Characterization of Ca^{2+} uptake and release by vesicles of skeletal-muscle sarcoplasmic reticulum

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Homogenization of muscle gives a preparation of sealed vesicles derived from the sarcoplasmic reticulum. In the presence of ATP these vesicles will initially accumulate Ca^{2+} from the external medium and then spontaneously release this Ca^{2+} in two phases, an initial slow phase and a faster second phase. By measuring ATP concentrations in parallel with measurements of external Ca^{2+} concentrations we have shown that the second phase of release occurs when the added ATP has been exhausted, but that the first phase of release occurs in the presence of ATP. A similar pattern of uptake and release has been observed in the presence of acetyl phosphate, showing that ADP generated by ATP hydrolysis is not essential for the release process. The temperature-dependence of both phases of release is similar to the temperature-dependence of ATPase activity. Release is dependent on pH over the same pH range as affects binding of Ca^{2+} to the ATPase. Therefore we propose that Ca^{2+} release from vesicles of sarcoplasmic reticulum actively loaded with Ca^{2+} is mediated by the same Ca^{2+} + Mg^{2+} -activated ATPase as is responsible for uptake of Ca^{2+} .

INTRODUCTION

Muscle can be homogenized to give a preparation of sealed vesicles derived from the sarcoplasmic reticulum (SR) that will take up Ca^{2+} from the external medium on addition of ATP (Weber, 1966; Hasselbach, 1981). Under the appropriate conditions of pH and temperature, these vesicles will spontaneously release some of this accumulated Ca^{2+} (Ohnishi & Ebashi, 1963; Nakamaru & Schwartz, 1970; Van Winkle, 1976; Sorenson & de Meis, 1977; Entman *et al.*, 1979; Gerdes & Møller, 1983). This spontaneous release appears to occur in two phases (Van Winkle, 1976; see Fig. 1). An initial relatively slow release of Ca^{2+} occurs apparently while ATP is still present in the system, and it has been suggested that the second, faster, phase of Ca^{2+} release is initiated when the ATP present in the system is exhausted (Gerdes & Møller, 1983). The spontaneous release of Ca^{2+} occurring in the presence of external Ca^{2+} and ATP has been likened to the Ca^{2+} release that can be induced in skinned muscle fibres by the addition of Ca^{2+} and referred to as ' Ca^{2+} -induced Ca^{2+} -release' (Endo, 1977).

As described in McWhirter *et al.* (1987), a number of reports have appeared of relatively fast efflux of Ca^{2+} from SR vesicles passively loaded with Ca^{2+} . It therefore seems likely that the spontaneous release of Ca^{2+} seen from actively loaded SR occurs through the same pathways as mediate Ca^{2+} efflux from passively loaded vesicles. In McWhirter *et al.* (1987) it was shown that Ca^{2+} efflux from passively loaded SR can be simulated by assuming that efflux is mediated by the Ca^{2+} + Mg^{2+} -activated ATPase responsible for uptake of Ca^{2+} . It should therefore be possible to combine the kinetic model for ATPase activity presented in Gould *et al.* (1986) and Stefanova *et al.* (1987) with that for Ca^{2+} efflux presented in McWhirter *et al.* (1987) to present a consistent model for the uptake and spontaneous release of Ca^{2+} by SR vesicles. In the present paper we

characterize the preparation of SR vesicles used in our experiments and their pattern of uptake and release of Ca^{2+} .

