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EVIDENCE FOR AN ELECTROGENIC ATPase IN MICROSOMAL VESICLES FROM PEA INTERNODES

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Summary

The transmembrane electropotential of microsomal vesicles from pea internode segments, monitored by equilibrium distribution of the permeant anion SCN^-, is strongly hyperpolarized when ATP is present in the incubation medium.

The stimulation of SCN^- uptake by ATP is rather specific with respect to the other nucleoside di- and triphosphates tested: ADP, GTP, CTP and UTP. ATP-stimulated SCN^- uptake is strongly inhibited by ATPase inhibitors such as *p*-chloromercuribenzenesulphonate and *N,N'*-dicyclohexylcarbodiimide and by 2.5% toluene/ethanol (1 : 4, v/v), the latter being a treatment which makes the vesicles permeable. On the contrary, oligomycin is almost ineffective in influencing ATP-induced SCN^- uptake. The proton conductor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone strongly inhibits ATP-stimulated SCN^- uptake. The effect of ATP on SCN^- uptake depends on the pH of the medium, the maximum being reached at about pH 7.0.

These data support the view that microsomal fractions from pea internodes contain membrane vesicles endowed with a membrane-bound ATPase coupling ATP hydrolysis to electrogenic transport of ions, probably H^+.

Abbreviations: $Tris_L$-Mes_L, buffer made by mixing solutions of equal concentrations of Tris and 2(*N*-morpholino)ethanesulphonic acid (Mes) to give the desired pH values: Hepes, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulphonic acid; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Mops, 4-morpholinopropanesulphonic acid; AMP-PNP, adenylyl imidodiphosphate; AMP-PCP, adenylyl(β,γ-methylene)diphosphonate; PCMBS, *p*-chloromercuribenzenesulphonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; p.d. transmembrane electropotential difference.

Introduction

An Mg^{2+}- and K^+-dependent ATPase has been demonstrated to be present in a number of plant materials. This enzyme appears to be located at the plasma membrane, and its wide distribution in different plant materials has suggested its possible use as a marker of plasma membranes in plant tissues [1]. Consistent indirect evidence, coming from both in vivo work on ion transport and in vitro work on ATPase activity, suggests that the Mg^{2+}- and K^+-dependent ATPase is involved in the electrogenic transport of H^+ and K^+ in plant cells [1—3].

A direct demonstration of the physiological role of this enzyme requires the study of its activity in isolated osmotically active membrane vesicles, which are capable of simulating the in vivo transport processes.

Isolated plasma membrane vesicles have been extensively and successfully used to elucidate the mechanism of transport processes in animals and bacteria [4,5].

In recent years, some attempts to study transport processes in isolated membrane vesicles from plant tissues have been reported [6—12]. Some evidence for an ATP-dependent transport of Na^+ and K^+ in membrane vesicles from bean cotyledons has been presented by Lai and Thompson [6]. Gross and Marmè [11] have reported more recently an ATP-dependent Ca^{2+} accumulation in microsomal vesicles prepared from various tissues. Scarborough [8] has demonstrated the presence of an electrogenic ATPase (presumably a proton-transporting ATPase) and a Ca^{2+}/H^+ antiport [9] in purified plasma membrane vesicles from *Neurospora*.

We have previously shown that a microsomal fraction from pea internode segments contains sealed vesicles capable of changing their volume in response to changes of the osmolarity of the medium. This fraction displayed very little contamination by mitochondria, as shown by the very low activities of both cytochrome *c* oxidase and oligomycin-sensitive ATPase. About 25% of the total membranes in this fraction are recognizable as plasma membranes on the basis of phosphotungstic acid-chromic acid staining [13].

The aim of the present work was to discover whether an electrogenic ATPase was active in this microsomal fraction from pea internodes. To this end, we have measured the effect of ATP on the equilibrium distribution of the permeant anion SCN^-, a parameter extensively used to monitor p.d. in in vitro systems [14].

