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EFFECT OF LONG CHAIN UNSATURATED FATTY ACIDS ON THE CALCIUM TRANSPORT OF SARCOPLASMIC RETICULUM

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The effect of arachidonic, oleic and linoleic acid on calcium uptake and release by sarcoplasmic reticulum isolated from longissimus dorsi muscle was investigated using a Ca^{2+} electrode. All three long chain fatty acids stimulated the release of Ca^{2+} from sarcoplasmic reticulum when added after exogenous Ca^{2+} was accumulated by the vesicles, and also inhibited Ca^{2+} uptake when added before Ca^{2+}. This inhibitory effect on the calcium transport by arachidonic, oleic and linoleic acid was prevented by bovine serum albumin through its ability to bind with the fatty acid. The order of effectiveness of the fatty acids in inhibiting calcium transport by isolated sarcoplasmic reticulum was arachidonic acid > oleic acid > linoleic acid. Similar inhibition of calcium uptake and induction of calcium release by arachidonic acid was observed in muscle homogenate sarcoplasmic reticulum preparations. Both arachidonic and oleic acid stimulated the (Ca^{2+} + Mg^{2+})-ATPase activity of sarcoplasmic reticulum at low concentrations, but inhibited the (Ca^{2+} + Mg^{2+})-ATPase activity at high concentrations. The maximal (Ca^{2+} + Mg^{2+})-ATPase activity observed with arachidonic acid was twice that obtained with oleic acid, but the concentration of arachidonic acid required was 3–4-times greater than that of oleic acid. The concentration of arachidonic acid required to give maximum stimulation of the (Ca^{2+} + Mg^{2+})-ATPase activity was 3.6-times greater than that needed for complete inhibition of calcium accumulation by the sarcoplasmic reticulum. With oleic acid, however, the concentration required to give maximum stimulation of the (Ca^{2+} + Mg^{2+})-ATPase activity inhibited the sarcoplasmic reticulum Ca^{2+} accumulation by 72%. The present data support our hypothesis that, in porcine malignant hyperthermia, unsaturated fatty acids from mitochondrial membranes released by endogenous phospholipase A_2 would induce the sarcoplasmic reticulum to release calcium (Cheah, K.S. and Cheah, A.M. (1981) Biochim. Biophys. Acta 634, 70–84).

Introduction

Malignant hyperthermia, a genetically determined frequently fatal condition, is triggered following anaesthesia with halothane [1–5]. The clinical symptoms are gross muscular rigidity, rapid rise in body temperature, metabolic acidosis and elevated levels of serum metabolites [6,7]. A sudden increase in the level of sarcoplasmic calcium has been postulated to induce malignant hyperthermia [8]. A similar condition, porcine stress syndrome, caused by physiological stress, also occurs in certain breeds of pigs, and is characterized by rapid muscle glycolysis immediately post-mortem [9], with the ultimate pH being attained while the muscle is still warm. Under these conditions, denaturation of myofibrillar and sarcoplasmic proteins occurs, resulting in the formation of pale, soft and exudative muscle [10]. In our recent work on porcine malignant hyperthermia [11] we reported that mitochondria from longissimus dorsi muscle contain a calcium-activated phospholipase A_2 (EC 3.1.1.4) which causes the release of fatty acids from the phospholipids of mitochondrial membranes. We suggested that such fatty acid would induce the sarcoplasmic reticulum to release calcium, contributing to the elevated sarcoplasmic calcium observed

during malignant hyperthermia [8]. The increase level of calcium would then stimulate glycolysis by activation of phosphorylase kinase [12] and myofibrillar ATPase, resulting in the production of excess lactic acid and a low muscle pH.

Various substances have been reported to affect sarcoplasmic reticulum calcium transport and (Ca^{2+} + Mg^{2+})-ATPase, including the fatty acid, oleic acid [13,14], several phospholipases [14–17], anaesthetics [18,19], monovalent cations [20,21], chaotropic anions [22] and Salygran [13,20]. Calcium uptake by sarcoplasmic reticulum was shown to be inhibited by oleic acid while the (Ca^{2+} + Mg^{2+})-ATPase was unaffected [13].

