On the Sidedness of Membrane Phosphorylation by Pi and ATP Synthesis during Reversal of the Ca\(^{2+}\) Pump of Sarcoplasmic Reticulum Vesicles*

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The membrane sidedness of Pi interaction in reactions which characterize reversal of the Ca\(^{2+}\) pump of sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle was investigated. Vesicles previously loaded with calcium [\(^{32}\)P]phosphate were incubated with 0.1 mM ADP and different concentrations of nonradioactive Pi. Alternatively, vesicles loaded with nonradioactive calcium phosphate were incubated in a medium containing \(^{32}\)Pi.

The rates of Ca\(^{2+}\) efflux and ATP synthesis were significantly activated only when Pi was included in the assay medium. Although the Pi contained by the vesicles crosses the membrane at a rate proportional to the Ca\(^{2+}\) efflux, [\(^{\gamma}\)-\(^{32}\)P]ATP was synthesized only when \(^{32}\)Pi interacted with the outer surface of the membrane. Similarly, ATP--\(^{32}\)Pi or ITP--\(^{32}\)Pi exchange could be measured only when the external pool of Pi was labeled.

Both for ATP synthesis and for the ITP--Pi exchange reaction, membrane phosphorylation by \(^{32}\)Pi was negligible unless the external pool of Pi was labeled.

The ionophore X-537 A increased the rate of Ca\(^{2+}\) efflux but inhibited the synthesis of ATP.

During reversal of the Ca\(^{2+}\) pump, Pi apparently interacts with the membrane only at the outer surface, and at a site different from that where Ca\(^{2+}\) crosses the membrane.

Fragmented sarcoplasmic reticulum vesicles SRV1 isolated from skeletal muscle actively take up calcium from the medium at the expense of ATP hydrolysis. Recently it has been shown that the calcium pump can be reversed. Evidence has been presented that the very same ATPase involved in calcium transport is able to promote the synthesis of ATP using the transmembrane calcium gradient as a source of energy (1-6).

When vesicles previously loaded with Ca\(^{2+}\) are incubated in a medium containing EGTA, a calcium chelating agent, a steep concentration gradient is formed across the vesicle membrane.

Another index of the reversal of the calcium pump is the ATP--Pi exchange reaction. When sarcoplasmic reticulum vesicles are incubated in a medium containing ATP, Mg\(^{2+}\), Mg\(^{2+}\), and calcium, calcium phosphate is accumulated by the vesicles (14) and a calcium concentration gradient is built up until a steady state is reached in which a slow Ca\(^{2+}\) efflux is balanced by an ATP-driven influx. When this condition is reached a steady rate of exchange between \(^{32}\)Pi and ATP is observed (4, 15-17). In previous papers (9, 14-16) evidence has been presented that this exchange is the result of the two reactions shown above, operating simultaneously forward (ATP hydrolysis) and backward (ATP synthesis from ADP and \(^{32}\)Pi).

The aim of this paper was to investigate whether \(^{32}\)Pi reacts at the inner or outer surface of the vesicle membrane for the formation of E-Pi, and whether newly formed ATP is released inside the vesicles or outside them.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as previously described (18).

\(^{32}\)Pi was obtained from the Brazilian Institute of Atomic Energy and purified by the procedure described by Boyer et al. (19, 20).

