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Effect of Post-mortem Electrical Stimulation on Ovine Sarcoplasmic Reticulum Vesicles

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Abstract

Sarcoplasmic reticulum (SR) vesicles from ovine skeletal muscle were iodinated with the use of immobilized lactoperoxidase to determine the location of proteins in the membrane and to observe any changes resulting from post-mortem electrical stimulation. The labelling pattern of the non-stimulated SR preparations was essentially the same as that observed previously for white muscle SR of rabbit. Most of the membrane proteins were labelled, except for the high-affinity calcium-binding protein. Electrical stimulation, however, resulted in an increased labelling of calsequestrin suggesting that this protein is more exposed as a result of such treatment. Certain activities of the adenosinetriphosphatase were affected by electrical stimulation. Both the steady-state concentration of phosphoenzyme and the ATP$\leftrightharpoons$$P_i$ exchange reaction were significantly reduced by electrical stimulation. It is not known if this alteration in the membrane is responsible for the reduced activity of the SR and thus the greater rate of post-mortem pH fall in electrically stimulated muscle.
[Other keywords: Membrane phosphorylation, muscle pH, cold-shortening.]

Introduction

Electrical stimulation of a carcass immediately after slaughter accelerates the onset of rigor mortis (de Fremery and Pool 1960; Carse 1973). The electrical stimulation increases glycolytic activity and high-energy phosphate turnover in the muscle, resulting in an increased rate of pH fall (Bendall 1976). Such electrical stimulation may allow the carcass to be cooled soon after slaughter without the likelihood of the muscles cold-shortening. In addition, meat from electrically stimulated carcasses is more tender than that from controls suggesting that electrical stimulation has an effect in its own right. Savell *et al.* (1977) suggested that electrical stimulation causes the muscle to be at a low pH while the temperature is still high, thus disrupting lysosomal membranes and releasing lysosomal enzymes which break down muscle components.

Because an increase in the concentration of Ca^{2+} ions in the sarcoplasm is required to initiate glycolysis and to activate certain adenosinetriphosphatases (ATPases), Bendall (1976) has suggested that electrical stimulation might affect the systems that control the Ca^{2+} ion concentration. It has previously been shown (Tume 1979*a*) that the enzyme of the sarcoplasmic reticulum (SR) responsible for transporting Ca^{2+} ions (the Ca^{2+}-dependent ATPase) is affected by electrical stimulation although, under the conditions used, no alteration in Ca^{2+} transport was observed. Purified membrane vesicles of the Ca^{2+}-dependent ATPase from electrically stimulated muscle were

shown to have a reduced activity as measured by ATP-splitting, phosphoenzyme formation from either ATP or inorganic orthophosphate (P_i), or by an ATP$\leftrightharpoons$$P_i$ exchange reaction. It was also suggested that the conformation of the ATPase or its association with the membrane lipids was altered by electrical stimulation because the ATPase was more susceptible to proteolytic digestion than was the control.

This paper describes the structural organization of the proteins in, and the effect of electrical stimulation on, the membranes of intact SR vesicles from sheep muscle.

Materials and Methods

Electrical Stimulation

Sheep (adult Merino wethers) were stunned and bled and the dressed carcasses were split into two sides. The side with the tail remnant attached was hung from an insulated hook by the Achilles tendon. Electrodes were attached to the tail remnant and shoulder muscles and a voltage was applied within 12 min of stunning. The power supply provided 2-ms pulses at a rate of 40 Hz. The following voltage–time sequence was found to accelerate the onset of rigor mortis. The voltage was increased stepwise over a 4-min period (0–10 s, 10V; 10–60 s, 50 V; 60–120 s, 75 V and 120–240 s, 110 V). At completion of electrical stimulation, the longissimus dorsi muscle was removed from each side (control and stimulated) and taken to the laboratory.

