Post-mortem Electrical Stimulation of Muscle and its Effects on Sarcoplasmic Reticulum Adenosine Triphosphatase

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Abstract
Sarcoplasmic reticulum (SR) was isolated from control muscles and from muscles which had been subjected to short-term post-mortem electrical stimulation. Both preparations had similar protein compositions but the SR from electrically stimulated muscle had a lower 'extra' ATPase activity. The ability of the SR preparations from electrically stimulated muscles to accumulate Ca\(^{2+}\) was about the same as the controls. There was, therefore, an apparently greater efficiency of Ca\(^{2+}\) transport in the isolated vesicles, the reason for which is not known, but an alteration in the 'leakiness' of the membrane may be involved. Purified ATPase isolated from control and stimulated SR contained, in addition to the ATPase protein, a polypeptide of molecular weight about 30000. The purified ATPase vesicles from electrically stimulated muscle had a reduced activity as measured by ATP splitting activity, phosphoenzyme formation from either inorganic orthophosphate (P\(_i\)) or ATP, or by an ATP \(\rightleftharpoons\) P\(_i\) exchange reaction. These reduced activities probably result from an alteration in the binding affinities of the ATPase for ATP and P\(_i\). The low affinity site for calcium binding was not affected by electrical stimulation. Purified ATPase vesicles from stimulated muscle were more susceptible to proteolytic attack, suggesting that the conformation of the protein or its association with the membrane lipids had been altered.

[Introductory keywords: Ca\(^{2+}\)-dependent ATPase, membrane phosphorylation, Ca\(^{2+}\) uptake, ATPase vesicles, sarcoplasmic reticulum vesicles.]

Introduction
An essential requirement for switching on the contraction of skeletal muscle fibres is an increase in the sarcoplasmic Ca\(^{2+}\) concentration (Ebashi and Endo 1968; Ebashi et al. 1969). Ca\(^{2+}\) is released from the sarcoplasmic reticulum (SR) in response to a stimulus and when the stimulus stops Ca\(^{2+}\) is pumped back into the SR, thus lowering the sarcoplasmic Ca\(^{2+}\) concentration. The exact mechanism by which the release and uptake is initiated is unknown but is thought to result from permeability changes of the membranes in response to a depolarizing stimulus.

Recently, considerable interest has developed in the electrical stimulation of freshly excised muscule or animal carcasses soon after slaughter. Electrical stimulation elicits muscle contraction presumably by direct stimulation as well as by electrical transmission of the impulse through the nerves. Depending on the conditions used for electrical stimulation it is possible to hasten the development of rigor mortis (de Fremery and Pool 1960; Carse 1973) due to an acceleration in the rate of muscle pH fall which results from increased glycolytic activity and high-energy phosphate turnover (Bendall 1976). Because the onset of glycolysis is dependent upon an increased concentration of sarcoplasmic Ca\(^{2+}\) (Ozawa et al. 1967) as are the activities of certain ATPases, it has been suggested that electrical stimulation could perma-
nently alter the controlling mechanisms in the muscle (Bendall 1976), in particular the SR membrane. Should the functioning of the SR be impaired, a higher sarcoplasmic Ca\(^{2+}\) concentration would remain after the stimulus is removed thus increasing the rate of the various post-mortem changes.

It was the purpose of the work reported here to study the enzymic properties of the Ca\(^{2+}\) pump protein of SR vesicles isolated from muscles previously subjected to electrical stimulation.

**Materials and Methods**

**Electrical Stimulation**

Sheep (adult Merino wethers) were stunned and bled. The dressed carcasses were split into two sides and the side with the tail still attached was hung from an insulated hook by the Achilles tendon. Electrodes were attached to the tail and shoulder muscles and a current was applied within 12 min of stunning. The voltage was increased stepwise over a 4-min period (0–10 s, 10 V; 10–60 s, 50 V; 60–120 s, 75 V and 120–240 s, 110 V). The power supply provided a pulsed direct current of 40 Hz with a pulse width of 2 ms. At completion of electrical stimulation the longissimus dorsi muscle was removed from each side (stimulated and control) and taken to the laboratory. The stimulated muscle had a pH of about 0.3 less than the control (pH 6.8 and 7.1 respectively).