Loaded Vesicles—Vesicle protein (28 mg) was incubated in 38 ml of a medium containing 20 mM Tris-maleate buffer (pH 7.0), 5 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.9 mM EGTA, 10 mM Pi, and 4 mM ATP. After 10 min of incubation at 30°, the suspension was centrifuged in the cold at 25,000 x g for 30 min. The pellet was washed by centrifugation with an ice-cold solution containing 5 mM MgCl\(_2\), 20 mM Tris-maleate buffer (pH 7.0), and 1 mM ATP. The final pellet was resuspended in 50 mM...
KCl and used immediately. Protein was measured by the biuret method. In these conditions, 65 to 75% of the \( Ca^{2+} \) contained in the loading mixture was accumulated by the vesicles. Phosphatase increases the \( Ca^{2+} \)-storing capacity of the vesicles by providing a sink for the entering calcium, which precipitates inside the vesicles as calcium phosphate (14). Once this precipitate is formed, the free calcium concentration inside the vesicles should remain constant as the ionic species in equilibrium with the calcium phosphate crystals flow out of the vesicles. For each experiment, the SRV were simultaneously loaded under conditions: 10 mm \( CaCl_2 \) and nonradioactive phosphate, and the other with nonradioactive \( CaCl_2 \) and \( ^{32}P \). The ratio of \( CaCl_2 \) accumulated by the vesicles in different SRV preparations tested varied between 1.4 and 2.0. In some experiments (Fig. 6), the vesicles were loaded with calcium oxalate. For these experiments, 5 mm ammonium oxalate was used instead of 10 mm \( P_2 \) in the loading medium. Oxalate has been shown to be more effective than \( P_2 \) in increasing the calcium uptake by sarcoplasmic reticulum vesicles; a smaller concentration of oxalate is required in the assay medium to increase maximally the rate of calcium uptake and the calcium storage capacity of the vesicles (1, 21-23). This is probably due to a lower solubility of calcium oxalate. Thus, oxalate anions would be more efficient than \( P_2 \) in decreasing the concentration inside the vesicles should remain constant as the ionic species in equilibrium with the calcium phosphate crystals flow out of the vesicles.

**RESULTS**

**ATP Synthesis**—When vesicles loaded with calcium phosphate were incubated in a medium containing EGTA but no ADP or \( P_2 \), a steady efflux of \( Ca^{2+} \) and \( ^{32}P \) was measured (Fig. 1, A and B). This represents the passive efflux, and its rate was determined by the concentrations of these two ionic species inside the vesicles. The addition of ADP to the medium promoted a slight increment in the efflux of both \( Ca^{2+} \) and \( ^{32}P \), which was more pronounced the longer the incubation time, this efflux was accompanied by the synthesis of a small amount of ATP (Fig. 1C). All the ATP synthesized was labeled with radioactive phosphate. If nonradioactive \( P_2 \) was included in the medium along with the ADP, both \( Ca^{2+} \) and \( ^{32}P \) effluxes sharply increased. This increment of efflux was coupled to the synthesis of a larger amount of ATP, which could be measured by the enzymatic method. However, only a very small fraction of the ATP synthesized was labeled with radioactive phosphate (Fig. 1C). This indicates that only the phosphate which reacted with the membrane at the outer surface of the vesicles was involved in the reversal of the calcium pump. The synthesis of ATP observed when ADP alone was added was probably promoted by the \( P_2 \), which left the vesicles by passive efflux and could then react with the outer surface of the membrane. This conclusion was substantiated by the experiments described in Figs. 2 to 4.

The data of Fig. 2A show that the rate of release of calcium and \( P_2 \) was progressively enhanced when the \( P_2 \) concentration of the medium was raised. In the absence of added \( P_2 \), the \( \gamma - { }^{32}P \)ATP synthesized was equal to the ATP measured enzymatically (Fig. 2B). Whereas the total ATP formed increased in parallel with the effluxes, the \( \gamma - { }^{32}P \)ATP formed decreased as the \( P_2 \) leaving the vesicles was diluted by increasing amounts of nonradioactive \( P_2 \) in the medium.

The apparent \( K_a \) for \( P_2 \) (Fig. 3) for both ATP synthesis and the increment of calcium efflux was 0.25 ± 0.10 (four experiments). This indicates that in the experiments in which only ADP was added to the efflux medium, the amount of \( P_2 \) released through the passive efflux in 1 min was sufficient to activate to some extent the reversal of the calcium pump. For the experiments of Fig. 4, the vesicles were loaded with

### Table I

**Membrane phosphorylation by \( ^{32}P \), under conditions for ATP synthesis and ITP—\( P_2 \) exchange**

| Condition | Conditions for ATP synthesis | Conditions for ITP—\( P_2 \) exchange |
|-----------|-------------------------------|------------------------------------|
| **SRV loading and incubation conditions** | \( A \): \( P_2 \) | \( A \): \( P_2 \) |
| \( P_2 \) | \( 0.46 \pm 0.01 \) | \( 0.55 \pm 0.04 \) |
| **\( P_2 \) accumulation** | \( 4.72 \pm 0.23 \) | \( 3.69 \pm 0.15 \) |
| **C** | \( 5.07 \pm 0.38 \) | \( 3.86 \pm 0.02 \) |

