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SYNTHESIS OF ATP COUPLED TO Ca^{2+} RELEASE FROM SARCOPLASMIC RETICULUM VESICLES

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SUMMARY

Ca^{2+} release from sarcoplasmic reticulum vesicles was enhanced by the addition of low concentrations of ADP and P_i. The effect of ADP and P_i was abolished in the presence of Mg^{2+}. These findings lead to a study of ATP synthesis coupled with Ca^{2+} release by ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid. It was demonstrated that one molecule of ATP is synthesized per two Ca^{2+} released from the sarcoplasmic reticulum. The high ratio of ATP synthesized per each Ca^{2+} released indicates that most of the Ca^{2+} leaves the vesicles through the pump system.

INTRODUCTION

Many biological membranes carry out an active transport of cations and create a large concentration gradient using the energy of ATP[1]. The chemiosmotic hypothesis predicts that these membranes would also be capable of using the osmotic potential for ATP synthesis during cation release[2]. Chemiosmotic syntheses of ATP were demonstrated with both chloroplast preparations, using a H^+ gradient[3], and erythrocyte ghosts, using a K^+ gradient[4]. The question whether the Ca^{2+} pump of muscle will also confirm the prediction of the chemiosmotic hypothesis is of a particular physiological importance. The contraction–relaxation cycle of muscle is mediated through changes in Ca^{2+} concentration within the muscle cells[5]. In skeletal muscle a nerve impulse causes release of Ca^{2+} from a special membrane structure known as the sarcoplasmic reticulum[5]. Although Ca^{2+} release is the key step that changes Ca^{2+} concentration in muscle, relatively few studies were directed to elucidating the mechanism that controls the release of Ca^{2+} from the sarcoplasmic reticulum vesicles. It seemed especially pertinent to test whether the sarcoplasmic reticulum can utilize the Ca^{2+} gradient and synthesize ATP during Ca^{2+} release. The vesicular membranes derived from the sarcoplasmic reticulum of skeletal muscle can be isolated in a pure state. The only function known to be located in these vesicles is the ATP-driven Ca^{2+} pump[6]. With the aid of such Ca^{2+} precipitating agents as oxalate and phosphate, large quantities of Ca^{2+} can be stored in the vesicles, thereby creating a concentration gradient of several thousand[7]. By the addition of ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), a specific chelator for Ca^{2+},

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

a steep concentration gradient would be maintained even when large quantities of Ca^{2+} are released. This work demonstrates that indeed stoichiometric amounts of ATP are synthesized during Ca^{2+} release from sarcoplasmic reticulum vesicles, indicating that most of the Ca^{2+} leaves *via* the Ca^{2+} pump. While this manuscript was in preparation MAKINOSE AND CO-WORKERS[7,8] published preliminary notes with some findings similar to those in the present paper.

MATERIALS AND METHODS

Isolation of sarcoplasmic reticulum vesicles and determination of ATPase activity were carried out as previously described[9].

Determination of Ca^{2+} uptake

Ca^{2+} uptake assay was performed employing filtration through Millipore filters, essentially as described by MARTONOSI AND FERETOS[10]. The reaction system contained: ATP, 4.5 mM; $MgCl_2$, 5 mM; imidazole chloride buffer (pH 7.0), 5 mM;KCl, 100 mM; phosphoenolpyruvate, 4 mM; pyruvate kinase, 75 μg/ml; $^{45}CaCl_2$, 0.1 mM containing $2 \cdot 10^4$ counts/min per ml. When indicated, oxalate was added to give a final concentration of 5 mM. Incubation was at 30°. The reaction was initiated by the addition of sarcoplasmic reticulum 50–500 μg protein/ml and was terminated by filtration of 0.2-ml aliquots through 0.45 μm Millipore filters using the 13-mm Swinnex Millipore filter holders. Ca^{2+} uptake was calculated from the difference in radioactivity between the unfiltered reaction mixture and the Millipore filtrate.

Determination of Ca^{2+} release

Ca^{2+} release was initiated by the addition of EGTA in large excess of the total Ca^{2+} in the system, 0.5–5 mM. The Millipore filter technique and determination of Ca^{2+} radioactivity in the filtrate were as described under *Determination of Ca^{2+} uptake*. In experiments in which ATP synthesis was measured, phosphoenolpyruvate and pyruvate kinase were not added to the Ca^{2+} uptake reaction mixture.