The data obtained suggest that the microsomal fraction from pea internodes contains a membrane-bound ATPase involved in the electrogenic transport of protons.

Materials and Methods

Isolation of microsomal vesicles. Pea (*Pisum sativum* L. cv. Alaska) internode segments, prepared as previously reported [13], were homogenized at 4°C in a Waring Blendor for 3 s with four vol. of 5 mM Hepes-Na (pH 7.8), 0.4 M sucrose, 0.1 mM $MgCl_2$ and 0.5% bovine serum albumin. All the following operations were performed at 0°C. The homogenate, filtered through eight

layers of cheesecloth, was centrifuged for 15 min at 20 000 × *g* in a Sorvall RC-5B centrifuge. The resulting supernatant was centrifuged in a Spinco L 50 centrifuge with a Type 30 rotor at 30 000 rev./min for 30 min. The supernatant was discarded and the pellet (microsomal membrane fraction) was resuspended (approx. 10 g fresh wt./ml) in 0.4 M sucrose, 0.1 mM $MgCl_2$, 0.5% bovine serum albumin and 1 mM $Tris_L$-Mes_L (pH 6.2 unless otherwise specified) using a Potter glass homogenizer.

$S^{14}CN^-$ uptake assay. To initiate the reaction, 50 µl (approx. 100 µg protein) of microsomal vesicle suspension were added to a test tube containing 0.12 mM $KS^{14}CN$ (59 mCi/mmol; Amersham) and the appropriate additions (see legends of figures and tables) in a total volume of 10 µl. Incubation was performed at 21°C for the desired time and terminated by dilution with 2 ml of 0.4 M sucrose plus 3 mM $MgSO_4$. Each sample was immediately filtered on a 25 mm Metricel GN6 filter (prewetted with washing solution) and washed twice with 5 ml of 0.4 M sucrose plus 0.5 mM $MgSO_4$. The second washing was performed without the upper funnel. The entire process lasted approx. 10 s and approx. 95% of the sample protein was retained on the filter. Zero-time controls were performed by adding the dilution medium immediately after the membrane suspension, and subsequently filtering and washing as described above. The radioactivity retained on the filter was approx. 0.2% of the total, and this value was subtracted from each $S^{14}CN^-$ uptake value.

The filters were dissolved in 10 ml of Filter Count ® (Packard) and radioactivity was measured in a Packard Tricarb scintillation counter.

Measurement of ATP hydrolysis rate. Assay conditions were the same as those described for SCN^- uptake assay, except that $KS^{14}CN$ was omitted. As the microsomal fraction from pea internodes contains very high activity of an enzyme which hydrolyzes sequentially ATP and ADP [15], measurements of P_i released do not give a proper estimate of the rate of ATP hydrolysis. Thus, we have measured directly the variation of ATP concentration in the medium. The reaction was terminated by addition of ice-cold $HClO_4$ at a final concentration of 0.8 M. After 5 min at 0°C, the samples were neutralized with KOH and $KClO_4$ was removed by centrifugation. Enzymic assay of ATP was performed according to the method of Lamprecht and Trautshold [16].

Protein assay. Proteins were determined according to the method of Lowry et al. [17].

All the experiments were run at least twice with 4-fold replications. The reported data represent the mean values ± S.E.

Chemicals. ATP, ADP, GTP, CTP, UTP, AMP-PNP and AMP-PCP were obtained from Boehringer Biochemia Robin. Metricel GN6 filters were obtained from Gelman Instruments S.p.A.

Results and Discussion

ATP-stimulated SCN^- uptake

SCN^- uptake is extensively used as monitor of p.d. in isolated membrane systems on the basis of the ability of this anion to permeate the membranes. SCN^- undergoes an asymmetrical distribution across the membrane as a function of p.d. [14].