Preparation of SR Vesicles

Preparation of SR vesicles from longissimus dorsi muscles of control and stimulated sides was performed according to the method of Martonosi *et al.* (1968) and commenced within 20 min of stunning. The resultant pellets were suspended in 0·25 M sucrose and 10 mM Tris–HCl, pH 7·5, at a protein concentration of about 20 mg/ml. For convenience, preparations from control sides are referred to as 'control SR' preparations and those from stimulated sides as 'stimulated SR' preparations.

Isolation of SR Fraction F3

20–30 mg of SR protein was applied to the top of a linear sucrose density gradient, ranging in concentration from 1·4 to 0·74 M sucrose. The gradient was centrifuged in a Beckman SW27 rotor at 22 000 rev/min for 16 h, resulting in the appearance of four distinct bands of vesicles. The third fraction from the bottom (F3) (density equivalent to about 1·0 M sucrose) was collected, diluted with cold (4°C) water, centrifuged at 135 000 *g* for 1 h and the pellet dispersed in 0·25 M sucrose and 10 mM Tris–HCl, pH 7·5. This fraction has been shown to be the most active of all the fractions in accumulating Ca^{2+} ions (R. P. Newbold and R. K. Tume, unpublished data).

Muscle pH

Longissimus dorsi muscles (30–40 g) from control and stimulated sides were kept in individual polyethylene bags at room temperature (20–23°C) and, at intervals, pieces of approximately 1 g were removed from each muscle and homogenized in 10 ml of 5 mM iodoacetate, pH 7·0. The pH of the homogenate was measured with the use of a glass electrode.

Phosphorylation of SR Vesicles by $^{32}P_i$

The steady-state concentration of phosphorylated enzyme (E$\sim$P) was measured by the procedure of Masuda and de Meis (1973) at room temperature (20–23°C). SR vesicles (0·5 mg protein) were added to a solution (0·5 ml) which contained 10 mM Tris–maleate, pH 6·0, 1 mM ethylene glycol bis(aminoethyl ether) tetraacetate, 10 mM $MgCl_2$ and 4 mM $^{32}P_i$ (approx. 10^7 cpm/μmol P_i). The reaction was terminated at 10 s by the addition of 0·5 ml of an ice-cold solution of 1 M perchloric acid which contained 2 mM P_i, and the E$\sim$P was determined as described previously (Tume 1979*a*).

ATP⇋P_i Exchange Reaction

The formation of [γ-^{32}P] ATP from $^{32}P_i$ was measured by the procedure described by de Meis and Carvalho (1974). The reaction mixture (0·5 ml) contained 20 mM Tris–maleate buffer, pH 7·0, 4 mM ATP, 15 mM $MgCl_2$, 0·1 mM $CaCl_2$, 6 mM $^{32}P_i$ (1–4 × 10^6 cpm/μmol P_i) and 0·25 mg SR protein. The reaction (at 37°C) was started by the addition of the SR to the reaction mixture and terminated by the addition of 0·5 ml of an ice-cold solution of 1 M perchloric acid which contained 2 mM P_i. The excess $^{32}P_i$ was extracted according to the method of Avron (1960) as modified by de Meis and Carvalho (1974). An aliquot of the aqueous solution was taken for liquid scintillation counting.

Iodination Procedure

Aliquots of the SR preparations (1 mg) were suspended in 25 mM Tris–maleate, pH 7·0, containing approximately 100 μC_i of carrier-free $Na^{125}I$, and 0·05 ml of Sepharose 4B-bound lactoperoxidase, in a total volume of 1 ml. Iodination was initiated at room temperature by the addition of 0·01 ml of freshly prepared 1·56 mM H_2O_2. Two further additions of H_2O_2 were made at 5-min intervals. At 15 min, the reaction was stopped by the addition of 0·3 ml of 50 mM sodium azide and 1 ml of 0·01 M sodium iodide then the mixture was diluted to about 5 ml with 0·1 M phosphate buffer, pH 7·0. The Sepharose 4B-bound lactoperoxidase was removed from the SR preparations by centrifugation at about 500 *g* for 5 min. The supernatant containing the SR membranes was removed and diluted to 10 ml with 0·1 M phosphate, pH 7·0, and then centrifuged at 135 000 *g* for 15 min. The pellet obtained was washed again in 10 ml of 0·1 M phosphate, pH 7·0, and the final pellet was resuspended in 1 ml of 1% (w/v) sodium dodecylsulfate (SDS), 0·01 M phosphate, pH 7·0, 1% (v/v) β-mercaptoethanol and boiled for 5 min. The samples were dialysed overnight against three changes of 1 litre of 0·1% (w/v) SDS, 0·01 M phosphate, pH 7·0, and 0·1% (v/v) β-mercaptoethanol. β-mercaptoethanol was omitted from the final change of dialysis buffer to prevent interference with the subsequent protein assay.

