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ATP ↔ P_i Exchange and Membrane Phosphorylation in Sarcoplasmic Reticulum Vesicles: Activation by Silver in the Absence of a Ca^{2+} Concentration Gradient†

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ABSTRACT: The activation of ATP ↔ P_i exchange, normally associated with a Ca^{2+} concentration gradient in sarcoplasmic reticulum vesicles, can be obtained in "leaky" vesicles in 4–10 m*M* $CaCl_2$. In the micromolar range, Ag^+ activates the ATP ↔ P_i exchange two- to fourfold. Similar concentrations of Ag^+ promote a parallel inhibition of Ca^{2+}-activated ATP hydrolysis and Ca^{2+} uptake in intact vesicles. Maximal inhibition of these activities by Ag^+ leaves the Mg^{2+}-dependent ATPase unaffected. No net synthesis of ATP was demonstrated in leaky vesicles. The effects of Ag^+ depend on the protein concentration and persist after removal of Ag^+ from the medium. Membrane phosphorylation from P_i or from ATP is respectively activated or inhibited by Ag^+ in reciprocal fashion.

Sarcoplasmic reticulum vesicles (SRV)[1] isolated from skeletal muscle actively take up Ca^{2+} from the medium in the presence of ATP and Mg^{2+}. Hydrolysis of ATP and the translocation of Ca^{2+} into the vesicles involve a transfer of the γ-phosphate of ATP to a membrane protein (E), forming an acylphosphoprotein (E~P). Accordingly, the following reaction sequence has been proposed (Makinose, 1969; Hasselbach, 1972).

$$E + ATP \xleftrightarrow{Ca^{2+}} E\sim P + ADP$$
$$E\sim P \longleftrightarrow E + P_i$$

Recently, it has been demonstrated that the entire process of Ca^{2+} transport can be reversed and that the enzymatic system of the SRV membrane is able to use the energy derived from a Ca^{2+} concentration gradient for the chemical synthesis of ATP (Barlogie et al., 1971; Makinose, 1971, 1972, 1973; Makinose and Hasselbach, 1971; Hasselbach et al., 1972; Panet and Selinger, 1972; Deamer and Baskin, 1972; Masuda and de Meis, 1973, 1974). The following data support this conclusion.

(a) *Net Synthesis of ATP.* When SRV previously loaded with calcium phosphate are incubated in a medium containing EGTA,[1] Mg^{2+}, ADP, and P_i, a fast release of Ca^{2+} coupled with ATP synthesis from ADP and P_i is observed (Makinose, 1972, 1973; Makinose and Hasselbach, 1971).

(b) *ATP ↔ P_i Exchange.* When intact SRV are incubated in a medium containing ATP, Mg^{2+}, [^{32}P]P_i, and Ca^{2+}, calcium phosphate is accumulated by the vesicles and a Ca^{2+} 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 P_i and the γ-phosphate of ATP is observed (Makinose, 1971; Racker, 1972; de Meis and Carvalho, 1974). This exchange implies that the two reactions shown above are operating simultaneously forward (ATP hydrolysis) and backward (ATP synthesis from ADP and P_i).

(c) If the SRV are made "leaky" by various means, the Ca^{2+} concentration gradient is abolished and both the net synthesis of ATP and ATP ↔ P_i exchange reaction are arrested (Makinose, 1971; de Meis and Carvalho, 1974).

When SRV are loaded with calcium phosphate, the formation of a gradient coincides with the establishment of three conditions: a high Ca^{2+} concentration (in the millimo-

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[1] Abbreviations used are: SRV, sarcoplasmic reticulum vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

lar range) inside the vesicles, a Ca^{2+} concentration in the micromolar range at the external surface of the vesicles, and a decrease in the rate of ATP hydrolysis due to the low Ca^{2+} concentration remaining in the assay medium (Hasselbach, 1972). In a previous study (de Meis and Carvalho, 1974), it was shown that solubilized or leaky SRV were still able to catalyze an ATP $\leftrightarrow$ P_i exchange in the presence of a high Ca^{2+} concentration (1–8 m*M*). However, in these conditions the exchange rate was three to five times lower than that measured with intact SRV with a Ca^{2+} concentration gradient.

In this paper it is shown that Ag^+ inhibits Ca^{2+} uptake and the Ca^{2+}-dependent ATPase of leaky SRV and at optimal Ca^{2+} concentrations activates two- to fourfold the rate of ATP $\leftrightarrow$ P_i exchange. Some of the data were presented in less detail at a symposium on biological membranes in Rio de Janeiro in June, 1974.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as previously described (de Meis and Hasselbach, 1971). [γ-^{32}P]ATP was prepared (de Meis, 1972) using [^{32}P]P_i obtained from the Brazilian Institute of Atomic Energy and purified by means of a column of Dowex AG1-X10 (de Meis and Carvalho, 1974).

