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Free fatty acids dissipate proton electrochemical gradients in pea stem microsomes and submitochondrial particles

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The effect of free fatty acids (FFA) and lysophosphatidylcholine-oleoyl (lyso-PC) on proton gradients of pea stem microsomes and submitochondrial particles was studied. Linolenic (18:3), linoleic (18:2), oleic (18:1), palmitic (16:0) and stearic (18:0) acids collapsed the proton gradient generated by addition of ATP or PP to microsomes. When an artificial ΔpH was generated by NaOH, FFA did not induce any effect, but the subsequent addition of valinomycin dissipated the proton gradient. FFA were also able to discharge the ΔpH built up by the oligomycin-sensitive H^+-ATPase of submitochondrial particles and the electrical potential generated by NADH oxidation in intact mitochondria. Free fatty acids stimulated NADH-dependent oxygen consumption by mitochondria and this effect was not abolished by ADP or carboxyatractyloside (CAtr). The effect of FFA increased with an increasing unsaturation of the acyl chain, while the length of the chain did not influence the activity. Lysophosphatidylcholine dissipated the proton gradient generated by H^+-PPase of microsomes and H^+-ATPase of submitochondrial particles, while the H^+-ATPase of microsomes was slightly affected. In addition, lyso-PC stimulated NADH-dependent oxygen uptake by mitochondria. Also in this case, neither ADP nor CAtr inhibited this stimulated O_2 consumption. These results show that FFA uncoupled oxidative phosphorylation of pea mitochondria and collapsed only proton electrochemical gradients in pea microsomes and submitochondrial particles. Therefore, in this regard FFA are similar to artificial protonophores, acting as proton carriers. The mechanism of action of lyso-PC appears to be more complex and different possible explanations are proposed.

Introduction

Aging, senescence and several environmental stresses alter membrane structure and function in plant cells. The phospholipids of the membrane bilayer in general are thought to be one of the primary targets for such alterations. Free fatty acids (FFA) are non-specifically released from phospholipids by the activity of lipolytic acyl-hydrolases. In addition, fatty acid oxidation products, generated by lipoxygenases, are also produced [1]. The high amount of free fatty acids in the membranes of plant cells increases microviscosity and enhances leakage of cytoplasmic solutes [2].

Since 1956, free fatty acids have been known as uncouplers of oxidative phosphorylation in animal mitochondria [3], although their mechanism of action is still obscure. In recent years, it has been suggested that uncoupling involves the ATP/ADP antiporter [4]. According to a first possible explanation, FFA allosterically stimulate the transfer of H^+ or OH^- by interacting with the exchanger. In a second hypothesis, protonated fatty acids diffuse via lipid bilayer, while the unprotonated form is recycled by the ATP/ADP antiporter [5]. However, the small inhibitory effect of carboxyatractyloside (CAtr), a specific inhibitor of this translocator, on the oleate-induced respiration and $\Delta\Psi$ dissipation in rat liver mitochondria, was interpreted as an evidence for a minor involvement of the translocator in the uncoupling action of FFA [6]. In the light of the

Abbreviations: AO, acridine orange; BSA, bovine serum albumin; CAtr, carboxyatractyloside; DTT, dithiothreitol; ΔpH, proton gradient; $\Delta\Psi$, transmembrane electrical potential; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluorometoxyphenylhydrazone; FFA, free fatty acids; Hepes, *N*-2-(hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid); lyso-PC, lysophosphatidylcholine-oleoyl; Oligo, oligomycin; TPP^+, tetraphenylphosphonium; Val, valinomycin.

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latter and other results [7], it has been suggested that FFA increase proton conductivity, probably by permeation of the protonated and unprotonated forms of FFA [6]. However, the low sensitivity of the oleate-stimulated respiration to CAtr may depend on the low content of the ADP/ATP translocator [8].

It has been reported that palmitate does not enhance H^+ and K^+ conductance of cytochrome *c* oxidase in proteoliposomes [9]. This means that FFA interact with different specific proteins of the membrane or, alternatively, that membrane proteins are not involved. Recently a direct effect of FFA on purified Na^+/K^+-ATPases of rabbit kidney outer medulla leading to inhibition and inactivation of the enzymes has been shown [10]. Inhibition caused by FFA was also demonstrated in purified H^+-ATPases of plasma membranes from rice. The effect increases with increasing acyl chain length and increasing unsaturation [11].

