A Phosphorylated Conformational State of the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase of Fast Skeletal Muscle Sarcoplasmic Reticulum Can Mediate Rapid Ca\(^{2+}\) Release*

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A rapid Ca\(^{2+}\) release from Ca\(^{2+}\)-loaded sarcoplasmic reticulum vesicles from fast skeletal muscle can be induced under conditions which permit the formation of a stable phosphorylated intermediate of the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase. Such a state can be achieved experimentally by phosphorylating the ATPase in the absence of Mg\(^{2+}\) ions, which otherwise would stimulate the dephosphorylation step(s). Also, quercetin stimulates the rapid release of Ca\(^{2+}\) if used in the concentration range which does not cause inhibition of phosphoenzyme formation but which inhibits phosphoenzyme dephosphorylation. The rapid efflux of Ca\(^{2+}\) ions proceeds as long as the low affinity Ca\(^{2+}\)-binding sites facing the lumen of the vesicles are saturated and as long as Ca\(^{2+}\) is removed from the catalytic sites facing the cytosol. A molecular mechanism of the phosphoenzyme-mediated Ca\(^{2+}\) release is proposed. This mechanism is based on a rapid shutting of the ATPase molecules between an ADP-sensitive and an ADP-insensitive phosphorylated state.

SR\(^{1}\) membranes are responsible for the fast regulation of cytosolic Ca\(^{2+}\) concentrations in skeletal muscle cells, and, hence, of the contraction-relaxation state of the fibers. The mechanism of the active Ca\(^{2+}\) translocation from the cytosol into the lumen of the SR membrane has been thoroughly investigated and several molecular aspects of the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase responsible for this reaction have been clarified (for a review, see Refs. 1 and 2). On the other hand, the sequence of events which couple the action potential at the sarcolemma to the massive Ca\(^{2+}\) release from SR is still poorly understood. It was soon recognized that the various steps involved in the active Ca\(^{2+}\) transport cycle are fully reversible (3, 4) and that, under particular conditions, a rapid efflux of Ca\(^{2+}\) ions coupled to the synthesis of ATP can be induced. However, such a rapid Ca\(^{2+}\) release is not likely to occur under physiological conditions (5). Many other conditions capable of releasing Ca\(^{2+}\) from Ca\(^{2+}\)-loaded fragmented SR vesicles have been described (6–9; for a review, see Ref. 10), but their physiological significance still remains dubious. In addition, no instance has the mechanism of the observed rapid release been clarified and it was never established which among the various SR proteins was responsible for the enhanced permeability to Ca\(^{2+}\) ions.

An interesting and possibly physiologically significant way of releasing Ca\(^{2+}\) was briefly described by Millman and Azari (11). They observed that the exposure of passively loaded SR vesicles to Mg\(^{2+}\)-free ATP induced a rapid transient release of Ca\(^{2+}\) ions. This observation implies that an ATP-binding protein, i.e. the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase or a kinase, might be involved in the process. In the present report, the ATP-induced Ca\(^{2+}\) release from fast skeletal muscle SR preparations was thoroughly characterized. It will be demonstrated that most of the observed release occurs through the ATPase molecules when they are in a phosphorylated state. The absence of Mg\(^{2+}\) is required to prolong the lifetime of the phosphorylated conformational state. On the basis of these results and of the known properties of the Ca\(^{2+}\)-pumping ATPase, a proposal for the observed rapid release of Ca\(^{2+}\) ions is made.

**MATERIALS AND METHODS**

SR membranes were isolated from white skeletal muscle of rabbit as previously described (12). Radioactively labeled [γ-32P]ATP and [14C]glucose were purchased from New England Nuclear. β,γ-methylenephosphate was obtained from Boehringer Mannheim and 8-bromo-ATP was from P. L. Biochemicals, Inc. Quercetin was purchased from Meck, Germany. All other reagents were of the best quality available.

Ca\(^{2+}\) uptake by SR membranes was measured with the Millipore filtration technique (13) using a cold isomotic solution containing 3 mM LaCl\(_3\) to quench the reaction (14).

The hydrolytic activity of the ATPase was studied either by a coupled enzyme assay with pyruvate kinase, lactate dehydrogenase, NADH, and phosphoenolpyruvate (15) or by determining the production of P, as described (16).

Ca\(^{2+}\) efflux from SR vesicles was investigated basically as described (11). SR vesicles were washed and resuspended (~10 mg/ml) in 100 mM KCl, 20 mM MOPS, pH 7.0, and 10 mM CaCl\(_2\). The vesicles were incubated overnight at 0 °C to allow full equilibration. Ca\(^{2+}\) release experiments were carried out at room temperature by diluting 40 times the loaded vesicles into a medium containing 100 mM KCl, 20 mM MOPS, pH 7.0, and 20 mM EGTA. When required, the dilution medium was supplemented with various amounts of nucleotides as indicated in the text. (The ADP contamination of the ATP containing solutions was routinely checked. Since ADP was found to inhibit the ATP-induced Ca\(^{2+}\) release, only solutions containing a ratio less than 0.01 of ADP to ATP were used.) At sequential intervals, aliquots were rapidly filtered and washed with a quenching solution consisting of 100 mM KCl, 20 mM MOPS, pH 7.0, and 3 mM LaCl\(_3\) (42).