ATP $\leftrightarrow$ P_i exchange was assayed by measuring the formation of [γ-^{32}P]ATP by the method of Avron (1960), slightly modified (de Meis and Carvalho, 1974). As a control, after extraction by the Avron method, the [γ-^{32}P]ATP formed in the different experimental conditions described in the Results section was identified enzymatically (Panet and Selinger, 1972) using hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.4.9.).

ATPase activity was assayed by measuring the release of [^{32}P]P_i from [γ-^{32}P]ATP. The [^{32}P]P_i was extracted from the assay medium as phosphomolybdate with isobutyl alcohol–benzene (de Meis and Carvalho, 1974). Two different ATPase activities can be distinguished in SRV. The Mg^{2+}-dependent ATPase requires only Mg^{2+} for its activation and is tested in the presence of EGTA to remove contaminating Ca^{2+}. The Ca^{2+}-activated ATPase requires Ca^{2+} and Mg^{2+} for full activity and is calculated by subtracting the Mg^{2+}-dependent activity from the total activity measured in the presence of Mg^{2+} and Ca^{2+} (see Table I). Only the Ca^{2+}-activated ATPase is intimately associated with SRV Ca^{2+} transport (Hasselbach, 1972), and the rate of this ATPase depends on the Ca^{2+} concentration in the assay medium. In experiments in which the total ATPase activity is measured as a function of the incubation time, the removal of Ca^{2+} from the assay medium by SRV results in a progressive decrease of the rate of the Ca^{2+}-activated component. Therefore, in order to obtain linear rates, all ATPase activities were measured using leaky SRV (Fiehn and Hasselbach, 1969).

Preparation of Leaky SRV. A suspension of SRV at 9 to 12 mg of protein/ml in 1 to 2 m*M* EGTA was adjusted to pH 9.2 by the addition of 1 *M* Tris. After 20 min at room temperature, the pH was readjusted to 7.0 by the addition of Tris-maleate buffer (pH 6.0), and the suspension was used immediately. This treatment increases the Ca^{2+} permeability of the SRV membranes, allowing any Ca^{2+} which might have accumulated inside the vesicles to flow out. As a control, Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity were measured in several preparations before and after this treatment. In agreement with earlier reports (Duggan and Martonosi, 1970; de Meis and Carvalho, 1974), the leaky vesicles no longer accumulated Ca^{2+}, although the ATPase activity is increased. Ca^{2+} uptake was measured by the use of ^{45}Ca and Millipore filters as previously described (de Meis, 1969, 1971).

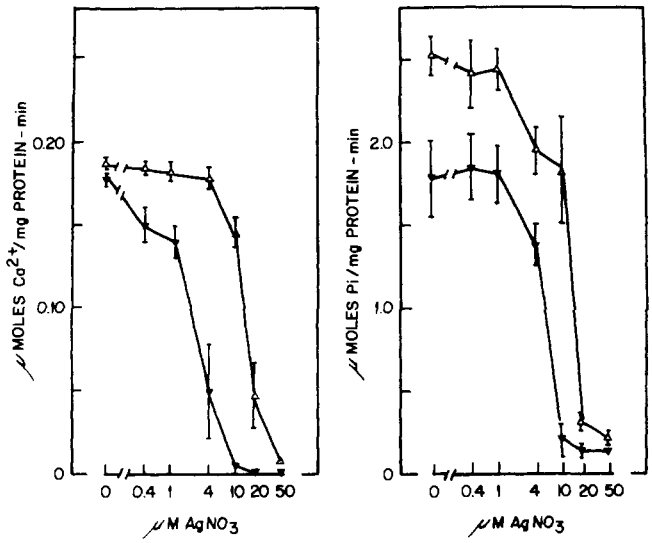

FIGURE 1: Inhibition of Ca^{2+} uptake and ATPase activity of SRV by Ag^+. For the Ca^{2+} uptake (left), the assay medium contained intact vesicles and 30 m*M* Tris-maleate buffer (pH 7.0), 20 m*M* $MgCl_2$, 5 m*M* ATP, 8 m*M* P_i, and 0.1 m*M* $^{45}CaCl_2$. For the total ATPase activity (right), leaky SRV, nonradioactive Ca, and [γ-^{32}P]ATP were used. The reaction was started by the addition of 0.05 (▼) or 0.20 (△) mg of SRV protein/ml. After 4 min incubation at 30° the reaction was stopped either by the removal of SRV with Millipore filters or by the addition of trichloroacetic acid. The values shown in the figure represent the average ± the standard error of seven (▼) or four (△) experiments.

