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ATP-DEPENDENT PHOSPHATE TRANSPORT IN SARCOPLASMIC RETICULUM AND RECONSTITUTED PROTEOLIPOSOMES

WILLIAM W. CARLEY and EFRAIM RACKER

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853 (U.S.A.)

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During uptake of Ca^{2+} by rabbit sarcoplasmic reticulum, about 1 μmol of $^{32}P_i$ was taken up per μmol $^{45}Ca^{2+}$ transported. The uptake of P_i was dependent on external Ca^{2+}, Mg^{2+} and ATP. Intravesicular Ca^{2+} did not substitute for external Ca^{2+}. In contrast to the accumulation of Ca^{2+} which was abolished by the ionophore A23187, the uptake of P_i continued to take place provided sufficient Ca^{2+} was present in the medium. Thus, a Ca^{2+} gradient did not seem to be required. Similar observations were made with proteoliposomes reconstituted with membrane preparations of sarcoplasmic reticulum and soybean phospholipids. However, when purified Ca^{2+}-ATPase was used for reconstitution, there was ATP-dependent Ca^{2+} uptake but no ATP-dependent P_i transport was observed. These data show that the mechanism of P_i transport cannot be a passive movement in response to a Ca^{2+} gradient but appears to be catalyzed by a specific protein, which is inactivated during purification of the Ca^{2+}-ATPase. A protein that catalyzes P_i transport in reconstituted vesicles has been solubilized by extraction of sarcoplasmic reticulum with sodium cholate.

Introduction

Ca^{2+} fluxes in sarcoplasmic reticulum participate in contraction and relaxation of skeletal muscle. The sarcoplasmic reticulum releases Ca^{2+} into the myoplasm in response to a neural signal, causing a cascade of events leading to contraction. Subsequently, relaxation is accomplished by the MgATP-dependent removal of Ca^{2+} back into the sarcoplasmic reticulum lumen by an ATPase residing in the reticular membrane.

The exact mechanisms for the storage and release of Ca^{2+} have remained elusive. Although there is now almost general agreement that calsequestrin and an acidic Ca^{2+}-binding protein appear to be localized in the lumen of sarcoplasmic reticulum, the total amount of Ca^{2+} taken up by isolated reticulum vesicles in the absence of either oxalate or P_i is small [1]. Liposomes reconstituted with purified Ca^{2+}-ATPase are impermeable to either oxalate or P_i. Ca^{2+} pumping does not take place unless a Ca^{2+}-precipitating anion is incorporated into the vesicles during reconstitution [2].

P_i seems to be a likely candidate as the natural co-ion in the Ca^{2+}-uptake process. Previous work by Hasselbach and Weber [1] has indicated that competition existsbetween P_i and oxalate for a common site of an anion channel and it was assumed that the transport of P_i and/or oxalate is a consequence of the ATP-energized accumulation of Ca^{2+}. We report in this paper that P_i transport, like Ca^{2+} transport, is dependent upon ATP, but that a Ca^{2+} gradient is not required for this process. Preliminary data on the solubilization of the P_i transporter are also reported.

Materials and Methods

Bovine albumin stock solution, Tris, H^+-Dowex 50W 50 × 8-100, sodium cholate and sucrose were

obtained from Sigma, St. Louis, MO. Cholic acid was recrystallized three times from 95% ethanol prior to use. Divalent cations (chloride form) were from Mallinckrodt Chemical Works, St. Louis, MO, and *N*-ethylmaleimide was from Schwarz/Mann, Orangeburg, NJ. A23187 was a gift from D.R. Hosley of Eli Lilly and Co., Indianapolis, IN. $^{32}P_i$ was obtained from ICN, Irvine, CA, and was hydrolyzed in 2 M HCl for 2 h at 95°C at 60–80 mCi/ml. H^+-Dowex 50W 50 × 8-100 was neutralized (pH 6.5) with Tris-HCl for the Ca^{2+}-transport assay. Anion exchange resin (AG1X8) from Bio-rad, Richmond, CA, was converted to the formate form for use in the P_i-transport assay (pH 6.0). Crude soybean phospholipids (asolectin) were obtained from Associated Concentrates, Woodside, NY. Liquiscint was from National Diagnostics, Sommerville, NJ. Protein was determined according to the method of Lowry et al. [3].