SDS–Polyacrylamide Gel Electrophoresis

Electrophoresis in SDS–polyacrylamide gels [5% (w/v) acrylamide, 0·135% (w/v) bis-acrylamide] was performed as described by Weber and Osborn (1969). Proteins were stained with Napthalene Black 10B (G. T. Gurr Ltd, London), the gels destained in 7·5% (v/v) acetic acid and then scanned at 500–550 nm in a Kipp and Zonen recording densitometer, model DD2.

The gels were cut into slices approximately 1·5 mm thick and the ^{125}I content of each slice was determined in a Packard gamma counter.

Sepharose 4B–Lactoperoxidase

Lactoperoxidase (Calbiochem, San Diego, California) was coupled to Sepharose 4B (Pharmacia, Uppsula, Sweden) as described by Cuatrecasas (1970). Lactoperoxidase (10 mg lyophilized powder) was reacted with 5 ml packed volume of activated Sepharose 4B. The coupled enzyme was kept at 1°C in an equal volume of 0·01 M phosphate buffer, pH 7·0, which contained a small crystal of thymol.

Protein Determination

Protein concentrations were determined using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Radiochemicals

Carrier-free $Na^{125}I$ was purchased from New England Nuclear, Boston, Mass. Prior to use, the iodide was diluted with an equal volume of 10 μM sodium sulfite solution (Morrison 1974).

$^{32}P_i$ was purchased from the Radiochemical Centre, Amersham, England.

Results and Discussion

Iodination Studies

Iodination of membrane components with the lactoperoxidase system provides a useful means of labelling those proteins which are exposed to some extent on the outer surface of the membrane (Phillips and Morrison 1971). Lactoperoxidase, being too large to penetrate the membrane, inserts radioactive iodine into the tyrosine and histidine residues of proteins exposed at the outer surface (Morrison 1974). Such a