In plant cells, the effect of FFA on mitochondria and other membrane activities appears to be poorly defined as in animals. In recent years, the interest in plant cell behaviour was stimulated by the fact that FFA and, in particular, phospholipids, lysophospholipids and sterols may be involved in the modulation of plasma membrane H^+-ATPase [11–21] and NADH-ferricyanide reductase activities of isolated vesicles [22]. In general, FFA are used as detergents to permeabilize the membrane vesicles, without inhibiting ATPase activity [19,21]. In some cases the stimulating effect on H^+-ATPases cannot be explained only by an unmasking of the latent active sites, since some phospholipids stimulate ATPase also in purified inside-out vesicles [21], or enhance the activity of purified enzymes [11,12,18,19].

In the present work we studied the effect of palmitic, stearic, oleic, linoleic, linolenic acids and lysophosphatidylcholine-oleoyl on proton gradients of plant mitochondria and microsomes, generated by the activity of H^+-ATPase (mitochondria and microsomes) and H^+-PPase (microsomes).

Materials and Methods

Plant material. Etiolated pea (*Pisum sativum* L., cv. Alaska) stems were obtained by growing plants for 7 days, in the dark, at 25 °C and 70% relative humidity.

Microsome preparation. Approx. 60–70 g of etiolated pea stems were ground in 250 ml 50 mM Tris-HCl (pH 8.0)/0.3 M sucrose/1 mM $MgCl_2$/1 mM DTT/3 mM ATP/0.5%(w/v) BSA by a mortar and pestle. The homogenate was filtered through eight layers of gauze and the filtrate was centrifuged at 15000 × *g* for 10 min by a Sorvall centrifuge, model RC-5B (rotor SS-34). The supernatant was centrifuged at 80000 × *g* for 30 min by a Beckman centrifuge, model L7-55 (rotor Ty 70ti). The pellet, washed in 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/1 mM ATP/1 mM $MgCl_2$/1 mM DTT/0.5% BSA, was recentrifuged as above. The pellet (microsomal fraction) was resuspended in approx. 4 ml 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/5 mM DTT/0.3% BSA with a Potter homogenizer, subdivided in four aliquots and stored at −40 °C for some weeks without loss of activity. This fraction, that contains several types of membrane, exhibits a high nitrate-sensitive, vanadate-insensitive H^+-ATPase activity, but is almost devoid of mitochondrial fragments [23].

Preparation of mitochondria. Pea stems were ground and filtered as above in the following homogenization medium: 250 ml 20 mM Hepes-Tris (pH 7.6)/0.4 M sucrose/5 mM Na-EDTA/25 mM potassium metabisulfite/0.1% BSA. The filtrate was centrifuged at 28000 × *g* for 5 min by a Sorvall RC-5B centrifuge. The pellet was resuspended in half of the initial volume of the above buffer by a Potter homogenizer. This fraction was centrifuged again at 2500 × *g* for 3 min and the supernatant recentrifuged at 28000 × *g* for 5 min. The pellet (mitochondrial fraction) was resuspended in approx. 3 ml of 20 mM Hepes-Tris (pH 7.5)/0.4 M sucrose.

Submitochondrial particle preparation. Mitochondria (3 ml) were diluted with 3 ml of 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/6 mM ATP/0.5% BSA and then sonically irradiated four times (100 W) for 0.5 min with 1 min intervals in an ice-bath by a Labsonic 1510 ultra-sound generator. The suspension obtained after sonic irradiation was centrifuged at 28000 × *g* for 5 min. The supernatant was centrifuged at 100000 × *g* for 30 min. The pellet was washed with 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/0.5% BSA and recentrifuged. The pellet was resuspended in 1 ml 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/0.3% BSA and stored at −40 °C.

Acridine orange measurements. The generation of proton gradients in microsomes and submitochondrial particles was monitored as uptake of AO, at room temperature, following the decrease of absorbance at 495 nm by a double beam Perkin-Elmer spectrophotometer, model 554 [23]. The medium was: 10 mM Hepes-Tris (pH 6.5 plus 150 mM KBr for microsomes; pH 7.5 plus 50 mM KCl and 0.25 M sucrose for submitochondrial particles), 5 mM $MgSO_4$, 1 mM EGTA, 5 μM AO and 50 μl submitochondrial particles (approx. 0.2 mg protein) or 25 μl microsomes (approx. 0.1 mg protein) in a final volume of 2 ml.