The reaction was started by addition of loaded vesicles to the assay medium and stopped after 10 s incubation at 30°. The location of the \( ^{32}P \), with respect to the lumen of the vesicles is indicated in the first column. Other additions and experimental conditions were as described under “Materials and Methods.” The values represent the average ± S.E. of three experiments.
Fig. 1. Synthesis of ATP coupled to Ca\(^{2+}\) efflux. The reaction was started by the addition of vesicles loaded with calcium phosphate and stopped after different incubation intervals at 30°C by removal of the vesicles with Millipore filters. A, Ca\(^{2+}\) efflux. SRV were previously loaded with \(^{45}\)Ca and nonradioactive P\(_i\). O—O, passive efflux; ADP and P\(_i\) were not added to the incubation medium; \(\bullet\)---\(\bullet\), with 0.1 mM ADP; \(\circ\)---\(\circ\), with 0.1 mM ADP plus 10 mM P\(_i\), B, P\(_i\) efflux. SRV were previously loaded with nonradioactive calcium and \(^{32}\)P\(_i\), \(\Delta\---\Delta\), passive efflux; \(\Delta\)---\(\Delta\), with 0.1 mM ADP; \(\Delta\)---\(\Delta\), with 0.1 mM ADP plus 10 mM nonradioactive P\(_i\). C, ATP synthesis. SRV were loaded as in B. Solid lines, total ATP as measured by the enzymatic method; dashed lines, synthesis of \([\gamma\-\text{P}]\)ATP. \(\bullet\)---\(\bullet\), with 0.1 mM ADP; \(\Delta\)---\(\Delta\), with 0.1 mM ADP plus 10 mM nonradioactive P\(_i\). Other additions and experimental conditions were as described under “Materials and Methods.”

Fig. 2 (left). Effect of external P\(_i\) on ATP synthesis. The reaction was started by the addition of vesicles loaded with calcium phosphate and stopped after 1 min incubation at 30°C by removal of SRV with Millipore filters. The ADP concentration was 0.1 mM and the concentration of nonradioactive P\(_i\) added to the medium is shown on the abscissa. Other additions and experimental conditions were as described under “Materials and Methods.” A, Ca\(^{2+}\) and P\(_i\) efflux. For the Ca\(^{2+}\) efflux (\(\circ\)---\(\circ\)), the vesicles were previously loaded with \(^{45}\)Ca and nonradioactive P\(_i\). For the P\(_i\) efflux (\(\bullet\)---\(\bullet\)), the vesicles were loaded with \(^{32}\)P\(_i\) and nonradioactive calcium. The values shown represent only the increment of efflux promoted by the addition of ADP and P\(_i\) to the assay medium. The passive efflux obtained in the absence of ADP and P\(_i\) has been subtracted from each point. B, ATP synthesis. SRV were previously loaded with nonradioactive calcium and \(^{32}\)P\(_i\). \(\bullet\)---\(\bullet\), total ATP as measured with the enzymatic method; \(\circ\)---\(\circ\), synthesis of \([\gamma\-\text{P}]\)ATP.

Fig. 3 (right). Apparent Km for P\(_i\). Lineweaver-Burk plot of the rate of ATP synthesis (\(O\)) as measured by the enzymatic method (\(\mu\text{mol/mg of protein min}^{-1}\)) and (\(\bullet\)) increment of the rate of Ca\(^{2+}\) efflux (\(\mu\text{mol/mg of protein min}^{-1}\)) promoted by 0.1 ADP and the P\(_i\) concentrations shown in the abscissa. Other additions to the assay medium and experimental conditions were as described under “Materials and Methods.” Essentially the same results were obtained in four different vesicle preparations tested.

Makinose and Hasselbach (5) reported that for the increment of Ca\(^{2+}\) efflux promoted by ADP and P\(_i\), the release of nonradioactive phosphate and incubated in media containing increasing concentrations of \(^{32}\)P\(_i\). The \([\gamma\-\text{P}]\)ATP formed was equal to the amount of ATP measured by the enzymatic method.