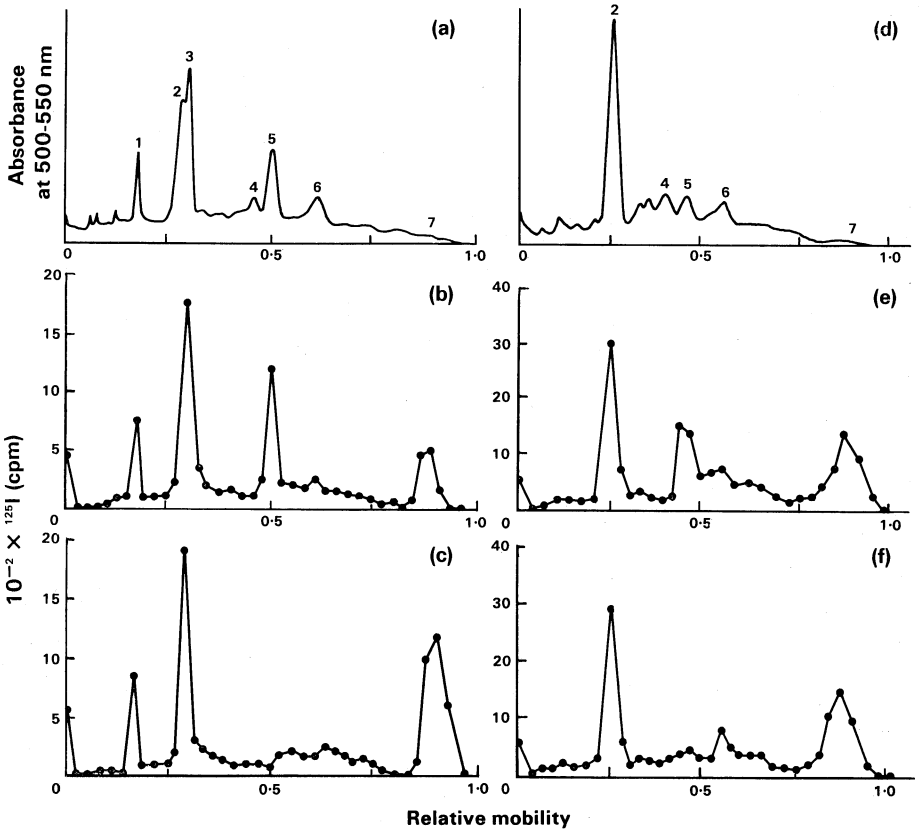

Fig. 1. Iodination of proteins of SR vesicles. Intact SR vesicles (*a–c*) and fraction F3 (*d–f*) were labelled with ^{125}I as described under Materials and Methods, and approximately 50-μg samples were subjected to electrophoresis on SDS–polyacrylamide gels. (*a*) and (*d*), Densitometric tracings of gels stained with Naphthalene Black 10B; (*b*) and (*e*), distributions of ^{125}I where reactions were performed without any further additions; (*c*) and (*f*), distributions of ^{125}I where 5 mM ATP, 5 mM $MgCl_2$ and 0·05 mM $CaCl_2$ were included in the reactions. Numbered peaks are identified as follows: *1*, unknown; *2*, Ca^{2+}-dependent ATPase (mol. wt 105 000); *3*, phosphorylase subunit (94 000); *4*, high-affinity calcium-binding protein (55 000); *5*, calsequestrin (47 000); *6*, acidic protein (30 000); *7*, phospholipid–proteolipid.

technique provides information on the location of proteins in a membrane, thus allowing certain predictions to be made regarding their function, and, in the case of SR, their role in the overall Ca^{2+} transporting process. Intact SR membranes were labelled with ^{125}I with the use of Sepharose 4B–lactoperoxidase and the components were dissociated and separated by SDS–polyacrylamide gel electrophoresis (Fig. 1).

Staining the gels with Naphthalene Black 10B showed the presence of Ca^{2+}-dependent ATPase (mol. wt 105 000), contaminating phosphorylase subunit (94 000), high-affinity calcium-binding protein (55 000), calsequestrin (47 000) and an acidic protein (30 000) (Fig. 1*a*). When the iodination reaction was done in the presence of 25 mM Tris–maleate buffer, pH 7·0, alone (Fig. 1*b*), most of the proteins were labelled, the exception being the high-affinity calcium-binding protein. Although there is relatively little of this protein in sheep SR preparations, its failure to become labelled was clearly established. In rabbit preparations where there is considerably more high-affinity calcium-binding protein as judged by SDS–polyacrylamide electrophoresis, no labelling is observed (Tume 1979*b*). This suggests that under the conditions used, the high-affinity calcium-binding protein of sheep SR, as in rabbit SR, is located within the membrane, and that none of the iodine-reactive groups are exposed to the surface. All the other proteins are likely to reside, at least in part, on the outer surface of the membrane.

Recently it has been shown with rabbit white muscle SR that the pattern of labelling can be altered by changing the composition of the reaction medium (Chyn and Martonosi 1977; Tume 1979*b*). Similar findings are reported here for sheep muscle SR. When sheep SR vesicles were pre-incubated with ATP, Ca^{2+} and Mg^{2+} and then subjected to iodination, the pattern of labelling was markedly different (Fig. 1*c*) from that found in the presence of 25 mM Tris–maleate buffer alone (Fig. 1*b*). In the presence of ATP, Ca^{2+} and Mg^{2+} there was virtually no labelling of calsequestrin or the high-affinity calcium-binding protein but the labelling of the ATPase remained about the same. There was a large increase in the labelling of the material running near the front of the gel, and on the basis of chloroform–methanol extraction and phase separation this is likely to represent labelling of the phospholipid–proteolipid fraction.