MATERIALS AND METHODS

AnalaR reagents and murexide were obtained from BDH Chemicals, Hepes (Ultrol) was from Calbiochem and Arsenazo III was from Sigma Chemical Co. SR was prepared largely in accordance with Warren *et al.* (1974) but with some modifications. White muscle was dissected from the hind legs of a female (New Zealand White) rabbit, this and all subsequent procedures being performed at 4 °C. After removal of fat and connective tissue, the muscle was finely chopped and homogenized in buffer (0.3 M-sucrose/20 mM-histidine/HCl/1 mM-dithiothreitol/5 μM -phenylmethanesulphonyl fluoride, pH 8.0) in a Waring Blendor for 15 s. The homogenate was spun at 8000 *g* for 20 min. The supernatant was then filtered through two layers of muslin and the pellet discarded. The supernatant was then spun at 37000 *g* for 90 min, the resulting supernatant discarded and the pellet resuspended in ice-cold buffer (0.3 M-sucrose/10 mM-histidine/HCl/0.6 M-KCl/1 mM-dithiothreitol/5 μM -phenylmethanesulphonyl fluoride, pH 8.0) in a homogenizer tube fitted with a pestle clearance of 0.32 mm. The suspension was left to stand for 30 min to precipitate actomyosin before centrifugation at 5000 *g* for 20 min. The supernatant was decanted off the soft actomyosin pellet and centrifuged at 37000 *g* for 90 min. The resulting pellet was rehomogenized as above in buffer (40 mM-Hepes/KOH/0.1 M-KCl/1 mM-dithiothreitol, pH 6.3) and made up to a volume of about 100 ml. This was again spun at 37000 *g* for 90 min to wash the pellet, and the wash step was repeated. The final pellet was resuspended in buffer (40 mM-Hepes/KOH/0.1 M-KCl/1 mM-dithiothreitol, pH 6.3) at

Abbreviations used: ATPase, Ca^{2+} + Mg^{2+} -activated ATPase; SR, sarcoplasmic reticulum.

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10–20 mg/ml and stored on ice for up to 3 days before use.

Protein was determined by using the absorption coefficient given by Hardwicke & Green (1974). ATPase activity was determined by using the coupled enzyme assay method described in Stefanova *et al.* (1987).

SR vesicles were fractionated on discontinuous sucrose gradients [5 ml of 25% (w/v), 10 ml of 35% (w/v), 15 ml of 40% (w/v), 5 ml of 60% (w/v)] at 4 °C, spinning at 85 000 *g* overnight. Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard. SDS/polyacrylamide gels were electrophoresed in accordance with the method of Laemmli & Faure (1973) and stained with Coomassie Blue G250. Gels were scanned with a Joyce-Loebel gel-scanning apparatus.

Ca²⁺ fluxes were monitored photometrically by using the dye murexide. Spectra were run on either Perkin-Elmer 356 or Shimadzu UV3000 dual-wavelength spectrometers with a wavelength pair of 507 nm–542 nm. Samples were stirred with a Cell-Spinbar magnet (Bel-Art Products, Radleys, Sawbridgeworth, Herts., U.K.) in the sample cuvette. Reagents were injected directly into the cuvette from Hamilton syringes through a modified lid, so that additions could be made without occluding the light-path. A saturated solution of murexide was prepared by addition of 2.4 mg of murexide to 1 ml of water, leaving the resulting suspension to stand on ice for 30 min, followed by filtration to give a clear solution. For Ca²⁺ assay, 160 µl of this stock solution was added to 3 ml of buffer containing SR membrane (usually at 0.3 mg of protein/ml) in the assay cuvette and incubated at the appropriate temperature for 10 min. To calibrate the optical signal, samples of a concentrated stock solution of CaCl₂ were added, usually to a final concentration of 100 µM: over the concentration range used here, the murexide response was linear with Ca²⁺ concentration. In this way, the response of murexide to Ca²⁺ was calibrated under the conditions to be used in the experiment. The use of murexide and other dyes to measure free Ca²⁺ concentrations is described by, for example, Ashley & Campbell (1979). Ca²⁺ uptake was initiated by addition of ATP; at the Mg²⁺ concentration usually employed (4 mM) the change in free Ca²⁺ concentration due to binding of Ca²⁺ to ATP was negligible. Ca²⁺ uptake was also measured by using Arsenazo III, freed from Ca²⁺ by the method of Dipolo *et al.* (1976), at a wavelength pair of 675 nm–685 nm.

ATP concentrations were monitored by using an LKB-Wallac analytical reagent kit, with firefly luciferin/luciferase bioluminescence as a measure of ATP concentration. ATP was added to SR samples contained in a temperature-controlled vessel to initiate Ca²⁺ uptake, the experiment being run in parallel with an identical sample being assayed for Ca²⁺ uptake as described above. Samples (100 µl) were taken at fixed times and mixed with 50 µl of 20% (v/v) HClO₄ to stop all reaction. Then 200 µl of 200 mM-Hepes/100 mM-KCl (adjusted with KOH so that addition of 200 µl to the HClO₄ mixture gave a final pH of 7.4 ± 0.1) was then added and the sample vortex-mixed. Samples were then incubated at 4 °C for 30 min before centrifugation at 10 000 *g* for 5 min to remove the precipitate of KClO₄. Portions (50 µl) were then dispensed into scintillation vials containing Tris/EDTA buffer (950 µl; 0.1 M-Tris/2.0 mM-EDTA adjusted to pH 7.75 with acetic