The formation of acid-stable phosphorylated intermediate species of the ATPase was followed under the same conditions used for the Ca\(^{2+}\) release assay. Vesicles were loaded with cold CaCl\(_2\) overnight and the reactions were started by a 40-fold dilution into 100 mM KCl, 20 mM MOPS, pH 7.0, 20 mM EGTA, and various amounts of [γ-32P]ATP at room temperature. Phosphorylation reactions were stopped by the addition of cold trichloroacetic acid (final concentra-
tion was 8%). An automated procedure allowing experimentation with incubation times as low as 0.3 s (17) was also used to study the phosphoenzyme formation. The quenched samples were rapidly centrifuged and resuspended in the sample buffer for electrophoresis in order to separate the labeled ATPase from all other radioactive material (see below). After electrophoresis, the gels were dried and autoradiographed on x-ray-sensitive films (Kodak XS-5) and with an intensifying screen (KYOKKO HS) at -70 °C. The autoradiographed films were then analyzed by a densitometric technique. Alternatively, the gels were rapidly stained with trace amounts of Coomassie brilliant blue in 50% methanol and 10% trichloroacetic acid (15 min) and destained (15 min). The ATPase bands, which were readily visible, were then cut out and incubated for 1 h in the presence of 1 ml of 100 mM Tris-Cl, pH 9.0, and then analyzed for radioactivity content in a scintillation mixture. Measured radioactivity could be corrected for the amount of protein present either by determining the protein concentration in a small aliquot of the solubilized material prior to electrophoresis or by determining the amount of ATPase present on the stained gels by a densitometric technique.

Phosphorylated SR vesicles were prepared by an acidic electrophoretic system at 0 °C. The denatured phosphorylated samples were solubilized in 100 mM Li phosphate, pH 5.5, 20% glycerol, 0.001% bromphenol blue, and 1% Li dodecyl sulfate. The samples (~50 μg of protein) were loaded onto a 1-mm thick slab gel consisting of 5.5% acrylamide, 0.15% bisacrylamide, 100 mM Li phosphate, pH 5.5, 1.5% polyacrylamide, 0.1% Li dodecyl sulfate, 0.2% ammonium persulfate, and 0.0005% tetramethylthelenediamine. Electrophoresis was carried out at 60 mA for 1 h. After that time, the ATPase bands were clearly separated from other bands. The gels were cut to separate the ATPase from the gel front, which contained most of the radioactivity present in the form of 32P, and [γ-32P]ATP and then further processed as described above.

Protein concentration was determined by the Lowry procedure (18) with bovine serum albumin as standard. The concentration of free Ca2+ and Mg2+ in the solutions was calculated with a computer program (19), taking into account binding to EGTA and ATP and competition with other cations.

RESULTS

The dilution of Ca2+-loaded SR vesicles into an isosmotic medium containing EGTA produced a slow passive efflux of Ca2+ ions (Fig. 1A). However, if the dilution medium was supplemented with 1 mM ATP, a transient rapid Ca2+ release could be observed, as first reported by Millman and Azari (11) (Fig. 1A). Fig. 1A also shows that the ATP analog 8-Br-ATP and GTP, which usually display low affinity for the ATP-binding site, had little effect on the Ca2+ release, at a concentration of 1 mM. The use of a La3+-quenching solution in the study of Ca2+ release (14, 42) has permitted us to establish that the rapid initial efflux rate decreased with time and reached the value of the slow passive efflux within 10–15 s. The ATP-induced release was apparently specific and not linked to a general increase of membrane permeability, since the efflux kinetics of [14C]sucrose from passively loaded vesicles was not affected by the presence of ATP in the dilution medium (Fig. 1B). Thus, the observation suggested that SR membranes might contain an ATP-responsive Ca2+ channel.

Millman and Azari (11) recognized that the rapid Ca2+ release could not be explained by the reversal of the Ca2+ pump, caused by contaminating ADP in the ATP-containing solutions. The conditions used in the release experiments described here (i.e. absence of Mg2+ and P i, high ATP to ADP ratio) could not have supported rapid Ca2+ release coupled to ATP synthesis by the pump (3, 4). Indeed, the presence of 1 mM ADP instead of ATP in the release solution did not affect the Ca2+ release rate (Fig. 1A). On the contrary, ADP was found to be a potent inhibitor of the rapid Ca2+ release induced by ATP (see below).

In order to clarify the mechanism of the rapid ATP-induced Ca2+ release, it is important to ascertain whether nucleotide binding is sufficient to induce Ca2+ efflux, or whether enzymatic utilization of the substrate is required, either by the ATPase itself or by an accessory protein kinase. Therefore, it was investigated whether any protein of the SR membrane became labeled with 32P when rapid Ca2+ release occurred. Experiments were carried out under the conditions described in Fig. 1A except that [γ-32P]ATP was added to the dilution medium so that the formation of phosphoenzyme could be followed. The reaction was quenched in acid in sequential time intervals. Phosphoenzyme levels were determined as described under "Materials and Methods."