Purification of sarcoplasmic reticulum on an anion-exchange column

Sarcoplasmic reticulum was purified from the nucleotide, phosphate and oxalate of the Ca^{2+} uptake reaction mixture on a Dowex-1 column. A membrane preparation that had accumulated more than 95 % of the total Ca^{2+} in the system in the presence of 5 mM oxalate was put on a 1cm × 2 cm column of Dowex AG 1 × 8 (100–200 mesh). Chromatography was carried out at 4°. The cloudy membrane fraction, usually of 5 ml, was collected within 1–2 min with essentially complete removal of ATP, ADP, and P_i. The accumulated Ca^{2+} was not released during the procedure, as the Millipore filtrates before and after the chromatography contained identical amounts of Ca^{2+}.

Measurements of $^{32}P_i$ incorporation into ATP during Ca^{2+} release

Ca^{2+} uptake was carried out in a system containing sarcoplasmic reticulum, 0.3–1 mg/ml; ATP, 4.5 mM; $MgCl_2$, 5 mM; KCl, 100 mM; imidazole chloride buffer (pH 7.0),5 mM; $CaCl_2$. 0.2 mM. Incubation time was 30 min at 30° leading to Ca^{2+} uptake of more than 95 % of the total Ca^{2+} in the system and hydrolysis of at least 99 % of the ATP in the system. Ca^{2+} release was initiated by the addition of EGTA to give a final concentration of 1 mM and $^{32}P_i$ to give a specific radioactivity of 10^5 counts/min per μmole, glucose, 5 mM and hexokinase, 50 μg/ml. Incubation was for 30 min at 30°. The reaction was terminated by the addition of an equal volume

of 10 % trichloroacetic acid and an aliquot of the supernatant was taken for extraction of P_i by the method of AVRON[11]. Extraction of the trichloroacetic acid supernatant with diethyl ether and electrophoresis for 4 h at 20 V/cm in a system of 9.5 % butyric acid and 0.1 % NaOH (ref. 12) established that all the organic phosphate was located in a spot with the electrophoretic mobility of glucose 6-phosphate.

ATP and ADP were products of Waldhof Co. Carrier free $^{32}P_i$ was obtained from the Nuclear Research Center, Negev, Israel. Phosphoenolpyruvate was synthesized according to the method of CLARK AND KIRBY[13] and was converted to a potassium salt. Pyruvate kinase hexokinase and glucose-6-phosphate dehydrogenase were obtained from Boehringer Co. Analytical grade Dowex 1 × 8 (Cl^-form; (100–200 mesh) was a Bio-Rad product. and a non-ionic detergent Nonidet P40 (a condensation product of ethylene oxide) was obtained from B.D.H.

RESULTS

Effect of EGTA on Ca^{2+} release from sarcoplasmic reticulum vesicles

Increasing the concentration of EGTA in the system causes a faster rate of Ca^{2+} release from sarcoplasmic reticulum vesicles (Fig. 1). It is also demonstrated that the vesicles purified from ATP, ADP, P_i and oxalate of a Dowex-1 column release Ca^{2+} at a much faster rate than do the unpurified vesicles. As EGTA is present