This paper extends our research on porcine malignant hyperthermia by investigating more fully the effect of oleic acid on sarcoplasmic reticulum [13], and comparing its action with that of arachidonic and linoleic acids. The results support the suggestion [11] that unsaturated fatty acids released from mitochondrial membranes of malignant hyperthermia-prone pigs by the action of phospholipase A_2 would induce the sarcoplasmic reticulum to release calcium. Arachidonic acid ($C_{20:4}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) were selected for investigation since they are the major long chain unsaturated fatty acids of the mitochondrial phospholipids [23] known to be released by the action of phospholipase A_2 [24].

Materials and Methods

Reagents. Antimycin A, ATP, bovine serum albumin, dithiothreitol, imidazole, oligomycin and the sodium salts of arachidonic, oleic and linoleic acids were obtained from Sigma Chemical Corp. All other reagents were analytical grade.

Methods. Sarcoplasmic reticulum was isolated from longissimus dorsi muscle of Pietrain-Hampshire pigs by modifying the method of Martonosi and Feretos [20]. The minced muscle was homogenised (15 000 rev./min for 45 s) using a Sorval Omnimixer (Model 17106) in 4 vol. ice-cold 100 mM KCl/40 mM imidazole chloride/1 mM dithiothreitol (pH 7.5). The muscle homogenate was centrifuged twice at 8000 $\times g$ for 20 min to remove the myofibrils and mitochondria, and the supernatant was then centrifuged at 80 000 $\times g$ for 20 min at 2°C. The 80 000 $\times g$ precipitate was resuspended in 600 mM KCl/10 mM imidazole chloride/1 mM dithiothreitol (pH 7.3), and centrifuged at 10 000 $\times g$ for 15 min. The 10 000 $\times g$ supernatant was recentrifuged at 80 000 $\times g$ for 20 min, and the resulting precipitate resuspended in the same medium was finally sedimented at 80 000 $\times g$ for 20 min. The sarcoplasmic reticulum pellet was suspended in 100 mM KCl/10 mM imidazole chloride/1 mM dithiothreitol (pH 7.3) to give a protein concentration of 20–25 mg/ml. 10% (w/v) muscle homogenate sarcoplasmic reticulum was prepared according to the method of Newman [25] in 300 mM sucrose and 10 mM Tris-HCl (pH 7.4).

Calcium uptake and efflux were measured in a temperature-controlled vessel using a Radiometer calcium selective electrode (F2112 Ca Selectrode) connected to a Radiometer PHM 64 research pH meter and a recorder. (Ca^{2+} + Mg^{2+})-ATPase activity was estimated at 35°C by determining the amount of liberated phosphate [26]. Protein was determined according to the method of Lowry et al. [27] using bovine serum albumin as standard.

Fatty acids were added as freshly prepared solutions. They were kept at 0°C in dark containers. Arachidonic acid was dissolved in distilled water, and oleic and linoleic acids in absolute alcohol.

Results

Effect of arachidonic acid on calcium uptake and release

Isolated sarcoplasmic reticulum vesicles can accumulate and retain calcium from a medium containing both calcium and ATP, to a concentration as high as 150 μmol Ca^{2+} per g protein in the absence of a precipitating anion [28]. When arachidonic acid is added to sarcoplasmic reticulum vesicles which have been allowed to accumulate calcium, an immediate loss of some or all the accumulated calcium occurs (Fig. 1). An initial loss of calcium is followed by a progressively slower loss until all the accumulated calcium is released (Fig. 1A). The amount of calcium released in the rapid initial phase depends upon the concentration of arachidonic acid used, with a complete release of all accumulated calcium observed at 0.53 μmol arachidonic acid per mg sarcoplasmic reticulum protein (Fig. 1A). At 0.08 μmol arachidonic acid per mg protein only a slow loss of calcium