Preparation of sarcoplasmic reticulum. Sarcoplasmic reticulum was prepared from rabbit skeletal muscle as described by MacLennen [4] except that 0.5 mM phenylmethanesulfonyl fluoride was added to the initial homogenate. The final sarcoplasmic reticulum pellet was resuspended in 0.3 M sucrose, 10 mM Tris-HCl (pH 8.0).

Reconstitution of sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles (100 μg protein) were mixed with 2 mg asolectin and 50 mM Tris-maleate, pH 7.5, 0.4 M potassium phosphate, pH 7.5, 2.5 mM dithiothreitol and brought to 100 μl with glass-distilled water. Freeze-thaw/sonication [5] as modified [6] was used for reconstitution.

P_i- and Ca^{2+}-transport assays. The $^{32}P_i$ uptake was assayed according to the method of Gasko et al. [7] and was initiated by the addition of 10 μg protein to 250 μl (35°C) containing 50 mM Tris-maleate, pH 7.5, 0.2 mM $CaCl_2$, 10 mM ATP (sodium salt), pH 7.5, 10 mM $MgCl_2$, 50 mM potassium [^{32}P]phosphate, pH 7.5 (1100 cpm/nmol P_i), and 0.13 M sorbitol. After 1.5 min, 200 μl were removed and passed through the anion-exchange resin column.

The $^{45}Ca^{2+}$-uptake assay was identical to that for P_i except ^{45}Ca (1788 cpm/nmol Ca^{2+}) was used rather than $^{32}P_i$ and a cation-exchange column was used (Dowex-Tris).

Results

Uptake of P_i in sarcoplasmic reticulum vesicles

As can be seen in Table I, the uptake of $^{32}P_i$ into sarcoplasmic reticulum vesicles required the presence of Mg^{2+}, Ca^{2+} and ATP. Deletion of any of these components eliminated the process, and addition of *N*-ethylmaleimide inhibited. The addition of 1% deoxycholate or Triton X-100 after the assay released all P_i.

Since these experimental conditions are identical to those required for the ATP-driven uptake of Ca^{2+}, the question arises as to whether the two processes are interdependent. It is clear from reconstitution experiments with the Ca^{2+}-ATPase [2] that cotransport of P_i is not required for Ca^{2+} uptake because Ca^{2+} transport occurs in vesicles that are incapable of P_i transport. However, the possibility that P_i is transported as a co-ion with Ca^{2+} needed further examination. As shown in Table II, $CoCl_2$ or $MnCl_2$ effectively substituted for Mg^{2+} in both systems. Table III shows that Sr^{2+}, which is known to be transported by sarcoplasmic reticulum vesicles [8], substituted for Ca^{2+} in the process of P_i transport. A parallel requirement for Ca^{2+} and P_i uptake was observed when the nucleotide specificity was explored (Table IV). ATP was the most effective trinucleotide supporting either Ca^{2+} or P_i transport. The re-

TABLE I

TRANSPORT IN SARCOPLASMIC RETICULUM VESICLES

P_i uptake was measured as described in Materials and Methods with additions and deletions as shown. Each value is the average of duplicates. Sarcoplasmic reticulum was incubated with 5 mM *N*-ethylmaleimide for 5 min at 35°C prior to assay.

Conditions	$^{32}P_i$ uptake (nmol/min per mg protein)
Complete system	605
$-Mg^{2+}$	41
−ATP	38
$-Mg^{2+}$, −ATP	41
$-Ca^{2+}$	86
+5 mM *N*-ethylmaleimide	58
(zero time)	85

TABLE II

Mg^{2+} REPLACEMENT BY DIVALENT CATIONS IN THE ATP-DEPENDENT P_i- AND Ca^{2+}-UPTAKE ASSAYS IN SARCOPLASMIC RETICULUM

The P_i- and Ca^{2+}-transport assays were as described in Materials and Methods except that 10 mM $MgCl_2$ was replaced by 10 mM of the chloride salt of the indicated divalent cation. Each value represents averaged duplicates of P_i and Ca^{2+} transport after subtracting a value obtained with sarcoplasmic reticulum vesicles that were heated to 60°C for 5 min. These blank values were 10% or less of the experimental values in all cases except with Cd^{2+} and Cu^{2+}, which gave rise to precipitation of P_i. Thus, the latter data are not as firm as the others because the blank values were similar to the experimental values.