**Preparation of SR Vesicles**

Preparation of SR vesicles from longissimus dorsi muscles from control and stimulated sides was commenced within 20 min of stunning according to the method of Martonosi et al. (1968). When preparations of SR vesicles were not to be used for ATPase isolation, the pellets were extracted twice (instead of once) with 0.6 M KCl, 20 mM histidine–HCl, pH 7.3, and 1 mM dithiothreitol to ensure complete removal of the contractile proteins. The final pellets were suspended in 0.25 M sucrose and 10 mM tris–HCl, pH 8.0, and stored at 1°C. These preparations were used within 3 h for the measurement of Ca\(^{2+}\)-accumulating activity or within 20 h for the measurement of ATPase activity. For convenience, preparations from control sides are referred to as ‘control’ preparations and those from stimulated sides as ‘stimulated’ preparations.

**Preparation of Purified ATPase Vesicles**

Purified ATPase vesicles were prepared from SR vesicles which had been kept overnight at 1°C. The method used was essentially that described by MacLennan (1974) for rabbit white muscle. The final ammonium acetate precipitate (containing the ATPase) was dissolved in 0.6 M sucrose, 45 mM tris–HCl, 0.9 mM histidine and 0.87 M ammonium acetate, pH 8.0, and dialysed against this solution for 16 h. The preparation, which was completely free of turbidity, was then passed through a Sephadex G50 (medium) column (2.5 by 45 cm) equilibrated with the above solution. The reformed vesicles emerged from the column in the void volume and were collected, diluted with one-third volume of ice-cold distilled water and centrifuged at 150,000 g for 60 min. The pellet was suspended in 0.6 M sucrose, 5 mM tris–HCl, 0.9 mM histidine and 0.87 M ammonium acetate, pH 8.0, at a protein concentration of 15–20 mg/ml and stored at −15°C until required.

**Ca\(^{2+}\) Accumulation by SR Vesicles**

Both the rate of Ca\(^{2+}\) accumulation by SR vesicles (measured in the presence of oxalate) and the total accumulation (measured in the absence of oxalate) was measured by the Millipore filtration method (Martonosi and Feretos 1964) using 0.22-μm pore filters. Aliquots of the filtrate were counted in 10 ml of scintillator (Gordon and Wolfe 1960) in a Packard liquid scintillation spectrometer, model 3385.

**ATPase Activity of SR Vesicles**

‘Basal’ ATPase activity (absence of Ca\(^{2+}\)) was measured at 37°C in the presence of 30 mM tris–maleate buffer, pH 7.0, 0.1 M KCl, 5 mM ATP, 5 mM MgCl\(_2\), 5 mM oxalate with 2 mM ethylene-glycolbis(aminoethyl ether)tetraacetate (EGTA) and 0.05–0.10 mg SR protein/ml reaction mixture.
For 'total' ATPase activity (presence of Ca\(^{2+}\)) the assay was performed as for 'basal' activity but with 1 mM EGTA and 1·1 mM CaCl\(_2\) in place of 2 mM EGTA. At different times 1·5-ml aliquots were taken and the reaction stopped by adding them to 0·5 ml of 20% (w/v) trichloroacetic acid solution. Inorganic orthophosphate (P\(_i\)) contents of the deproteinized samples were measured by the method of Fiske and Subbarow (1925). 'Extra' ATPase activity was estimated by subtracting the 'basal' from the 'total' ATPase activity.

**ATPase Activity of Purified ATPase Vesicles**

The procedure was the same as that described for intact SR vesicles except that oxalate and EGTA were not added and the Ca\(^{2+}\) concentration was 50 μM. ATPase protein was approximately 0·03 mg/ml reaction mixture.