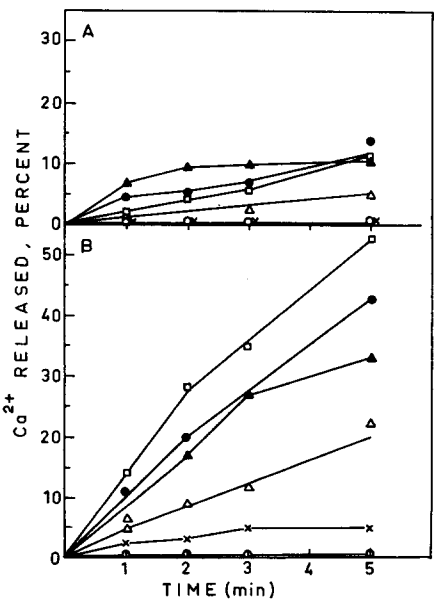


Fig. 1. [Effect of EGTA on Ca^{2+} release from sarcoplasmic reticulum vesicles. Ca^{2+} uptake was carried out in the standard Ca^{2+} uptake system in the presence of 5 mM oxalate and 0.3 mg/ml reticulum protein. After 90% of the Ca^{2+} was taken up by the vesicles (0.6 μmole Ca^{2+} per mg protein) part of the system was purified from ADP, ATP, P_i and oxalate on a Dowex-1 column (see MATERIALS AND METHODS). A. Unpurified sarcoplasmic reticulum. B. Sarcoplasmic reticulum purified on Dowex-1 column. At zero time EGTA was added to the system to give the following concentrations: ○—○, no EGTA; ×—×, 0.05 mM; △—△, 0.1 mM; ▲—▲, 0.5 mM; ●—●, 1 mM; □—□, 5 mM. Ca^{2+} release was measured as described under MATERIALS AND METHODS.

TABLE I

THE EFFECT OF ADP AND P_i ON Ca^{2+} RELEASE FROM SARCOPLASMIC RETICULUM

Ca^{2+} uptake and purification of the Ca^{2+}-loaded sarcoplasmic reticulum on Dowex-1 column were carried out as described under MATERIALS AND METHODS. The purified sarcoplasmic reticulum vesicles containing 0.3 μmole Ca^{2+} per mg protein were suspended in 50 mM imidazole–chloride buffer (pH 7.0) to give a concentration of 0.1 mg protein per ml. At zero time EGTA and EDTA were added to give final concentrations of 0.5 and 5 mM, respectively. When inidcated, ADP and P_i were added with the EGTA and EDTA. Ca^{2+} release is expressed as a percent of total Ca^{2+} in the system.

Time (min)	*Ca^{2+} released (%)*						
	No addition	*+ 1 μM P_i*	*+10 μM P_i*	*+ 1 μM ADP*	*+ 10 μM ADP*	*+ 10 μM ADP and 10 μM P_i*	*+10 μM ATP*
1	17	21	35	38	28	32	19
2	29	36	44	44	37	44	29
3	39	41	48	48	49	59	41

TABLE II

THE EFFECT OF Mg^{2+} ON Ca^{2+} RELEASE FROM SARCOPLASMIC RETICULUM VESICLES

The experimental conditions are those of Table I. When indicated, Mg^{2+} was added in 5 mM excess of the EDTA in the system.

Time (min)	*Ca^{2+} released (%)*							
	No addition	*+5 mM $MgCl_2$*	*+20 μM ADP*	*+20 μM ADP and 5 mM $MgCl_2$*	*+20 μM P_i*	*+ 20 μM P_i and 5 mM $MgCl_2$*	*+ 0.5 mM ATP*	*+ 0.5 mM ATP and 5 mM $MgCl_2$*
2	17	6	29	7	29	7	18	6
5	33	11	43	16	42	19	35	11

in the system in a large excess of Ca^{2+}, it is possible that part of its effect is due to chelation of Mg^{2+} (*cf.* ref. 14).

Effect of ADP and P_i on Ca^{2+} release in the presence and absence of Mg^{2+}

When Mg^{2+} is chelated by 5 mM EDTA, the addition of 1 μM ADP or 10 μM P_i causes a 2-fold increase in the initial rates of Ca^{2+} release during the first minute (Table I). The effect of P_i and ADP is not additive; addition of both ADP and P_i does not increase the rate of Ca^{2+} release over that achieved by either one separately. ATP fails to cause a significant increase in Ca^{2+} release. If Mg^{2+} is added in 5 mM excess over EDTA, no effect of ADP and P_i can be demonstrated and the rate of Ca^{2+} release is greatly reduced (Table II).

Incorporation of $^{32}P_i$ into ATP during Ca^{2+} release

The addition of 1 mM EGTA to sarcoplasmic reticulum vesicles loaded with Ca^{2+} phosphate results in an incorporation of $^{32}P_i$ into ATP (Table III). Detergent added to the system either prior to or after the uptake of Ca^{2+} completely abolished

the incorporation of $^{32}P_i$ into ATP, indicating that this phenomenon is dependent on the integrity of the vesicles during Ca^{2+} release. When Ca^{2+} accumulation was carried out in the presence of oxalate a lower yield of $^{32}P_i$ incorporation into ATP was observed. Therefore all further experiments were carried out in the absence of oxalate. The incorporation of $^{32}P_i$ into ATP is in good correlation with the net increase in ATP measured with the aid of hexokinase and glucose-6-phosphate dehydrogenase (Table IV). The results indicate that the incorporation of $^{32}P_i$ into ATP represents a net synthesis of ATP and not an exchange reaction between $^{32}P_i$ and ATP.