Divalent cations (10 mM)	$^{32}P_i$ uptake (nmol/min per mg protein)	^{45}Ca uptake (nmol/min per mg protein)
None	72	130
Mg^{2+}	780	1155
Ba^{2+}	33	65
Cd^{2+}	0	70
Co^{2+}	871	1050
Cu^{2+}	0	31
Mn^{2+}	618	630
Sr^{2+}	14	6

quirement for Ca^{2+} for P_i uptake and a titration of P_i concentrations are shown in Figs. 1 and 2. Under the given conditions, calcium phosphate precipitation did not occur until the Ca^{2+} concentration was above 400 μM. The K_m value for ATP, as well as for Mg^{2+}, was about 2 mM, 50 μM for Ca^{2+} and 15 mM for P_i. The pH optimum for P_i uptake was 7.0–8.0.

The first clue pointing to a difference between these two processes was observed when the effect of A23187 was examined. As can be seen from Table V (Expt. 1), this ionophore, which eliminates Ca^{2+} accumulation, was much less effective in suppressing P_i uptake, particularly when the Ca^{2+} concentration was raised to 1 mM.

Uptake of P_i in reconstituted vesicles

Reconstitution of P_i transport into phospholipid vesicles was achieved by freeze-thaw/sonication as described in Materials and Methods. It can be seen from Table VI that basically the same requirements for P_i were observed

TABLE III

EFFECT OF DIVALENT CATIONS IN REPLACING Ca^{2+} IN ATP-DEPENDENT UPTAKE OF P_i IN SARCOPLASMIC RETICULUM VESICLES

The P_i-transport assay was performed as described in Materials and Methods except that 0.2 mM $CaCl_2$ was replaced by 0.2 mM of the chloride salt of the divalent cations indicated. All values represent the average of duplicates after subtracting a value obtained with sarcoplasmic reticulum heated to 60°C for 5 min, which was consistently less than 10% of the experimental value.

Divalent cations (0.2 mM)	P_i uptake (nmol/min per mg protein)
Ca^{2+}	616
Ba^{2+}	115
Cd^{2+}	127
Co^{2+}	131
Cu^{2+}	91
Mn^{2+}	123
Sr^{2+}	497

in the reconstituted vesicles as in the intact sarcoplasmic reticulum vesicles. As shown in Table V (Expt. 2), there was little effect by A23187 on P_i accumulation, while Ca^{2+} accumulation measured under identical conditions was greatly suppressed. Thus, much more P_i was retained than

TABLE IV

ATP-DEPENDENT P_i AND Ca^{2+} TRANSPORT IN SARCOPLASMIC RETICULUM: ENERGY SOURCE DEPENDENCE

The P_i- and Ca^{2+}-transport assays were as described in Materials and Methods except ATP was replaced by the indicated compound. Each value is the average of duplicates.

Energy source	P_i uptake (nmol/min per mg protein)	Ca^{2+} uptake (nmol/min per mg protein)
−ATP	33	6
ATP	527	505
UTP	129	135
GTP	121	119
ITP	133	121
CTP	126	59
Phospho*enol*-pyruvate	16	3
Acetyl phosphate	48	50

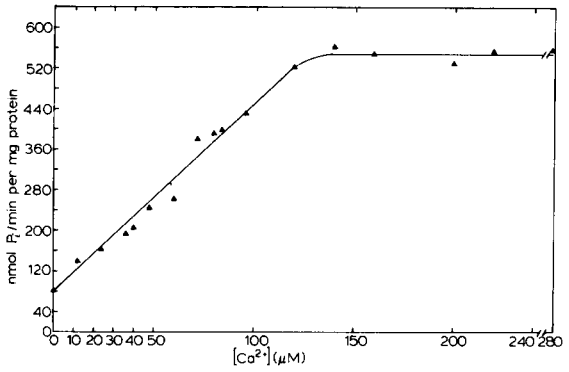

Fig. 1. The effect of Ca^{2+} on ATP-dependent P_i uptake in sarcoplasmic reticulum. The P_i-transport assay was as described in Materials and Methods except increasing amounts of Ca^{2+} were added to the assay as indicated. Each value is the average of duplicates.