**Phosphorylation of ATPase Vesicles by \(^{32}\)P\(_1\)**

The steady-state concentration of phosphoenzyme (E~P) formed from P\(_1\) was measured at room temperature (20–23°C) (Masuda and de Meis 1973). The reaction was started by the addition of 0·5-ml ATPase protein to a solution which contained 10 mM tris-maleate buffer, pH 6-0, 1 mM EGTA, 10 mM MgCl\(_2\) and 4 mM \(^{32}\)P\(_1\) (approximately 10\(^7\) cpm/μmol P\(_1\)). The total volume of the reaction mixture was 0·5 ml and the reaction was terminated at 10 s by the addition of 0·5 ml of an ice-cold solution of 1 mM perchloric acid which contained 2 mM P\(_1\). The denatured protein was collected by centrifugation at 5000 g for 10 min at 1–5°C, resuspended in 1 ml of ice-cold 125 mM perchloric acid which contained 2 mM P\(_1\), and was applied to a glass fibre filter (Whatman GF/C, 2·5 or 2·1 cm diameter) for washing. The filter containing the denatured protein was washed with 20 ml of the resuspending solution, air-dried at room temperature and then placed in 10 ml of Instagel (Packard Instrument Co. Inc., Downers Grove, Ill. U.S.A.) in a counting bottle for liquid scintillation counting. Non-specific binding of \(^{32}\)P\(_1\) was taken into account by the use of controls in which perchloric acid was included in the reaction mixture prior to the addition of ATPase vesicles (zero time). Data were corrected for this non-specific binding. In those experiments where a range of specific activities of \(^{32}\)P\(_1\) were used a zero time control was performed for each P\(_1\) concentration.

**Phosphorylation of ATPase Vesicles by [γ-\(^{32}\)P]ATP**

The steady-state level of E~P formed from ATP was measured with the use of a reaction mixture similar to that used for the measurement of the ATPase activity of purified vesicles. The reaction mixture (0·5 ml) consisted of 30 mM tris-maleate buffer, pH 7-0, 0·1 mM KCl, 5 mM [γ-\(^{32}\)P]ATP (approximately 0·5 × 10\(^7\) cpm/μmol ATP), 5 mM MgCl\(_2\) and 0·5 mg ATPase protein. The reaction was started by the addition of ATPase and the mixture was incubated for 10 s at room temperature (20–23°C). An equal volume of an ice-cold solution of 1 mM perchloric acid which contained 2 mM P\(_1\) and 1 mM ATP was used to terminate the reaction. The denatured protein was treated as described above for phosphorylation by P\(_1\), except that the resuspending solution also contained 1 mM ATP.

**ATP ↔ P\(_1\), Exchange Reaction**

The formation of [γ-\(^{32}\)P]ATP from \(^{32}\)P\(_1\) was measured with the use of 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\(_2\), 4 mM CaCl\(_2\), 6 mM \(^{32}\)P\(_1\) (1 × 10\(^7\) cpm/μmol P\(_1\)) and 0·25 mg ATPase protein. The reaction was started by the addition of the ATPase to the reaction mixture and terminated 5 min later by the addition of 0·5 ml of an ice-cold solution of 1 mM perchloric acid which contained 2 mM P\(_1\). The reaction was performed at 37°C and was linear for at least 5 min. The excess \(^{32}\)P\(_1\) was extracted according to the method of Avron (1960) as modified by de Meis and Carvalho (1974). An aliquot of the final aqueous solution was taken for liquid scintillation counting.

**Pronase Treatment of ATPase Vesicles**

ATPase vesicles (3·0 mg protein) were incubated at 37°C in 0·4 ml of 25 mM tris-maleate buffer, pH 7-0. Prior to the addition of Pronase, a 0·05-ml aliquot was removed and added to a solution which contained 1% (w/v) sodium dodecyl sulfate, 10 mM phosphate buffer, pH 7-0, and 1% (v/v) β-mercaptoethanol and heated at 100°C for 5 min. At zero time, 0·04 ml of a freshly prepared Pronase solution [1 mg Pronase-CB (Calbiochem, San Diego, California) in 10 ml H\(_2\)O] was added to the reaction mixture, and 0·05-ml aliquots were taken at various time intervals and treated as described above. The solubilized proteins were subjected to gel electrophoresis as described below.
Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Electrophoresis in sodium dodecyl sulfate–polyacrylamide gels (5% acrylamide, 0.135% bis-acrylamide) was performed as described by Weber and Osborn (1969). Proteins were stained with Coomassie Brilliant Blue R, the gels were destained and then scanned at 500–550 nm in a Kipp and Zonen recording densitometer, model DD2.

Radiochemicals

$^{32}$P, was purchased from the Australian Atomic Energy Commission (Lucas Heights, New South Wales) and was further purified by the method described by Kanazawa and Boyer (1973).

$[^32P]ATP$ was prepared as described by de Meis (1972). $^{45}$CaCl$_2$ was purchased from the Radiochemical Centre, Amersham, England.

