Biochimica et Biophysica Acta, 693 (1982) 287–295
Elsevier Biomedical Press

BBA 71433

ACTIVE TRANSPORT OF Ca^{2+} IN MEMBRANE VESICLES FROM PEA

EVIDENCE FOR A H^+/Ca^{2+} ANTIPORT

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(Received April 13th, 1982)
(Revised manuscript received August 2nd, 1982)

Key words: Ca^{2+} transport; H^+/Ca^{2+} antiport; Membrane vesicle; (P. sativum)

Two non mitochondrial systems involved in ATP-dependent Ca^{2+} accumulation have been described and characterized in two membrane fractions from pea internodes purified on a metrizamide-sucrose discontinuous gradient. In the lighter membrane fraction an ATP-dependent Ca^{2+} accumulation system, which shows the characteristics of an ATP-dependent H^+/Ca^{2+} antiport, predominates. This system is inhibited by FCCP and nigericin and stimulated by 50 mM KCl. It is saturated by 0.8–1.0 mM $MgSO_4$-ATP, strictly requires ATP and is severely inhibited by an excess of free Mg^{2+} or Mn^{2+}. A second system of ATP-dependent Ca^{2+} accumulation, recovered mainly in the heavier membrane fraction, is insensitive to FCCP, is saturated by 8–10 mM $MgSO_4$-ATP, can utilize also ITP or other nucleoside triphosphates although at lower rate than ATP and is only scarcely affected by an excess of free Mg^{2+} or Mn^{2+}. This system is interpreted as corresponding to the (Ca^{2+} + Mg^{2+})-ATPase described by Dieter, P. and Marmé, D. ((1980) Planta 150, 1–8).

Introduction

The concentration of Ca^{2+} in the cytoplasm of living cells is kept very low (about 10^{-7}–10^{-6} M) and strictly controlled as transient variations of it can dramatically influence a number of metabolic processes. Cytoplasmic Ca^{2+} concentration is regulated on this low level by means of both Ca^{2+} extrusion through the plasma membrane and, in eukaryotic cells, Ca^{2+} accumulation within intracellular organelles [1,2].

Two kinds of Ca^{2+}-pumping systems are presently recognized in animal cells and bacteria: (i) systems which directly use the energy of hydrolysis of ATP, such as the (Ca^{2+} + Mg^{2+})-ATPases of the endoplasmic reticulum and of the plasma membranes of a number of animal cells [3,4]; (ii) systems which dissipate the electrical, the chemical or the electrochemical gradient of another ion, such as the electrophoretic Ca^{2+} pump of mitochondria, the H^+/Ca^{2+} antiporters of bacteria and the Na^+/Ca^{2+} antiporters of plasma membranes of animal cells [5–8].

Only recently have 'in vitro' systems suited to the study of transport phenomena also been made available from plant materials: so, while the Ca^{2+}

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; $Hepes_L$-$Bistris_L$, buffer made by mixing solutions of equal concentrations of Hepes and of Bistris to give the desired pH values; EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

0005-2736/82/0000–0000/$02.75

pump of plant mitochondria has been described in some detail (Ref. 9 and references therein), little is known about non mitochondrial Ca^{2+} transport systems of plant cells.

Scarborough and co-workers [10,11] have shown that plasma membrane vesicles from *Neurospora crassa* are endowed with a H^+/Ca^{2+} antiporter which dissipates the chemical proton gradient built up by a proton translocating electrogenic ATPase.

An ATP-dependent Ca^{2+} accumulation has been described in microsomal vesicles from higher plants; this active transport of Ca^{2+} is insensitive to protonophores and seems to be driven by a calmodulin-regulated $(Ca^{2+} + Mg^{2+})$-ATPase [12,13].

In the present paper we show that membrane vesicles from pea internodes are endowed with at least two non mitochondrial systems involved in the active transport of Ca^{2+}: a H^+/Ca^{2+} antiport * which utilizes the proton gradient built up by an electrogenic proton translocating ATPase, similar to that described in plasma membranes of *Neurospora*, and a protonophore-resistant ATP-dependent Ca^{2+} transport system, similar to that described by Marmé and co-workers [12,13].

A preliminary report of this work was presented elsewhere [14].

