Volume 307, number 3, 267–271 FEBS 11348 August 1992

Electrical potential dissipation induced by free fatty acids in pea stem mitochondria

E. Petrussa, E. Braidot, G. Nagy, A. Vianello and F. Macri

Section of Plant Physiology and Biochemistry, Institute of Plant Protection, University of Udine, via Cotonificio 108, I-33100 Udine, Italy

Received 23 April 1992; revised version received 1 June 1992

Linolenic, linoleic, oleic, palmitic and stearic acids (FFA) collapse the electrical potential of pea stem mitochondria in the absence or in the presence of 0.5 mM Mg^{2+}. Higher concentrations of this cation (5 mM) lower the rate of dissipation caused by linoleic, oleic and palmitic acids, while abolishing that induced by stearic acid. Carboxyatractyloside and ADP do not reverse the FFA-induced collapse both in the presence or absence of Mg^{2+}. EDTA, EGTA or BHT do not influence the dissipation caused by FFA that, in addition, is not linked to lipid peroxidation evaluated as malondialdehyde or conjugated diene formation. Only linolenic acid sustains a peroxidation which, however, appears to be caused by its own oxidation catalysed by lipoxygenases rather than by membrane lipoperoxidation induced by this free fatty acid. These results suggest that neither the ATP/ADP exchanger nor lipid peroxidation appear to be involved in FFA-induced uncoupling in pea stem mitochondria.

Fatty acid; Mitochondrion; ATP/ADP antiporter; Lipid peroxidation; *Pisum sativum* L.

1. INTRODUCTION

It has been known for a long time that free fatty acids (FFA) uncouple oxidative phosphorylation of animal mitochondria [1] and, albeit uncompletely known, their mechanism of action appears to be different from that postulated for classical protonophore uncouplers [2].

In 1988 Skulachev and coworkers suggested the fatty acid circuit hypothesis to explain the FFA-induced uncoupling [3]. The protonated form of the FFA penetrates through the internal mitochondrial membrane, while the unprotonated one fails to cross the phospholipid bilayer. The anionic species would thus utilize protein(s) that facilitate the transport of these forms through the inner membrane. Two proteins accomplishing this transfer have been hitherto identified: the ATP/ADP antiporter in several animal tissues and thermogenin in brown fat [2].

The involvement of the ATP/ADP exchanger is based on the following observations: (i) inhibition of the FFA-induced uncoupling by CAtr or ADP [3]; (ii) restoration of the FFA-collapsed potential by CAtr, ADP, atractyloside, bongkrekic acid, palmitoyl-CoA or pyridoxal phosphate [4,5]; (iii) correlation between the content of the ATP/ADP antiporter in mitochondria of different tissues and the ability of CAtr to reverse the effect of FFA [6]. Thermogenin seems to act similarly to the ATP/ADP exchanger because of several evidences indicating strict structural and functional relationships between the two proteins [7,8].

In plants, our knowledge of the effects of FFA is still limited, although it has been demonstrated that they inhibit the inducible proton cotransport system for hexose analogues in *Chlorella vulgaris* [9] and uncouple chloroplasts [10,11]. In a recent paper [12], we have shown that FFA stimulate oxygen consumption linked to NADH oxidation in pea stem mitochondria, and that this stimulation is associated to a dissipation of the ATP-dependent proton gradient in submitochondrial particles and to a collapse of the NADH-generated electrical potential in intact mitochondria. CAtr or ADP neither inhibit the FFA-stimulated oxygen uptake, nor restore the FFA-collapsed electrical potential of intact mitochondria. In analogy to that, the ATP or PP_i-dependent proton gradient of pea stem microsomes, that do not possess the ATP/ADP antiporter, is also dissipated by FFA.

These results led us to suggest a mechanism of action different from that postulated for animal mitochondria. The difference might however be due to the high Mg^{2+} concentration (5 mM) used in our experiments. In fact, magnesium salts of ADP or CAtr fail to relieve the FFA-induced uncoupling [4]. Another possible explanation, involving the formation of non-specific pores due to phospholipid peroxidation, may also be invoked to explain our results [4,13,14].

Abbreviations: BHT, butylated hydroxytoluene; CAtr, carboxyatractyloside; FFA, free fatty acid(s); FCCP, carbonyl cyanide *p*-(triphluoromethoxy)phenylhydrazone; MDA, malondialdehyde; TBA, thiobarbituric acid.