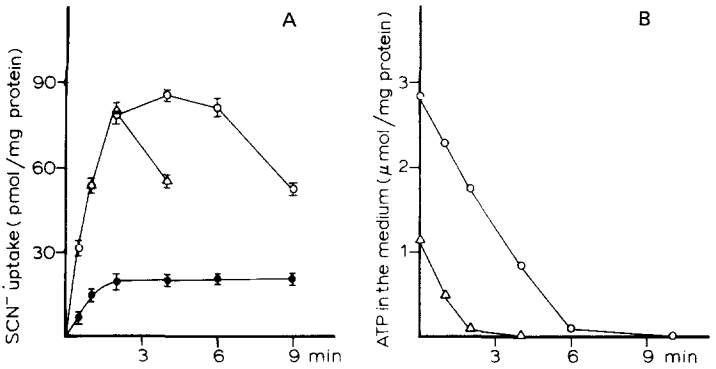

Fig. 1. Time course of ATP-stimulated SCN^- uptake and ATP hydrolysis in microsomal vesicles. (A) 50 μl of membrane suspension were incubated at 21°C for the desired times with 10 μl of 0.4 M sucrose, 0.12 mM $KS^{14}CN$, 30 mM $MgSO_4$, with or without 12 or 30 mM ATP, buffered at pH 6.2 with 120 mM $Tris_L$-Mes_L. Points represent the mean values of ten replicates; vertical bars indicate S.E. (B) As in A, but without $KS^{14}CN$. Points represent the mean values of four replicates. At zero time, ATP concentration was: zero (●), 2 mM (△), 5 mM (○).

Fig. 1 shows the time course of $S^{14}CN^-$ uptake in microsomal vesicles, in the presence or absence of 2 or 5 mM ATP; the time course of ATP hydrolysis under the same experimental conditions is also reported. The results show that SCN^- equilibrates across the membrane in approx. 2 min (Fig. 1A). SCN^- distribution is strongly influenced by ATP, which brings about a substantial increase in the amount of SCN^- retained in the vesicles. When 5 mM ATP is added, the effect of ATP on SCN^- uptake is maximal within 4 min and rapidly decays after 6 min; when ATP is added at a concentration of 2 mM, ATP-stimulated SCN^- accumulation decreases rapidly after the first 2 min.

The time course of the decrease in ATP concentration in the incubation medium (Fig. 1B) shows that the rate of ATP hydrolysis is nearly constant until the ATP level in the medium reaches very low values, i.e., it is constant for approx. 6 min when 5 mM ATP is added and for approx. 2 min when 2 mM ATP is added. The time course of P_i release in the medium, measured under the same experimental conditions, matches quite closely that of ATP hydrolysis but the values of P_i released are roughly double those expected from ATP hydrolysis to ADP and P_i (data not shown). This finding is in agreement with the results reported by Tognoli and Marrè [15] indicating that the microsomal fraction from pea internodes contains high activity of an enzyme which hydrolyzes sequentially ATP and ADP.

Substrate specificity of ATP-stimulated SCN^- uptake

The stimulating effect of ATP on SCN^- uptake is rather specific, as compared with that of other nucleoside di- and triphosphates. As shown in Table I, SCN^- uptake is maximal in the presence of ATP; the effect of UTP is about one-half that of ATP; CTP, GTP and ADP are almost ineffective. AMP-PNP and AMP-PCP, non-hydrolyzable analogs of ATP, do not induce any detectable effect.

TABLE I

SPECIFICITY OF ATP-STIMULATED SCN^- UPTAKE IN MICROSOMAL VESICLES

50 μl of membrane suspension were incubated at 21°C for 2 min with 10 μl of 0.4 M sucrose, 30 mM $MgSO_4$, 0.12 mM $KS^{14}SN$ with or without the specified nucleoside di- and triphosphates at 30 mM, buffered at pH 6.2 with 120 mM $Tris_L$-Mes_L. Data represent the mean of eight samples ± S.E.