Materials and Methods

Fractionation of membrane vesicles

Pea (*Pisum sativum*, L. cv Alaska) internode segments, prepared as previously described [15] were chopped with razor blades in two volumes of extraction medium (0.25 M sucrose, 10 mM Hepes-Bistris (pH 7.6), 4 mM $MgSO_4$ and 0.5% bovine serum albumin), gently ground in a mortar and strained through four layers of cheesecloth. 10 ml of the homogenate were layered on top of a discontinuous gradient prepared as follows: 16 ml of 50% (w/w) sucrose, 6 ml of 7% (w/w) metrizamide plus 5% (w/w) sucrose and 6 ml of 9% (w/w) sucrose. All gradient solutions contained 5 mM Hepes-Bistris (pH 7.6), 4 mM $MgSO_4$ and 0.5% bovine serum albumin. Gradients were run for 60 min at 27000 rev./min in a Spinco SW27 rotor. Visible membrane bands were removed from the 9% sucrose/7% metrizamide plus 5% sucrose interface (fraction L) and from the 7% metrizamide plus 5% sucrose/50% sucrose interface (fraction H), using a glass capillary connected to a peristaltic pump. Membrane fractions were diluted 2–4-times with 0.5% bovine serum albumin and centrifuged for 30 min at $150000 \times g$ in a Spinco 50Ti rotor. Pellets were resuspended (0.5–1 mg protein/ml) in 0.25 M sucrose, 1 mM $Hepes_L$-$Bistris_L$ (pH 7.0), 0.1 mM $MgSO_4$ and 0.5% bovine serum albumin. All the procedure was carried out at 0–4°C. Presence of bovine serum albumin throughout all the isolation and purification steps, as well as in the final membrane suspension, was important to obtain membrane vesicles able, for a long time, to build up an electrochemical proton gradient upon addition of ATP [21].

Assay of marker enzymes

Glucan synthetase I and II were assayed according to Ray [16]. NADPH-cytochrome-*c* reductase was assayed according to Hodges and Leonard [17] and cytochrome-*c* oxidase as previously described [15].

Assay of proteins and of phospholipids

Proteins were determined according to Lowry et al. [18] after removal of bovine serum albumin by repeated dilution and precipitation. Phospholipids were extracted from membrane fractions according to Wilson and Rinne [19]. Phosphate of phospholipids was determined according to the method of Ames [20].

$^{45}Ca^{2+}$, $S^{14}CN^-$ and [^{14}C]imidazole uptakes

The uptake of $^{45}Ca^{2+}$, $S^{14}CN^-$ and [^{14}C]imidazole by the vesicles was determined by the filtration technique on 25 mm Metricel GN6 filters essentially as described (14, 21).

$^{45}Ca^{2+}$ uptake was measured on 25 µl of membrane suspension (10–20 µg of proteins) in 1 ml of 0.25 M sucrose, 40 mM $Hepes_L$-$Bistris_L$ (pH 7.0), 0.5% bovine serum albumin, 10 µM $CaCl_2$ labelled with 0.1 µCi of $^{45}Ca^{2+}$ (30 mCi/mg, Amersham) and $MgSO_4 \pm$ ATP at the concentrations specified in the legends of the tables. Incubation was run at 21°C for the time specified in the legends of tables

* The term H^+/Ca^{2+} does not imply a specific stoichiometry.

and figures. Reaction was terminated by dilution with 3.5 ml of 0.25 M sucrose, 2 mM EGTA and 3 mM $MgSO_4$ (pH 7.0) and rapid filtration. The filter was washed three times with 5 ml of 0.25 M sucrose, 2 mM EGTA and 0.5 mM $MgSO_4$ (pH 7.0). The whole procedure lasted approx. 10 s.

$S^{14}CN^-$ uptake was measured on 25 μl of membrane suspension in a final volume of 120 μl in the presence of 40 mM $Hepes_L$-$Bistris_L$ (pH 7.0), 0.5% bovine serum albumin, 10 μM $KS^{14}CN$ (59 mCi/mmol, Amersham), 1 mM $MgSO_4 \pm 1$ mM ATP. Incubation was run for 3 min at 21°C. The reaction was terminated by dilution with 3.5 ml of 0.25 M sucrose plus 3 mM $MgSO_4$ and rapid filtration. The filter was washed twice with 5 ml of 0.25 M sucrose plus 0.5 mM $MgSO_4$. The whole procedure lasted less than 10 s.

[^{14}C]Imidazole uptake was measured on 100 μl of membrane suspension in 2 ml of 0.25 M sucrose, 10 mM $Hepes_L$-$Bistris_L$ (pH 7.0), 0.5% bovine serum albumin, 30 μM [^{14}C]imidazole (1.32 mCi/mmol, California Bionuclear Corporation), 1 mM $MgSO_4 \pm 1$ mM ATP. Incubation was run for 15 min at 21°C. The reaction was terminated by dilution with 7 ml of 0.25 M sucrose plus 3 mM $MgSO_4$ and rapid filtration. The filter was washed three times with 7 ml of 0.25 M sucrose plus 0.5 mM $MgSO_4$. The whole procedure lasted approx. 15 s.