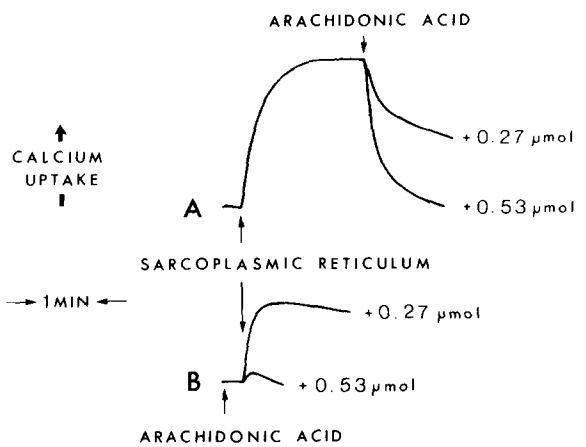

Fig. 1. Typical calcium electrode tracings showing that arachidonic acid causes the release (A) and inhibits accumulation (B) of calcium by sarcoplasmic reticulum from pig LD muscle at 35°C. The reaction medium (pH 6.8) contained 40 mM imidazole chloride, 100 mM KCl, 4 mM $MgSO_4$, 1.15 mM ATP and 53 μM $CaCl_2$. Volume = 5.0 ml. Total protein = 1.56 mg. Concentration of arachidonic acid shown adjacent to each trace is expressed in μmol/mg protein. LD muscle, longissimus dorsi muscle.

is observed, and some calcium is still retained by the sarcoplasmic reticulum after 15 min at 35°C. Arachidonic acid also inhibits calcium accumulation when added prior to the sarcoplasmic reticulum (Fig. 1B). Total inhibition occurs at 0.53 μmol arachidonic acid per mg protein. When lower concentrations of arachidonic acid were used, accumulation of some calcium by the sarcoplasmic reticulum is observed, but the sarcoplasmic reticulum is unable to retain the calcium under these conditions. This is in contrast to the situation in the absence of arachidonic acid, when all the accumulated calcium is retained by the sarcoplasmic reticulum.

It is well established that bovine serum albumin binds fatty acids, 6–13 molecules fatty acid being bound per molecule of bovine serum albumin [29]. In order to demonstrate that bovine serum albumin would bind the exogenous fatty acid and thus remove the inhibitory effect on the sarcoplasmic reticulum, the experiments shown in Fig. 1 were repeated using medium containing bovine serum albumin. 0.08 μmol bovine serum albumin per mg protein was added to the reaction medium, and the sarcoplasmic reticulum was then allowed to accumulate exogenous calcium. An addition of 0.53 μmol arachidonic acid per mg sarcoplasmic reticulum induces the release of only 7% of the accumulated calcium, but a second addition of a similar amount of arachidonic acid resulted in the release of all the accumulated calcium. Bovine serum albumin added to the medium also counteracted the inhibition of calcium accumulation of sarcoplasmic reticulum by arachidonic acid.

Comparative studies of the effect of long chain unsaturated fatty acids on calcium uptake and release

The effects of arachidonic acid, oleic acid and linoleic acid on the calcium release and accumulation by pig skeletal muscle sarcoplasmic reticulum were compared in Figs. 2 and 3. All three unsaturated fatty acids cause the release of some of the calcium accumulated by the sarcoplasmic reticulum (Fig. 2) and also inhibit accumulation when added before calcium (Fig. 3). The effectiveness of the three fatty acids in inhibiting calcium uptake is illustrated in Fig. 4. The