Correspondence address: F. Macri, Sezione di Fisiologia e Biochimica Vegetale, Istituto di Difesa delle Piante, Università di Udine, via Cotonificio 108, I-33100 Udine, Italy. Fax: (39) (432) 558784.

In this paper we examine the effect of FFA on electrical potentials in pea stem mitochondria in the presence of low (0.5 mM) concentration of Mg^{2+} or in its absence. The possible involvement of lipid peroxidation in the FFA-induced uncoupling is also shown.

2. MATERIALS AND METHODS

Mitochondria from etiolated pea (*Pisum sativum* L., cv. Alaska) stems were prepared as previously described [12], except that BSA in the grinding medium was 0.3%. The protein concentration was determined by the biuret method [15], using BSA as a standard.

Electrical potential difference was measured by the safranin method [16], following changes of dye fluorescence [17] by a Perkin-Elmer fluorescence spectrometer, model LS-3, at 25°C. The excitation and emission wavelengths were 495 and 586 nm, with a slitwidth of 5 nm. The incubation medium contained 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose, 0, 0.5 or 5 mM $MgCl_2$, 5 mM Na/K phosphate, 5 μM safranin 0, 5 μg/ml oligomycin and 100 μl of mitochondria (ca. 0.7 mg protein) in a final volume of 2 ml. Succinate (4 mM) or NADH (0.5 mM) were used as substrates.

Oxygen uptake was determined by a Clark-type electrode in the same incubation medium, without safranin O, used for electrical potential determinations, at 25°C.

When mitochondria were used to evaluate lipoperoxidation, they were washed in 20 mM HEPES-Tris (pH 7.5), 0.125 M KCl and resuspended in the latter buffer which was also used as incubation medium. Lipid peroxidation was estimated, at 25°C, either as formation of MDA by the TBA method [18], or as absorbance changes at 232 nm, due to conjugated diene formation, by a Perkin-Elmer spectrophotometer, model λ15.

Na-palmitate and Na-stearate were dissolved in absolute ethanol; Na-oleate, Na-linoleate and linolenic acid (free acid) were dissolved in 5 mM MES-Tris (pH 8.9), 0.1 mM EDTA, 0.1 mM DTT, 9.6% (v/v) ethanol to give in all cases 5 mM stock solutions.

3. RESULTS AND DISCUSSION

To better compare our data with previous results obtained by others with animal mitochondria [3,6], we assayed the effect of CAtr and ADP on the dissipated electrical potential or the stimulated oxygen uptake by a low concentration (10 μM) of palmitic acid in pea stem mitochondria resuspended in a medium without or with 0.5 mM $MgCl_2$. As can be seen in Fig. 1, neither CAtr nor ADP reversed the palmitate-dissipated electrical potential generated by NADH (traces A and B) or succinate (results not shown). In agreement, the palmitate-induced stimulation of NADH-dependent oxygen uptake was not inhibited by CAtr or ADP (traces C and D).

According to our preceding [12] and above results, several FFA dissipated the electrical potential of pea stem mitochondria generated by the oxidation of NADH, in the presence of a low concentration of Mg^{2+} (0.5 mM) or in its absence (Fig. 2). Oleic, stearic and linolenic acids were the most effective, while CAtr and ADP again did not reverse the FFA-collapsed potential. The dissipation induced by linolenic acid, after a lag period, was spontaneously and partially relieved and a new gradient, collapsed by FCCP, was built up. Again, CAtr or ADP did not affect this restoration. Conversely, BHT, propyl gallate or iodoacetate completely prevented the recovery of the gradient. The same behaviour was observed by using succinate as a substrate. However, in the presence of 5 mM Mg^{2+} (dashed lines)