Fig. 1. Effect of ATP on SR permeability. A, effect of nucleotides on the Ca2+ release from SR vesicles. SR vesicles (about 10 mg/ml) were passively loaded with radioactively labeled Ca2+ by overnight incubation at 0 °C in 100 mM KCl, 20 mM MOPS, pH 7.0, and 10 mM 4CaCl2. Efflux experiments were initiated at room temperature by diluting the vesicles into 100 mM KCl, 20 mM MOPS, pH 7.0, 20 mM EGTA (O), and, when required, 1 mM ATP (C), or 1 mM ADP (A), or 1 mM 8-Br-ATP (■), or 1 mM GTP (□). The efflux was measured using the Millipore filtration technique as described under "Materials and Methods." B, effect of ATP on [14C]sucrose release from SR vesicles. Efflux experiments were carried out as described in A. The incubation and dilution media, however, were both supplemented with 10 mM sucrose. [14C]Sucrose was added to the incubation medium. Efflux was started upon dilution in the presence (C) or absence (O) of 1 mM ATP.

ATPase itself or by an accessory protein kinase, therefore, it was investigated whether any protein of the SR membrane become labeled with 32P when rapid Ca2+ release occurred. Experiments were carried out under the conditions described in Fig. 1A except that [γ-32P]ATP was added to the dilution medium and the vesicles were loaded with cold CaCl2. The results obtained could show that only one protein having Mr ~ 110,000 was labeled and that all radioactivity disappeared from it upon treatment with hydroxylamine (20). This indicated that an acylphosphate intermediate of the ATPase was formed during the rapid Ca2+ release. This phosphorylation reaction was absolutely Ca2+ dependent and occurred in spite of the presence of high EGTA concentrations in the [γ-32P] ATP-containing solution. This finding was not surprising since it was previously demonstrated that Ca2+ removal from
the high affinity binding sites on the ATPase is a relatively slow process with respect to the Ca\(^{2+}\) dependent formation of phosphoenzyme intermediate (14, 21, 22). Fig. 2 shows that, after dilution of Ca\(^{2+}\)-loaded vesicles into a medium containing EGTA and \([\gamma-\text{P}]\text{ATP}\), phosphoenzyme was rapidly formed up to maximal levels of 0.6-0.7 nmol/mg of protein (under optimal phosphorylating conditions, the levels of phosphoenzyme can be as high as 4-5 nmol/mg of protein). Thereafter, the phosphoenzyme slowly decayed with a half-time of approximately 4-6 s. This time dependency strongly suggested a correlation between the phosphoenzyme level and the rapid release of Ca\(^{2+}\). By means of \([\gamma-\text{P}]\text{ATP}\) chase experiments, it could be established that the phosphoenzyme was formed only once during the process and that no turnover of the ATPase occurred. It may be concluded, therefore, that under the specific experimental conditions which induced rapid Ca\(^{2+}\) release, a long-lived phosphoenzyme was formed. Detail studies were therefore carried out on the correlation of the two phenomena.

**ATP Dependence of Ca\(^{2+}\) Release and of Phosphoenzyme Levels**—The rate and total amount of Ca\(^{2+}\) rapidly released from loaded SR vesicles depended on the ATP concentration in the dilution medium. Fig. 3 shows that 120-150 µM ATP was required to induce half-maximal release rate, indicating that sites of relatively low ATP affinity were involved. It is known that the \(K_{n}\) (Mg-ATP) of the catalytic sites of the ATPase is, under optimal conditions, in the micromolar range (23), but that, in the absence of divalent cations, the affinity of these sites decreases drastically (24). Indeed, it is shown in Fig. 3 that half-maximal levels of phosphoenzyme, as measured under the conditions required to induce rapid Ca\(^{2+}\) release, were formed at relatively high ATP concentrations (\(K_{1/2} = 120-150 \mu M\)). The correlation between the ATP concentration dependence of the two reactions illustrated in Fig. 3 indicated strongly that Ca\(^{2+}\) release occurred through a mechanism mediated by phosphoenzyme formation or, at least, that ATP binding to the catalytic sites of the ATPase was required.