ATPase and PPase activity determination. These activities were assayed in 1 ml (final volume) of the buffer used for AO experiments with the addition of 100 μM molybdate. The reactions were started by 1 mM ATP and 100 μM PP, respectively, and proceeded for 10 min at 37 °C. Inorganic phosphate released was determined as described in Ref. 24.

Oxygen consumption determination. Oxygen uptake was monitored at room temperature by a platinum electrode of the Clark type. The medium was 20 mM

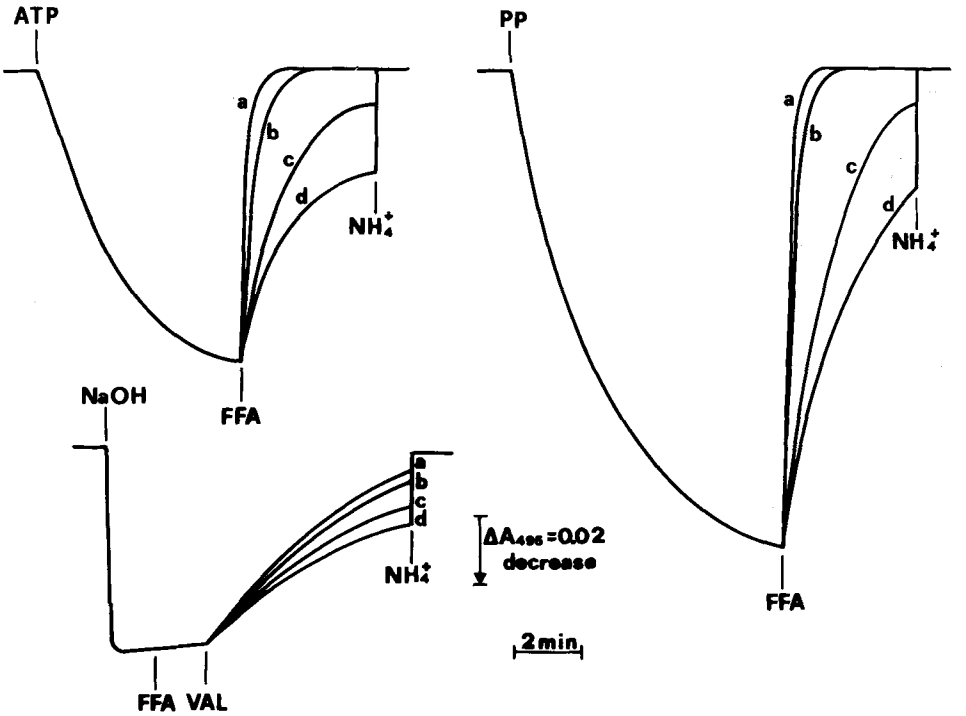

Fig. 1. Effect of free fatty acids (FFA) on ATP- or PP-dependent and NaOH-induced decrease in AO absorbance by pea stem microsomes. Additions were: 0.5 mM MgATP, 200 μM PP, 50 μM FFA. (a) Linolenic acid (18:3) or linoleic acid (18:2); (b) oleic acid (18:1); (c) palmitic acid (16:0); (d) stearic acid (18:0). 20 μl 1 M NaOH and 10 μl of a saturated solution of $(NH_4)_2SO_4$.

Hepes-Tris (pH 7.5), 0.4 M sucrose, 5 mM $MgSO_4$, 5 mM Na/K phosphate and 200 μl of mitochondria (approx. 0.75 mg protein) in a final volume of 2 ml.

TPP^+ electrode measurements. TPP^+ uptake was followed at room temperature by a hand-made specific electrode prepared according to Kamo et al. [25], with a