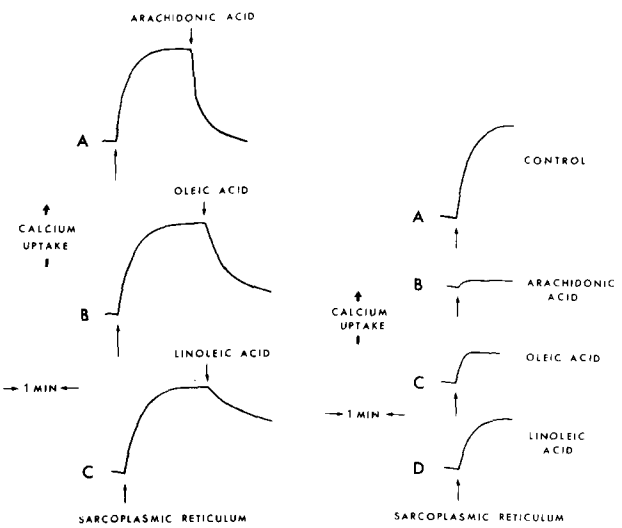

Fig. 2. (left) Release of calcium from sarcoplasmic reticulum of pig LD muscle, caused by three different unsaturated fatty acids. Experimental conditions are given in the legend to Fig. 1. Fatty acids were added as indicated in the appropriate tracings. Final concentration of fatty acids (μmol/mg protein): A, arachidonic acid 0.53; B, oleic acid 0.56; C, linoleic acid 0.48.

Fig. 3. Effect of the three unsaturated fatty acids, arachidonic acid (B), oleic acid (C) and linoleic acid (D) on calcium uptake (A) of sarcoplasmic reticulum from pig LD muscle. Experimental conditions are as described in the legend to Fig. 2.

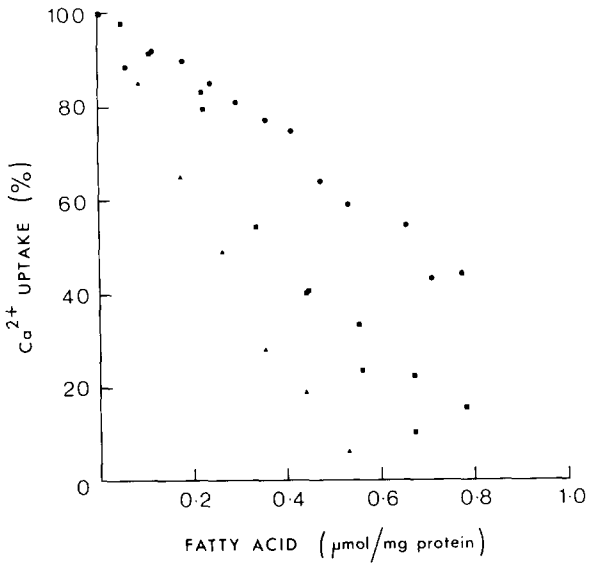

Fig. 4. Inhibition of calcium uptake of sarcoplasmic reticulum from pig LD muscle by unsaturated fatty acids. Experimental conditions as described in Fig. 1 except that various concentrations of three unsaturated fatty acids were used. ▲, arachidonic acid; ■, oleic acid; ●, linoleic acid.

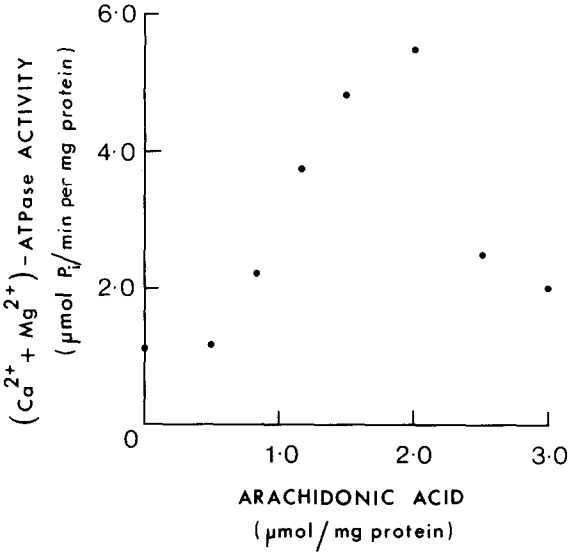

Fig. 5. Effect of arachidonic acid on the $(Ca^{2+} + Mg^{2+})$-ATPase activity of pig LD muscle sarcoplasmic reticulum at 35°C. The reaction medium (pH 7.2) contained 40 mM imidazole chloride, 100 mM KCl, 4 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 0.8 mg sarcoplasmic reticulum protein, and appropriate concentration of arachidonic acid in a total volume of 2 ml. The reaction was started by addition of 4 mM ATP, and stopped after 1 min by addition of 2 ml perchloric acid (4%).