Additions	SCN^- uptake (pmol/mg protein)
None	21.5 ± 1.2
ATP	85.1 ± 1.5
ADP	23.4 ± 0.9
CTP	38.1 ± 1.9
GTP	31.4 ± 1.9
UTP	55.9 ± 2.0
AMP-PNP	22.5 ± 0.9
AMP-PCP	22.5 ± 1.0

Effect of ATPase inhibitors on ATP-stimulated SCN^- uptake

The stimulating effect of ATP on SCN^- uptake in microsomal vesicles might depend: (i) on an interaction of ATP with the membranes such as to modify membrane characteristics and/or p.d., independently of ATP hydrolysis; and (ii) on an increase in p.d. linked to ATP hydrolysis catalyzed by a membrane-bound ATPase. If the latter hypothesis is true, ATP stimulation of SCN^- uptake should be inhibited by treatments which inhibit ATPase activity.

We have tested the inhibitory effect on ATP-stimulated SCN^- uptake of PCMBS, a sulphydryl reactive agent, which also inhibits plasma membrane ATPases [18], of DCCD, a known inhibitor of proton-conducting ATPases, which has been shown to be (at high concentrations) a powerful inhibitor of plant plasma membrane ATPase [1,18,19], and of oligomycin, the well known inhibitor of mitochondrial ATPase, which is ineffective on plasma membrane ATPase [1,19]. ATP-stimulated SCN^- uptake is strongly inhibited by PCMBS and by DCCD, but only very slightly affected by oligomycin; PCMBS, DCCD and oligomycin do not affect SCN^- uptake in the absence of ATP (Table II).

TABLE II

EFFECT OF ATPase INHIBITORS ON ATP-STIMULATED SCN^- UPTAKE IN MICROSOMAL VESICLES

50 μl of resuspended membranes, preincubated for 10 min at 0°C with the specified inhibitors, or with 0.25% ethanol (control) were incubated at 21°C for 2 min with 10 μl of 0.4 M sucrose, 0.12 mM $KS^{14}CN$, 30 mM $MgSO_4$, with or without ATP (30 mM), buffered at pH 6.2 with 120 mM $Tris_L$-Mes_L. Data represent the mean of eight samples ± S.E.

	SCN^- uptake (pmol/mg protein)	
	—ATP	+ATP
Control	24.2 ± 0.9	92.9 ± 1.9
PCMBS (0.1 mM)	24.0 ± 1.0	41.1 ± 1.4
DCCD (0.5 mM)	23.5 ± 0.7	52.4 ± 1.4
Oligomycin (2 μg/ml)	22.9 ± 0.8	81.1 ± 1.7
2.5% toluene/ethanol (1 : 4, v/v)	18.9 ± 0.8	46.6 ± 1.3

TABLE III

INHIBITION OF ATP-STIMULATED SCN^- UPTAKE BY FCCP

50 μl of resuspended membranes, preincubated for 10 min with FCCP at the specified concentrations or with 0.25% ethanol (control), were incubated as described in the legend of Table II.

	SCN^- uptake (pmol/mg protein)	
	−ATP	+ATP
Control	22.6 ± 0.8 (12)	91.8 ± 2.0 (12)
10^{-6} M FCCP	22.4 ± 0.8 (8)	63.5 ± 1.7 (8)
10^{-5} M FCCP	22.3 ± 0.9 (12)	36.2 ± 0.9 (12)

The inhibitory effect of PCMBS and of DCCD on ATP-stimulated SCN^- uptake indicate that ATP hydrolysis rather than ATP per se is responsible for the increase in SCN^- uptake in microsomal vesicles. These data thus support the view that the increase in SCN^- uptake upon addition of ATP reflects the effective building up of an interior-positive p.d. in the vesicles, brought about by the activity of an electrogenic ATPase coupling ATP hydrolysis to electrogenic ion transport.