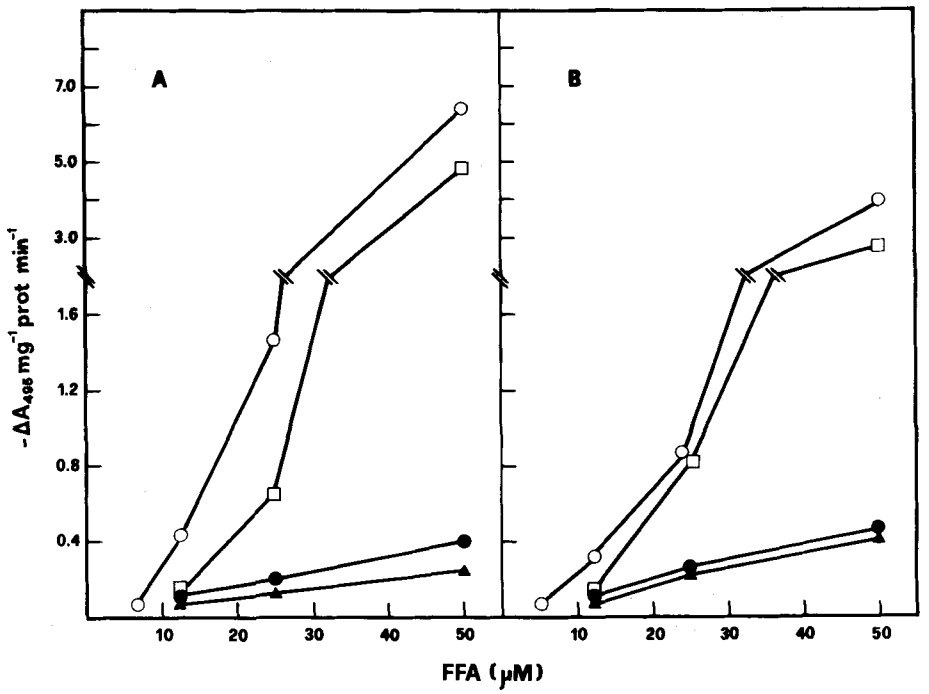

Fig. 2. Effect of concentration of linolenic acid (○), oleic acid (□), palmitic acid (●) and stearic acid (▲), on the initial rate of FFA-induced increase in AO absorbance by pea stem microsomes, after the ATP- (panel A) or PP- (panel B) dependent decrease of AO absorbance had reached a steady state.

combined glass pH electrode as a reference. The medium was as in oxygen uptake experiments, with the addition of 2.5 μM TPP^+ and 300 μl mitochondria (approx. 1.1 mg protein).

Protein determination. Protein was determined by the biuret method described by Gornall et al. [26], after washing the samples with 5 mM $MgSO_4$ to remove the BSA present in the resuspending medium, when necessary.

Chemicals. Palmitic, stearic, oleic and linoleic acids (sodium salts), linolenic acid (free acid) and lysophosphatidylcholine-oleoyl, MgATP, ADP, NADH, valinomycin, carboxyatractyloside and acridine orange were purchased from Sigma Co., St. Louis, MO, U.S.A. Sodium pyrophosphate was obtained from Merck, Darmstadt, F.R.G. Palmitate and stearate were dissolved in absolute ethanol; the other FFA and lyso-PC were dissolved in 5 mM Mes-Tris (pH 8.9)/0.1 mM EDTA/0.1 mM DTT/9.6% ethanol to give stock solutions of 5 mM.

Results

Effect of FFA on electrogenic and electroneutral proton gradients of pea microsomal vesicles

Pea stem microsomes exhibit a marked nitrate-sensitive, vanadate-insensitive ATP-dependent (H^+-ATPase) and vanadate- and nitrate-insensitive, cation-stimulated PP-dependent (H^+-PPase) proton pumping [27,28]. They are, therefore, useful to study the effect of substances with protonophoric activity.

Fig. 1 shows that the addition of ATP to pea microsomes built up a proton gradient which was collapsed by the addition of FFA. Linoleic and linolenic acids were the most effective, completely dissipating the gradient in less than 0.5 min. Oleic acid was also very effective, while the effect of palmitic and stearic acids was lower: even after 2 min they did not completely release the gradient, as shown by the subsequent addition of ammonium sulfate. The same pattern was observed on the proton gradient generated by H^+-PPase activity. This gradient is larger than that generated by H^+-ATPase and was collapsed with the same order of effectiveness by all the assayed FFA. The complete relationship between these proton pumping activities and FFA concentration is shown in Fig. 2. The results confirm that linolenic and oleic acids were more effective than palmitic and stearic acids in dissipating proton gradients. The half-maximal inhibition for these acids was approx. 25–30 μM. The extent of the FFA-dissipated proton gradient was dependent on the degree of unsaturation. One double bond (oleic acid) in the acyl chain strongly increased the collapsing ability. The latter was further increased by the presence of two double bonds (linolenic acid), while no difference was found between FFA with two or three double bonds. Conversely, the length of the acyl chain did not influence this capacity.