When the SRV were loaded, the ratio of \(^{45}\)Ca: \(^{32}\)P\(_i\) accumulated by the vesicles varied in different preparations between 1.4 and 2.0. Since the same ratio as that of Ca: P\(_i\) accumulated was maintained in a given vesicle preparation for the initial rates of \(^{45}\)Ca and \(^{32}\)P\(_i\) effuxes, the effuxes appear to be controlled by the internal concentrations of these two species. The increment of \(^{32}\)P\(_i\) efflux promoted by the addition of ADP and P\(_i\) to the medium was probably not an active process like that of Ca\(^{2+}\), but rather a passive efflux. The rapid solubilization of the calcium phosphate crystals inside the vesicles which was promoted by the rapid draining of Ca\(^{2+}\) from the vesicles should result in a sharp increase of the P\(_i\) concentration inside the vesicles, and hence an increment of the passive efflux of P\(_i\). The conclusion that the P\(_i\) efflux was not involved in the ATP synthesis was substantiated by the finding that an increment of the Ca\(^{2+}\) efflux coupled with ATP synthesis was also found in vesicles loaded with calcium oxalate (1-4) (Fig. 6).

Makinose and Hasselbach (5) reported that for the increment of Ca\(^{2+}\) efflux promoted by ADP and P\(_i\), the release of
two Ca\(^{2+}\) ions was coupled to the synthesis of 1 mol of ATP. In our experimental conditions we found a ratio varying from 1.4 to 2.0.

Several reports have shown that the ATP synthesis is initiated by the phosphorylation of the SRV ATPase by Pi, forming an acylphosphoprotein (E-P). Subsequently the phosphate of the phosphoprotein is transferred to ADP, forming ATP (6-9, 11-13). The data of Table I show that under conditions similar to those of Figs. 2 and 4 the formation of E-P from \(^{32}\)Pi, occurred only when the radioactive phosphate was outside the vesicles. Radioactive phosphate inside the vesicles did not contribute to the total amount of E-P formed.

**NTP-P, Exchange**—For these experiments, preloaded vesicles were incubated in a medium which, in addition to Pi, and NDP, contained 5 mM ATP or ITP. In these conditions there was no net efflux, as the Ca\(^{2+}\) which leaves the vesicles was reaccumulated at the expense of the triphosphonucleotide hydrolysis. Consequently, the Ca\(^{2+}\) concentration gradient was preserved and no net synthesis of NTP was observed. But because the Ca\(^{2+}\) pump was operating simultaneously forward and backward, Pi was steadily incorporated into the NTP pool (4, 15-17). Fig. 5 shows that the ITP formed during the steady state exchanges was labeled only when the radioactive phosphate was present outside the vesicles. When the vesicles were preloaded with calcium and \(^{32}\)Pi, and ITP-Pi exchange was measured in media with or without 10 mM nonradioactive phosphate, no [\(\gamma-^{32}\)P]ITP was detected. The amount of \(^{32}\)P, which left the vesicles during the experiment was quite small (inset, Fig. 5), and practically no Ca\(^{2+}\) efflux could be measured (data not shown). Essentially the same results as those shown in Fig. 5 were obtained when ATP and ADP were used as substrates.

In a previous paper (15) it was shown that the apparent $K_m$ for Pi in the ATP-Pi exchange was in the range of 3 to 4 mM, i.e., severalfold higher than the apparent $K_m$ for Pi, for the
reversal of the Ca\(^{2+}\) pump (Fig. 3). Notice that in Fig. 5 (inset), the \(^{32}\)P, which leaked out of the vesicles containing calcium \(^{32}\)Posphate contributed less than 0.1 mm to the medium, and thus was not sufficient to activate a measurable incorporation of \(^{32}\)P into the ITP pool.

As shown for the experiments accompanied by net synthesis of ATP, the formation of E-P from \(^{32}\)P, in the conditions of Fig. 5 was much greater when the \(^{32}\)P, was outside the vesicles (Table I). For these experiments, ETP was used as energy-donating substrate, since in a previous report (10) it was shown that the yield of enzyme phosphorylated by \(^{32}\)P, was small when ATP and ADP were included in the assay media.