SR fraction F3 was investigated as it is essentially free of phosphorylase and consists mainly of the Ca^{2+}-dependent ATPase (mol. wt 105 000) with small amounts of the other proteins present in SR vesicles (Fig. 1*d*). Iodination of fraction F3 in the presence of buffer alone resulted in the labelling of the ATPase, calsequestrin, the acidic proteins and the phospholipid–proteolipid fraction (Fig. 1*e*). Again, when the reaction was carried out on vesicles pre-incubated with ATP, Ca^{2+} and Mg^{2+}, the labelling of the calsequestrin was almost completely inhibited (Fig. 1*f*). The high-affinity calcium-binding protein was not labelled under either condition.

These studies suggest that in intact SR vesicles or purified fraction F3, iodinated in the presence of buffer alone, the high-affinity calcium-binding protein is either buried within the membrane or located on the internal surface of the vesicle. All the other major proteins are at least partially exposed on the outer surface of the membrane. Because labelling of calsequestrin was inhibited by the presence of ATP, Ca^{2+} and Mg^{2+}, it appears that under these conditions the surface of the vesicle is changed so that the iodine-reactive groups of calsequestrin have an internal location. Major structural changes are also indicated by the increased ($P < 0{\cdot}01$) labelling of the phospholipid–proteolipid fraction under these conditions (see Table 1).

Effect of Electrical Stimulation on Muscle pH

Electrical stimulation at the relatively low voltages used in this study did not result in any significant temperature change of the longissimus dorsi muscles. When

small pieces were excised and set aside for pH measurement, the temperature of the pieces from electrically stimulated muscles and controls fell to room temperature (20–23°C) in less than 1 h.

The effect of electrical stimulation on the rate of pH fall is given in Fig. 2. Each line is the regression obtained from 12 animals, with four or five measurements of pH for each animal made at times ranging from 0·35 h to 6·0 h post mortem. Statistical analysis indicates that the slopes of the regression lines for the control ($-0·109 \pm 0·006$) and stimulated ($-0·142 \pm 0·007$) carcasses were significantly different ($P < 0·01$). Thus, relatively low voltage electrical stimulation of sheep carcasses resulted in the immediate fall in the pH of the longissimus dorsi muscle of about 0·34 units and in the following 6-h period the rate of pH fall was faster than in the control. The pH values at 24 h were the same for control and stimulated muscles.

Table 1. Effect of electrical stimulation on the percentage distribution of ^{125}I in protein and lipid components of SR

SR component	Preparation incubated in buffer alone		Preparation incubated in ATP, Ca^{2+}, Mg^{2+}[A]	
	Control	Stimulated	Control	Stimulated
	SR preparation[B]			
Unknown	6·9±1·0	6·6±0·4	8·1±0·6	7·2±0·7
ATPase[C]	27·7±1·0	24·3±1·2	25·9±1·2	22·6±0·9
High-affinity calcium-binding protein	1·5±0·2	1·5±0·2	1·5±0·2	1·5±0·1
Calsequestrin	12·1±0·5	17·9±0·7	2·3±0·1	2·7±0·3
Acidic protein	4·7±0·7	4·2±0·3	5·6±0·2	5·0±0·4
Phospholipid–proteolipid	10·6±0·9	11·9±1·6	19·6±2·3	20·8±1·7
	SR–F3 preparation			
Unknown	—	—	—	—
ATPase	18·2	19·7	20·3	23·0
High-affinity calcium-binding protein	1·9	1·7	1·8	1·4
Calsequestrin	10·1	16·0	3·2	3·2
Acidic protein	7·8	7·7	6·4	6·0
Phospholipid–proteolipid	22·1	24·0	24·3	18·6

[A]In addition to the 25 mM Tris–maleate buffer, pH 7·0, the reaction mixture contained 5 mM ATP, 0·05 mM Ca^{2+} and 5 mM Mg^{2+}.