Incorporation of $^{32}P_i$ into ATP at various concentrations of $CaCl_2$

The rate and total yield of $^{32}P_i$ incorporation into ATP as a function of initial

TABLE III

THE EFFECT OF DETERGENT ON $^{32}P_i$ INCORPORATION INTO ATP

Ca^{2+} uptake was carried out in a system containing 1 mg/ml of sarcoplasmic reticulum protein. $^{32}P_i$ incorporation, Ca^{2+} uptake, and ATPase activity were determined as described under MATERIALS AND METHODS. When indicated, Nonidet P40 was added to give a final concentration of 1 mg/ml. Ca^{2+} uptake and ATPase activity are expressed as a percent of the activity in the system to which detergent was not added. Under the experimental conditions essentially all the Ca^{2+} has been taken up. 0.2 μmole Ca^{2+} per mg of sarcoplasmic reticulum protein. The Ca^{2+} storing capacity under these conditions (measured at a lower protein concentration) is 0.4 μmole Ca^{2+} per mg protein.

Treatment	*Ca^{2+} uptake* (%)	*ATPase activity* (%)	*$^{32}P_i$ incorporation into ATP* (*μmole/mg protein*)
None	100	100	0.104
Nonidet P40 prior to Ca^{2+} uptake	0	113	0.002
Nonidet P40 prior to Ca^{2+} release	100	108	0.001

TABLE IV

THE CORRELATION BETWEEN $^{32}P_i$ INCORPORATION AND GLUCOSE 6-PHOSPHATE FORMATION AT VARIOUS Ca^{2+} CONCENTRATIONS

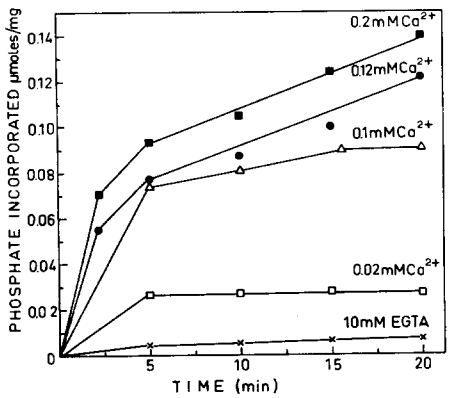
The experimental conditions and $^{32}P_i$ incorporation were those described under MATERIALS AND METHODS using 0.7 mg/ml sarcoplasmic reticulum protein. Glucose 6-phosphate was determined by coupling with glucose-6-phosphate dehydrogenase (40 μg/ml) NADP (7 mM), and Mg^{2+} (10 mM). The amount of glucose 6-phosphate was calculated from the absorption of NADPH at 340 mM.

Ca^{2+} concn. during uptake (*mM*)	*Glucose 6-phosphate formed** (*μmole/mg protein*)	*$^{32}P_i$ incorporation* (*μmole/mg protein*)
0.05	0.101	0.090
0.1	0.140	0.150
0.2	0.184	0.187

* In the absence of EGTA, 0.2 μmole glucose 6-phosphate per ml were formed. This blank amount was subtracted from the amount formed in the presence of EGTA.

Ca^{2+} concentration during uptake is shown in Fig. 2. At a higher initial Ca^{2+} concentration, leading presumably to a greater concentration of Ca^{2+} inside the vesicles, a higher yield of $^{32}P_i$ incorporation into ATP is achieved. Addition of EGTA at a concentration of 10 mM before initiation of Ca^{2+} uptake prevents Ca^{2+} accumulation and accordingly abolishes $^{32}P_i$ incorporation into ATP (Fig. 2).

Ca^{2+} concentration equal to, or slightly greater than, the total capacity for Ca^{2+} accumulation in this system gives rise to the highest yield of $^{32}P_i$ incorporation into ATP. A further increase in Ca^{2+} concentration results in an inhibition of $^{32}P_i$ incorporation into ATP (Fig. 3).