Pretreatment with $AgNO_3$. In most experiments, the effect of $AgNO_3$ was assessed by adding SRV to a complete assay medium containing the $AgNO_3$. In some experiments, the SRV were tested after preincubation with $AgNO_3$ at concentrations specified in the results: SRV (0.35–0.5 mg of protein/ml) were incubated for 3–5 min at room temperature in 5 m*M* Tris-maleate buffer (pH 7.0) containing $AgNO_3$. The pellet formed after 30 min of centrifugation at 20,000*g* was resuspended in 5 m*M* Tris-maleate buffer (pH 7.0) before being added to the assay testing medium. Essentially the same results were obtained if the pellet was washed once with 40 ml of buffer before being resuspended for use. If the pretreatment with $AgNO_3$ was performed in a more complete assay medium, the SRV behaved quite differently (see text).

Membrane phosphorylation from either [γ-^{32}P]ATP or [^{32}P]P_i was assayed as previously described and corrected for nonspecific binding (de Meis and Masuda, 1974).

Results

Inhibition of Ca^{2+} Transport by $AgNO_3$. Micromolar concentrations of $AgNO_3$ promote a parallel inhibition of the Ca^{2+} uptake and the ATPase activity of SRV (Figure 1). This inhibition is observed when either oxalate or phosphate is present in the assay medium to increase the Ca^{2+} concentrating ability of the SRV (Hasselbach, 1972; de Meis et al., 1974). For both the Ca^{2+} uptake and the ATPase activity, the concentration of $AgNO_3$ required for 50% inhibition increases with the SRV protein concentration (Figure 1). The data of Table I show that in the range of $AgNO_3$ concentrations used, only the Ca^{2+}-activated AT-

Table I: Effect of Ag^+ on SRV ATPase Activity and ATP ↔ P_i Exchange.[a]

Additions to Assay Medium or SRV Treatment	ATPase Act., μmol of P_i/mg of Protein, 5 min					ATP ↔ P_i Exchange, μmol of ATP ↔ P_i/mg of Protein, 5 min	
	Mg^{2+}-Dependent EGTA	Total		Ca^{2+}-Activated			
		Low Ca^{2+}	High Ca^{2+}	Low Ca^{2+}	High Ca^{2+}	Low Ca^{2+}	High Ca^{2+}
(A) None	0.98 ± 0.05	10.7 ± 0.4	2.25 ± 0.08	9.71 ± 0.40	1.47 ± 0.18	0	0.10 ± 0.01
(B) $AgNO_3$ added to assay medium (5×10^{-5} *M*)	0.84 ± 0.02	0.87 ± 0.04	0.92 ± 0.08	0.03 ± 0.02	0.08 ± 0.08	0.01 ± 0.01	0.42 ± 0.04
(C) SRV preincubated in 5×10^{-5} *M* $AgNO_3$	1.09 ± 0.13	1.11 ± 0.12	1.28 ± 0.11	0.02 ± 0.02	0.08 ± 0.08		0

[a] A and B are paired experiments on four preparations of leaky SRV at 30° m*M* Tris-maleate buffer (pH 7.0), 5 m*M* ATP, 0.5 m*M* ADP, 6 m*M* P_i, 20 m*M* $MgCl_2$, and 5 m*M* EGTA or 0.1 m*M* $CaCl_2$ or 4.0 m*M* $CaCl_2$. The Ca^{2+}-activated ATPase was calculated for each experiment as described under Materials and Methods. The experiments in C were performed on 11 preparations of SRV pre-treated with $AgNO_3$ and buffer as described under Materials and Methods, and tested at 30° in an assay medium without $AgNO_3$ but containing 1 m*M* ATP, 1 m*M* $MgCl_2$, 20 m*M* KCl, 20 m*M* NH_4Cl, and 30 m*M* Tris-maleate buffer (pH 7.0). For the Mg^{2+}-dependent activity the EGTA concentration was 2 m*M*. Low Ca^{2+} was 30 μ*M* (0.5 m*M* EGTA and 0.47 m*M* $CaCl_2$) and high Ca^{2+} was 5 m*M* $CaCl_2$. Reactions were started by the addition of SRV (0.2 or 0.3 mg of protein/ml) and stopped after 5 min with trichloroacetic acid. ATPase activity and ATP ↔ P_i exchange were determined as described under Materials and Methods, using parallel assays containing [γ-^{32}P] ATP or [^{32}P] P_i, respectively. Values represent the average ± the standard error.