[B]Values are the mean±s.e. for five experiments.

[C]For SR preparations, ATPase values include contribution from phosphorylase. SR–F3 preparations are essentially free of phosphorylase.

Effect of Electrical Stimulation on Certain SR Activities

Previous work has shown that purified Ca^{2+}-dependent ATPase (the enzyme responsible for pumping Ca^{2+} across the membrane) is greatly affected by electrical stimulation, whereas the same enzyme in the intact SR vesicle is somewhat less affected (reduced by 76 and 36% respectively; see Tume 1979*a*). Likewise, in the present study with intact SR vesicles, the enzymic activities investigated below were each reduced by electrical stimulation. The steady-state concentration of E∼P

formed from P_i for each preparation is shown in the following tabulation (values in nmol E~P/mg protein and are means ± s.e. for six preparations):

Control	0·74±0·04
Stimulated	0·63±0·04 ($P<0·1$)

The ATP⇌P_i exchange reaction of SR vesicles was also significantly reduced by electrical stimulation (Fig. 3). With intact SR vesicles, the rate becomes significant only when the low affinity Ca^{2+}-binding site of the ATPase becomes saturated, that is, when the internal Ca^{2+} concentration reaches about 3–5 mM (de Meis and Carvalho 1974). Both types of preparations gave almost straight lines between 1 and 6 min, the mean rate per minute for the control being 0·062 μmol ATP⇌P_i/mg protein compared with 0·045 for stimulated ($P<0·1$).

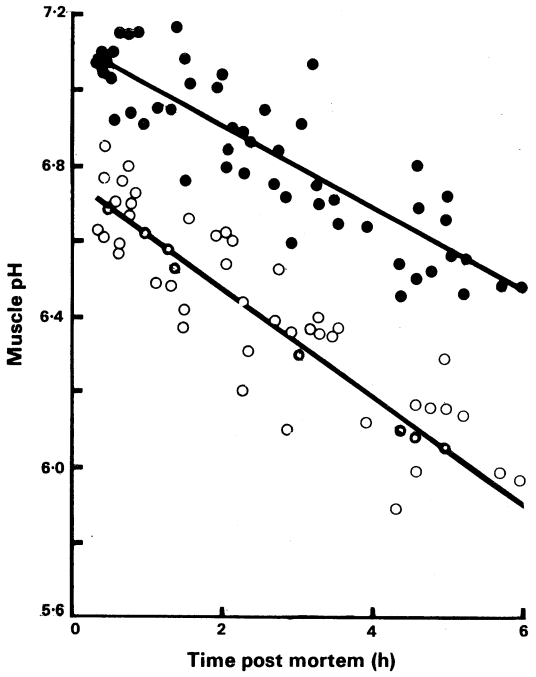

Fig. 2. Effect of electrical stimulation on muscle pH fall. ● Control. ○ Stimulated. Lines are calculated regression equations for each group.

Effect of Electrical Stimulation on Membrane Structure

Previous work has shown that the ATPase of reformed ATPase vesicles from electrically stimulated muscles has a greater susceptibility to proteolytic attack by Pronase, suggesting that the conformation of the ATPase itself, or its association with the membrane phospholipids, had been altered (Tume 1979*a*). In the present study, possible structural alterations to the membrane were investigated with the use of the ^{125}I labelling technique. As only exposed proteins were labelled, any change in the pattern of labelling would indicate that some modification had occurred. The specific activity of iodination was about the same for both control and stimulated preparations ($\simeq 4\times10^5$ cpm/mg protein), both in the presence and absence of ATP, Ca^{2+} and Mg^{2+} (see Table 1). When the reaction was done in the presence of Tris–maleate buffer alone, electrical stimulation produced a significant increase