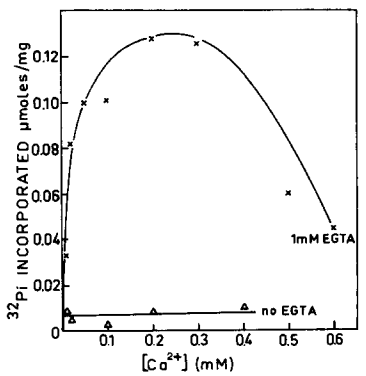


Fig. 2. Incorporation of $^{32}P_i$ into ATP at various concentrations of $CaCl_2$. Ca^{2+} uptake was carried out in the same system as described under MATERIALS AND METHODS, using 0.7 mg/ml sarcoplasmic reticulum and the indicated initial concentrations of $CaCl_2$. Incubation was 5 min at 30°. Initiation of Ca^{2+} release, termination of the reaction, and determination of $^{32}P_i$ incorporation into ATP were the same as described under MATERIALS AND METHODS, except that Ca^{2+} release was carried out at 20°.

Fig. 3. Incorporation of $^{32}P_i$ into ATP at various concentrations of $CaCl_2$. The experimental conditions were those described in Fig. 2, except that $^{32}P_i$ incorporation was determined after 30 min of Ca^{2+} release. The Ca^{2+} storing capacity of the sarcoplasmic reticulum preparation tested under these conditions was 0.2 µmole Ca^{2+} per mg protein.

The stoichiometry between Ca^{2+} release and $^{32}P_i$ incorporation into ATP

Previous work had shown that for each molecule of ATP that is hydrolyzed, two Ca^{2+} are translocated into the sarcoplasmic reticulum vesicles[7]. It was therefore of interest to measure the stoichiometry between Ca^{2+} release and $^{32}P_i$ incorporation into ATP. At various concentrations of sarcoplasmic reticulum, a release of an average of 2.2 Ca^{2+} results in an incorporation of one molecule of $^{32}P_i$ into ATP (Fig. 4). This shows that most of the Ca^{2+} is released from the vesicles through the ATP-driven Ca^{2+} pump. Thus the energy used to create the osmotic potential is highly preserved by the sarcoplasmic reticulum vesicles. Incorporation of $^{32}P_i$ into ATP during Ca^{2+} release can be demonstrated with all sarcoplasmic reticulum preparations that are capable of Ca^{2+} uptake. The yield of $^{32}P_i$ incorporation into ATP is directly related to the Ca^{2+} storing capacity under the experimental conditions. An aged sarcoplasmic reticulum preparation that stores 0.15 µmole Ca^{2+} per mg protein gives rise to one-third of the $^{32}P_i$ incorporation into ATP of that of a preparation that stores 0.40 µmoles Ca^{2+} per mg protein (Fig. 5).

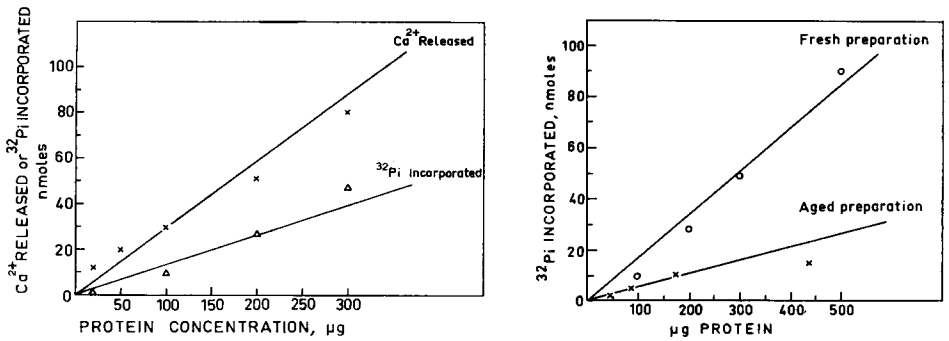


Fig. 4. The stoichiometry between Ca^{2+} release and $^{32}P_i$ incorporation into ATP. The experimental conditions are those as described under MATERIALS AND METHODS with the indicated concentrations of sarcoplasmic reticulum protein. Ca^{2+} release was determined as described under MATERIALS AND METHODS. The specific radioactivity of Ca^{2+} was determined by measuring total Ca^{2+} concentration by atomic absorption spectrometry.