order of effectiveness of the three fatty acids in inhibiting calcium accumulation and stimulating its release is arachidonic acid > oleic acid > linoleic acid. Thus, at a fatty acid concentration of 0.5 μmol per mg protein, arachidonic acid inhibits calcium accumulation by 88% and induces the release of 70% of calcium previously accumulated by the sarcoplasmic reticulum. Oleic acid inhibits calcium accumulation by 66% and induces the release of 57% of accumulated calcium, while linoleic acid inhibits calcium accumulation by 39% and also induces the release of 39% of accumulated calcium. Complete inhibition of calcium accumulation occurs with 0.53 μmol arachidonic acid per mg protein, but the same concentration of oleic acid and linoleic acid inhibits calcium accumulation by only 72% and 42%, respectively.

Effect of arachidonic and oleic acids on $(Ca^{2+} + Mg^{2+})$-ATPase activity

At low concentrations both arachidonic acid and oleic acid stimulate the $(Ca^{2+} + Mg^{2+})$-ATPase activity of the sarcoplasmic reticulum, but inhibit the enzyme activity when used in high concentrations. The effect of arachidonic acid on the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase activity is illustrated in Fig. 5. 2.0 μmol arachidonic acid per mg protein increase the $(Ca^{2+} + Mg^{2+})$-ATPase activity by 4-times that observed in the absence of the fatty acid. Oleic acid also stimulates the $(Ca^{2+} + Mg^{2+})$-ATPase activity, but with this fatty acid the maximum activity is only double that observed in the absence of oleic acid, and maximum stimulation of the enzyme activity occurs with 0.53 μmol oleic acid per mg sarcoplasmic reticulum. With both fatty acids, further increase in concentration leads to inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity.

Effect of fatty acids on calcium uptake and release in muscle homogenate sarcoplasmic reticulum preparations

The effect of fatty acid on sarcoplasmic reticulum was repeated using muscle homogenate preparations to determine whether the same amount of fatty acid would produce results similar to those obtained with isolated sarcoplasmic reticulum. In whole muscle homogenate, the effect of fatty acid would create a

more realistic situation in assessing their effects in vivo. Arachidonic acid and oleic acid were selected for comparative studies between isolated sarcoplasmic reticulum and whole muscle homogenate preparations, since there are the most potent of the unsaturated fatty acids employed in the present studies.

Fig. 6 illustrates the typical effect of arachidonic acid on calcium release (A) and uptake (B) in whole muscle homogenate preparations of sarcoplasmic reticulum. When arachidonic acid is added to muscle homogenate sarcoplasmic reticulum preparations which have been allowed to accumulate calcium in the presence of 10 mM oxalate, release of calcium occurs. The amount of calcium released depends on the concentration of arachidonic acid used. Arachidonic acid also inhibits calcium accumulation when added prior to the muscle homogenate sarcoplasmic reticulum (B). 50% inhibition of calcium uptake by muscle homogenate sarcoplasmic reticulum was observed with 2.5 nmol arachidonic acid per mg muscle (Fig. 7), and for the same extent of inhibition of calcium uptake in isolated sarcoplasmic reticulum 0.23 μmol arachidonic acid per mg protein (Fig. 4) was required. Similarly, 4.5 nmol oleic acid per mg muscle homogenate and 0.39 μmol oleic acid per mg protein isolated sarcoplasmic reticulum were needed to give 50% inhibition of calcium uptake by muscle homogenate and isolated sarcoplasmic reticulum preparations. Assuming a sarcoplasmic reticulum protein content of 10 mg per gram muscle [28], the effect produced by both arachidonic and oleic acid in whole muscle homogenate closely resembled those obtained with isolated sarcoplasmic reticulum (Fig. 1).