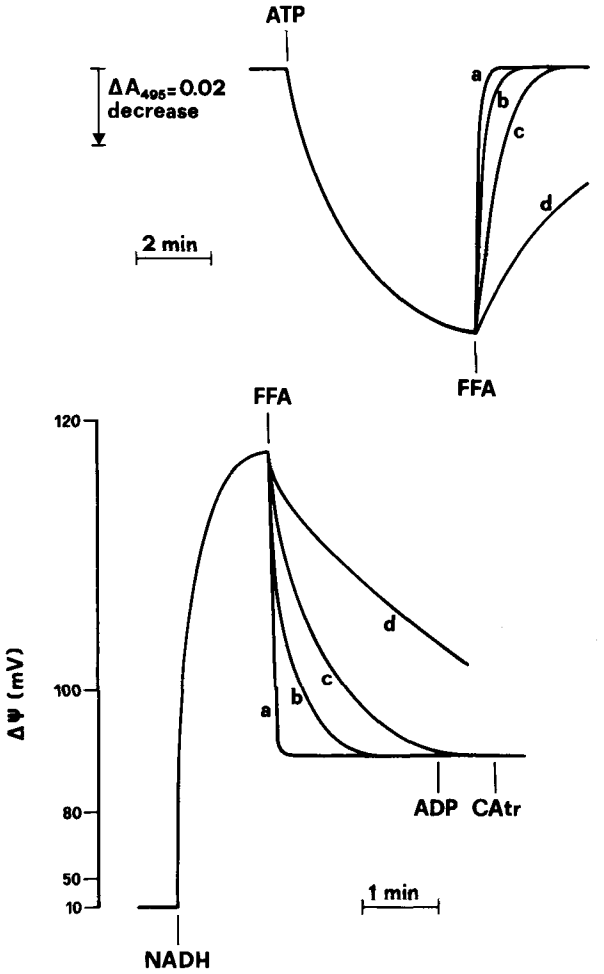

Fig. 3. Effect of FFA on ATP-dependent decrease in AO absorbance by pea stem submitochondrial particles, and on TPP^+ uptake by pea stem mitochondria. Additions were: 0.5 mM MgATP, 50 μM FFA. (a) Linolenic acid (18:3) or linoleic acid (18:2); (b) oleic acid (18:1); (c) palmitic acid (16:0); (d) stearic acid (18:0), 1 mM NADH, 0.15 mM ADP, 50 μM CAtr.

Similarly to FCCP, FFA were not capable of dissipating electroneutral proton gradients, generated by the addition of NaOH to pea microsomal vesicles (Fig. 1). However, when valinomycin was subsequently added, the proton gradient was released with the same order of effectiveness exhibited on ATP- or PP-generated proton gradients, but with a slower rate. Therefore, free fatty acids were able to dissipate only electrogenic proton gradients, but in the presence of an ionophore as valinomycin they could accomplish an electroneutral H^+/K^+ exchange which mimicked the effect of nigericin.

Effect of FFA on proton gradient of pea submitochondrial particles and on electrical potential of intact mitochondria

The ability of FFA to collapse proton gradients was also checked in a different system of membranes. Fig. 3 shows that FFA markedly dissipated the proton gradi-