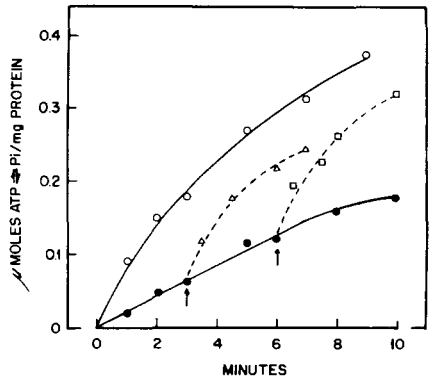

FIGURE 2: Time course of activation of ATP ↔ P_i exchange by Ag^+. All assays contained 30 m*M* Tris-maleate buffer (pH 7.0), 5 m*M* ATP, 0.5 m*M* ADP, 6 m*M* [^{32}P]P_i, 20 m*M* $MgCl_2$, and 4 m*M* $CaCl_2$, at 30°. The reactions were started by the addition of leaky SRV (0.3 mg of protein/ml) and aliquots were mixed with trichloroacetic acid at the times indicated. Formation of [γ-^{32}P]ATP was analyzed as described under Materials and Methods: (●) control (no $AgNO_3$); (○) 20 μ*M* $AgNO_3$ added before SRV; (△,□) 20 μ*M* $AgNO_3$ added 3 or 6 min after SRV, as shown by arrows.

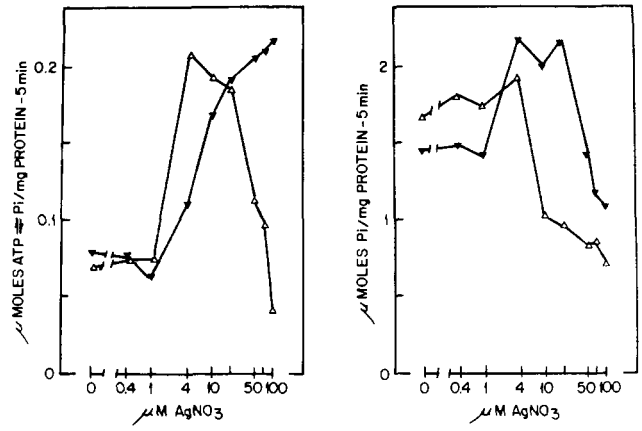

FIGURE 3: Effects of Ag^+ on exchange and hydrolysis at different SRV concentrations. The P_i concentration was 8 m*M* and $AgNO_3$ was present as indicated. Other components of the assay medium and experimental conditions were as described for the control medium in Figure 2. The figure shows a typical experiment. With 0.1 mg of leaky SRV protein/ml, the concentration of $AgNO_3$ required for maximum activation of the exchange reaction varied from 4 to 20 $\times 10^{-6}$ *M* in five different SRV preparations tested: (left) ATP ↔ P_i exchange; (right) total ATPase activity; (△) leaky SRV, 0.1 mg of protein/ml; (▼) 0.3 mg of protein/ml.

Pase is inhibited, while the Mg^{2+}-dependent ATPase is not modified.

Activation of the ATP ↔ P_i Exchange Reaction by $AgNO_3$. In contrast to the inhibitory effect shown in Figure 1, $AgNO_3$ activates ATP ↔ P_i exchange in leaky vesicles in the presence of a high Ca^{2+} concentration. In the experiment of Figure 2, the addition of 20 μ*M* $AgNO_3$ augmented the rate of exchange to similar levels whether it was present in the medium before the SRV or added several minutes after the reaction had begun. The degree of activation at optimal concentrations of $AgNO_3$ was similar to that seen when comparing leaky vesicles with intact vesicles having a Ca^{2+} concentration gradient (Table II). The rate of ATP ↔ P_i exchange as well as the degree of its activation by $AgNO_3$ varied considerably among preparations, even at the same protein concentration (cf. Tables I and II). However, the addition of $AgNO_3$ at its optimal concentration to the assay medium never failed to activate ATP ↔ P_i exchange at least twofold. Potassium nitrate in the range of 1–100 μ*M* had no effect on exchange.

The concentration of Ag^+ required for maximal activation (Figure 3, left). In the experiments of Figure 1, a parallel inhibition of the ATPase activity and Ca^{2+} uptake was shown. A similar parallelism was not found in leaky vesicles in the presence of a high Ca^{2+} concentration for the effects on ATPase activity and ATP ↔ P_i exchange. With 0.1 mg of protein/ml, for example (Figure 3), the ATP ↔ P_i exchange reaction was fully activated at 4 μ*M* $AgNO_3$, whereas the ATPase activity was not modified. When the $AgNO_3$ was raised to 10 and 20 μ*M*, the rate of exchange remained high but the total ATPase activity was inhibited to the level of the Mg^{2+}-dependent ATPase. With higher concentrations of SRV, low concentrations of $AgNO_3$ activated hydrolysis and higher concentrations inhibited it.