acid). The luciferin/luciferase assay was begun by addition of ATP monitoring reagent (20 µl) followed by rapid mixing. Light output was measured in a Phillips PW4700 liquid-scintillation counter. A calibration curve was established by using the same procedure but with solutions of known ATP concentration.

RESULTS

Homogenization of muscle gives a preparation of sealed vesicles derived from SR that can take up Ca²⁺ from the external medium in the presence of ATP. Meissner (1975) has shown that this vesicle preparation is heterogeneous and can be separated by sucrose-gradient centrifugation into light, intermediate and heavy SR vesicles. As shown in Table 1, SR vesicles prepared by the procedures outlined above are predominantly a mixture of intermediate and heavy vesicles, in contrast with those prepared using the procedure of Meissner (1975), which are 75% intermediate vesicles with only 15% heavy vesicles. In all fractions the three major protein components are the Ca²⁺ + Mg²⁺-activated ATPase, calsequestrin and the M55 glycoprotein, with minor amounts of the acidic Ca²⁺-binding proteins (for nomenclature of protein components see Campbell, 1986). As reported by Meissner (1975), the concentration of calsequestrin is highest in the heavy SR fraction. In all subsequent experiments unfractionated SR vesicles were used.

Addition of ATP to a suspension of SR vesicles leads to uptake of Ca²⁺, which can be monitored spectrophotometrically by using murexide to determine the concentration of Ca²⁺ in the external medium (Fig. 1): the rate of uptake of Ca²⁺ is too fast to be measured in these experiments. Following uptake of Ca²⁺, Ca²⁺ is spontaneously released in two phases, an initial slow phase and a faster second phase. At the completion of the second phase of release, there is a small and variable difference between the initial and final external Ca²⁺ concentrations. Blayney *et al.* (1977) have suggested that the spontaneous release of Ca²⁺ seen in experiments such as that shown in Fig. 1 is an artifact attributable to the binding of murexide to the SR vesicles. Entman *et al.* (1979), however, have presented a series of experiments that suggest that this is unlikely. We have also monitored

Table 1. Composition of SR vesicles

SR vesicles were fractionated on sucrose gradients as described in the text. The protein composition of fractionated vesicles was determined from Coomassie Blue-stained gels, and all fractions showed small contributions attributable to acidic Ca²⁺-binding proteins. For nomenclature of protein bands see Campbell (1986).

SR fraction . . .	Light	Intermediate	Heavy
% of total SR protein*	20.0 ± 6.1	43.5 ± 10.6	34.5 ± 3.0
% of protein in SR fraction as:			
ATPase	90	77	69
Calsequestrin	7	12	24
M55 protein	2	4	4

* Means ± s.d. for three preparations

Fig. 1. Uptake and release of Ca²⁺ by SR vesicles monitored spectrophotometrically

The uptake and release of Ca²⁺ following addition of ATP (500 μM) to SR vesicles (0.3 mg of protein/ml) in buffer (40 mM-Tris/HCl/40 mM-maleate/5 mM-MgSO₄, pH 6.0) at 30 °C was monitored by the change in absorbance of murexide (0.45 mM) at the wavelength pair 507 nm–542 nm. The absorbance change was calibrated by addition of two portions of a concentrated stock solution of CaCl₂, to give Ca²⁺ concentrations of 50 and 100 μM respectively. Uptake was initiated by addition of ATP. Following uptake of Ca²⁺, spontaneous release occurred in two phases, an initial slow phase (S) and a faster second phase (F). At the completion of the second phase of release, a residual difference (D) was sometimes observed between the initial and final concentrations of Ca²⁺. Also shown is the definition of the maximal extent of Ca²⁺ uptake (U_{max}).