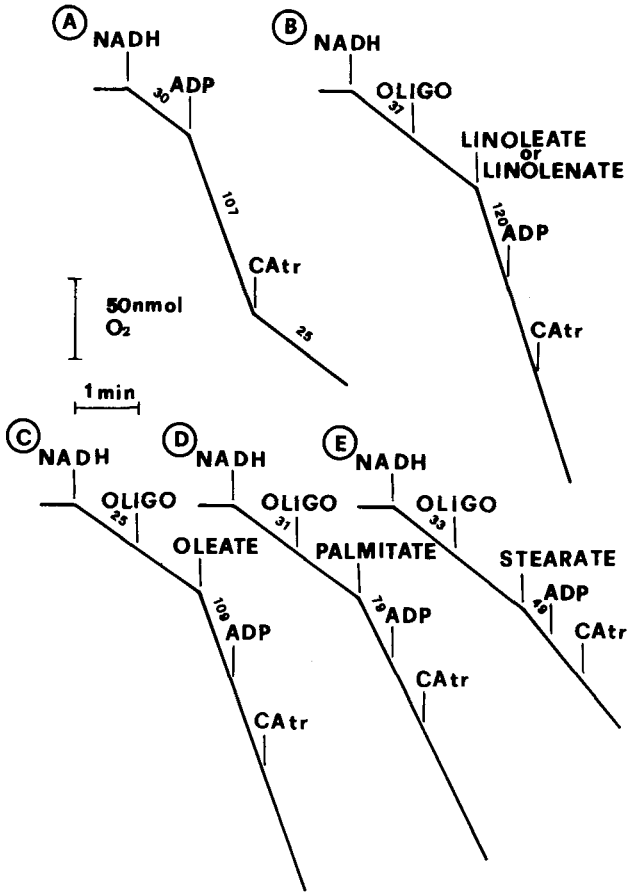

Fig. 4. Effect of free fatty acids on oxygen uptake by pea stem mitochondria. Additions were; 1 mM NADH, 0.15 mM ADP, 50 μM CAtr, 2 μg/ml oligomycin, 50 μM palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids.

ent generated by the oligomycin-sensitive H^+-ATPase of submitochondrial particles. The assayed fatty acids collapsed the proton gradient to the same extent observed in microsomes, but palmitic acid was more effective. They were also capable of dissipating $\Delta\Psi$ of pea mitochondria and these effects were not reversed by the subsequent addition of ADP or CAtr.

Effect of FFA on oxygen consumption of pea mitochondria

Recently, it was shown that carboxyatractyloside inhibits the FFA-stimulated O_2 uptake by animal mitochondria [4,5], which led to the suggestion that the ATP/ADP antiporter is crucial in the mechanism of FFA-induced uncoupling of oxidative phosphorylation. In plant mitochondria, there is no available evidence for a similar effect. Therefore, we assayed the effect of FFA on O_2 consumption (Fig. 4). Trace A shows that NADH induced an O_2 uptake in pea mitochondria which was stimulated by ADP (RCR 4). Carboxyatractyloside abolished this increase, suggesting that this type of mitochondrion possesses an ATP/ADP translocator sensitive to this inhibitor. Traces B–E show that, in the presence of oligomycin, all free fatty acids increased oxygen consumption and that neither ADP nor CAtr inhibited this FFA-stimulated oxygen uptake. In addition, CAtr was unable to inhibit FFA-stimulated O_2 consumption even in the absence of ADP (result not shown). These results show that FFA uncoupled oxidative phosphorylation in plant mitochondria. The lack of inhibition on FFA-stimulated O_2 uptake and the lack of effect on FFA-dissipated electric potential (see Fig. 3) of ADP or CAtr, suggest that the ATP/ADP exchanger was not involved in the FFA-induced uncoupling. Again, the effect of any single fatty acid depended on its degree of unsaturation.

Effect of lysophosphatidylcholine-oleoyl (lyso-PC) on pea stem microsomes and mitochondria

Lyso-PC is commonly used as a detergent in studies on the latency of H^+-ATPase of plant plasma membranes. This lysophospholipid has been shown to stimulate ATPase activity, evaluated as release of inorganic phosphate [11,12,15,16,21], and ATP-dependent proton pumping [16]. This was interpreted as a possible mechanism of modulation of the enzyme activity.

Fig. 5 shows that lyso-PC slightly released the ΔpH built up by H^+-ATPase of microsomal vesicles. However, lyso-PC, added before ATP supply, markedly inhibited such an activity. Conversely, the proton gradient generated by H^+-PPase of microsomes and H^+-ATPase of submitochondrial particles, was sharply dissipated by lyso-PC. In addition, the lyso-PC stimulated O_2 uptake in pea stem mitochondria, although with slower kinetics. Also in this case, ADP or CAtr did not inhibit the lyso-PC-stimulated O_2 uptake.

Effect of FFA and lyso-PC on ATPase and PPase activity of pea microsomal vesicles

Oleic, linoleic, linolenic acids and lyso-PC were able to stimulate ATPase and PPase activity, evaluated as release of inorganic phosphate. The effect on the latter was higher than that recovered on the former. Palmitic and stearic acids significantly stimulated only PPase activity. Again, unsaturation was important in amplifying the effect of FFA on such activities (Table I).