0.5% Bovine serum albumin does not affect the initial rate of ATP-dependent FCCP-sensitive Ca^{2+} uptake and ATP-dependent SCN^- or imidazole accumulation and only scarcely affects (-10 or -15%) the initial rate of ATP-dependent FCCP-resistant Ca^{2+} accumulation, but it improves the reproducibility of results in long time experiments.

The filters were dissolved in 5 ml of Filter Count^R (Packard) and radioactivity was measured in a Prias Liquid Scintillation counter.

All the experiments were run at least three times with three or more replicates; concentration of ATP in the incubation media did not diminish more than 10–15% during the incubation.

Chemicals

Essentially fatty acid free bovine serum albumin, metrizamide, EGTA, cytochrome *c*, vanadium free ATP, CTP, ITP, UTP, GTP and ADP were purchased from Sigma Chemicals Co. UDPGlc and FCCP were obtained from Boehringer Biochemia Robin. A23187 was purchased from Calbiochem. Metricel GN6 filters were obtained from Gelman Instruments SpA. Nigericin was a gift of Dr. R.L. Hamill (Lilly Research Laboratories). UDP[U^{14}C]glucose (240 mCi/mmol) was obtained from Amersham Radiochemical Center.

Results and Discussion

(1) Distribution of ATP-dependent Ca^{2+} uptake on a discontinuous metrizamide-sucrose gradient

The data presented in this paper were obtained on two membrane fractions from pea internodes separated by means of a two step metrizamide-sucrose gradient: one fraction was collected on top of a 7% metrizamide plus 5% sucrose layer (fraction L) and a second one on top of a 50% sucrose cushion (fraction H). The distribution of marker enzymes for a number of membrane systems in

TABLE I

MARKER-ENZYME ACTIVITIES IN TWO MEMBRANE FRACTIONS SEPARATED ON A DISCONTINUOUS METRIZAMIDE-SUCROSE GRADIENT

Data from a representative experiment run with three replicates.

	Fraction	
	L	H
Protein (mg/g fresh wt.)	0.12	0.48
Phospholipids (μmol/g fresh wt.)	0.11	0.45
Cytochrome-*c* oxidase (μmol/g fresh wt. per min)	0.02	0.61
NADPH-cytochrome-*c* reductase (nmol/g fresh wt. per min)	1.00	21.72
Glucan synthetase I (pmol/g fresh wt. per 15 min)	2.51	57.12
Glucan synthetase II (nmol/g fresh wt. per 15 min)	10.54	46.93
Ca^{2+} uptake [a] (nmol/g fresh wt. per 30 min)		
+ 1 mM $MgSO_4$	0.20	0.44
+ 1 mM $MgSO_4$ + 1 mM ATP	0.83	1.43

[a] The reaction was terminated as described in Materials and Methods, but dilution and washing media did not contain EGTA.

these two fractions is shown in Table I. The reported data come from one representative experiment: the distribution of the various enzyme activities was substantially constant in five independent experiments. Table I shows that fraction L contains about 20% of total membranes, as monitored by the amount of proteins and/or phospholipids. It contains only traces of mitochondria, Golgi and endoplasmic reticulum, as monitored respectively by cytochrome *c* oxidase, glucan synthetase I (high affinity) and NADPH-cytochrome-*c* reductase and about 20% of plasma membranes, monitored by the activity of glucan synthetase II (low affinity). Fraction L results enriched in plasma membranes with respect to Golgi, endoplasmic reticulum and mitochondria, as compared to fraction H or to a crude microsomal fraction. In fact a crude microsomal fraction contains about the same amount of plasma membranes but about four times as much Golgi and 15 times as much endoplasmic reticulum (data not shown). However the specific activity of glucan synthetase II in fraction L is not higher than in fraction H, indicating that fraction L must contain some other membrane system not identified by any of the markers tested.

The last two lines of Table I show the effect of ATP on the uptake of Ca^{2+} in the two membrane fractions. ATP strongly increases the amount of Ca^{2+} taken up by membrane vesicles of both L and H fractions. This stimulating effect of ATP on Ca^{2+} uptake is unaffected by oligomycin (not shown) which in this material completely blocks mitochondrial ATPase [22]. This result indicates that in our experimental conditions the contribution of mitochondrial Ca^{2+} uptake is negligible also in fraction H which contains the bulk of mitochondria, as expected on the basis of the stringent requirement of Ca^{2+} uptake for inorganic phosphate in plant mitochondria [9].