($P<0{\cdot}001$) in the labelling of calsequestrin, suggesting that the location of this protein became more exposed on the surface of the membrane vesicle. This was also true for the SR fraction F3 from stimulated muscle (Table 1). The relative changes in the other components were not significantly different. In the presence of ATP, Ca^{2+} and Mg^{2+}, the labelling of calsequestrin was very small (Fig. 1*c*) and was not significantly changed by electrical stimulation (Table 1). The significant increase in labelling that occurred in the phospholipid–proteolipid fraction of SR preparations when ATP, Ca^{2+} and Mg^{2+} were present was also found in the electrically stimulated samples. The concentrations of label in only the major peaks is presented in Table 1; the remaining 30–40% was distributed essentially as shown in Fig. 1.

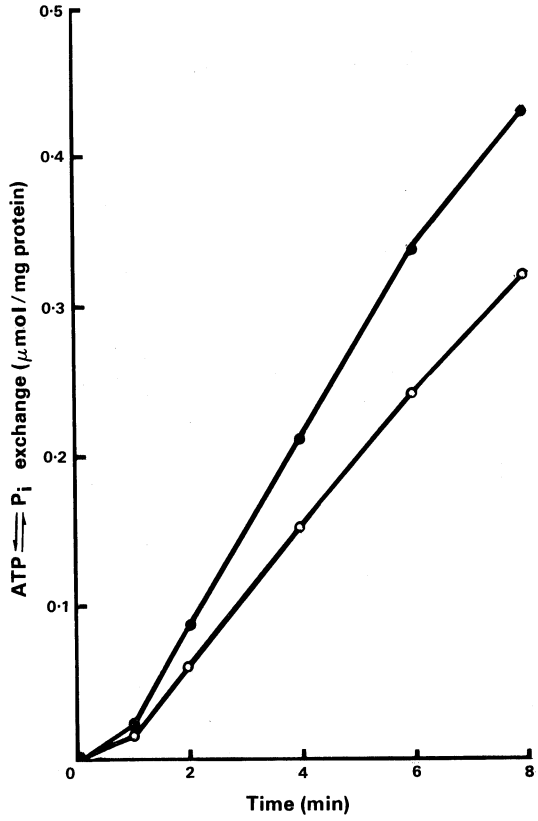

Fig. 3. Effect of electrical stimulation on the ATP⇋P_i exchange reaction of SR vesicles. Experimental conditions were as described under Materials and Methods. ● Control. ○ Stimulated.

It is apparent from previous work (Tume 1979*b*) and also from that described here that the labelling of calsequestrin in the membrane is extremely sensitive to the conditions used for the reaction. These studies have shown that it is possible to reduce the labelling of calsequestrin by addition of ATP, Ca^{2+} and Mg^{2+}. However, in the presence of buffer alone, electrical stimulation increases the label in this protein, suggesting that calsequestrin is more exposed to the environment. It appears that electrical stimulation has no irreversible effect on the position of calsequestrin because when ATP, Ca^{2+} and Mg^{2+} were present calsequestrin was virtually unlabelled, indicating its internal location.

It is not known if this apparent movement of calsequestrin is responsible for the reduced biochemical activities observed in preparations from electrically stimulated muscle. To date, the role of calsequestrin in Ca^{2+} uptake by SR is unknown, although it has been suggested by some workers (MacLennan and Wong 1971; Ikemoto *et al.* 1974) that calsequestrin (located internally) acts as a storage site for accumulated Ca^{2+} ions. Calsequestrin is not required for the actual transport of Ca^{2+} ions as phospholipid vesicles containing purified Ca^{2+}-dependent ATPase are able to pump Ca^{2+} ions against a concentration gradient (Racker 1972).

Thus, electrical stimulation of muscle does have an effect on the structural organization of the isolated SR membrane. This is further supported by earlier work (Tume 1979*a*) which showed that the ATPase of purified ATPase vesicles from SR of electrically stimulated muscle was more susceptible to Pronase digestion.

Acknowledgments

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