\(\text{Ca}^{2+}\) Efflux and ATP Synthesis—The utilization of a cation gradient for ATP synthesis was first reported in mitochondria by Cockrell et al. (28). These authors were able to show synthesis of ATP coupled to \(\text{K}^{+}\) efflux in valinomycin-treated mitochondria. This was interpreted in terms of reversal of a cation pump. An alternative explanation was that the \(\text{K}^{+}\) efflux was electrogenic, giving rise to a membrane potential which in turn drove the ATP synthesis (29). This would imply that in order to activate the ATP synthesis, \(\text{K}^{+}\) must not necessarily leave the mitochondria through a specific membrane site. In fact, in these experiments, the synthesis of ATP was observed when the membrane permeability to \(\text{K}^{+}\) was artificially increased with the use of the ionophore valinomycin. With these possibilities in mind we measured ATP synthesis and \(\text{Ca}^{2+}\) efflux in vesicles loaded with calcium oxalate in the presence of X-537 A, an ionophore which increases the membrane \(\text{Ca}^{2+}\) permeability (30). Fig. 6 shows that while the \(\text{Ca}^{2+}\) efflux was increased by the addition of 12 \(\mu\)g of X-537 A/ml, the ATP synthesis was sharply inhibited. These effects of X-537 A were graded with its concentration down to 3 \(\mu\)g/ml (data not shown). This effect of X-537 A suggests that for the ATP synthesis in SRV, \(\text{Ca}^{2+}\) must leave the vesicle through the pump itself.

\section*{Discussion}

The data presented indicate that during reversal of the \(\text{Ca}^{2+}\) pump, \(\text{P}^{32}\) interacts with the outer surface of the vesicle membrane. The pool of \(\text{P}^{32}\), inside the vesicles, as well as the fraction of \(\text{P}^{32}\), which enters the membrane as it leaves the vesicles is not involved in the ATP synthesis. It is not utilized for the membrane phosphorylation and is not incorporated into the ATP synthesized. The same reasoning applies for the ATP-\(\text{P}^{32}\), or ITP-\(\text{P}^{32}\), exchange. This correlates well with a previous suggestion that for the forward reaction (\(\text{Ca}^{2+}\) accumulation and ATP hydrolysis) the \(\text{P}^{32}\), derived from the ATP hydrolysis is liberated at the outer surface of the vesicle membrane (31). The data of Fig. 4 indicated that the ATP synthesized is also released from the outer surface of the SRV. Otherwise one would expect that some ATP should be trapped inside the vesicles, and more ATP should be found in the medium when the reaction was arrested by acid denaturation than when the vesicles were removed by filtration.

On the grounds of these data, it is proposed that ATP hydrolysis and/or ATP synthesis would take place at a site different from that at which \(\text{Ca}^{2+}\) crosses the membrane. In previous reports, it has been shown that the membrane phosphorylation by \(\text{P}^{32}\), which is the first step of the reversal of the \(\text{Ca}^{2+}\) pump, could take place when \(\text{Ca}^{2+}\) was absent from both sides of the membrane (9, 11-13, 20). This was achieved by incubating leaky vesicles in a medium containing an excess of EGTA. However, in these conditions no ATP synthesis could be measured (13). The transfer of the phosphate of the phosphoenzyme to ADP, both for ATP-P, exchange and for net synthesis of ATP, takes place only when the inner surface of the membrane is exposed to a high \(\text{Ca}^{2+}\) concentration (12, 13, 15-17). This suggests a membrane configuration in which a site on the outer surface is phosphorylated by \(\text{P}^{32}\), and another site on the inner surface binds \(\text{Ca}^{2+}\). Since there is no active translocation of \(\text{Ca}^{2+}\), during the process of ATP synthesis and/or hydrolysis, these observations corroborate the conclusion that \(\text{P}^{32}\), and \(\text{Ca}^{2+}\) interact at different sites. During reversal, \(\text{P}^{32}\), phosphorylates the outer surface, whereas \(\text{Ca}^{2+}\) must interact first with the inner surface of the membrane.

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