The effect of sequential EGTA and ATP additions on Ca\(^{2+}\) Release—Ca\(^{2+}\)-loaded SR vesicles were diluted in a release medium containing high EGTA and then, after a few seconds, ATP was added to the mixture. During the reincubation time in the presence of EGTA, all Ca\(^{2+}\) bound to the high affinity sites on the ATPase enzyme therefore could be completely removed. Even though no phosphoenzyme could be formed under these conditions (Fig. 4B), a small but significant rapid Ca\(^{2+}\) release from the vesicles was observed (Fig. 4A). These results indicated that a portion of the ATP-induced Ca\(^{2+}\) release occurred through a mechanism which did not require the formation of a phosphoenzyme. We investigated, therefore, the effect of the nonhydrolyzable ATP analog AMP-PCP on the Ca\(^{2+}\) release kinetics. Fig. 5 shows, indeed, that AMP-PCP also could induce a portion of the rapid Ca\(^{2+}\) release with a half-maximal effect in the concentration range between 100 and 150 µM. However, the amount of Ca\(^{2+}\) released was absolutely not dependent on whether the nucleotide was present in the EGTA-containing solution at zero dilution time, or whether it was added after 15 s, indicating that under both experimental conditions the release mechanism operated by AMP-PCP binding was optimally working. From these results, it is apparent that the rapid phase of the ATP-induced Ca\(^{2+}\) release (Fig. 4A) was composed of two distinct components. Part of the rapid release, which can be induced also by AMP-PCP, required only binding of the nucleotide to the catalytic sites. The amplitude of this component was dependent on the nucleotide concentration. The other rapid component was observed only under conditions which permitted the formation of a phosphorylated intermediate of the ATPase and was obviously absent when the nonhydrolyzable AMP-PCP was used instead of ATP. In this report, we have focused our attention on the latter component of the ATP-induced Ca\(^{2+}\) release, which we shall call phosphoenzyme-mediated Ca\(^{2+}\) release to distinguish it from the other component. In all experiments to be described below,

![Fig. 3. ATP concentration dependence of phosphoenzyme formation and Ca\(^{2+}\) release.](image)

![Fig. 4. Effect of sequential EGTA and ATP additions on Ca\(^{2+}\)-release (A) and phosphoenzyme level (B).](image)
the phosphoenzyme-independent rapid Ca\(^{2+}\) efflux was determined by adding ATP 15 s after dilution of the vesicles into the EGTA-containing solutions. The averaged rapid efflux portion at zero time was then subtracted from the rapid Ca\(^{2+}\) efflux data obtained when ATP was present at zero time. The resulting corrected curve was considered to represent phosphoenzyme-mediated Ca\(^{2+}\) release.

**Kinetic Analysis of the Phosphoenzyme-mediated Ca\(^{2+}\) Release**—In spite of the La\(^{3+}\)-quenching technique used in the efflux experiments, we were unable to resolve with sufficient accuracy the rapid transient phase of Ca\(^{2+}\) release induced by ATP to attempt a thorough kinetic analysis of the phenomenon. Moreover, phosphoenzyme-induced Ca\(^{2+}\) release curves were calculated as the difference between total ATP-induced and phosphoenzyme-independent Ca\(^{2+}\) release as shown in Fig. 4A, and this might introduce additional uncertainty. However, semilogarithmic plots of corrected phosphoenzyme-mediated Ca\(^{2+}\) release could still provide some qualitative information. Fig. 6 clearly illustrates, for instance, the transient character of the rapid phase, which then levels off to the normal slow passive rate. Interestingly, the approximate half-time of this rapid phase was in the range between 5 and 10 s and nicely correlates with the half-time of phosphoenzyme decay (Fig. 2).

**ADP Inhibition of Phosphoenzyme-mediated Ca\(^{2+}\) Efflux**—The rapid phosphoenzyme-induced Ca\(^{2+}\) release was inhibited by low amounts of ADP. When 100 \(\mu\)M ADP was added to the dilution medium, the rapid Ca\(^{2+}\) efflux was reduced to less than 50% (Fig. 7A). The inhibitory effect of ADP could be due to interference with the availability of phosphoenzyme. ADP could compete with ATP for the binding sites and prevent phosphoenzyme formation, or stimulate the decomposition of the phosphoenzyme (i.e. ADP-induced shift of the phosphorylation equilibrium \(E\cdot\text{ATP} \rightleftharpoons E \rightleftharpoons P + \text{ADP}\) in the direction of E-ATP) and thus close the pathway for Ca\(^{2+}\) efflux. The experiment presented in Fig. 7B is in favor of the first alternative. 100 \(\mu\)M ADP competed efficiently with 200 \(\mu\)M ATP for the binding sites on the ATPase, since it reduced quantitatively the maximal level of phosphorylation. This observation implied that the affinity of the catalytic sites for ADP is considerably higher than for ATP, in good agreement with the binding study of nucleotides to the ATPase in the absence of divalent cations (24).

According to the interpretation given here, it would follow that the addition of ADP after maximal levels of phosphoenzyme were reached should not affect the phosphoenzyme-mediated Ca\(^{2+}\) release mechanism. Indeed, this was shown by the ADP chase experiments illustrated in Fig. 9. 100 \(\mu\)M ADP, added 2 s after dilution of the Ca\(^{2+}\)-loaded vesicles into the ATP-containing medium, did not inhibit phosphoenzyme-mediated Ca\(^{2+}\) efflux (Fig. 9A), nor did it affect the dephosphorylation kinetics of the phosphoenzyme (Fig. 9B).

**Mg\(^{2+}\) Inhibition of Phosphoenzyme-mediated Ca\(^{2+}\) Efflux**—The presence of Mg\(^{2+}\) ions in the dilution medium prevented

![Image](http://www.jbc.org/)

**Ca\(^{2+}\) Release from SR Vesicles**

**Fig. 5. Effect of AMP-PCP on Ca\(^{2+}\) release.** Ca\(^{2+}\) release from SR vesicles loaded with 10 mM \(^{45}\)CaCl\(_2\) was measured as described in Fig. 1A. \(\times\), control passive efflux. Closed symbols, AMP-PCP was present in the EGTA dilution medium at zero time. Open symbols, AMP-PCP was added 15 s after dilution of the vesicles into the EGTA-containing medium. \(\bullet\), \(100 \mu\)M; \(\Delta\), \(200 \mu\)M; and \(\triangledown\), \(500 \mu\)M AMPPCP.