To ascertain to what extent ATP-dependent Ca^{2+} uptake reflects Ca^{2+} accumulation within the vesicles we measured the amount of Ca^{2+} released upon addition of the Ca^{2+}-ionophore A23187 [23]. Preliminary experiments run on fraction L showed that the effect of A23187 on Ca^{2+} release was greatly increased in the presence of $(NH_4)_2SO_4$, so that Ca^{2+} equilibration occurred in less than two min. This effect of $(NH_4)_2SO_4$ is likely due to its known capability to collapse an interior acid pH gradient. In fact, as A23187 facilitates an electroneutral H^+/Ca^{2+} exchange, A23187 induced release of Ca^{2+} accumulated could be limited by the parallel accumulation of H^+ within the vesicles. Table II shows the results of an experiment in which membrane vesicles of both membrane fractions, which had been allowed to take up Ca^{2+} in the presence of ATP for 30 min, were treated with $(NH_4)_2SO_4 \pm$ A23187 for 2 min. Data show that in the absence of the Ca^{2+}-ionophore the addition of $(NH_4)_2SO_4$ elicits, in 2 min, the loss of about 10% of Ca^{2+} taken up upon addition of ATP by membrane vesicles in both fractions; subsequently the release of Ca^{2+} from the vesicles continues but at very low rate (not shown) as expected on the basis of the low permeability of membranes to Ca^{2+}. In contrast, if also A23187 is supplied together with $(NH_4)_2SO_4$ virtually all of the Ca^{2+} taken up is released from the vesicles within 2 min. The difference between the amount of Ca^{2+} retained in the vesicles after treatment with $(NH_4)_2SO_4$ and that retained after treatment with $(NH_4)_2SO_4$ and A23187 seems conveniently interpreted as representing free and/or rapidly exchangeable Ca^{2+} actually transported inside the vesicles upon addition of ATP.

Another experimental approach was utilized to evaluate the portion of Ca^{2+} really transported inside the vesicles: namely a short treatment with

TABLE II

RELEASE OF Ca^{2+} TAKEN UP UPON ADDITION OF ATP BY TREATMENT WITH A23187 AND WITH EGTA

Ca^{2+} uptake was measured after 30 min of incubation in the presence of 1 mM $MgSO_4 \pm 1$ mM ATP for fraction L and of 5 mM $MgSO_4 \pm 5$ mM ATP for fraction H (control). At the end of this period the samples were treated for 2 min with 5 mM $(NH_4)_2SO_4 \pm 10$ μM A23187 or with 1 mM EGTA. The reaction was terminated as in Table I. The figures represent the difference between Ca^{2+} uptake in the presence and that in the absence of ATP. Data, expressed as nmol/mg protein per 30 min, represent the mean of nine samples $\pm$ S.E.

	Ca^{2+} uptake	
	fraction L	fraction H
Control	6.1 ± 0.1	4.2 ± 0.1
NH_4^+	5.4 ± 0.1	3.8 ± 0.1
A23187 + NH_4^+	0.7 ± 0.2	0.4 ± 0.1
EGTA	5.2 ± 0.2	3.0 ± 0.1

TABLE III

EFFECT OF ATP ON Ca^{2+} UPTAKE IN THE L AND IN THE H MEMBRANE FRACTION

Data (nmol/mg protein per 10 min) represent the mean of nine samples ± S.E.

	Ca^{2+} uptake			
	1 mM $MgSO_4$	1 mM $MgSO_4$ + 1 mM ATP	5 mM $MgSO_4$	5 mM $MgSO_4$ + 5 mM ATP
Fraction L	0.36 ± 0.09	2.35 ± 0.15	0.25 ± 0.04	1.25 ± 0.10
Fraction H	0.46 ± 0.10	1.82 ± 0.11	0.32 ± 0.08	2.40 ± 0.14

the Ca^{2+}-chelating agent EGTA, which was shown to wash out Ca^{2+} externally bound without affecting Ca^{2+} accumulated within the vesicles [24]. About 85% or 70% of Ca^{2+} taken up by the vesicles upon addition of ATP in the L and in the H fraction is retained after short treatment with EGTA (last line of Table II).