Results

Crude SR Vesicle Preparations

Preparations of SR vesicles from control and electrically stimulated muscles were essentially identical in terms of yield (approximately 1 mg protein/g muscle) and protein composition (Fig. 1a, stimulated not shown). The appearance of a polypeptide of molecular weight 95 000 (Fig. 1a, peak 2) suggested the presence of phosphorylase and this was confirmed by measuring phosphorylase activity (L. M. Small, unpublished data).

The effects of electrical stimulation on the ATPase activities of SR vesicles are shown in the following tabulation (values are means ± s.e. for six preparations):

<table>
<thead>
<tr>
<th>ATPase activity (μmol P$_1$ per mg protein per min)</th>
<th>Basal</th>
<th>Total</th>
<th>Extra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.32±0.06</td>
<td>1.00±0.11</td>
<td>0.69±0.06</td>
</tr>
<tr>
<td>Stimulated</td>
<td>0.42±0.13</td>
<td>0.86±0.15</td>
<td>0.44±0.03</td>
</tr>
</tbody>
</table>

While the ‘total’ ATPase activity appeared to be higher in the controls and the ‘basal’ activity higher in the stimulated preparations, these differences were not significant.
However, the ‘extra’ ATPase was significantly greater in the controls than in the stimulated preparations.

$\text{Ca}^{2+}$ accumulation by SR vesicles was investigated under conditions that allowed either the rate or total amount of accumulation to be determined. Furthermore, each

**Table 1. Effect of electrical stimulation on the rate of $\text{Ca}^{2+}$ accumulation by SR vesicles**

SR vesicles (0.02–0.05 mg protein/ml reaction mixture) were added to a medium composed of 0.1 M KCl, 20 mM histidine–HCl buffer, pH 6.4, 5 mM ATP, 5 mM MgCl$_2$, 2.5 mM phosphoenolpyruvate, 8 units of pyruvate kinase (EC 2.7.1.40) per ml, 5 mM azide, 5 mM oxalate, and $\text{Ca}^{2+}$ and EGTA concentrations as indicated. A tracer amount of $^{45}\text{Ca}^{2+}$ was added to each incubation. The mixture was incubated at 22°C.

<table>
<thead>
<tr>
<th>Total $\text{Ca}^{2+}$ concn (μM)</th>
<th>EGTA concn (mM)</th>
<th>Free $\text{Ca}^{2+}$ concn (μM)</th>
<th>Rate of $\text{Ca}^{2+}$ accumulation (nmol per mg protein per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>c. 100$^A$</td>
<td>300</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>c. 20$^A$</td>
<td>187$^B$</td>
</tr>
<tr>
<td>185</td>
<td>1.0</td>
<td>c. 0.1–1.0</td>
<td>106$^C$</td>
</tr>
</tbody>
</table>

$^A$ No account taken of $\text{Ca}^{2+}$ binding to other ions.

$^B$ Mean of two preparations.

$^C$ Mean of three preparations.

was performed using several concentrations of free external $\text{Ca}^{2+}$ so that the range likely to be present *in situ* could be covered. The data on the rate of $\text{Ca}^{2+}$ accumulation given in Table 1 demonstrates that at any of the concentrations of free external $\text{Ca}^{2+}$ tested the rates were identical for the control and stimulated preparations. The $\text{Ca}^{2+}$-accumulating abilities of the SR vesicle preparations studied at two different concentrations of free external $\text{Ca}^{2+}$ are shown in Fig. 2. At both high and low $\text{Ca}^{2+}$ concentrations all stimulated preparations tested were able to remove more $\text{Ca}^{2+}$ from the medium than the controls, i.e. $\text{Ca}^{2+}$ accumulation ceased when a higher internal concentration had been reached.

![Fig. 2. Calcium accumulation in the absence of oxalate. SR vesicles (0.1–0.2 mg protein/ml reaction mixture) were added to a medium composed of 0.1 M KCl, 20 mM histidine–HCl buffer, pH 6.4, 5 mM ATP, 5 mM MgCl$_2$, 2.5 mM phosphoenolpyruvate, 8 units of pyruvate kinase per ml, 5 mM azide and either 25 μM $\text{Ca}^{2+}$ with no EGTA (○) or 25 μM $\text{Ca}^{2+}$ with 68 μM EGTA (▲). This concentration of EGTA was required to give a free $\text{Ca}^{2+}$ concentration of approximately 1 μM (Katz et al. 1970). A tracer amount of $^{45}\text{Ca}^{2+}$ was added to the unlabelled $\text{Ca}^{2+}$ prior to the addition of the EGTA. The amount of SR vesicles was adjusted so that at maximum uptake only 20–40% of the total $\text{Ca}^{2+}$ in the medium had been accumulated. Incubation was at 22°C. ○,▲ Control. ○,▲ Stimulated. Low external free $\text{Ca}^{2+}$ (c. 1 μM)—mean of three experiments; high external free $\text{Ca}^{2+}$—mean of two experiments.](image-url)
Purified ATPase Vesicles