**Fig. 6. Kinetic analysis of the phosphoenzyme-mediated Ca\(^{2+}\) release.** SR vesicles were loaded with 16 mM \(^{45}\)CaCl\(_2\) and then diluted into a Ca\(^{2+}\) release medium as described in Fig. 1A. Release was measured in the absence (●) or in the presence (○) of 150 \(\mu\)M ATP. The curve obtained in the presence of ATP (○) was corrected as shown in Fig. 4A in order to get the phosphoenzyme-mediated portion. The fraction of Ca\(^{2+}\) retained by the vesicles relative to zero time (obtained by extrapolation of the passive Ca\(^{2+}\) efflux in the absence of ATP) is presented on a semilogarithmic scale. The extrapolated portion of the slow component (broken line) was subtracted from the experimental points (○) to obtain the difference (x).

**Fig. 7. Inhibition of phosphoenzyme formation and rapid Ca\(^{2+}\) release by ADP.** A, effect of ADP on the phosphoenzyme-mediated Ca\(^{2+}\) release. Rapid "Ca\(^{2+}\) efflux from passively loaded SR vesicles was investigated as described in Fig. 1A, with ADP present in the dilution medium. All curves obtained in the presence of ADP were corrected in a similar way as described for Fig. 4A. B, effect of ADP on the phosphoenzyme formation during the rapid release. Phosphoenzyme was measured as described in Fig. 2 with 150 \(\mu\)M [\(\gamma\)\(^{32}\)P]ATP (●), or 150 \(\mu\)M [\(\gamma\)\(^{32}\)P] ATP and 100 \(\mu\)M ADP (○) in the dilution medium.
both the phosphoenzyme-dependent and independent portions of the ATP-induced Ca\(^{2+}\) release. Low levels of free Mg\(^{2+}\) (70 \(\mu\)M) were sufficient to obtain half-maximal inhibition of the rapid Ca\(^{2+}\) efflux, while the slow passive efflux (obtained in the absence of ATP) was minimally affected. This observation rules out the possibility that a protein kinase might be involved in the ATP-induced Ca\(^{2+}\) release phenomenon, since kinases are known to require Mg\(^{2+}\) for catalytic activity. Fig. 6A shows the effect of 125 \(\mu\)M free Mg\(^{2+}\) on the phosphoenzyme-mediated Ca\(^{2+}\) release. The effect of Mg\(^{2+}\) on the phosphoenzyme level was also investigated in parallel experiments. The maximal levels of phosphoenzyme formed were slightly enhanced in the presence of Mg\(^{2+}\) (Fig. 8B), probably owing to the presence of Mg-ATP in the medium, which is a better and faster substrate for the ATPase reaction than ATP alone. However, the most striking effect of Mg\(^{2+}\) was on the rate of phosphoenzyme decomposition. It is known that Mg\(^{2+}\) ions are required for the dephosphorylation step(s) of the ATPase reaction cycle (25, 26) and Fig 8B shows that the half-time of phosphoenzyme decay was indeed decreased from 4–6 s, in the absence of Mg\(^{2+}\), down to less than 1 s in the presence of 125 \(\mu\)M free Mg\(^{2+}\). Our analysis indicated, therefore, that Mg\(^{2+}\) ions inhibited the phosphoenzyme-mediated rapid Ca\(^{2+}\) release because they increased the phosphoenzyme decay rate. Mg\(^{2+}\) chase experiments provided additional evidence for the hypothesis on the correlation between formation of phosphoenzyme and activation of the Ca\(^{2+}\)-release mechanism. Accordingly, Mg\(^{2+}\) ions were added to the reaction mixture 2 s after dilution of the vesicles into the ATP-containing medium. Fig. 9B shows that normal levels of phosphoenzyme were formed during the 2 s following dilution of the vesicles and that, after the addition of 125 \(\mu\)M free Mg\(^{2+}\), the phosphoenzyme rapidly dissipated. In parallel, the phosphoenzyme-mediated portion of the rapid Ca\(^{2+}\) release became inhibited after addition of Mg\(^{2+}\) (Fig. 9A).

**Inhibition by Free Ca\(^{2+}\) outside the Vesicles—**When the concentration of Ca\(^{2+}\) ions in the dilution medium was adequate to saturate the high affinity binding sites of the ATPase (submiconomal range), almost complete inhibition of the phosphoenzyme-induced Ca\(^{2+}\) release was observed (Fig. 8A). Our experiments also demonstrated that a rapid 45Ca-Ca exchange phenomenon could not occur under our dilution conditions. The inhibition occurred in spite of the high levels of phosphoenzyme which could be formed in the presence of Ca\(^{2+}\) ions outside the vesicles (not shown). Evidently, the pathway for rapid Ca\(^{2+}\) release was blocked when the high affinity sites were occupied by Ca\(^{2+}\) ions. The inhibition, however, was reversible, since the rapid release could be initiated again by chelation of the Ca\(^{2+}\) ions outside the vesicles. This required, however, that a significant amount of phosphoenzyme was still present at the time of Ca\(^{2+}\) removal.