Discussion

Similarly to animal mitochondria [3], free fatty acids uncoupled oxidative phosphorylation of plant mitochondria and the effect seems to be linked to their capacity of permeabilizing the membranes to protons [21]. The protonophoric activity of FFA appears to be exerted only on electrogenic proton gradients of microsomal vesicles and submitochondrial particles, since the electroneutral ΔpH was unaffected. In agreement with this result, it has been shown that oleic acid markedly inhibits the proton pumping capacity of inside-out

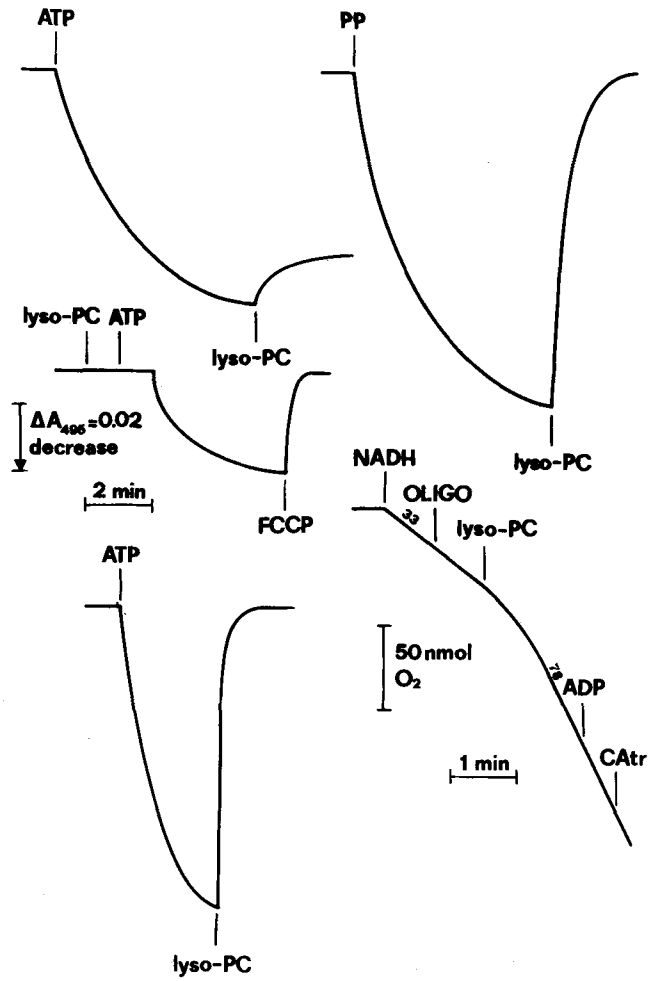

Fig. 5. Effect of lyso-PC on ATP- or PP-dependent decrease in AO absorbance by pea stem microsomes, ATP-dependent decrease in AO absorbance by pea stem submitochondrial particles and on oxygen uptake by mitochondria. Additions were: 0.5 mM MgATP, 200 μM PP, 50 μM lyso-PC, 1 mM NADH, 0.15 mM ADP, 50 μM CAtr, 2 μg/ml oligomycin.

plasma membrane vesicles [21]. The half-maximal inhibition found in the latter work is 5 μM, which is lower than that found by us (25–30 μM). The difference may depend on the different fatty acid/protein ratio used in the experiments. The increase in the unsaturation of the acyl chain appears to be very important in amplifying the protonophoric capacity. We can exclude that these effects may be a consequence of a direct inhibition of FFA on the ATPase [11], because the free fatty acids tested do not inhibit or stimulate ATPase and PPase activity, similarly to that observed by others on the H^+-ATPase activity of plasma membrane vesicles (evaluated as release of P_i) [15,21]. In all cases these stimulatory effects may be explained with the uncoupling caused by FFA which discharge the proton gradient.

TABLE I

Effect of free fatty acids and lyso-PC on ATPase and PPase activity of pea stem microsomes

Data are means of three replicates ± S.D.