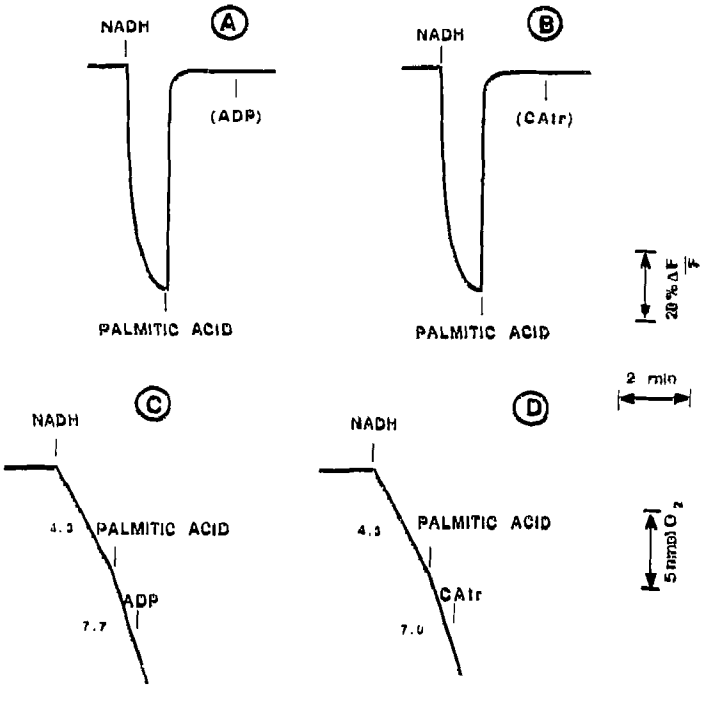

Fig. 1. Effect of CAtr and ADP on the palmitate-dissipated NADH- dependent electrical potential (traces A and B) or the palmitate-stimulated NADH-dependent oxygen uptake (traces C and D) in pea stem mitochondria. Additions were: 10 μM palmitic acid; 10 μM CAtr; 0.5 mM ADP. Figures next to traces C and D are expressed as nmol O_2/mg protein × min.

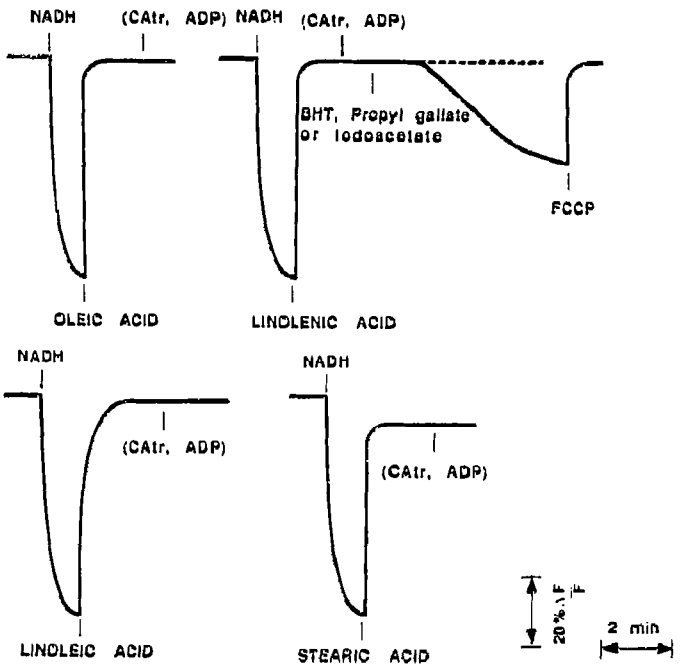

Fig. 2. Effect of FFA on the NADH-dependent electrical potential in pea stem mitochondria. Additions were: 50 μM linoleic, oleic, linolenic or stearic acids; 10 μM CAtr; 0.5 mM ADP; 5 μM FCCP; 0.5 mM NADH. Other additions (dashed line) were: 1 mM BHT; 10 mM propyl gallate; 0.1 mM iodoacetate.

the rate of the dissipation of the electrical potential induced by oleic, linoleic and palmitic acids, was lowered, while that caused by stearic acid was completely prevented. Conversely, the effect of linolenic acids was unaffected by this concentration of Mg^{2+} that instead inhibited the spontaneous restoration of the potential (Fig. 3).

To clarify such a discrepancy in the mode of action of free fatty acids, it has been demonstrated that the effect of palmitic acid on the electrical potential differ-