**Dynamics of the Phosphoenzyme-induced Ca\(^{2+}\) Release on the Ca\(^{2+}\) Load of the Vesicles—**SR vesicles were passively loaded with various amounts of 45CaCl\(_2\) presumably yielding different free Ca\(^{2+}\) concentrations inside the vesicles, and then diluted in the EGTA-containing medium in the presence of 100 \(\mu\)M ATP. A low ATP concentration was chosen in order to limit the amount of rapid Ca\(^{2+}\) release occurring in a phosphoenzyme-independent way, so that sufficient amounts of Ca\(^{2+}\) ions were retained by the vesicles to analyze the phosphoenzyme-mediated portion of Ca\(^{2+}\) release also at low Ca\(^{2+}\)-loading conditions. Fig. 10 shows that the rapid phosphoenzyme-mediated release of Ca\(^{2+}\) ions was highly dependent on the concentration of free Ca\(^{2+}\) ions inside the vesicles, indicating that the occupation of low affinity sites by internal Ca\(^{2+}\) (K\(_D\) between 2 and 3 mM) was necessary to observe the rapid release phenomenon. It should be noted that the ATPase displays Ca\(^{2+}\)-binding sites facing the lumen of the vesicles with a similar low affinity (1, 2).

We also investigated whether the rapid phosphoenzyme-induced release was peculiar to a specific region of the SR network. The vesicular preparation used in the present study was a mixture of membranes derived from both the cisternae and the longitudinal system of the SR. We subfractionated the preparation, therefore, into heavy and light vesicular components (27), which are derived mainly from the cisternae and from the longitudinal system, respectively. The main difference between the two fractions was the presence of high amounts of calsequestrin in the former. When the two preparations were loaded with 10 mM CaCl\(_2\), a marked phosphoenzyme-
enzymes-dependent portion of the rapid Ca\(^{2+}\) release obtained in a medium containing 100 mM KCl, 20 mM MOPS, pH 7. Efflux experiments were carried out as usual (see Fig. 4A) and the phosphoenzyme-dependent portion of the rapid Ca\(^{2+}\) release obtained in the presence of ATP in the dilution medium was determined as shown in Fig. 4A. The amount of Ca\(^{2+}\) released within 5 s was considered as an indication of the rate of the phosphoenzyme-dependent Ca\(^{2+}\) release. It was assumed that the concentration of free Ca\(^{2+}\) inside the vesicles (free Ca\(_{\text{free}}\)) was the same as that of the incubation mixture. Such a concentration was calculated after subtraction of the fraction of Ca\(^{2+}\) bound to the vesicles (as judged from the Ca\(^{2+}\)-content of the vesicles after extrapolation of the release curves to zero time) from the total amount of Ca\(^{2+}\) added to load the vesicles.

\(\text{Effect of Quercetin—It has been recently reported that quercetin is a potent inhibitor of the ATPase activity of SR vesicles (28). The inhibition mechanism has been investigated in detail and it was found that several steps of the reaction cycle are affected by the drug (28). Interestingly, at the appropriate quercetin concentration (80–120 \(\mu\)M), the steps leading to phosphoenzyme formation from ATP were not inhibited while the dephosphorylation of the enzyme and, thus, the turnover rate of the cycle were strongly reduced (28). The effect of quercetin was tested under similar conditions on the phosphoenzyme-induced Ca\(^{2+}\) release. It was found indeed, that phosphoenzyme was formed as usual and that its half-time was prolonged with respect to control experiments (\(t_{1/2} = 6–8\) s in the presence and 4–6 s in the absence of 100 \(\mu\)M quercetin). Concomitantly, a small but significant increase (15–20%) of the phosphoenzyme-mediated Ca\(^{2+}\) release from the vesicles was observed.}

\(\text{Identification of the Phosphorylated Intermediate Responsible for the Rapid Ca\(^{2+}\) Release—The results presented so far have shown that a phosphorylated intermediate of the ATPase with an unusually long time of decay was formed under the special conditions used to induce rapid Ca\(^{2+}\) release. Such a concentration was calculated after subtraction of the fraction of Ca\(^{2+}\) bound to the vesicles (as judged from the Ca\(^{2+}\)-content of the vesicles after extrapolation of the release curves to zero time) from the total amount of Ca\(^{2+}\) added to load the vesicles.}