Because the enzyme responsible for the transport of Ca\(^{2+}\) across the membrane could be obtained in almost pure form, a detailed study was made of the effect of electrical stimulation of this component alone. Fig. 1b shows a densitometric scan after gel electrophoresis of the purified enzyme obtained from a control muscle preparation. The enzyme from electrically stimulated muscle was identical to that from the control in protein composition and gave essentially the same yield. The purification technique removed most of the calsequestrin and much of the phosphorylase, but the proportion of the protein of molecular weight 30000 remained in the same proportion to the ATPase protein as was present initially. Alteration of the concentrations of ammonium acetate used in the fractionation (MacLennan 1974) did not free the ATPase from the component of molecular weight 30000. The purified ATPase had an activity of approximately 2·5 compared with 0·7 μmol ATP split per mg protein per min for the 'extra' ATPase of SR vesicles (Table 2 and tabulation above).

| Table 2. Effect of electrical stimulation on various parameters of purified ATPase vesicles |
|----------------------------------|-----------|-----------|
| Values expressed are means±s.e. and the numbers of preparations are shown in parentheses |
|                                  | Control   | Stimulated |
| ATPase activity (μmol P\(_i\) per mg protein per min) | 2.46±0.27 (5) | 0.58±0.20 (5) |
| Steady-state E\(\sim\)P level (nmol per mg protein) | 0.57±0.13 (5) | 0.22±0.08 (5) |
| (1) From \([\gamma^{32}P]\)ATP | 0.59±0.04 (6) | 0.16±0.03 (6) |
| (2) From \(^3\)P, ATP \(\rightleftarrows\) P\(_i\) exchange (nmol per mg protein per 5 min) | 106±44 (6) | 18.2±9.8 (6) |

Properties of Ca\(^{2+}\)-dependent ATPase Vesicles

For all of the functions assayed the ATPase vesicles isolated from the stimulated muscle were significantly less active than those from the control muscle (Table 2).

The ATPase activity of the control preparation was about six times greater than that of the stimulated preparation (Table 2). Virtually no activity was observed in the absence of Ca\(^{2+}\) with either preparation (data not shown).

The steady-state level of E\(\sim\)P was the same whether formed from ATP or from P\(_i\) under the conditions described in the Methods section. Preparations from stimulated muscle gave about one-third of the concentration of E\(\sim\)P compared to those from control muscle.

The ATP \(\rightleftarrows\) P\(_i\) exchange reaction was measured since it provides information on forward and backward reactions occurring simultaneously. In each experiment the control preparation was nearly six times as active as that obtained from stimulated muscle (Table 2). The reason for the large range of absolute values from one experiment to another is not known. It is not likely to be the result of different limiting concentrations of endogenous ADP because it has been shown that the ADP formed from ATP during the course of the reaction is adequate for maximal exchange (de Meis and Carvalho 1974).
**Effect of Ca\(^{2+}\) Concentration on ATP ⇄ P\(_i\) Exchange**

The experiments showing large differences between control and stimulated preparations for the ATP ⇄ P\(_i\) exchange were measured at Ca\(^{2+}\) concentrations of 4 mM. In order to determine whether or not the relative activities of the control and stimulated preparations were the same at other Ca\(^{2+}\) concentrations, the affinity of the preparations for Ca\(^{2+}\) was measured. The effect of Ca\(^{2+}\) concentration is shown in Fig. 3, from which it can be seen that at all concentrations tested the stimulated ATPase had less activity. Both preparations, however, exhibited maximum activity close to 3 mM Ca\(^{2+}\). The concentration required for 50\% of maximum activation was about 1 mM for both preparations.