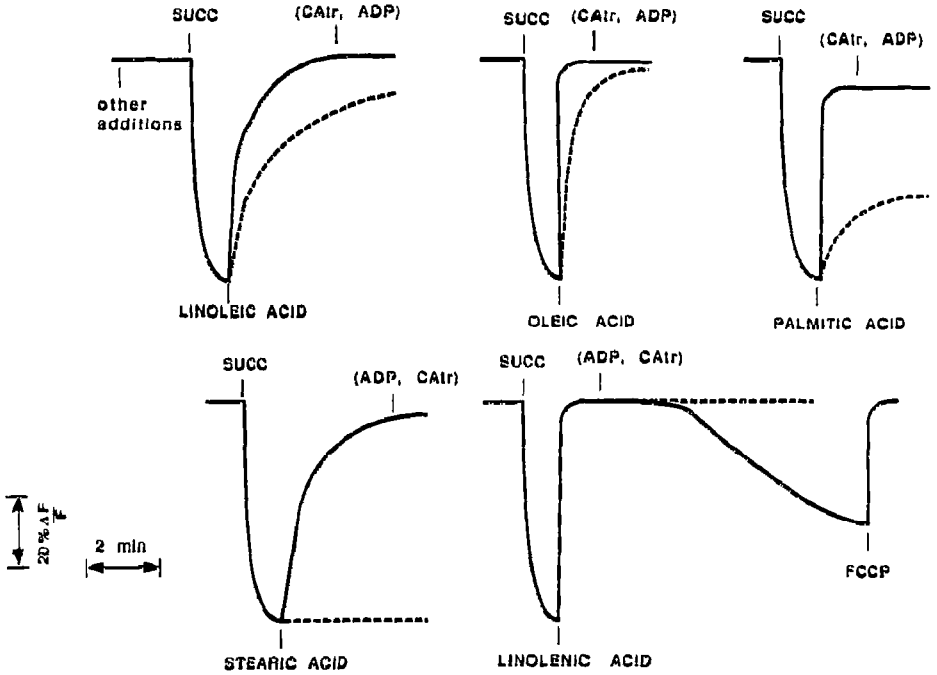

Fig. 3. Effect of FFA on the succinate-dependent electrical potential in pea stem mitochondria. Additions were: 50 μM linoleic, oleic, palmitic, stearic or linolenic acids; 10 μM CAtr; 0.5 mM ADP; 5 μM FCCP; 4 mM succinate. Other additions were: 1 mM EDTA; 1 mM EGTA; dashed line refers to experiments made in the presence of 5 mM $MgCl_2$.

ence depends on the medium in which the mitochondria are incubated, although the reason for that remains obscure [19]. In medium A [20] the electrical potential of FFA-treated mitochondria decreases much more than in medium B [21], while the rate of respiration is equally stimulated in both media. The main difference between the two incubation media is the presence of 5 mM $MgCl_2$ in medium B. Therefore, the observed lack of effect of palmitic acid on the electrical potential can be attributed to the ability of Mg^{2+} in lowering or preventing the collapsing effect induced, in particular, by saturated fatty acids (palmitic and stearic acids).

We found that ADP or CAtr do not reverse the FFA-dissipated electrical potential in mitochondria incubated with 5 mM Mg^{2+} [12]. From the above results, it appears that the lack of this effect cannot be due to the formation of Mg-ADP or Mg-CAtr complexes that, as known, do not interact with the ADP/ATP translocator [4]. On the other hand, a specific system for the removal of Mg^{2+} ions in the vicinity of the plant mitochondrial carrier has been suggested, since it is probable that ATP or ADP molecules, in the cytosol and in this organelle, chelate this divalent cation [22].

Another possible mechanism of the FFA-mediated uncoupling may be related to non-specific pore formation induced by several coordinated events, including lipid peroxidation and requiring Ca^{2+} [4,13,14]. However, EGTA, EDTA or BHT did not affect the FFA-induced dissipation of the electrical potential generated by succinate (Fig. 3) and, therefore, lipid peroxidation does not appear to be linked to such a dissipation. Besides linolenic acid, none of the tested FFA triggered lipid peroxidation evaluated either as MDA or conjugated diene formation (results not shown). The lipoperoxidation caused by linolenic acid and monitored by both methods, may be attributed to the oxidation of the latter rather than to a membrane lipid peroxidation elicited by the free fatty acid. In particular, the conjugated diene formation induced by linolenic acid was not inhibited by EDTA, suggesting the involvement of lipoxygenases that utilize such free fatty acid as a typical substrate [23]. In the light of the last result it is also possible to explain the spontaneous recovery of the electrical potential occurring after linolenic acid-induced dissipation (Figs. 2 and 3). The activity of lipoxygenases, with the free fatty acid as a substrate, permitted the regeneration of the electrical potential. BHT, an antioxidant and propyl gallate or iodoacetate, two lipoxygenase inhibitors, abolished the restoration by inhibiting the activity of the latter enzyme. Similarly, Mg^{2+} inhibited the recovery of the potential because divalent cations are known to inhibit lipoxygenase activity [23].