Thus the results of both these experimental approaches converge in indicating that ATP induces a real accumulation of Ca^{2+} inside the vesicles in the L as well as in the H membrane fraction.

Since adsorbed Ca^{2+} is washed out also by simply adding 2 mM EGTA in the dilution and washing media (not shown), in all the following experiments washing with EGTA was routinely performed at the end of the uptake period, so that the reported data chiefly represent Ca^{2+} accumulated within the vesicles.

Table III shows the effect of increasing $MgSO_4$-ATP concentration from 1 to 5 mM on ATP-induced Ca^{2+} accumulation in L and H fractions. In fraction L Ca^{2+} accumulation is higher in the presence of 1 mM $MgSO_4$-ATP than in the presence of 5 mM $MgSO_4$-ATP. In contrast the increase of $MgSO_4$-ATP concentration from 1 to 5 mM stimulates ATP-induced intravesicular accumulation of Ca^{2+} in fraction H by about 50%. This different response of ATP-induced Ca^{2+} accumulation in the two membrane fractions to the increase of $MgSO_4$-ATP concentration suggests that the two membrane fractions are enriched in two distinct systems involved in ATP-dependent Ca^{2+} accumulation. The significance of the inhibiting effect of 5 mM $MgSO_4$-ATP on ATP-dependent Ca^{2+} accumulation observed in fraction L will be discussed below.

(2) Evidence for a H^+/Ca^{2+} antiport mechanism

In an attempt to further discriminate between the two systems of ATP-induced intravesicular Ca^{2+} accumulation suggested by the data of Table III, we tested the effect of FCCP on ATP-dependent Ca^{2+} accumulation in L and H fractions. Table IV shows that FCCP inhibits ATP-induced Ca^{2+} accumulation in the two membrane fractions, but to a very different extent: namely in fraction L it inhibits ATP-induced Ca^{2+} accumulation by 76% and in fraction H by 28%. The sensitivity of ATP-induced Ca^{2+} accumulation to FCCP suggests the existence of a Ca^{2+} transport system which depends not only on ATP but also on the electrochemical gradient of protons. The finding that FCCP, at a concentration * which collapses the ATP-induced electrochemical proton gradient [21], only partially inhibits ATP-induced Ca^{2+} accumulation supports the view that indeed more than one system of ATP-induced Ca^{2+} accumulation exists in membrane vesicles from pea internodes. Moreover the different sensitivity to FCCP of ATP-induced Ca^{2+} accumulation in the two membrane fractions indicates that the FCCP-sensitive and the FCCP-resistant system of Ca^{2+} transport separate in this kind of gradient. Thus, ATP-induced Ca^{2+} accumulation in fraction L mainly reflects the activity of the FCCP-sensitive system while that in fraction H mainly reflects the activity of the FCCP-resistant one.

* The concentration of FCCP used is that which completely collapses the ATP-induced electrochemical proton gradient in the presence of 0.5% bovine serum albumin: this concentration is about one order of magnitude higher than that necessary to obtain the same result in the absence of bovine serum albumin (data not shown).

TABLE IV

EFFECT OF PROTONOPHORES ON ATP-INDUCED Ca^{2+} UPTAKE IN THE L AND H MEMBRANE FRACTIONS

Figures represent the differences between Ca^{2+} uptake measured in the presence of 1 mM $MgSO_4$ and 1 mM ATP and that measured in the presence of 1 mM $MgSO_4$ (nmol/g fresh wt. per 10 min). Na_2SO_4 was added at 5 mM, FCCP at 20 μM and nigericin at 5 μM. Fraction L and fraction H contained, respectively, 0.11 and 0.47 mg of protein/g fresh wt. Data represent the mean of 12 samples ± S.E.; n.d., not determined.

	Ca^{2+} uptake	
	fraction L	fraction H
Control (*a*)	0.25 ± 0.01	0.64 ± 0.02
Na_2SO_4	0.24 ± 0.01	n.d.
FCCP (*b*)	0.06 ± 0.01	0.49 ± 0.02
Na_2SO_4 + nigericin	0.05 ± 0.01	n.d.
Na_2SO_4 + nigericin + FCCP	0.05 ± 0.01	n.d.
FCCP-sensitive (*a* − *b*)	0.19	0.15
FCCP-resistant	0.06	0.49

The FCCP-resistant system of ATP-induced Ca^{2+} accumulation most likely corresponds to the (Ca^{2+} + Mg^{2+})-ATPase already described in higher plants [12,13].