**Effect of P\(_i\) Concentration on ATP ⇄ P\(_i\) Exchange**

The effect of P\(_i\) concentration on the exchange reaction was determined over the range 1·0–10·0 mM. The control and stimulated preparations all gave straight-line, double reciprocal plots (Fig. 4). In the experiment shown, the maximum velocity of the control was about 130 compared with 70 nmol per mg protein per 5 min for the
stimulated preparation. Also, the apparent $K_m$ for $P_i$ was considerably greater for the stimulated than for the control preparation (18 mM compared with 10 mM), demonstrating a reduction in $P_i$ affinity.

**Effect of $P_i$ Concentration on $E\sim P$ Formation**

The involvement of $P_i$ in the $ATP\rightleftharpoons P_i$ exchange reaction begins with the formation of $E\sim P$, therefore the affinity of the enzyme for $P_i$ was assessed directly by measuring the steady-state amount of $E\sim P$ at $P_i$ concentrations ranging from 0.5 to 8.0 mM. Fig. 5 shows the reciprocal of the steady-state level of $E\sim P$ plotted against the reciprocal of the $P_i$ concentration. A straight line was obtained for the stimulated and control preparations. At very high $P_i$ concentrations the amount of $E\sim P$ formed by stimulated and control ATPase appeared to approach the same concentration. However, the affinity for $P_i$ is much lower for the stimulated ATPase (approximately 10–15 mM $P_i$ compared with 2–3 mM $P_i$ for the control; range for three experiments).

**Effect of $Mg^{2+}$ Concentration on $E\sim P$ Formation**

Phosphorylation of ATPase by $P_i$ is dependent upon the presence of $Mg^{2+}$ ions (Kanazawa and Boyer 1973; Masuda and deMeis 1973). Stimulated and control preparations had the same $Mg^{2+}$ requirements (maximum activity with 4–6 mM $Mg^{2+}$) (data not given).

**Effect of ATP Concentration on ATP Splitting**

ATPase activity was investigated over a wide range of ATP concentrations with a constant $Mg^{2+}$ concentration of 5 mM. An ATP regenerating system was included in the reaction mixture to prevent inhibition by ADP (Inesi et al. 1967). At all ATP concentrations tested the control exhibited greater activity (Fig. 6), the maximum splitting occurring at about 0.3 mM. In the case of the stimulated preparation, maximum splitting had not been reached even at 5 mM ATP. Fig. 6 reveals an ATP concentration of about 0.05 mM for 50% maximum activity for the control. From
the data it is not possible to put a similar value on the stimulated preparations as saturating concentrations had not been reached, but it is likely to be at least 0.05 mm. Fig. 6 (inset) provides some information on ATP affinity of the enzyme preparations at very low ATP concentrations. The high ATP concentration values were not plotted as they are crowded near the y axis. An apparent $K_m$ of 1 $\mu$m for control compared with 9 $\mu$m for stimulated preparations was indicated, demonstrating the higher affinity of the control enzyme for the substrate.

![Graph showing ATPase activity](image)

**Fig. 6.** Effect of ATP concentration on ATP splitting by purified ATPase vesicles. ATPase activity was determined as described under Methods except that 0.5 mm phosphoenolpyruvate and pyruvate kinase (8 units/ml) were included in the reaction mixture. Inset shows data from the same experiments (ATP concentration range 1–30 $\mu$m) but presented as a double reciprocal plot. Mean of two experiments. ● Control. ○ Stimulated.

*Pronase Treatment*

To test the possibility that after stimulation the protein might be organized in a different way in the membrane, ATPase vesicles were subjected to Pronase treatment for up to 30 min at 37°C. Figs 7a and 7b show that the ATPase from stimulated muscle was more rapidly broken down than the control ATPase. Even though the stimulated ATPase was more susceptible, hydrolysis of the polypeptides occurred at the same points yielding fragments having the same molecular weight.