Fig. 2. Effect of maleate on uptake and release of Ca²⁺ by SR vesicles

The uptake and release of Ca²⁺ are shown following the addition of ATP (500 μM) to SR vesicles (0.3 mg of protein/ml) in buffer (40 mM-Tris/HCl/5 mM-MgSO₄, pH 6.0) at 30 °C in the presence of potassium maleate at concentrations of 0 mM (trace A), 20 mM (trace B), 40 mM (trace C) and 60 mM (trace D). K⁺ concentration was kept constant at 100 mM by the addition of KCl. The times of addition of ATP are shown by the arrows.

Fig. 3. Effect of temperature on uptake and release of Ca²⁺ by SR vesicles

The uptake and release of Ca²⁺ are shown following addition of ATP (500 μM) to SR vesicles (0.3 mg of protein/ml) in buffer (40 mM-Tris/HCl/40 mM-potassium maleate/5 mM-MgSO₄, pH 6.0) at an initial external Ca²⁺ concentration of 100 μM as a function of temperature. Note that the time scale for the experiment at 37 °C is half that at the other temperatures. The times of addition of ATP are shown by the arrows.

Ca²⁺ uptake and release in these systems using a Ca²⁺ electrode or using Arsenazo III as dye (results not shown) and by measuring the fluorescence intensity of chlorotetracycline as a measure of Ca²⁺ concentration within the SR vesicles (H. I. Stefanova & A. G. Lee, unpublished work), and, using all these techniques, we observe spontaneous release of Ca²⁺, as shown in Fig. 1. We conclude, therefore, that murexide can be used to monitor external Ca²⁺ concentrations in these systems, and that technique was utilized in all experiments described below.

Sorenson & de Meis (1977) have reported that uptake of Ca²⁺ by SR vesicles is markedly increased by the addition of anions, particularly bivalent anions such as maleate. Fig. 2 illustrates the effect on Ca²⁺ uptake of the addition of up to 60 mM-maleate at pH 6.0 and 30 °C. Under these conditions the major effect of maleate is to increase the extent of uptake and the rate of release of Ca²⁺ both in the initial slower phase and in the faster second phase: as a consequence, the two-phase nature of release is more apparent in the presence of maleate than in its absence.

As illustrated in Fig. 3, Ca²⁺ release is markedly temperature-sensitive, and at 5 °C is barely detectable (results not shown). The extent of Ca²⁺ uptake is relatively insensitive to temperature in the range 20–30 °C, but decreases significantly outside this range. The extent of Ca²⁺ uptake and the rate of release are also pH-sensitive, as shown in Fig. 4. Berman *et al.* (1977) have reported that incubation of SR vesicles at pH 5.5–6.0 leads to an irreversible loss of Ca²⁺ uptake. We, however, find that effects of pH are fully reversible.

Fig. 4. Effects of pH on Ca^{2+} uptake and release and ATP concentrations in SR vesicles

The effects of pH on Ca^{2+} uptake and release (continuous traces) and ATP concentrations (broken lines) were measured in parallel experiments following the addition of ATP ($500 \mu\text{M}$) to SR vesicles (0.3 mg of protein/ml) in buffer (40 mM -Hepes/KOH/ 100 mM -KCl/ 5 mM - MgSO_4) at 30°C . The initial external Ca^{2+} concentration was $100 \mu\text{M}$. The times of addition of ATP are shown by the arrows.

Fig. 5. Effect of temperature on ATP concentrations in SR vesicles

Ca^{2+} uptake and release (continuous traces) and ATP concentrations (broken lines) were determined in parallel experiments following the addition of ATP to SR vesicles at pH 6 under the conditions of Fig. 4 at 37°C and 25°C . The times of addition of ATP are shown by the arrows.

Thus SR vesicles incubated at pH 6.0 for 10 min at 30°C show normal uptake and release when assayed at pH 7.2. Similarly, incubation of SR vesicles at pH 7.4 has no effect on the uptake and release of Ca^{2+} measured subsequently at pH 6.2 (results not shown).

Fig. 6. Effect of ATP concentration on the time to the second phase of Ca^{2+} release by SR vesicles

The time from the addition of ATP to the start of the second phase of Ca^{2+} release is shown as a function of the concentration of ATP.