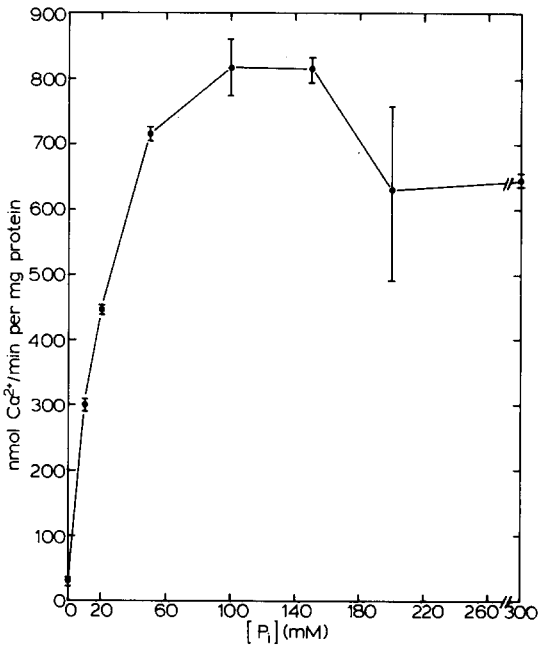

Fig. 2. The effect of phosphate on ATP-dependent Ca^{2+} uptake in sarcoplasmic reticulum. The Ca^{2+}-transport assay was as described in Materials and Methods except increasing amounts of P_i were added to the assay as indicated. Each value is the average of duplicates.

Ca^{2+} (615 as compared to 95 nmol/min per mg protein, respectively) under identical conditions. To ensure that these results were not an artifact of the A23187 and the ion-exchange column removing Ca^{2+} from inside the vesicles after the assay, we carried out similar experiments using the Sephadex column assay [9]. As in the ion-exchange assay much more $^{32}P_i$ (560 nmol P_i/min per mg protein) was retained than $^{45}Ca^{2+}$ (23 nmol Ca^{2+}/min per mg protein) in the presence of A23187 in the Sephadex assay, indicating no dif-

TABLE V

EFFECT OF A23187 ON P_i AND Ca^{2+} TRANSPORT IN SARCOPLASMIC RETICULUM VESICLES

Ca^{2+}- and P_i-transport assays were as described in Materials and Methods. A 0.25 μl aliquot of A23187 (5 mg/ml Me_2SO) was added to the assay where indicated. An equal amount of Me_2SO had no effect in the assay. The reconstitution was as described in Materials and Methods. Each value is the average of duplicates with the ATP value minus the value without ATP.

Assay conditions	P_i uptake (nmol/min per mg protein)	Ca^{2+} uptake (nmol/min per mg protein)
Expt. 1		
Sarcoplasmic reticulum	702	825
Sarcoplasmic reticulum + A23187 (5 μg/ml)	133	2
Sarcoplasmic reticulum + 1 mM $CaCl_2$ + A23187	480	6
Expt. 2		
Reconstituted sarcoplasmic reticulum	695	520
Reconstituted sarcoplasmic reticulum + A23187 (5 μg/ml)	615	95

TABLE VI

P_i TRANSPORT IN RECONSTITUTED SARCOPLASMIC RETICULUM

P_i transport was assayed as described in Materials and Methods with deletions as shown. Each value is the average of duplicates. *N*-Ethylmaleimide was incubated with the vesicles prior to assay. The sarcoplasmic reticulum vesicles were reconstituted as described in Materials and Methods.

Assay conditions	P_i uptake (nmol/min per mg protein)
Complete	702
$-Mg^{2+}$	345
−ATP	146
$-Mg^{2+}$, −ATP	131
$-Ca^{2+}$	257
Complete + 10 mM *N*-ethylmaleimide (10 min, 35°C)	86
Complete (zero time)	98

ference between the assays and eliminating the possibility of an artifact caused by the column assay.