FFA (50 μM)	Activity (μmol P_i/mg prot. per h)	
	ATPase	PPase
Control	61.9 ± 1.3	26.7 ± 2.5
Palmitic acid	62.5 ± 1.4	32.4 ± 1.5
Stearic acid	62.0 ± 2.5	28.5 ± 3.0
Oleic acid	70.0 ± 4.5	38.3 ± 0.9
Linoleic acid	68.3 ± 3.0	38.5 ± 1.4
Linolenic acid	71.9 ± 1.9	37.0 ± 2.5
Lyso-PC	72.4 ± 2.1	46.8 ± 1.9

The results presented in this paper are in conflict with the postulated mechanism of decoupling, suggested to explain the effect of FFA on animal mitochondria [29]. Recently, it has been proposed a second hypothesis in which the ATP/ADP antiporter appears somehow involved [4]. In this case the protonated (neutral) form of a fatty acid (RCOOH) freely permeates the phospholipid bilayer, while the transfer of the anionic species of such fatty acid ($RCOO^-$) would be mediated by the ATP/ADP exchanger [5]. Our results are not compatible with such conclusion. Two orders of experimental findings are in disagreement with it: first, the ΔpH of microsomes, which do not contain the exchanger, was also markedly collapsed by FFA; second, CAtr, a specific inhibitor ATP/ADP antiporter, did not inhibit the FFA-stimulated O_2 uptake and did not release the FFA-collapsed electric potential in pea stem mitochondria. In accord with another recent paper [6], we postulate that proton transport in plant plasma membrane may occur through the lipid bilayer, as suggested for artificial protonophoric uncouplers which possess a pK_a (4.1–6.8) very similar to that of FFA (4.7–5.8). There is no doubt that protonated fatty acids can permeate the lipid bilayer, while the unprotonated forms may have some difficulty due to their negative charges. However, lipophilicity of the acyl chain may help FFA to overcome this hindrance. This possibility is supported by the observation that the addition of fatty acids increases proton conductance in phospholipid bilayer membranes, although their efficiency is lower than that of classical protonophores. Fatty acids simply would therefore act as proton carriers (A^- type) [30].

The modulation of ATPases from many animal sources by lipids has been recognized and discussed in some reviews [31,32] and the same has been claimed for plant plasma membrane bound ATPases [20]. There is a general agreement on the stimulating effect of phospholipids, in particular lysophosphatidylcholine on ATPase activity of plant plasma membranes [11,12,15,16,21]. This effect does not appear to be related to the ability of lyso-PC to act as a detergent, because it is also able to stimulate ATPase in purified inside-out vesicles [21] and to enhance the activity of the purified enzyme as well [11,12]. However, the effects of lyso-PC were mainly

found on ATPase activity, evaluated as release of inorganic phosphate. When lyso-PC was assayed on the proton pumping activity of ATPases, some conflicting results were obtained: in oat root plasma membrane vesicles, lyso-PC stimulates of about 85% the rate of Mg-ATP-dependent H^+ accumulation without affecting the passive permeability to protons [16]; on the other hand, with inside-out plasma membrane vesicles from sugar beet leaves such an effect cannot be demonstrated [21]. The latter authors attribute these differences to the presence of lyso-PC-degrading enzymes (e.g., lysophospholipase), that release free fatty acids, the true responsible of the decrease in proton pumping activity. Our results show that lyso-PC slightly collapsed the proton gradient generated by H^+-ATPases of microsomes, whereas it markedly dissipated the ΔpH generated by both H^+-PPase of microsomes and H^+-ATPases of submitochondrial particles. In addition, we have also shown that lyso-PC uncoupled oxidative phosphorylation, although to a lower extent. The latter effect may be rationalized by considering the above suggested possibility [21]. The effect of lyso-PC would depend on the liberation of FFA (oleic acid) from this phospholipid, but this rationale does not explain the rapid collapse of ΔpH generated by H^+-PPase of microsomes and H^+-ATPase of submitochondrial particles. Therefore, we suggest that, in addition to the effect caused by the liberation of FFA, lyso-PC may per se act on the phospholipid 'anulus' of H^+-PPase and H^+-ATPase of submitochondrial particles, modifying the specific lipid environment in which these enzymes are embedded.

The main fatty acids in lipids of plant plasma membranes are palmitic, linoleic and linolenic acids [33]. Therefore, in the early stages of senescence, characterized by a high lipolytic activity, these acids may be the major degradation products. It has been observed that palmitate and linoleate enhance solute leakage, alter phase properties and promote fusion of fully hydrated model membranes [2]. These effects may all account for the symptoms caused by several environmental stresses in plants, although, in the light of the present results, a most precocious injury may be due to the protonophoric capacity of the FFA.

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