gordon, R. A. et al. eds) pp. 238–270, Charles C. Thomas Publishers, Springfield.
[8] Van den Hende, C., Lister, D., Muylle, E., Ooms, L. and Oyaert, W. (1976) Brit. J. Anaesth. 48, 821–829.
[9] Britt, B. A. and Kalow, W. (1970) Can. Anaesth. Soc. J. 17, 293–315.
[10] Campion, D. R. and Topel, D. G. (1975) J. Anim. Sci. 41, 779–786.
[11] McIntosh, D. B., Berman, M. C. and Kench, J. E. (1977) Biochem. J. 166, 387–398.
[12] Cheah, K. S. (1978) in: Proc. 24th Eur. Meat Workers Meet., Kulmbach, E2:1–E2:11.
[13] Rensburg, A. J., Cheah, K. S. and Cheah, A. M. (1978) in: Proc. 24th Eur. Meat Workers Meet., Kulmbach, E5:1–E5:5.
[14] Cheah, K. S. (1970) FEBS Lett. 10, 109–112.
[15] Mela, L. and Chance, B. (1968) Biochemistry 7, 4059–4063.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Melchior, D. L., Morowitz, H. J., Sturtavant, J. M. and Tsong, T. Y. (1970) Biochim. Biophys. Acta 219, 114–122.
[18] Raison, J. K., Lyons, J. M., Melhorn, R. J. and Keith, A. D. (1971) J. Biol. Chem. 246, 4036–4040.
[19] Haslam, J. M., Cobon, G. S. and Linnane, A. W. (1973) Biochem. Soc. Trans. 2, 207–209.
[20] Haslam, J. M. and Fellows, N. F. (1977) Biochem. J. 166, 565–570.
[21] Raison, J. K. (1973) Bioenergetics 4, 285–309.
[22] Lyons, J. M. and Raison, J. K. (1970) Plant Physiol. 45, 386–389.
[23] Vazquez-Colon, L., Ziegler, F. D. and Elliott, W. B. (1966) Biochemistry 5, 1134–1139.
[24] Weinbach, E. C., Garbus, J. and Claggett, C. E. (1966) J. Biol. Chem. 241, 3708–3717.
[25] Haslam, J M., Spithill, T. W., Linnane, A. W. and Chappell, J. B. (1973) Biochem. J. 134, 949–957.
[26] Akerman, K. E. O. (1977) J. Bioenerg. Biomembr. 9, 65–72.
[27] Sechi, A. M., Cabrini, L., Landi, P., Pasquali, and Lenaz, G. (1978) Arch. Biochem. Biophys. 186, 248–254.