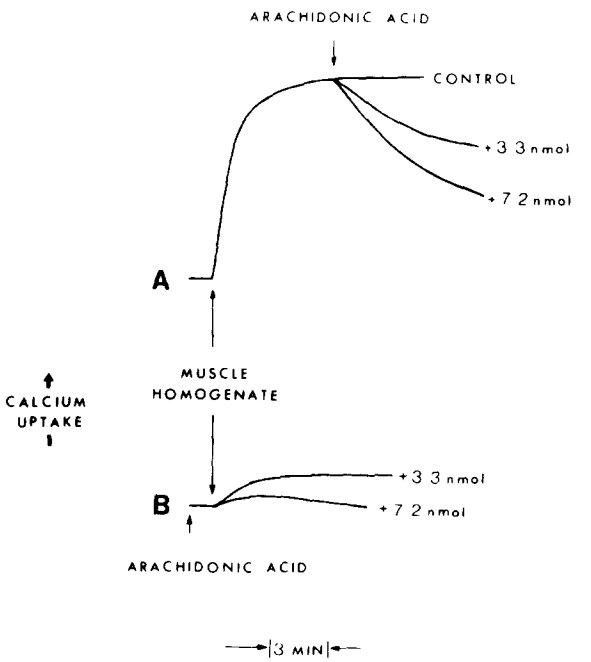

Fig. 6. Effect of arachidonic acid on calcium release (A) and uptake (B) in longissimus dorsi muscle homogenate sarcoplasmic reticulum. The reaction medium (pH 7.4) contained 20 mM imidazole chloride, 100 mM KCl, 5 mM $MgCl_2$, 10 mM sodium oxalate, 5 mM ATP and 5 mM sodium azide. The reaction was started by addition of 73 μM $CaCl_2$, 1 μg antimycin A, 2 μg oligomycin and finally 0.25 ml muscle homogenate sarcoplasmic reticulum (25 mg protein). Calcium uptake by mitochondria was eliminated by the combined additions of oligomycin, antimycin A and sodium azide. Concentrations of arachidonic acid shown adjacent to each trace are expressed in nmol/mg wet weight muscle. Trace A shows the typical effect of arachidonic acid inducing calcium release when added after calcium uptake was complete. Trace B shows the inhibition of calcium uptake by arachidonic acid. Total volume, 5.0 ml; temperature, 35°C.

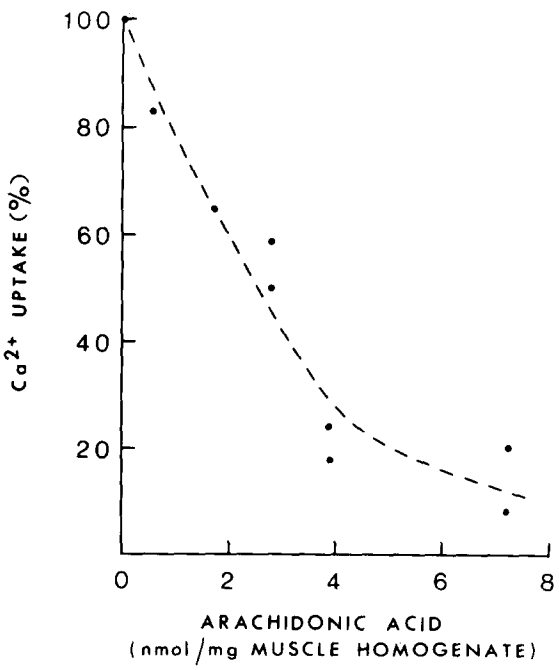

Fig. 7. Inhibition of calcium uptake of muscle homogenate sarcoplasmic reticulum by arachidonic acid. Experimental conditions are as described in Fig. 6. Data obtained from two pigs.

TABLE I

INTERACTION BETWEEN MITOCHONDRIA AND SARCOPLASMIC RETICULUM

Both mitochondria and sarcoplasmic reticulum were isolated from longissimus dorsi muscle of malignant hyperthermia-prone and normal pigs. The experiments were conducted with a calcium electrode at 35°C in a total volume of 5.0 ml. The reaction medium (pH 6.8) contained 40 mM imidazole hydrochloride, 100 mM KCl, 4 mM $MgSO_4$, 1.15 mM ATP, 1.0 mM azide, 2 μg antimycin A and 2 μg oligomycin. The reaction was started by additions, in sequence, of 53 μM Ca^{2+}, 0.5 mg mitochondrial protein and 1.0 mg sarcoplasmic reticulum. *n* refers to the number of pigs used.