The FCCP-sensitive system of ATP-induced Ca^{2+} accumulation presents the characteristics of a H^+/Ca^{2+} antiport, similar to that described in *Neurospora* plasma membranes and bacteria [6,7,10], driven by the electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) built up by a proton pumping ATPase [14,21,25–29]. This conclusion is supported by the finding that the FCCP-sensitive system of ATP-induced Ca^{2+} accumulation is completely inhibited also by another treatment affecting $\Delta\bar{\mu}_{H^+}$ such as nigericin in the presence of Na_2SO_4 (Table IV). As nigericin facilitates the electroneutral exchange of H^+ and Na^+ [23], the inhibiting effect of nigericin on ATP-induced Ca^{2+} accumulation suggests that the H^+/Ca^{2+} antiport is driven by the chemical component of $\Delta\bar{\mu}_{H^+}$. However in our experimental conditions nigericin inhibits also ATP-induced hyperpolarization (monitored as SCN^- accumulation, not shown) and thus does not allow us to rule out an involvement of the electrical component of $\Delta\bar{\mu}_{H^+}$ as a driving force for H^+/Ca^{2+} antiport.

The electrical component of $\Delta\bar{\mu}_{H^+}$ can be collapsed by high concentration of a permeant anion. Cl^-, which easily permeates membrane vesicles from higher plants [15] has been shown to be useful for this purpose [25–27] as it does not inhibit membrane ATPases of higher plants which are inhibited by high concentrations of other permeant anions such as SCN^- [30]. The effect of 50 mM KCl on ATP-dependent $\Delta\psi$ (monitored by SCN^- accumulation), Δ pH (monitored by imidazole accumulation) and FCCP-sensitive Ca^{2+} accumulation in fraction L is reported in Table V. Addition of KCl (or NaCl, not shown) drastically lowers ATP-dependent SCN^- accumulation and strongly stimulates ATP-dependent accumulation of imidazole, indicating that in our material Cl^- collapses indeed the electrical component of $\Delta\bar{\mu}_{H^+}$ and increases the chemical one. Addition of KCl (or NaCl, not shown) stimulates ATP-dependent FCCP-sensitive intravesicular Ca^{2+} accumulation by about 50%, indicating that the chemical component of $\Delta\bar{\mu}_{H^+}$ is really the driving force for Ca^{2+} accumulation mediated by the H^+/Ca^{2+} antiporter.

TABLE V

EFFECT OF KCl ON ATP-DEPENDENT $\Delta\psi$, ΔpH AND FCCP-SENSITIVE Ca^{2+} UPTAKE IN FRACTION L

Figures represent the differences between the uptakes measured in the presence of 1 mM $MgSO_4$ and 1 mM ATP and those measured in the presence of 1 mM $MgSO_4$. FCCP-sensitive Ca^{2+} uptake is the difference between ATP-induced Ca^{2+} uptake measured in the absence and in the presence of 20 μM FCCP. Incubation lasted 3 min for SCN^- uptake and 15 min for imidazole and Ca^{2+}. In the absence of ATP, SCN^- and imidazole uptakes were, respectively, 0.04 and 0.27 nmol/mg protein. Data are expressed as nmol/mg protein ± S.E.; figures in brackets represent the number of replicates.

	SCN^- uptake	Imidazole uptake	FCCP-sensitive Ca^{2+} uptake
Control	1.13 ± 0.02(10)	0.45 ± 0.02(14)	2.32 ± 0.11(9)
50 mM KCl	0.12 ± 0.01(10)	0.87 ± 0.05(14)	3.53 ± 0.18(9)

(3) Biochemical characterization of the two systems involved in ATP-dependent Ca^{2+} accumulation

Characterization of biochemical properties of FCCP-sensitive and of FCCP-resistant ATP-dependent system of Ca^{2+} uptake was performed, respectively, on L and on H membrane fraction.

Fig. 1 shows the time courses of ATP-dependent Ca^{2+} accumulation by both the FCCP-sensitive and the FCCP-resistant systems. The rate of ATP-dependent Ca^{2+} accumulation is practically constant for about 15 min for the FCCP-sensitive system and for about 10 min for the FCCP-resistant one.

Fig. 2 shows the initial rates of ATP-dependent Ca^{2+} uptake by the two systems as a function of the concentration of $MgSO_4$-ATP. The two Ca^{2+} transport systems reach saturation at very different concentrations of $MgSO_4$-ATP. Namely the FCCP-sensitive system seems saturated between 0.8 and 1.0 mM $MgSO_4$-ATP, while the FCCP-resistant one reaches saturation only at 8 to 10 mM $MgSO_4$-ATP concentration.