The present results are not compatible with the mechanism of uncoupling induced by FFA, involving the ADP/ATP antiporter and, in addition, cannot be explained by events including lipid peroxidation and requiring Ca^{2+}. As recently suggested, there are other possible effects of fatty acid which can result in uncoupling: (i) a proton gradient dissipation caused by diffusion of the protonated fatty acids via-phospholipid bilayers; (ii) an increase change in the inner dielectric constant of the membrane due to the transported anion; (iii) a transport of the fatty acids anion by proteins different from the ATP/ADP antiporter or thermogenin [2]. Each possibility is more or less compatible with our results, but, considering that protonated FFA can freely cross the membranes, we suggest that in plant mitochondria the unprotonated form of FFA is outwardly translocated by systems different from the ADP/ATP antiporter or thermogenin. In particular, this system should also be present in microsomal membranes and in the plasmalemma, to account for the effects of FFA found with this type of membrane [12,24].

Acknowledgements: We thank Prof. V.P. Skulachev, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, for very useful suggestions. We also thank Prof. L. Galzigna, Department of Biochemistry, University of Padua, for the critical reading of the manuscript. This work was supported by M.U.R.S.T. (60% fund) - U.O. (Angelo Vianello) - U.S.U. (1990). G. Nagy, Department of Plant Physiology, University of Szeged, is a student of the TEMPUS program of the European Economic Community.

REFERENCES

[1] Pressman, B.C. and Lardy, H.A. (1956) Biochim. Biophys. Acta 21, 458–466.
[2] Skulachev, V.P. (1991) FEBS Lett. 294, 158–162.
[3] Andreyev, A.Yu., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P. and Volkov, N.I. (1987) FEBS Lett. 226, 265–269.
[4] Andreyev, A.Yu., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Tsofina, L.M., Volkov, N.I. and Vygodina, T.V. (1989) Eur. J. Biochem. 182, 585–592.
[5] Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Starkov, A.A., Arrigoni-Martelli, E. and Bobyleva, V.A. (1991) FEBS Lett. 295, 51–54.
[6] Schönfeld, P. (1990) FEBS Lett. 264, 246–248.
[7] Aquila, H., Link, T.A. and Klingenberg, M. (1985) EMBO J. 4, 2369–2376.
[8] Bouilland, F., Weissenbach, J. and Ricquier, D. (1986) J. Biol. Chem. 261, 1487–1490.
[9] Decker, M. and Tanner, W. (1975) FEBS Lett. 60, 346–348.
[10] McCarthy, R.E. and Jagendorf, A.T. (1965) Plant Physiol. 40, 725–735.
[11] Pick, U., Weiss, M. and Rottemberg, H. (1987) Biochemistry 26, 8295–8302.
[12] Macri, F., Vianello, A., Braidot, E. and Zancani, M. (1991) Biochim. Biophys. Acta 1058, 249–255.
[13] Hunter, D.R., Haworth, R.A. and Southard, J.H. (1976) J. Biol. Chem. 251, 5069–5077.
[14] Dragunova, S.F., Novgorodov, S.A., Sharyshev, A.A. and Yaguzhinsky, L.S. (1981) Biokhimiya 46, 1242–1247 (Russ.).
[15] Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766.
[16] Moore, A.L. and Bonner Jr., W.D. (1982) Plant Physiol. 70, 1271–1276.
[17] Holden, M.J. and Sze, H. (1989) Plant Physiol. 91, 1296–1302.
[18] Buege, J.A. and Aust, S.D. (1978) Methods Enzymol. 52, 302–310.

[19] Luvisetto, S., Buso, M., Pietrobon, D. and Azzone, G.F. (1990) J. Bioenerg. Biomembr. 22, 635–643.
[20] Luvisetto, S., Pietrobon, D. and Azzone, G.F. (1987) Biochemistry 26, 7332–7338.
[21] Rottemberg, H. and Hashimoto, K. (1986) Biochemistry 25, 1747–1755.
[22] Vignais, P.V. (1976) Biochim. Biophys. Acta 456, 1–38.
[23] Galliard, T. and Chan, H.V.-S., in: The Biochemistry of Plants. Lipids: Structure and Functions (P.K. Stumpf and E.E. Conn, Eds.) Vol. 4, Academic Press, New York, 1980, pp. 131–161.
[24] Palmgren, M.G., Sommarin, M., Ulvskov, P. and Larsson, C. (1990) Biochim. Biophys. Acta 1021, 133–140.