Mitochondria	Sarcoplasmic reticulum	Inhibition (%) of Ca^{2+} uptake by sarcoplasmic reticulum
Malignant hyperthermia	Malignant hyperthermia	14 (n = 3)
Malignant hyperthermia	Normal	16 (n = 3)
Normal	Normal	0 (n = 3)
Normal	Malignant hyperthermia	0 (n = 2)

Influence of mitochondria on Ca^{2+} uptake by sarcoplasmic reticulum

Table I summarizes the influence of mitochondria on the Ca^{2+} uptake of sarcoplasmic reticulum. Only mitochondria isolated from longissimus dorsi muscle of malignant hyperthermia-prone pigs can inhibit the Ca^{2+} uptake of isolated sarcoplasmic reticulum from longissimus dorsi muscle of both malignant hyperthermia-prone and normal pigs. Mitochondria from normal pigs, however, have no influence on the sarcoplasmic reticulum from either type of pigs.

Discussion

The results show that the calcium transport system of sarcoplasmic reticulum of longissimus dorsi muscle is inhibited by arachidonic, oleic and linoleic acids. The (Ca^{2+} + Mg^{2+})-ATPase activity of isolated sarcoplasmic reticulum is stimulated by low concentrations of arachidonic acid and oleic acid, but the enzyme is inhibited with high concentrations of both fatty acids. The concentration of arachidonic acid giving maximum stimulation of the (Ca^{2+} + Mg^{2+})-ATPase is 3.6-times higher than that at which the calcium transport is completely inhibited. Calcium transport is completely inhibited by a concentration of arachidonic acid which has almost no effect on the (Ca^{2+} + Mg^{2+})-ATPase activity. Maximum stimulation of the (Ca^{2+} + Mg^{2+})-ATPase by oleic acid occurs at a concentration which almost completely inhibits calcium transport by the sarcoplasmic reticulum. Inhibition of calcium transport by these fatty acids cannot therefore be attributed to inhibition of the (Ca^{2+} + Mg^{2+})-ATPase, and is probably due to dissociation of calcium accumulation from (Ca^{2+} + Mg^{2+})-ATPase activity and increased calcium permeability of the sarcoplasmic reticulum [16].

Waite and Sisson [24] showed that oleic, linoleic and arachidonic acids are released from liver mitochondrial membranes by the action of phospholipase A_2; 54% of the fatty acid released was linoleic acid, 26% was oleic acid and 15% was arachidonic acid. Arachidonic acid is also released from mitochondrial membranes during endotoxic shock [30]. In a recent paper [11] we showed that mitochondria isolated from longissimus dorsi muscle of malignant hyperthermia-prone pigs exhibit a calcium-stimulated phospholipase A_2 activity which liberates fatty acids from the phospholipids of mitochondrial membranes. The present paper clearly demonstrates that in the presence of such unsaturated fatty acids the calcium-accumulating capacity of the sarcoplasmic reticulum is decreased and the ability to retain calcium is abolished. This would contribute in vivo to the enhanced level of sarcoplasmic calcium observed in porcine malignant hyperthermia. Furthermore, only mitochondria isolated from longissimus dorsi muscle of malignant hyperthermia-prone pigs can inhibit calcium accumulation by sarcoplasmic reticulum isolated from both malignant hyperthermia-prone and normal pigs, while mitochondria from normal pigs have no effect on the sarcoplasmic reticulum. The inhibitor responsible could be unsaturated fatty acids released from mitochondria of malignant hyperthermia-prone pigs through the action of the calcium-stimulated phospholipase A_2 [11]. The present studies with fatty acids on isolated sarcoplasmic reticulum and muscle homogenate sarcoplasmic reticulum preparations thus substantiate our hypothesis [11] that mitochondria of pigs with malignant hyperthermia can influence the sarcoplasmic reticulum to liberate its calcium.

Acknowledgments

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