This conclusion is strengthened by the effect of a treatment such as that with toluene/ethanol, which dissipates p.d. by rendering the membranes leaky to ions [20], on ATP-stimulated SCN^- uptake (last line of Table II). Toluene/ethanol inhibits SCN^- uptake both in the absence and much more so in the presence of ATP. Toluene/ethanol-induced inhibition of basal SCN^- uptake is probably due to a modification of the diffusion potential brought about by increased membrane permeability to ions. The increased membrane permeability to ions implies an enhancement of the tendency to redistribute across the membrane according to the electrochemical gradient of the ion electrogenically transported by the membrane-bound ATPase, and thus accounts for the strong inhibition by toluene/ethanol on ATP-stimulated SCN^- uptake.

As oligomycin completely blocks oxidative phosphorylation and strongly inhibits ATPase activity of pea mitochondria [21], the finding that ATP-stimulated SCN^- uptake is only slightly inhibited by oligomycin indicates that at least most of the ATP-induced increase in p.d. does not depend on the activity of mitochondrial ATPase, as expected on the basis of the very low mitochondrial contamination of the microsomal fraction [13].

Effect of FCCP on ATP-stimulated SCN^- uptake

The inhibiting effect of DCCD on ATP-stimulated SCN^- accumulation in microsomal vesicles, although evident only at high DCCD concentrations, is consistent with the hypothesis that ATP-induced hyperpolarization of p.d. depends on electrogenic transport of protons mediated by a membrane-bound ATPase. To check further this hypothesis we have studied the effect of FCCP, which dissipates the electrochemical gradient of protons, on ATP-stimulated SCN^- accumulation.

The results of Table III show that, in the absence of ATP, FCCP does not affect SCN^- uptake, which was expected as the incubation was carried out at the same pH at which the vesicles were equilibrated. On the contrary, FCCP

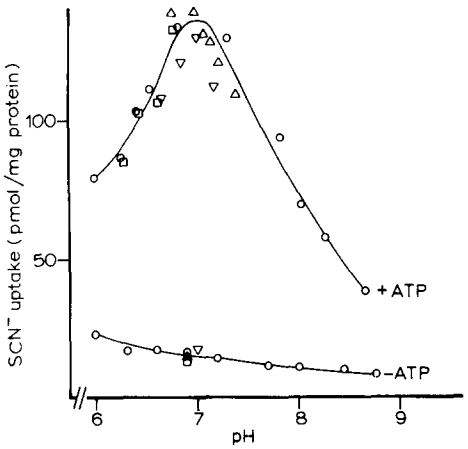

Fig. 2. pH dependence of ATP-stimulated SCN^- uptake in microsomal vesicles. 50 µl of membranes, resuspended at pH 7.0, were incubated at 21°C for 2 min with 10 µl of 0.4 M sucrose, 0.12 mM $KS^{14}CN$, 30 mM $MgSO_4$, with or without 30 mM ATP, buffered at the desired pH values with 240 mM of: $Tris_L$-Mes_L (○), Hepes-Na (△), Bis-Tris-H_2SO_4 (□), Mops-Na (▽). Each point represents the mean value of four replicates.

drastically inhibits ATP-stimulated SCN^- accumulation. The inhibitory effect of FCCP increases with increasing FCCP concentration up to 10^{-5} M.

These data support the hypothesis that at least a large portion of ATP-induced hyperpolarization of p.d. in our microsomal vesicles depends on ATP-dependent electrogenic transport of protons.

pH dependence of ATP-stimulated SCN^- accumulation

Fig. 2 shows the effect of varying the pH of the incubation medium from pH 6 to 9 on ATP-stimulated SCN^- accumulation in microsomal vesicles from pea internodes. Basal SCN^- uptake is influenced by pH in the sense that SCN^- uptake decreases with increasing pH of the medium. This is what is expected as the membrane vesicles had all been equilibrated at the same pH (7.0): under these conditions a change in the pH of the medium determines a change of the diffusion potential of protons, which becomes more positive inside as the pH of the medium decreases and vice versa.

ATP-stimulated SCN^- uptake is strongly dependent on the pH of the incubation medium, the maximum being reached at about pH 7: at this pH, ATP increases SCN^- accumulation approx. 10-times over the controls.