Fig. 7. Effects of sequential addition of ATP on uptake and release of Ca^{2+} by SR vesicles

Ca^{2+} uptake and release are shown following the addition of ATP (0.5 mM) to SR vesicles (0.3 mg of protein/ml) in 40 mM -Hepes/KOH/ 100 mM -KCl/ 4 mM - MgSO_4 , pH 6.0, at 30°C at an initial external Ca^{2+} concentration of $100 \mu\text{M}$. Following the initial addition of ATP, a second portion of ATP (0.5 mM) was added at 5 min (A), 9 min (B) or 14 min (C).

ATP concentrations were measured in parallel with measurements of external Ca^{2+} concentration as described in the Materials and methods section. As shown in Fig. 4, the onset of the faster second phase of Ca^{2+} release appears to be correlated with a decrease in the concentration of ATP to a low level. In Fig. 4 this is illustrated for samples in the absence of maleate: the same correlation is observed in the presence of 40 mM -maleate (results not shown). As shown in Fig. 5, the increase in the delay between the addition of ATP and the start of the second phase of release seen with decreasing temperature also correlates with a longer time for depletion of ATP. As expected from the above results, the time to the second phase of release increases as the ATP concentration increases: as illustrated in Fig. 6, there is an approximately linear relationship between the concentration of ATP added and the time to the second phase.

The importance of ATP concentration was also

Fig. 8. Effect of acetyl phosphate on Ca²⁺ uptake and release by SR vesicles

Ca²⁺ uptake and release are shown following the addition of acetyl phosphate (traces A) at 0.5 mM (a) and 1.0 mM (b) and (trace B) at 0.5 mM to SR vesicles (0.3 mg of protein/ml) in 40 mM-Hepes/KOH/100 mM-KCl/4.0 mM-MgSO₄, pH 6.0 (traces A) or pH 7.0 (trace B), both at 30 °C. The times of addition of acetyl phosphate are shown by the arrows.

Fig. 9. Effects of an ATP-regenerating system and of P_i on Ca²⁺ uptake and release by SR vesicles

Ca²⁺ uptake and release are shown following the addition of ATP (500 μM) to SR vesicles (0.3 mg of protein/ml) in 40 mM-Hepes/KOH/100 mM-KCl/4 mM-MgCl₂, pH 6.0, at 30 °C. For trace A the system contained an ATP-regenerating system consisting of pyruvate kinase (10 units) and phosphoenolpyruvate (1 mM), and for trace B 5 mM-potassium phosphate. The times of addition of ATP are shown by the arrows.

demonstrated by addition of portions of ATP during the release process, as shown in Fig. 7. Addition of ATP during the initial phase of Ca²⁺ release had no effect on the rate of release, confirming that this release occurs in the presence of ATP. However, addition of ATP during the faster second phase of release leads to re-uptake of Ca²⁺, confirming that the second phase of release only occurs when the ATP concentration is low: the relatively low extent of re-uptake of Ca²⁺ achieved following the final addition of ATP can be attributed to the presence of ADP generated by the ATPase (see below).

It is known that under the appropriate conditions (high internal and low external Ca²⁺ concentrations and low ATP concentrations), addition of ADP to SR vesicles can lead to efflux of Ca²⁺ from the vesicles, by a reversal of the ATPase (de Meis, 1981). This is unlikely to account for the spontaneous release of Ca²⁺ observed here, since release occurs when the external Ca²⁺ concentration is high. A possible role for ADP in release can be eliminated by using acetyl phosphate to drive uptake. As shown in Fig. 8, the pattern of uptake and spontaneous release of Ca²⁺ seen with acetyl phosphate at pH 6 is very similar to that seen with ATP, and again with acetyl phosphate release was more marked at pH 6 than at pH 7 (Fig. 8). In fact, the major effect of addition of ADP to SR vesicles in the presence of ATP is to decrease the maximal extent of uptake of Ca²⁺. Effects of an ATP-regenerating system on Ca²⁺ uptake and release are complex, with initial release being followed by further uptake (Fig. 9). However, as shown in Fig. 9, and as discussed below, this effect can in part be attributed to the formation of P_i in the system, since inclusion of P_i leads to a marked change in the pattern of Ca²⁺ uptake and release.

Varying the external Ca²⁺ concentration had no effect on the extent of Ca²⁺ uptake, as long as the initial external Ca²⁺ concentration was not so low that the external Ca²⁺ became depleted before maximal Ca²⁺ uptake was achieved. The external Ca²⁺ concentration did, however, have a small effect on release, rates of release increasing slightly with decreasing external Ca²⁺ concentrations (results not shown).