**Discussion**

The $Ca^{2+}$ pump ATPase of intact SR vesicles and ATPase vesicles is responsible for the translocation of $Ca^{2+}$ across the membrane. As a result of recent studies (Masuda and de Meis 1973; de Meis and Carvalho 1974; de Meis and Masuda 1974; Ikemoto 1974, 1975; Kanazawa 1975; Knowles and Racker 1975; Carvalho et al. 1976; de Meis and Tume 1977) it has been proposed that the ATPase can exist in
two conformations, one having a high and the other a low affinity for Ca\(^{2+}\), and a fully reversible reaction sequence has been described for the transport of Ca\(^{2+}\) (Carvalho et al. 1976). Under conditions which are suitable for Ca\(^{2+}\) accumulation the enzyme binds Ca\(^{2+}\) and is phosphorylated by ATP (high affinity conformation), releasing ADP to the external medium. The Ca\(^{2+}\) is then translocated across the membrane (the enzyme assumes low affinity conformation) and is released. The enzyme is dephosphorylated and then undergoes a conformational change back to the original form ready for the cycle to be repeated. In intact SR vesicles, however, as Ca\(^{2+}\) accumulates within the vesicle the low affinity Ca\(^{2+}\) binding site may approach saturation and tend to drive the reaction in the reverse direction leading to an inhibition of the ATP splitting activity. These individual reactions must be taken into account in the study of the overall process of Ca\(^{2+}\) transport.

Isolated intact SR vesicles from electrically stimulated muscle appear to be able to pump Ca\(^{2+}\) at the same rate as control preparations and, in fact, in those experiments where total accumulation was measured rather than rate, the stimulated preparations were able to reduce the external Ca\(^{2+}\) concentration to an even lower level than were the controls. Because the activity of the 'extra' ATPase was decreased by stimulation and yet the same uptake rate was attained, it is possible that the membrane itself becomes less leaky to accumulated Ca\(^{2+}\) after stimulation. The larger amount of Ca\(^{2+}\) accumulated by the stimulated vesicles might also suggest that the enzyme in the low affinity form is less sensitive to the internal Ca\(^{2+}\) concentration and therefore a larger Ca\(^{2+}\) concentration could be reached before the uptake was switched off. However, this is not in keeping with the finding that the ATP ⇌ P\(_i\) exchange reaction of purified ATPase vesicles from both the control and stimulated muscles showed the same Ca\(^{2+}\) concentration requirements (Fig. 3).

![Fig. 7. Susceptibility of purified ATPase vesicles to Pronase treatment. (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified ATPase vesicles subjected to Pronase for the times indicated. (b) Breakdown of the ATPase as a function of time, based on the area under the curve obtained from densitometric scans. ● Control. ○ Stimulated. Details of incubations are given under Methods.](image-url)
Although no decrease in the Ca\(^{2+}\)-transporting ability of the intact SR vesicles from electrically stimulated preparations was observed, under the conditions used, it does not necessarily mean that the vesicles were unaffected by such treatment, since the amount of Ca\(^{2+}\) accumulated is the net result of inward pumping and outward efflux. A better method of investigating Ca\(^{2+}\) transport by these membranes is to determine the activity of the enzyme responsible for the pumping of Ca\(^{2+}\), namely the Ca\(^{2+}\)-dependent ATPase.

The technique used for preparing purified ATPase was essentially that described by MacLennan (1974) for rabbit white muscle. When this method was applied to sheep longissimus dorsi muscle, a mixed muscle composed of red and white fibres, the final preparation contained a protein of molecular weight 30,000 in addition to the ATPase. Margreth et al. (1975) reported that purified ATPase from rabbit slow (red) muscles prepared by the method of Meissner et al. (1973) contained an additional protein with an apparent molecular weight of 30,000 whereas preparations from rabbit fast (white) muscles did not. It is likely that the protein of molecular weight 30,000 in sheep muscle preparations is the same as that in rabbit slow muscle preparations. The purified ATPase vesicles prepared by this method (MacLennan 1974) from either control or electrically stimulated muscle are unable to accumulate Ca\(^{2+}\) since Ca\(^{2+}\) leaks out as fast as it is pumped in by the ATPase. The ATP splitting activity of these preparations, however, is considerably higher than that of the intact SR preparations. The various activities measured for the sheep muscle preparations are low compared with those of rabbit white muscle preparations, but are similar to those reported for red muscle preparations (Margreth et al. 1975).

Electrical stimulation has a marked effect on all the parameters of ATPase vesicles measured. The results show that the forward reactions as measured by the ATP splitting activity and by phosphorylation from ATP, the overall reaction measured by the ATP $\rightleftharpoons$ P\(_i\) exchange reaction and the reverse reaction measured by phosphorylation from P\(_i\) are all affected by electrical stimulation.