Ca^{2+} Concentration Dependence. In a previous report (de Meis and Carvalho, 1974), no ATP ↔ P_i exchange was measured in leaky SRV in the presence of 0.1 m*M* Ca^{2+}, whereas ATP hydrolysis was fully activated. Raising the Ca^{2+} concentration of the medium from 0.1 to 8 m*M* pro-

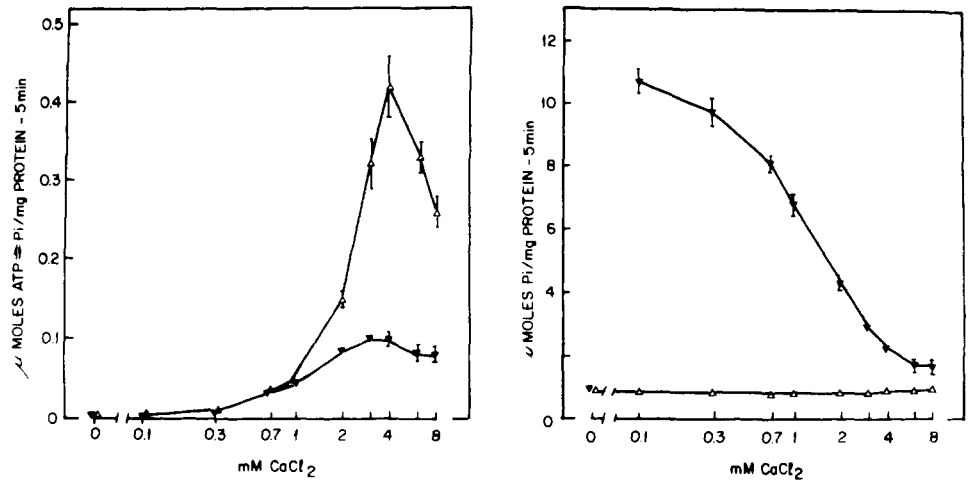

FIGURE 4: Ca^{2+} dependence of ATP ↔ P_i exchange and ATPase activity in the presence of $AgNO_3$. For ATP ↔ P_i exchange (left), the assay medium composition was 30 m*M* Tris-maleate buffer (pH 6.8), 5 m*M* ATP, 0.5 m*M* ADP, 6 m*M* [^{32}P]P_i, 20 m*M* $MgCl_2$, and different $CaCl_2$ concentrations as shown in the figure. For zero Ca^{2+}, $CaCl_2$ was omitted and 5 m*M* EGTA was added. For the total ATPase activity (right), nonradioactive P_i and [γ-^{32}P]ATP were used. The reaction was started by the addition of leaky SRV (0.2 or 0.3 mg of protein/ml) and stopped after 5-min incubation at 30° by the addition of trichloroacetic acid. The values shown represent the average ± the standard error of four experiments: control, without $AgNO_3$ (▼); with 5×10^{-5} *M* $AgNO_3$ (△).

Table II: Comparison of Activation of ATP ⇌ P_i Exchange by $AgNO_3$ with That by a Ca^{2+} Concentration Gradient.[a]

SRV Treatment	μmol of ATP ⇌ P_i/mg of Protein, 5 min
(A) Leaky	0.064 ± 0.013 (5)
(B) Leaky + $AgNO_3$	0.202 ± 0.033 (5)
(C) Intact, with gradient	0.200 ± 0.034 (4)

[a] The assay medium contained 30 m*M* Tris-maleate buffer (pH 7.0), 5 m*M* ATP, 20 m*M* $MgCl_2$, 6 m*M* [^{32}P]P_i, 4 m*M* $CaCl_2$ (A and B), or 0.1 m*M* $CaCl_2$ (C), and 0.2 mg of SRV protein/ml, at 30°. A and B contained 0.5 m*M* ADP. For experiment C, ADP was omitted and the rate of ATP ↔ P_i exchange was measured after 99% of the Ca^{2+} of the assay medium had been removed by the SRV (de Meis and Carvalho, 1974). For B, each preparation was tested in a range of $AgNO_3$ concentrations as in Figure 3, and the maximal rate of exchange in each case was used to calculate the values shown. The values represent the average ± the standard error of the number of experiments indicated in parentheses.

gressively inhibited ATPase activity, at the same time activating ATP ↔ P_i exchange. In a similar experiment (Figure 4 and Table I), the addition of 50 μM $AgNO_3$ fully inhibited the Ca^{2+}-activated ATPase at all Ca^{2+} concentrations, while the ATP ↔ P_i exchange reaction was activated two- to fourfold at Ca^{2+} concentrations above 1 m*M*. Both in the presence and in the absence of $AgNO_3$, the Ca^{2+} concentration required for 50% of the maximal activation of the exchange reaction ranged from 1.7 to 2.4 m*M*.

P_i Concentration Dependence. The rate of ATP ↔ P_i exchange varies with the P_i concentration of the assay medium (de Meis and Carvalho, 1974). The addition of 50 μM Ag^+ activated exchange in ten preparations under conditions similar to those of Figure 2 using from 1 to 8 m*M* P_i. This is the maximum of P_i that can be used with 4 m*M* $CaCl_2$ without causing precipitation of calcium phosphate. The average activation was threefold at 1 m*M* P_i and twofold at 8 m*M* P_i.