Fig. 5. $^{32}P_i$ incorporation into ATP by sarcoplasmic reticulum preparations with different Ca^{2+} storing capacities. The experimental conditions of Ca^{2+} uptake and $^{32}P_i$ incorporation into ATP are those described under MATERIALS AND METHODS. ×—×, an aged sarcoplasmic reticulum preparation stored at −20° for 6 months had a Ca^{2+} storing capacity of 0.15 µmole Ca^{2+} per mg protein: ○—○, sarcoplasmic reticulum preparation stored at −20° for 1 month had a Ca^{2+} storing capacity of 0.4 µmole Ca^{2+} per mg protein.

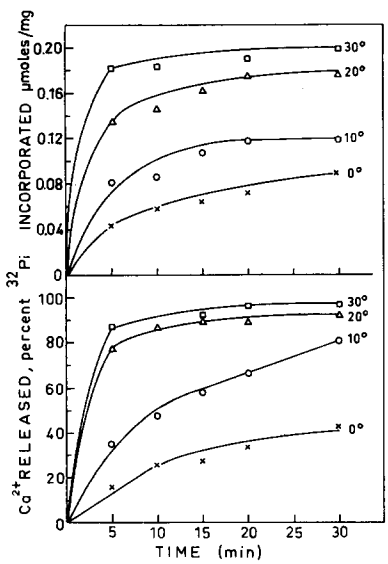


Fig. 6. The effect of temperature on the release of Ca^{2+} and incorporation of $^{32}P_i$ into ATP. Ca^{2+} uptake was carried out as described under MATERIALS AND METHODS, using a concentration of 0.5 mg sarcoplasmic reticulum per ml. During the uptake period the sarcoplasmic reticulum vesicles had accumulated 0.4 µmole Ca^{2+} per mg protein. Aliquots were then transferred to separate tubes and Ca^{2+} release was initiated at the indicated temperatures. Ca^{2+} release was determined as described under MATERIALS AND METHODS and the release of Ca^{2+} is expressed as a percent of total Ca^{2+} in the system.

The effect of temperature on $^{32}P_i$ incorporation into ATP

Ca^{2+} release and $^{32}P_i$ incorporation can be demonstrated at temperatures between 0–30° (Fig. 6). Increased temperatures result in higher rates of Ca^{2+} release and $^{32}P_i$ incorporation in a parallel manner. Under the experimental conditions of this work, part of the Ca^{2+} is in the form of calcium phosphate precipitate[15]. From the data of Figs. 4 and 6, it must be realized that both the free and the phosphate precipitated Ca^{2+} can be used for the generation of ATP during Ca^{2+} release.

DISCUSSION

The sarcoplasmic reticulum system is visualized by some investigators as a cation exchanger that binds Ca^{2+} more strongly in the presence of ATP[5,17]. The other mechanism that was put forth by HASSELBACH[6] describes the Ca^{2+} uptake as an active transport process. The results of the present work indicate that the amount of $^{32}P_i$ incorporation into ATP greatly exceeds the stoichiometric amount of phosphoprotein that can be formed in the system[18]. It is therefore much more difficult to ascribe a net synthesis of ATP to the mere dissociation of bound Ca^{2+}. Two lines of evidence support a chemio-osmotic mechanism for ATP synthesis coupled with Ca^{2+} release. The first is the decisive role played by the structural integrity of the vesicular membranes in ATP synthesis (*cf.* Table III). The second is based on the finding that Ca^{2+} accumulating inside the vesicles as an osmotically inert calcium phosphate precipitate[15] can also serve as a driving force for ATP synthesis (Fig. 6). Therefore, the sarcoplasmic reticulum has the advantage of being able to store a large osmotic potential in the form of an osmotically inert calcium phosphate precipitate. Addition of EGTA, in turn, releases Ca^{2+} gradually and gives very high yields of ATP synthesis. The sarcoplasmic reticulum seems to be unique in that it demonstrates the potential energy in an apparently osmotically inert calcium phosphate precipitate. This system seems to be superior to other ion transport systems with respect to the yield of ATP and the simplicity of conditions under which it is demonstrated. The high ratio of ATP synthesized per Ca^{2+} released indicates that most of the Ca^{2+} leaves the vesicles through the pump system. It is therefore possible that such a release mechanism also operates *in vivo*.

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