To explore the possibility that P_i transport is simply driven by the inside positive potential generated by the electrogenic ATP-dependent Ca^{2+} translocation [6], valinomycin (2 μg/ml) was added to the assay. No significant difference in P_i transport was seen. In the presence of ATP, 615 nmol P_i/min per mg protein, and in the absence of ATP 121 nmol P_i/min per mg protein were transported.

Effect of internal and external Ca^{2+} on transport

To examine more directly the effect of a Ca^{2+} gradient on P_i uptake, we passively loaded sarcoplasmic reticulum vesicles with various amounts of $CaCl_2$ and then assayed P_i uptake in the presence or absence of ATP. In these experiments the Ca^{2+}-loaded vesicles were diluted in the assay, so that more Ca^{2+} was present inside the vesicles than outside. As shown in Table VII, even at 20 mM $CaCl_2$ inside the vesicles, P_i uptake still required 200 μM external Ca^{2+} as well as ATP for a maximal rate (see also Fig. 1). Moreover, ATP hydrolysis was necessary, since nonhydrolyzable adenylyl imidodiphosphate did not substitute for

TABLE VII

THE EFFECT OF PASSIVELY LOADING SARCOPLASMIC RETICULUM VESICLES ON P_i TRANSPORT

Sarcoplasmic reticulum vesicles (40 mg/ml of 0.3 M sucrose, 10 mM Hepes, pH 7.3) were loaded with $CaCl_2$ by incubation for 20 h at 0–4°C with the indicated amount of $CaCl_2$. Each value is the average of duplicates. The P_i transport was assayed in the presence (Expt. 1) and absence of ATP (Expt. 2) as described in Materials and Methods.

$CaCl_2$ outside (μm)	$CaCl_2$ inside (mm)	P_i transport (nmol/min per mg protein)
Expt. 1		
0	0	84 ± 14
0.6	0.5	90 ± 5
2.4	2	98 ± 1
12	10	218 ± 8
24	20	182 ± 31
100	0	388 ± 26
200	0	548 ± 19
Expt. 2		
1.2	1.0	14
12	10	19

ATP in either $CaCl_2$-loaded or unloaded sarcoplasmic reticulum vesicles.

Similar experiments were performed with reconstituted vesicles prepared in the presence or absence of Ca^{2+} or P_i. It can be seen from Table

TABLE VIII

EFFECT OF Ca^{2+} AND P_i LOADING OF RECONSTITUTED SARCOPLASMIC RETICULUM ON Ca^{2+} AND P_i UPTAKE

Sarcoplasmic reticulum was reconstituted as described in Materials and Methods with either 10 mM $CaCl_2$ or 0.4 M potassium phosphate, pH 7.5, present. P_i- and Ca^{2+}-transport assays were as described in Materials and Methods. Each value represents an averaged duplicate.

	Ca^{2+} uptake (nmol/min per mg protein)	P_i uptake (nmol/min per mg protein)
(A) Ca^{2+} loading (10 mM $CaCl_2$)		
−ATP	40	32
+ATP	442	490
(B) P_i loading (0.4 M potassium phosphate, pH 7.5)		
−ATP	31	60
+ATP	532	551

TABLE IX

SOLUBILIZATION OF ATP-DEPENDENT P_i AND Ca^{2+} TRANSPORT FROM SARCOPLASMIC RETICULUM

Sarcoplasmic reticulum (8 mg/ml) was solubilized with the indicated amounts of sodium cholate in the presence of 0.1 M sucrose, 50 mM Tris-maleate, pH 7.5, and 1 M KCl for 10 min at 4°C. The suspension was centrifuged at 200000 × *g* for 20 min. The supernatant was diluted 20-fold with 0.1 M sucrose, 0.05 M KCl and 10 mM Tris-maleate, pH 7.5, and centrifuged at 200000 × *g* for 1 h. The pellet was suspended in 100 μl of the dilution buffer and 100 μg of protein were reconstituted with soybean phospholipids as described in Materials and Methods. The P_i and Ca^{2+} assays were as described in Materials and Methods. Each value represents averaged duplicates less the rate of uptake without Mg^{2+} and ATP (which was less than 10% of the active transport). Total units = specific activity (nmol/min per mg protein) × mg protein recovered from the supernatant.