We have found that the maximal extents of Ca²⁺ accumulation and the times to the second phase of release vary among preparations of SR. The highest extents of uptake that we observe (160–250 nmol/mg of protein) are comparable with those reported by Sreter (1979) and Sorenson & de Meis (1977). Preparations with such uptakes show a Ca²⁺+Mg²⁺-activated ATPase activity of 0.5–1.0 μmol of P_i/min per mg of protein at 37 °C when assayed at pH 7.2 and ATP at 2 mM. Preparations are also obtained, however, that show a smaller extent of maximal Ca²⁺ uptake (typically 90–120 nmol of Ca²⁺/mg of protein) but that are also comparable with published data (Meissner, 1975; Endo, 1977). We find that such preparations also show a higher rate of spontaneous release of Ca²⁺ and an increased ATPase activity of approx. 2–3 μmol of P_i/min per mg of protein. We believe that these effects can be attributed, at least in part, to oxidative damage. Thus we have shown that exclusion of dithiothreitol from the final wash medium used in the preparation of the SR vesicles leads to a significantly higher rate of Ca²⁺ release in both the first and second phases of release (results not shown).

DISCUSSION

Muscle can be homogenized to give a preparation of sealed vesicles derived from the SR that can accumulate Ca^{2+} from the external medium in the presence of ATP. The population of vesicles is heterogeneous, and can be separated into light, intermediate and heavy fractions by sucrose-gradient centrifugation (Meissner, 1975). It is believed that the light SR fraction is derived from the longitudinal SR and the heavy SR fraction from the terminal SR (Meissner, 1975; Campbell, 1986). The relative proportions of these fractions in a preparation of SR vesicles depend on the centrifugation procedures used in the preparation: the relatively slow spins that we utilize give a preparation that is composed predominantly of intermediate and heavy SR with little light SR (Table 1). As shown in Table 1, the protein compositions of the SR fractions differ, the content of calsequestrin being greatest in the heavy SR fraction, as reported by Meissner (1975). Meissner (1984) has shown that the various SR fractions also differ in their rates of Ca^{2+} efflux, fastest release being observed from the heaviest fractions. Our preparation of SR is comparable with that used by Meissner (1984) to obtain 'fast-release' fractions of SR.

It is well established that, following uptake of Ca^{2+} , SR vesicles will spontaneously release a fraction of the accumulated Ca^{2+} (Ohnishi & Ebashi, 1963; Nakamaru & Schwartz, 1970; Van Winkle, 1976; Sorenson & de Meis, 1977; Entman *et al.*, 1979; Gerdes & Møller, 1983). As shown in Fig. 1, this spontaneous release occurs in two phases, an initial slow phase followed by a faster second phase. It was our aim in the studies reported here to characterize the properties of these two phases of release.

It can be expected that Ca^{2+} will be released from SR when the ATP in the external medium is exhausted (Hasselbach, 1979). Therefore we measured ATP concentrations in the external medium in parallel with measurements of Ca^{2+} uptake. As shown in Figs. 4 and 5, there is a good correlation between the start of the second phase of Ca^{2+} release and the decrease in ATP concentrations to low levels ($5 \mu\text{M}$ or less), at various pH values, temperatures and ionic conditions. It is clear, however, that the initial slower phase of Ca^{2+} release occurs while the concentration of ATP in the external medium is high. If the second phase of release indeed corresponds to Ca^{2+} efflux after the ATP has become exhausted, then it would be expected that the time to the second phase of release would increase with increasing ATP concentration. This is, in fact, what is observed (Fig. 6). Gerdes *et al.* (1983) have made a similar observation but in the presence of an ATP-regenerating system. As described below, the pattern of Ca^{2+} release is affected by the presence of maleate in the buffer (Fig. 2). With increasing concentration of maleate, the time to the second phase of release is seen to decrease (Fig. 2): the decrease in the time to the second phase of release with increasing maleate concentration is correlated with an increase in ATPase activity (results not shown). We conclude, therefore, that the second phase of release does indeed correspond to release under conditions where ATP is exhausted.