Membrane Phosphorylation from ATP and P_i. In the process of ATP hydrolysis by the Ca^{2+}-activated ATPase, the γ-phosphate of ATP is covalently bound to a membrane protein. This phosphoprotein represents an intermediate product in the sequence of reactions leading to Ca^{2+} transport and P_i liberation (Yamamoto and Tonomura, 1967; Makinose, 1969; de Meis and de Mello, 1973). In a previous report, little phosphorylation from [^{32}P]P_i was found in the presence of Ca^{2+} and ATP (de Meis and Masuda, 1974). Apparently, the phosphorylation sites are preferentially phosphorylated by ATP (de Meis, 1972; de Meis and de Mello, 1973). Under similar conditions (Figure 5), the membrane phosphorylation from [γ-^{32}P]ATP is inhibited by $AgNO_3$. Concomitantly, membrane phosphorylation from [^{32}P]P_i is enhanced. These effects are seen both at low and at high Ca^{2+} concentrations, although only in the latter case does $AgNO_3$ activate ATP ↔ P_i exchange (Figure 4).

Preincubation of SRV with $AgNO_3$. The aim of the following set of experiments was to ascertain whether the effect of $AgNO_3$ is related to the formation in the assay medium of different substrate species, such as a silver-nucleotide complex, or whether it is consistent with binding of Ag^+ to the SRV membrane. The dependence of the effects of $AgNO_3$ on the SRV protein concentration (Figures 1 and 3) lends support to the second possibility.

In the first set of experiments, SRV were preincubated as described under Materials and Methods in a buffer solution containing different $AgNO_3$ concentrations (10^{-8} to 10^{-4} *M*) before testing the rates of exchange and hydrolysis in 4 m*M* $CaCl_2$ under the conditions of the control experiments of Figure 4. In vesicles pretreated with 10^{-8} to 4×10^{-6} *M* $AgNO_3$, neither the exchange reaction nor the Ca^{2+}-activated ATPase activity was modified. With pretreatment in higher $AgNO_3$ concentrations, the Mg^{2+}-dependent ATPase remained unimpaired but both of the other activities were progressively inhibited, being fully inhibited at 5×10^{-5} *M* $AgNO_3$. These data reinforce those of Table I showing that the Ca^{2+}-activated ATPase is more sensitive to $AgNO_3$ than is the Mg^{2+}-dependent ATPase. Incubating SRV with buffer and $AgNO_3$ alone did not produce the activation of the ATP ↔ P_i exchange reaction seen when $AgNO_3$ was added to the complete assay medium (Table I).

In a second set of experiments, leaky SRV were preincubated with $AgNO_3$ in a medium that was optimal for the exchange reaction (Figure 4) except that it lacked Mg^{2+}. After centrifugation and resuspension of the pellet in the same medium but without $AgNO_3$, the activity of the prep-

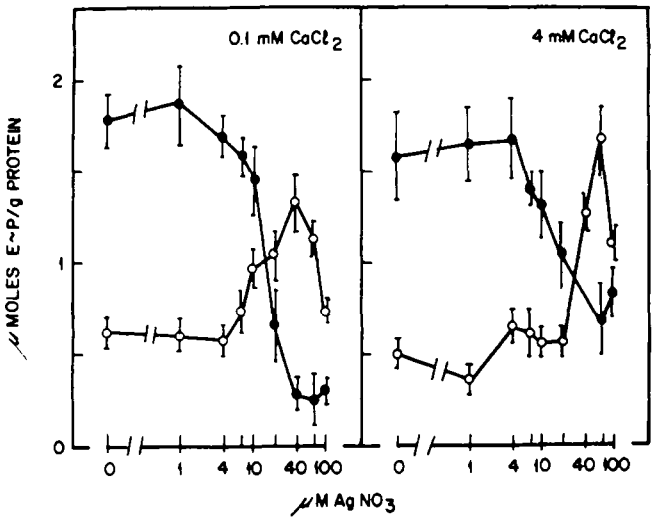

FIGURE 5: Membrane phosphorylation by [^{32}P]P_i and [γ-^{32}P]ATP. The assay medium composition was 30 mM Tris-maleate buffer (pH 7.0), 5 mM ATP, 0.5 mM ADP, 6 mM P_i, 20 mM $MgCl_2$, and 0.1 (left) or 4.0 mM (right) $CaCl_2$. The reaction was started by the addition of leaky SRV (0.33 mg of protein/ml) and stopped after 20- or 40-sec incubation at 30° by the addition of trichloroacetic acid to a final concentration of 20% (w/v). The degree of membrane phosphorylation from either compound was essentially the same for either of these times, indicating that the steady state was reached within the first 20 sec: (●) [γ-^{32}P]ATP and nonradioactive P_i; (○) [^{32}P]P_i and nonradioactive ATP. The values shown in the figure represent the average ± the standard error of four or seven experiments, respectively.