Fraction reconstituted	P_i uptake (nmol/min per mg protein)	Total units	Ca^{2+} uptake (nmol/min per mg protein)	Total units
Sarcoplasmic reticulum	544	2176	860	3440
0.2% cholate extract	255	284	281	284
0.4% cholate extract	285	662	715	1645
0.6% cholate extract	236	684	684	1984
0.8% cholate extract	354	1239	406	1421

VIII that neither P_i nor Ca^{2+} uptake was significantly influenced by these intravesicular ions. ATP was still required and a Ca^{2+} gradient was not. It should be emphasized that in these experiments, sarcoplasmic reticulum membranes were incorporated into liposomes without solubilization and fractionation. In these vesicles external P_i was capable of driving Ca^{2+} transport as in sarcoplasmic reticulum vesicles. On the other hand, incorporation of solubilized and purified preparations of the Ca^{2+}-ATPase [2] gave vesicles that did not catalyze ATP-dependent P_i uptake yet catalyzed extensive Ca^{2+} transport provided P_i or oxalate was inside.

Solubilization of P_i transporter

Experiments on the solubilization of the P_i transporter with various concentrations of sodium cholate are shown in Table IX. Over 50% of the total P_i-transport activity was solubilized with 0.8% cholate. Extraction with 1% cholate or fractionation of the extract obtained with 0.8% cholate by salting out with ammonium sulfate inactivated the transporter. Attempts to solubilize the P_i transporter with deoxycholate also led to inactivation, explaining the previous observation [2] that there is no P_i transport in the Ca^{2+} pump reconstituted with an ATPase extracted with this detergent.

Discussion

There are two lines of evidence for the existence of a distinct P_i transporter in sarcoplasmic reticulum vesicles. The first is based on kinetic studies on the effect of P_i concentrations and competition with oxalate as previously described [1]. The second is based on reconstitution experiments. Purified ATPase preparations incorporated into liposomes do not catalyze Ca^{2+} uptake in the presence of external P_i but do respond to internal P_i [2]. The rate of P_i transport in sarcoplasmic reticulum vesicles is very high (over 500 nmol/min per mg protein) compared to P_i transport in sarcolemma membrane vesicles and mitochondria which were 5 and 2 nmol P_i/min per mg protein, respectively. It is obvious, therefore, that these membranes cannot contribute significantly as contaminants to the P_i transport measured in sarcoplasmic reticulum preparations.

The question of the mode of action of the P_i transporter of sarcoplasmic reticulum vesicles and the role of ATP needs to be elucidated. Attempts to detect any P_i-stimulated ATPase activity have failed thus far. Similarly, no evidence for the formation of a modified transporter by ATP and a protein kinase could be established. The fact that the ATP-driven P_i uptake took place in the pres-

ence of A23187 under conditions that suppressed net Ca^{2+} uptake indicates that the ATP requirement is independent of the formation of a Ca^{2+} gradient. Ca^{2+}-loading experiments support this conclusion. Moreover, a dependence on ATP was seen in reconstituted vesicles which contained a large reservoir of P_i to dilute and trap the isotopically labeled anion. Thus, ATP-independent P_i-P_i exchange contributes little to the uptake of $^{32}P_i$ and ATP-driven P_i transport occurs against a P_i gradient.

The most curious aspect of this P_i uptake is its physiological significance. It would be attractive to view the transporter as a collaborator of the Ca^{2+} pump, insuring the deposition of sufficient Ca^{2+} inside the vesicles to serve as a proper Ca^{2+} reservoir. Yet the affinity data reported here for P_i are difficult to reconcile with such a role. Although the estimates of intracellular P_i vary considerably with different investigators, methods and tissues, the overal intramuscular P_i concentration appears to be too low to support Ca^{2+} entry into sarcoplasmic reticulum. It is conceivable, however, that in the intact muscle there is a high local P_i concentration at the sarcoplasmic reticulum membrane generated by ATP hydrolysis during initial Ca^{2+} transport. On the other hand, the possibility should be considered that the affinity for P_i may be under the control of other factors, e.g., secondary messengers, and further explorations of these appear warranted.

Acknowledgements

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