Unfortunately, the use of an ATP-regenerating system to maintain a constant ATP concentration creates problems because of the P_i generated (Fig. 9). The

pattern of uptake and release of Ca^{2+} seen in the presence of an ATP-regenerating system is distinctly different to that seen in its absence, initial uptake of Ca^{2+} being followed by a second phase of uptake. As shown in Fig. 9, the addition of P_i to the system also results in an increased extent of uptake. The effect of P_i has been attributed to the precipitation of $\text{Ca}_3(\text{PO}_4)_2$ within the SR vesicles (Weber *et al.*, 1964; Mermier & Hasselbach, 1976). Lau (1983), however, has shown that removal of ADP by the regenerating system also has important effects on the pattern of Ca^{2+} uptake. We have shown that addition of ADP diminishes the overshoot in Ca^{2+} uptake seen at pH 6.0, and decreases the extent of Ca^{2+} uptake (results not shown). These effects of ADP are discussed in more detail in Gould *et al.* (1987).

It is known that, under the appropriate conditions, the Ca^{2+} + Mg^{2+} -activated ATPase can be driven backwards, moving Ca^{2+} out of the SR with the formation of ATP from ADP and P_i (de Meis, 1981). For the following reasons such a mechanism is unlikely to explain the Ca^{2+} efflux observed here. First, Ca^{2+} efflux occurs when the external Ca^{2+} concentration is high, but external Ca^{2+} is known to inhibit reversal of the ATPase (de Meis, 1981). Secondly, addition of ADP to the system does not increase the rate of Ca^{2+} release (results not shown), and a similar pattern of spontaneous release is seen with either ATP or acetyl phosphate (Fig. 8), showing that ADP is not important. Lastly, Ca^{2+} release occurs in the presence of ATP (Figs. 4, 5 and 7), whereas ATP inhibits reversal of the ATPase (de Meis, 1981).

Apart from reversal of the ATPase, a number of other possible mechanisms have been suggested for the initial phase of Ca^{2+} release observed when ATP is still present in the system and for the second phase of release when ATP is exhausted (Gerdes *et al.*, 1983; Gerdes & Møller, 1983; Chu *et al.*, 1983a,b; Morii *et al.*, 1985; Meszaros & Ikemoto, 1985). In McWhirter *et al.* (1987) we have suggested that the efflux of Ca^{2+} observed from SR vesicles passively loaded with Ca^{2+} is mediated by the ATPase. If this is indeed the case, then it would seem likely that the two phases of spontaneous release of Ca^{2+} observed from actively loaded SR vesicles are also mediated by the ATPase. Spontaneous release of Ca^{2+} would then be expected to share a number of properties in common with ATPase activity. Since it is known that the ATPase is affected by temperature (Hidalgo, 1985), we studied the effect of temperature on Ca^{2+} release. Fig. 3 shows that the rates of Ca^{2+} release decrease with decreasing temperature both in the initial release phase in the presence of ATP and in the second phase, where ATP is exhausted. The effects of temperature were fully reversible. Plots of Ca^{2+} uptake and release show discontinuities at 15–20 °C, which can be compared with discontinuities in ATPase activity at approx. 22 °C (Inesi *et al.*, 1973; Lee *et al.*, 1974). Although discontinuities in Arrhenius plots of ATPase activity were initially attributed to phase changes in the phospholipid component of the SR membrane (Inesi *et al.*, 1973; Lee *et al.*, 1974), it now seems more likely that they correspond to conformational changes in the ATPase itself (see Hidalgo, 1985). The similar temperature profiles of Ca^{2+} uptake, Ca^{2+} efflux and ATPase activity are consistent with the ATPase itself mediating Ca^{2+} efflux.

As shown in Fig. 4, the rate of Ca^{2+} release and the maximal extent of Ca^{2+} accumulation both decrease

with increasing pH, as reported by Nakamaru & Schwartz (1970) and Sorenson & de Meis (1977). Berman *et al.* (1977) claimed that incubation of SR vesicles at pH 5.5–6 led to irreversible inactivation. We find, however, that effects of low pH are completely reversible in agreement with Tate *et al.* (1981). Tate *et al.* (1981) also found that incubation of SR vesicles at high pH led to irreversible inhibition of Ca²⁺ uptake, if the vesicles were incubated in the absence of Ca²⁺; in the presence of Ca²⁺ only slight inhibition was observed. We find that, under the conditions of our experiments, the effects of high pH are reversible. Importantly, the effects of pH occur over the same range as do effects of pH on the binding of Ca²⁺ to the sites of high and low affinity on the E1 and E2 conformations of the ATPase respectively (Gould *et al.*, 1986), consistent with the suggestion that these same Ca²⁺-binding sites are involved in Ca²⁺ efflux.