\(\text{Since this phosphoenzyme might represent a special, still unidentified phosphorylated conformational state of the ATPase, it was investigated whether the known phosphoenzyme species, formed during the normal reaction cycle, would also support the rapid Ca\(^{2+}\) release. SR vesicles (10 mg/ml) were passively loaded with 10 mM \(^{45}\)CaCl\(_2\) and then exposed to 10 mM ATP for 2 s, whereby high steady state levels of phosphoenzyme were formed (3–4 nmol/mg of protein). The reaction mixture was then diluted into release medium containing 20 mM EGTA and a rapid massive release of Ca\(^{2+}\) occurred. The efflux rate was too fast for the Millipore technique, indicating that all the phosphoenzyme existing prior to dilution was involved in the release process. It appears, therefore, that the fundamental requirement to induce Ca\(^{2+}\) release is not the formation of a particular species of phosphorylated intermediate, but the inhibition (e.g., by absence of Mg\(^{2+}\)) of phosphoenzyme decomposition. Accordingly, it should also be possible to induce rapid Ca\(^{2+}\) release from SR vesicles which are actively transporting Ca\(^{2+}\) ions. This is indeed clearly shown in Fig. 11. SR vesicles were allowed to accumulate Ca\(^{2+}\) ions in the presence of ATP and in the absence of Mg\(^{2+}\). Under these conditions, a suboptimal transport rate was observed; however, a considerable amount of Ca\(^{2+}\) ions could be actively translocated (Fig. 11). After a subsequent addition of EGTA to the uptake reaction mixture to remove Ca\(^{2+}\) from the high affinity cytosolic sites, a sudden release of part of the accumulated Ca\(^{2+}\) ions occurred (Fig. 11). If Mg\(^{2+}\) was added together with EGTA, the rapid release of Ca\(^{2+}\) was inhibited.}

\(\text{DISCUSSION}
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Our investigation of the characteristics of the ATP-induced Ca\(^{2+}\) release from loaded SR vesicles has shown that the phenomenon consists of two separate fast components. One component does not require utilization of the substrate and can be induced also by the nonhydrolyzable ATP analog AMP-PCP (39). The other component only occurs when a phosphorylated intermediate of the ATPase is formed. In this report, we investigated in detail this latter component of the ATP-induced Ca\(^{2+}\) release in order to better understand some characteristics of the phosphorylated intermediate forms of...
the ATPase. The results obtained have led to the following conclusions. (i) The release is a specific mechanism, since the characteristics of the vesicles in permeability to sucrose are not affected (Fig. 1). (ii) The rapid efflux occurs only if a phosphorylated intermediate of the ATPase is formed. The mechanism of phosphoenzyme formation is immaterial; however, it is required that the phosphoenzyme does not undergo the normal rapid cycle of the ATPase reaction coupled to Ca\(^{2+}\) uptake, but that it is in a "frozen" state (the life span of the phosphoenzyme can be prolonged experimentally by working in the absence of Mg\(^{2+}\) ions, which are required to stimulate the dephosphorylation steps (25, 26)). (iii) Ca\(^{2+}\) release depends on the concentration of Ca\(^{2+}\) ions on both sides of the membrane. The Ca\(^{2+}\) concentration on the inside of the vesicles must be elevated so that the low affinity binding sites are occupied (Fig. 10); on the other hand, Ca\(^{2+}\) outside the vesicles must be removed (i.e. by EGTA) from the high affinity binding sites. In summary, under specific conditions (i.e. absence of Mg\(^{2+}\), high Ca\(^{2+}\) inside and low Ca\(^{2+}\) outside the vesicles, and slow dephosphorylation rate), a phosphorylated intermediate of the ATPase can mediate rapid Ca\(^{2+}\) release, and we therefore refer to it as the phosphoenzyme-mediated Ca\(^{2+}\) release.

From the information presented and some known characteristics of the Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase, it is possible to envision a molecular mechanism to explain the release process. This is shown in the following scheme

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\begin{align*}
\text{Ca}^{2+} & \rightarrow \text{E} & \text{P} \rightarrow \text{E},
\end{align*}
\]

where \(\text{E} \sim \text{P}\) is the ADP-sensitive form and \(\text{E} \sim \text{P}\) is the ADP-insensitive form of the phosphoenzyme. In the scheme, only the interconversion between phosphorylated forms of the ATPase is taken into account. It is envisaged that the enzyme can be phosphorylated by ATP only when the high affinity binding sites of the molecule facing the cytosol (out) are occupied by Ca\(^{2+}\) ions (30). Once the ATPase becomes phosphorylated, the acylphosphate formed can exist in several conformational states, which are in equilibrium. It is known that the presence of specific divalent cations (i.e. Ca\(^{2+}\) and Mg\(^{2+}\)) in the two compartments separated by the membrane can affect considerably both the relative distribution of the ATPase molecules into the possible phosphoenzyme conformations and the rate of interconversion between these intermediate species. Particularly relevant for the understanding of the proposed mechanism are recent results obtained by investigations on the properties of the phosphoenzyme in the presence of Mg\(^{2+}\) ions (31-34). Accordingly, it was observed that phosphorylation of the vesicles by ATP in the presence of Ca\(^{2+}\) ions and in the absence of Mg\(^{2+}\) leads to the formation of a phosphorylated intermediate with bound Ca\(^{2+}\) ions, which are still exposed to the cytosolic face. This phosphoenzyme, which is represented in the scheme by \(\text{E} \sim \text{P}\), can transfer, under appropriate conditions, its phosphate group to ADP to form ATP (ADP-sensitive phosphoenzyme). Lowering of the Ca\(^{2+}\) concentration in the external medium (i.e. by addition of EGTA) causes dissociation of the bound Ca\(^{2+}\) from the ADP-sensitive phosphoenzyme, which is then transformed into an ADP-insensitive form (\(\text{E} \sim \text{P}\) in the scheme). On the other hand, the ADP-insensitive phosphoenzyme (\(\text{E} \sim \text{P}\)) is characterized by having low affinity Ca\(^{2+}\)-binding sites facing the lumen of the vesicles (2, 35). Provided that the Ca\(^{2+}\) concentration inside the vesicles is high enough to occupy these sites, the phosphoenzyme becomes ADP sensitive (\(\text{Ca}^{2+} \sim \text{E} \sim \text{P} \rightarrow \text{E} \sim \text{P} \rightarrow \text{Ca}^{2+} \sim \text{E} \sim \text{P}\)). A vectorial translocation of the bound Ca\(^{2+}\) through the osmotic barrier is coupled to the phosphoenzyme transition, and concomitantly, the affinity of the sites for Ca\(^{2+}\) is increased several orders of magnitude. Ca\(^{2+}\) ions can be released to the external medium in the presence of a Ca\(^{2+}\)-buffering system (EGTA) and the phosphoenzyme becomes ADP insensitive again (\(\text{E} \sim \text{P} \rightarrow \text{E} \sim \text{P}\)).