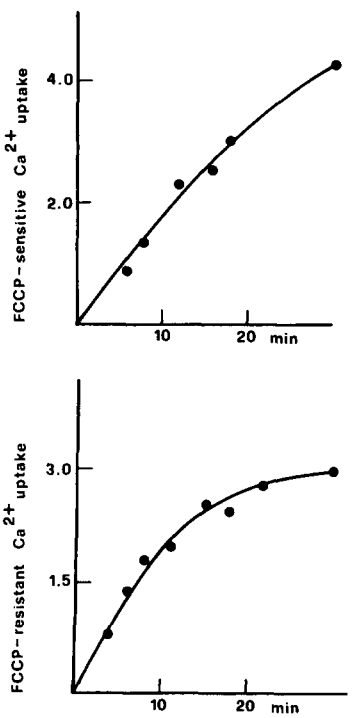

Fig. 1. Time-courses of FCCP-sensitive and of FCCP-resistant ATP-dependent Ca^{2+} uptake. FCCP-sensitive Ca^{2+} uptake (measured in fraction L) represents the difference between Ca^{2+} taken up in the presence of 1 mM $MgSO_4$-ATP and that taken up in the presence of 1 mM $MgSO_4$-ATP plus 20 μM FCCP. FCCP-resistant Ca^{2+} uptake (measured in fraction H) represents the difference between Ca^{2+} taken up in the presence of 5 mM $MgSO_4$-ATP plus 20 μM FCCP and that taken up in the absence of ATP. Data are expressed as nmol/mg protein.

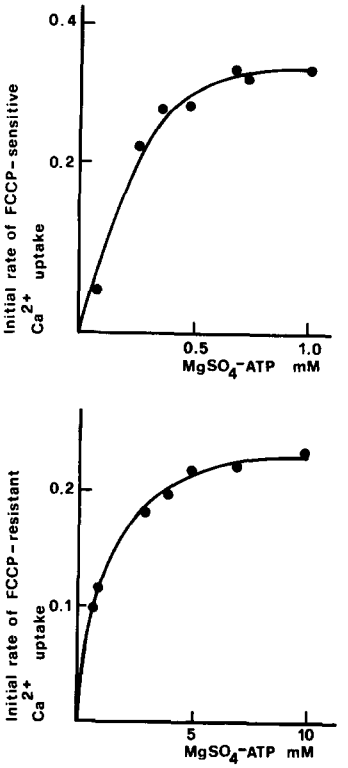

Fig. 2. Initial rates of FCCP-sensitive and FCCP-resistant Ca^{2+} uptake as a function of $MgSO_4$-ATP concentration. FCCP-sensitive Ca^{2+} uptake (i.e. the difference between Ca^{2+} taken up in the presence of equimolar $MgSO_4$ and ATP and that taken up in the presence of equimolar $MgSO_4$ and ATP plus 20 μM FCCP) was measured in fraction L. FCCP-resistant Ca^{2+} uptake (i.e. the difference between Ca^{2+} taken up in the presence of equimolar $MgSO_4$ and ATP plus 20 μM FCCP and that taken up in the same conditions in the absence of ATP) was measured in fraction H. Data are expressed as nmol/mg protein per min. Incubation was run for 8 min at 21°C.

The two systems of ATP-dependent Ca^{2+} accumulation differ also in their sensitivity to an excess of divalent cations. Table VI shows that the FCCP-sensitive system of Ca^{2+} transport is severely inhibited by 1 mM $MgSO_4$ and more so by 1 mM $MnSO_4$, in agreement with what reported for H^+/Ca^{2+} antiports of bacteria [7]. On the contrary the FCCP-resistant system is unaf-

TABLE VI

EFFECT OF AN EXCESS OF Mg^{2+} AND OF Mn^{2+} ON FCCP-SENSITIVE AND ON FCCP-RESISTANT Ca^{2+} UPTAKE

FCCP-sensitive Ca^{2+} uptake was measured in fraction L in the presence of 1 mM $MgSO_4$ and 1 mM ATP± 20 μM FCCP; FCCP-resistant Ca^{2+} uptake was measured in fraction H in the presence of 20 μM FCCP and 5 mM $MgSO_4$ ±5 mM ATP. Incubation was run for 10 min.