The pH optimum for ATP-induced hyperpolarization is therefore higher than the pH used in all the experiments reported in this paper. Nevertheless, the results of a few experiments run at pH 7.0 in the presence of inhibitors such as DCCD, FCCP and oligomycin indicate that raising the pH from 6.2 to 7.0 does not affect the relative responses of ATP-induced hyperpolarization to inhibitors.

Conclusions

The results reported in this paper show that microsomal preparations from pea internodes contain a population of tight membrane vesicles able to accumulate SCN^- upon addition of ATP.

The sensitivity of ATP-stimulated SCN^- uptake to ATPase inhibitors and to treatments which increase membrane permeability suggests that the ATP effect on SCN^- uptake does not depend on ATP per se but on ATP hydrolysis and on the structural and functional integrity of the membranes. As ATP does not induce any detectable change of vesicle volume (A_{546nm} of microsomal vesicle suspension does not change upon addition of ATP), ATP-stimulated SCN^- uptake reflects an increase in p.d. in microsomal vesicles, which become more interior positive.

The possibility that ATP-induced hyperpolarization of p.d. depends on the change of ionic composition of the incubation medium, consequent to ATP hydrolysis, is ruled out by the high specificity of ATP-stimulated SCN^- uptake for ATP with respect to other nucleoside triphosphates and ADP. In fact, the microsomal fraction used in these experiments shows a very high ATPase and ADPase activity, hydrolyzing with similar efficiencies also GTP, UTP and CTP [15], but only ATP (and to a much lesser extent UTP) effectively stimulates SCN^- uptake. Thus, ATP-induced hyperpolarization of p.d. of membrane vesicles seems to depend on electrogenic transport of ions, coupled to ATP hydrolysis mediated by a membrane-bound ATPase.

The data obtained so far do not allow us to determine unequivocally which is the electrogenically transported ionic species. However, the inhibitory effect of DCCD and, more so, that of FCCP on ATP-stimulated SCN^- uptake support the view that ATP-induced hyperpolarization of the p.d. of membrane vesicles depends on ATP-driven electrogenic transport of protons into the vesicles.

The microsomal fraction used in these experiments contains membrane vesicles originating from various cellular membrane systems (plasma membrane, Golgi, endoplasmic reticulum, tonoplast, etc.). The low mitochondrial contamination of the microsomal fraction [13] and more so the finding that ATP-stimulated SCN^- uptake is nearly unaffected by oligomycin, which completely blocks oxidative phosphorylation in pea mitochondria [21], allow us to exclude the possibility that the observed ATP-dependent hyperpolarization of p.d. depends on the activity of the mitochondrial ATPase.

The finding that substrate specificity of ATP-stimulated SCN^- uptake in microsomal vesicles resembles closely that reported for ATPase of purified pea plasma membrane [22] is consistent with the hypothesis that the electrogenic ATPase described in this paper is located on plasma membrane, which represents approx. 25% of total membranes of the microsomal fraction used in these experiments [13]. The pH optimum of the ATP effect on SCN^- uptake is slightly higher than that reported for plant plasma membrane ATPase [1,2,18]; however, the pH optimum for p.d. hyperpolarization might well be shifted from the actual pH optimum of the ATPase involved in the process, inasmuch as a change in the pH of the incubation medium could affect the characteristics of membrane permeability to protons.

A direct demonstration of the location of the electrogenic ATPase requires the isolation of purified membrane fractions. Unfortunately, the capability of our microsomal vesicles to build up a membrane potential on addition of ATP decays quite rapidly: the half-life of ATP-stimulated SCN^- uptake is fairly variable and never longer than $1\frac{1}{2}$ h, similar to that previously reported for the osmotic behaviour of the microsomal vesicles [13]. The simplest interpretation

of these results is that the activities of hydrolytic enzymes produce a modification of membrane composition [7,23] such as to change the permeability characteristics of the membranes.

Further work is thus necessary in order to obtain microsomal vesicles which maintain unaltered their permeability characteristics for longer times, so as to allow separation of the different membrane systems.

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