The ATP $\rightleftharpoons$ P\(_i\) exchange reaction proceeds at optimal rate only when the Ca\(^{2+}\) binding site of low affinity approaches saturation (de Meis and Carvalho 1974). Because electrical stimulation causes no alteration in Ca\(^{2+}\) requirements compared with the control, it can be concluded that the low affinity Ca\(^{2+}\) binding site is not affected. However, differences in P\(_i\) affinity for the exchange reaction were observed, the enzyme from stimulated muscle having a lower affinity for P\(_i\). It should be pointed out that in these exchange experiments the reaction was at pH 7 in the presence of Ca\(^{2+}\) and ATP, and therefore the apparent P\(_i\) affinity is a net effect of the overall reactions under the conditions used. The phosphorylation of the enzyme by P\(_i\) occurs very rapidly [40–60 ms for \(t_f\) (Boyer et al. 1977)] and therefore it is easier to measure the steady-state concentration than the rate of formation of E $\sim$ P. A measure of E$\sim$P concentration at different P\(_i\) concentrations allows a direct P\(_i\) affinity to be determined under conditions optimal for this step. Again preparations from stimulated muscle have a low P\(_i\) affinity and, as would be expected, both preparations show a higher affinity than when measured under conditions for the exchange reaction.

The Mg\(^{2+}\) requirement for E$\sim$P formation was the same for both types of preparations and was similar to that found previously for rabbit preparations (Masuda and de Meis 1973).

The reduced affinity of the enzyme for P\(_i\) suggests that ATP affinity might also be reduced because ATP and P\(_i\) are thought to bind at the same site of the enzyme (de
Meis and Masuda 1974), the preference for one over the other depending on the Ca\textsuperscript{2+} concentration. This is indeed the case. Whilst there appears to be two types of binding sites the kinetic binding data can only be ascertained for the high affinity sites and the preparation from stimulated muscle has a somewhat lower affinity for ATP than does the control.

The different susceptibility of the protein to Pronase suggests that these changes in affinity of the enzyme for the substrates following stimulation are brought about by some conformational change. At this stage it is not possible to say whether there is a conformational change in the protein itself or if there is a change in the relationship of the protein to its lipid environment. The reason for the large differences in ATPase activity observed after purification is not known. For full activity, however, each molecule of ATPase protein must be associated with a lipid annulus comprising about 30 phospholipid molecules (Warren et al. 1974). Should this lipid annulus be disturbed by electrical stimulation, the effect of stimulation may not be markedly apparent until the preparation has been treated with deoxycholate during the purification procedure. Disturbance of the lipid annulus would possibly render the protein more susceptible to proteolytic attack by Pronase.

As part of a more general study on the relationship between excitation and contraction, a number of workers (Van der Kloot 1966; Lee et al. 1966; Turina and Jenny 1968; Miyamoto and Kasai 1973) have electrically stimulated suspensions of SR vesicles loaded with Ca\textsuperscript{2+} in vitro, in an attempt to show an effect of electrical stimulation on Ca\textsuperscript{2+} release. Although under certain conditions release of Ca\textsuperscript{2+} was observed (Lee et al. 1966; Turina and Jenny 1968), the SR was shown to be irreversibly damaged (Lee et al. 1966; Miyamoto and Kasai 1973). Miyamoto and Kasai (1973) repeated the work of Lee et al. (1966) and found that the release of Ca\textsuperscript{2+} could be explained by the inactivation of the vesicles caused by chlorine gas generated at the anode. It has not been possible to show an effect on the SR itself by this approach as the results are overshadowed by this inactivation described.

Conclusions

The evidence provided shows that electrical stimulation results in an alteration in the ATPase of SR. It is apparent that the part of the ATPase which binds ATP and P\textsubscript{i} is modified by stimulation, resulting in reduced binding affinities. The low affinity site for Ca\textsuperscript{2+} binding is not affected. It is therefore this reduced binding affinity for ATP and P\textsubscript{i} that results in the decreased activities observed in the preparations from stimulated muscle.

Acknowledgments

This work was supported in part by a grant from the Australian Meat Research Committee. I am grateful to Mr F. D. Shaw and Mr S. B. Penna for carrying out the electrical stimulation of the sheep, and to Mr B. W. Arantz for assistance with the Ca\textsuperscript{2+} accumulation studies.

References


Manuscript received 13 July 1978