	ATP-induced Ca^{2+} uptake (relative activity)	
	FCCP-sensitive	FCCP-resistant
Equimolar $MgSO_4$-ATP	100	100
Equimolar $MgSO_4$-ATP+ 1 mM $MgSO_4$	27	93
Equimolar $MgSO_4$-ATP+ 1 mM $MnSO_4$	9	72

fected by addition of 1 mM $MgSO_4$ and only scarcely inhibited by 1 mM $MnSO_4$. The inhibiting effect of an excess of free Mg^{2+} on the H^+/Ca^{2+} antiport could account for the inhibiting effect of an increase of $MgSO_4$-ATP concentration from 1 to 5 mM observed in fraction L (Table III) which is enriched in H^+/Ca^{2+} antiport. In fact, at 5 mM $MgSO_4$-ATP, the concentration of free Mg^{2+} is about 0.5 mM, while at 1 mM $MgSO_4$-ATP it is about 0.2 mM. The inhibition by high concentrations of $MgSO_4$-ATP can be reversed by increasing Ca^{2+} concentration in the incubation medium from 10^{-5} to 10^{-4} M (data not shown) suggesting a possible competition between Ca^{2+} and Mg^{2+} for the H^+/Ca^{2+} antiporter. The inhibiting effect of an excess of Mg^{2+} on FCCP-sensitive ATP-dependent Ca^{2+} accumulation may explain why no sensitivity to uncouplers was detectable in microsomal fractions from various plant materials, in the experiments by Marmè and co-workers [12,13] which were run in the presence of 1 mM ATP and 5 mM $MgCl_2$.

Both the FCCP-sensitive and the FCCP-resistant systems of ATP-dependent Ca^{2+} accumulation prefer ATP to other nucleoside triphosphates and ADP (Table VII). However, the FCCP-sensitive system is virtually inactive if other nucleoside triphosphates are supplied, while the

TABLE VII

SUBSTRATE SPECIFICITY OF FCCP-SENSITIVE AND FCCP-RESISTANT Ca^{2+} UPTAKE

Experimental conditions as in Table VI; but incubation lasted 30 min; n.d., not determined.

	ATP-induced Ca^{2+} uptake (relative activity)	
	FCCP-sensitive	FCCP-resistant
ATP	100	100
CTP	6	26
ITP	11	44
UTP	10	35
GTP	9	24
ADP	0	n.d.

FCCP-resistant one is partially active also with other nucleoside triphosphates (from 24% with GTP to 44% with ITP).

Conclusions

The data reported in this paper show the existence of two distinct ATP-dependent systems of active transport of Ca^{2+} in membrane vesicles from pea. The activity of these systems is completely insensitive to oligomycin, thus indicating that none of them reflects the activity of the mitochondrial Ca^{2+} pump. Both the systems bring about true intravesicular Ca^{2+} accumulation, as shown by the finding that about 80 to 90% of Ca^{2+} taken up upon addition of ATP is rapidly released by treatment with the Ca^{2+}-ionophore A23187. The two systems are easily distinguishable on the basis of their sensitivity to FCCP and differ for a number of biochemical characteristics such as dependence on the concentration of $MgSO_4$-ATP, specificity for ATP, sensitivity to an excess of divalent cations. They separate on a discontinuous metrizamide-sucrose gradient, so that fraction L is highly enriched in the FCCP-sensitive system, while the major part of the FCCP-resistant one is recovered in fraction H.

As to the FCCP-resistant system of ATP-induced Ca^{2+} accumulation, our data do not allow us to elucidate the relationships between ATP hydrolysis and Ca^{2+} uptake. However, available

data are consistent with the view that it corresponds to the $(Ca^{2+} + Mg^{2+})$-ATPase already described in membrane vesicles from a number of higher plants [12,13].

The FCCP-sensitive system of ATP-induced Ca^{2+} accumulation presents the characteristics of a H^+/Ca^{2+} antiporter, similar to that described in bacteria and in *Neurospora* [6,7,10]. The energy for Ca^{2+} accumulation by this system is provided by the chemical component of $\Delta\bar{\mu}_{H^+}$ built up by a proton pumping ATPase [11,21,25–29]. In fact H^+/Ca^{2+} antiport is completely inhibited by nigericin in the presence of Na_2SO_4 and it is stimulated by 50 mM KCl, which collapses ATP-induced $\Delta\psi$ and increases ATP-induced intravesicular acidification. Indirect evidence for a H^+/Ca^{2+} antiport has been recently reported also for microsomal vesicles from maize coleoptiles [31].

Acknowledgements

The authors wish to thank Professor E. Marré for his interest in their work. This work was partially supported by the Research Project 'Chimica Fine e Secondaria' (C-a) of the Italian Research Council.

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