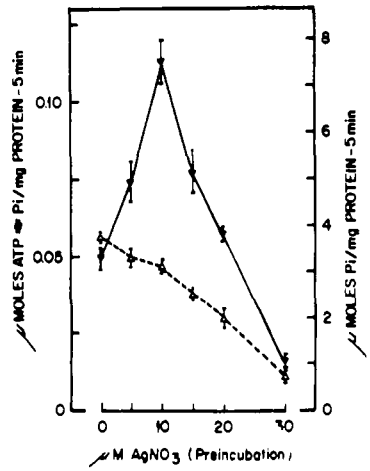

FIGURE 6: Preincubation of SRV with $AgNO_3$. The assay medium contained 30 mM Tris-maleate buffer (pH 7.0), 5 mM ATP, 0.5 mM ADP, 6 mM P_i, 4 mM $CaCl_2$, and 20 mM $MgCl_2$. Leaky SRV at 0.5 mg of protein/ml were preincubated for 3 min at room temperature in the same medium but without $MgCl_2$ as described in the text. ATP ↔ P_i exchange and total ATPase activity were tested at a final concentration of about 0.5 mg of protein/ml. After 5-min incubation at 30° the reaction was arrested by the addition of trichloroacetic acid. Other conditions are described under Materials and Methods. The values shown in the figure represent the average ± the standard error of four experiments: (▼) ATP ↔ P_i exchange; the medium contained [^{32}P]P_i and nonradioactive ATP; (△) total ATPase activity; the medium contained [γ-^{32}P]ATP and nonradioactive P_i.

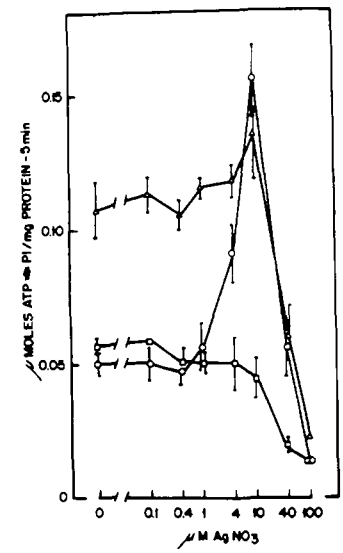

FIGURE 7: Effect of $AgNO_3$ on SRV preincubated with $AgNO_3$. Preincubation of leaky SRV with $AgNO_3$, assay medium, and experimental conditions were as described for Figure 6, differing only in the addition of $AgNO_3$ to the assay medium in the concentrations shown. The values represent the average ± the standard error of four experiments. The concentrations of $AgNO_3$ in the preincubation were: (○) zero (control); (△) 10^{-5} M; and (□) 2×10^{-5} M.

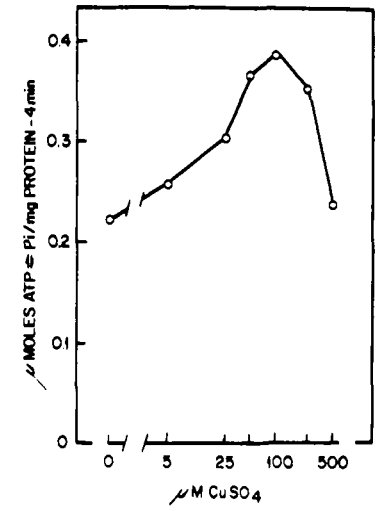

FIGURE 8: Effect of $CuSO_4$ on the ATP ↔ P_i exchange. The assay medium contained 30 mM Tris-maleate buffer (pH 7.0), 5 mM ATP, 2 mM ADP, 8 mM [^{32}P]P_i, 20 mM $MgCl_2$, 4 mM $CaCl_2$, and the specified concentrations of $CuSO_4$. The reaction was started by the addition of leaky SRV (0.3 mg of protein/ml) and stopped after 4-min incubation at 37° by the addition of trichloroacetic acid. Essentially the same results were obtained in three different SRV preparations tested.

aration was tested with Mg^{2+} added to the assay medium. Figure 6 shows that the exchange reaction was activated and the ATPase inhibited by the pretreatment with $AgNO_3$ (cf. Figure 3). Figure 7 shows that when SRV were treated with the optimal concentration of $AgNO_3$ in the preincubation, subsequent addition of $AgNO_3$ to the assay medium did not activate further the rate of ATP ↔ P_i exchange. Similarly, SRV which were treated with an excessive concentration of $AgNO_3$ in the preincubation were no longer activated by the addition of $AgNO_3$ to the assay medium. These data and those of Figures 1 and 3 lead to the conclusion that the effect of $AgNO_3$ is consistent with its binding to a membrane component, and that one or more ligands, except Mg^{2+}, involved in the exchange reaction (ATP, ADP, Ca^{2+}, and P_i) somehow protect the enzyme from a nonspecific inhibitory effect of $AgNO_3$.