As reported by Sorenson & de Meis (1977) and Chu *et al.* (1983a,b), the pattern of Ca²⁺ uptake and release is markedly affected by the anions present in the system. As shown in Fig. 2, the addition of maleate increases the maximal extent of Ca²⁺ accumulation, increases the rate of Ca²⁺ release and, by increasing ATPase activity, decreases the time to the second phase of release; as a consequence, spontaneous release of Ca²⁺ is much more distinct in the presence of maleate than in its absence. As reported by Chu *et al.* (1983b), succinate has effects comparable with those of maleate (results not shown). In the following paper (Gould *et al.*, 1987) we show that the effects of maleate can be understood in terms of effects on the concentration of free Ca²⁺ within the SR.

The rate of Ca²⁺ efflux is only slightly affected by external Ca²⁺ concentration (results not shown) and occurs at Ca²⁺ concentrations where the Ca²⁺-binding sites on the E1 conformation of the ATPase will be saturated (Gould *et al.*, 1986). Similarly, it was shown by Meissner (1984) that Ca²⁺ efflux will occur from SR vesicles passively loaded with Ca²⁺ when the external Ca²⁺ concentration is high. Gerdes & Møller (1983) have argued that this demonstrates that the Ca²⁺-binding sites on the Ca²⁺ + Mg²⁺-activated ATPase cannot be involved in release. However, it is known that, on phosphorylation of the ATPase and transformation into the E2 form, the Ca²⁺-binding sites change from high to low affinity (Gould *et al.*, 1986). In the E1–E2 change, the Ca²⁺-binding sites, as well as transforming from high to low affinity, also change their orientation from facing the external medium to facing the interior of the SR vesicle. We have shown that Ca²⁺ efflux from passively loaded vesicles can be simulated if a conformation of the ATPase is proposed in which the Ca²⁺-binding sites have the same affinity as in E2 but are outward-facing rather than inward-facing (McWhirter *et al.*, 1987). The evidence presented here is, we believe, also consistent with the ATPase being involved in both the initial slow phase and the faster second phase of spontaneous Ca²⁺ release from actively loaded vesicles. In the following paper (Gould *et al.*, 1987) we show that release can indeed be simulated in terms of our previously developed models for Ca²⁺ uptake (Gould *et al.*, 1986; Stefanova *et al.*, 1987) and Ca²⁺ release (McWhirter *et al.*, 1987).

During uptake and release of Ca²⁺ by SR vesicles, counter-movement of other ions is necessary to prevent the build-up of an inhibitory membrane potential across the SR membrane. Experiments have shown that the SR membrane contains efficient channels for, for example,

K⁺ and Cl[−], and that these prevent the build-up of any but a transient membrane potential (Meissner, 1983). Madeira (1978) has, however, suggested that the SR membrane is impermeant to H⁺ and that an H⁺ gradient forms during ATP hydrolysis; such a gradient could have significant effects on Ca²⁺ fluxes. The possible effects of charge and H⁺ gradients were tested by measuring Ca²⁺ uptake and release in the presence of NO₃[−] (100 mM) as lipid-soluble anion and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (7.5 μM) to collapse any H⁺ gradients: these had no effects on Ca²⁺ uptake and release (results not shown), showing that, if any charge or H⁺ gradients formed, they were too transient to have any significant effects.

In summary, we believe that the pattern of Ca²⁺ release observed from SR vesicles actively loaded with Ca²⁺ is consistent with release being mediated by the Ca²⁺ + Mg²⁺-activated ATPase. In the following paper (Gould *et al.*, 1987) we show that Ca²⁺ uptake and release by SR vesicles can be simulated in terms of models of ATPase activity (Gould *et al.*, 1986; Stefanova *et al.*, 1987) and Ca²⁺ release (McWhirter *et al.*, 1987).

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