In the last decade, extensive experimentation has been carried out in order to obtain rapid Ca\(^{2+}\) release from isolated SR vesicles (for a review, see Ref. 10). It is interesting to discuss whether the Ca\(^{2+}\) release mechanism described in this report could have accounted for some of the effects observed in the past. The rapid Ca\(^{2+}\) release which is obtained during reversal of the Ca\(^{2+}\)-pumping cycle, coupled to ATP synthesis, is a well known phenomenon which requires the formation of a phosphoenzyme. Our results, however, have shown that the phosphoenzyme-mediated Ca\(^{2+}\) release is not related to such a mechanism, since it is inhibited by Mg and ADP, ligands...
which are necessary for activation of Ca\(^{2+}\) release involving ATP synthesis. One can also exclude any analogy, for instance, with the reports on the so-called Ca\(^{2+}\)-induced Ca\(^{2+}\) release (9, 38) for the obvious reason that micromolar free Ca\(^{2+}\) outside the vesicles inhibits, rather than stimulates, Ca\(^{2+}\) release induced by ATP. Similarly, the Ca\(^{2+}\)-releasing action of AMP-PCP previously described by Ogawa and Ebashi (39) was also occurring via a mechanism distinct from that observed under our experimental conditions, since the release described by these authors was apparently insensitive to free Mg\(^{2+}\) and Ca\(^{2+}\) ions outside the vesicles. On the other hand, for some other experiments previously described whose requirements for Ca\(^{2+}\) release, on first examination, appear quite diverse, the interesting possibility exists that they might share a common phosphoenzyme-mediated Ca\(^{2+}\) release mechanism. Good candidates for such a mechanism are, for instance, the depolarization-induced (40) and pH-induced (41) Ca\(^{2+}\) release experiments, which were carried out in the presence of ATP, so that a phosphoenzyme was present. In the former case (40), the addition of specific ions was used to induce a transient change of the membrane and/or surface potential of the SR vesicles. Such a treatment might have altered the dephosphorylation kinetics of the phosphoenzyme present (as discussed above). In the other experiments (41), a sudden increase in pH was shown to produce a rapid Ca\(^{2+}\) release and we have observed that dilution of Ca-loaded vesicles into a more alkaline ATP-containing medium (ΔpH = 0.3–0.5) produced a marked increase of Ca\(^{2+}\) release. The possibility that the dephosphorylation rate of the phosphoenzyme might be affected by the experimental conditions described in those reports (40, 41) should be investigated. Finally, it was recently reported (5) that the depolarization-induced contraction of skinned fibers is not inhibited by 50–100 μM quercetin, while parallel experiments could show that quercetin, in a similar concentration range, completely blocked the Ca\(^{2+}\) efflux coupled to ATP synthesis in isolated SR preparations (5, 28). This observation does not exclude the involvement of a Ca\(^{2+}\) release mechanism like that described in this communication, since the quercetin concentration used on skinned fibers was not sufficient to affect the level of phosphoenzyme present. Actually, the drug concentration used was shown to inhibit the dephosphorylation rate and it is interesting to note that both phosphoenzyme-mediated Ca\(^{2+}\) release and onset of contraction in the skinned fiber system are stimulated by quercetin. This might indicate that a common release mechanism might have been involved. A main point of concern regarding the physiological significance of this and other Ca\(^{2+}\) release mechanisms so far reported is the inadequacy of the measured release rates to account for the rapidity of excitation-contraction coupling in vivo.

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A phosphorylated conformational state of the (Ca\textsuperscript{2+}-Mg\textsuperscript{2+})-ATPase of fast skeletal muscle sarcoplasmic reticulum can mediate rapid Ca\textsuperscript{2+} release.

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