Specificity. Copper sulfate is also able to activate the ATP ↔ P_i exchange, although somewhat less effectively than $AgNO_3$ (Figure 8). KNO_3, zinc acetate, $FeCl_3$,

$FeSO_4$, $PtCl_6$, $CoCl_2$, $PdCl_2$, and $CdCl_2$ in the concentration range 10^{-6} to 10^{-3} M had no effect on the ATP $\leftrightarrow$ P_i exchange reaction.

Discussion

In this paper it is shown that the binding of Ag^+ to leaky SRV membranes activates the rate of ATP $\leftrightarrow$ P_i exchange to a level similar to that measured in vesicles having a transmembrane Ca^{2+} concentration gradient. This indicates that the gradient is not necessary as an energy source for the reaction (Makinose, 1971; Makinose and Hasselbach, 1971; de Meis and Carvalho, 1974), but probably favors one or more of the intermediate steps involved in the overall exchange reaction.

Exchange of phosphate between the medium and ATP implies formation of a phosphoprotein from P_i, but it has not been possible to demonstrate this correlation in intact vesicles in the presence of ATP and Ca^{2+} (Kanazawa and Boyer, 1973; de Meis and Masuda, 1974). Figure 6 shows that this can be done in the presence of Ag^+ and provides additional information which can be related to the exchange reaction in untreated vesicles.

(a) The phosphorylation from P_i was equally activated by Ag^+ at a low and at a high Ca^{2+} concentration. Therefore, the role of a high Ca^{2+} concentration in promoting the exchange reaction (Figure 4) is not related to the phosphorylation step, but to the transfer of this phosphate to ADP. This implies that formation of E~P is followed by formation of Ca:E~P. We are presently attempting to distinguish these two forms of the phosphoprotein by other means.

(b) The activation by Ag^+ of the membrane phosphorylation from P_i coincides with an inhibition of the membrane phosphorylation from ATP. Evidence has been presented that ATP and P_i are substrates of a common site in the membrane and that the binding of Ca^{2+} to its outer surface dictates a preference for ATP in the phosphorylation reaction (Kanazawa and Boyer, 1973; Masuda and de Meis, 1973; de Meis and Masuda, 1974). The binding of Ag^+ apparently can override this control mechanism and imitate the effect of low external Ca^{2+} concentrations in intact vesicles by favoring the phosphorylation from P_i, decreasing phosphorylation from ATP, and consequently inhibiting the Ca^{2+}-dependent ATPase activity.

With the information presented here and in previous papers (Kanazawa and Boyer, 1973; Masuda and de Meis, 1973; de Meis and Masuda, 1974; de Meis and Carvalho, 1974; Kanazawa, 1975) the following hypothesis is proposed. The binding of Ca^{2+} to the internal surface of the membrane allows the transfer of the phosphate from the phosphoenzyme to ADP, while the binding of Ca^{2+} to the outer surface of the SRV modulates the rate of ATP $\leftrightarrow$ P_i exchange by regulating the number of sites phosphorylated from ATP or P_i. The Ca^{2+} binding site on the outer surface of membrane would be saturated in micromolar Ca^{2+} concentrations (Makinose, 1969; Hasselbach, 1972; de Meis and de Mello, 1973), while the binding site at the inner surface would only be saturated in the millimolar range (de Meis and Carvalho, 1974).

Finally, the Ca^{2+}-dependent ATPase of SRV is similar to the $(Na^+ + K^+)$-ATPase provided that Ca^{2+} and Na^+ are assigned corresponding roles for the two enzymes. Both enzymes are phosphorylated from ATP or P_i, both catalyze ADP $\leftrightarrow$ ATP, HOH $\leftrightarrow$ P_i, and ATP $\leftrightarrow$ P_i exchanges, and both enzymes have a similar primary structure in the neighborhood of the active site of phosphorylation (Bastide et al., 1973; Dahms and Boyer, 1973; de Meis and Masuda, 1974; Hasselbach, 1972; Kanazawa and Boyer, 1973; Masuda and de Meis, 1973; Post et al., 1975; Taniguchi and Post, 1975).

Acknowledgments

We are grateful to Dr. Robert L. Post for a stimulating discussion of the manuscript. The excellent technical assistance of Mr. Isaltino R. Soares and Mr. Valdecir Antunes Suzano is acknowledged with thanks.

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