FEBS Letters 371 (1995) 258–260 FEBS 15969

Effect of cyclosporin A on energy coupling in pea stem mitochondria

A. Vianello*, F. Macri, E. Braidot, E.N. Mokhova**

Department of Biology and Agro-industrial Economics, Section of Plant Biology, University of Udine, via del Cotonificio, 108 I-33100 Udine, Italia

Received 19 July 1995

Abstract Effect of cyclosporin A on energy coupling in pea stem mitochondria is studied. It is found that incubation of mitochondria with 100 nM FCCP and/or CAtr, oligomycin, $CaCl_2$, palmitate and ADP results, after some lag phase, in a collapse of $\Delta\psi$ generated by succinate oxidation in the presence of rotenone. Cyclosporin A (0.2–0.8 nmol/mg mitochondrial protein) markedly increases the lag phase. The cyclosporin A effect requires dithioerythritol to be added to the isolation medium. Metabisulphite fails to substitute for dithioerythritol. The relationships between these effects and cyclosporin A-sensitive mitochondrial permeability transition in animal mitochondria are discussed.

Key words: Uncouplers; Permeability transition; Pora; Transmembrane electrical potential; Cyclosporin A; Carboxyatractylate; Plant mitochondria

1. Introduction

In animal mitochondria, some proteins were found to be involved in the uncoupler-induced increase in the inner membrane permeability. These proteins, localized in the inner mitochondrial membrane, are thermogenin in brown fat (uncoupling by fatty acids [1]), the ATP/ADP antiporter in other tissues (uncoupling by fatty acids and dinitrophenol [2]), and a still unknown protein(s) acting as steroid hormone receptor(s) in liver and heart mitochondria (FCCP and related uncouplers) [3–5].

Some of these effects are also inherent in plant mitochondria. Indeed, indications of the involvement of the ATP/ADP-antiporter in free fatty acid-induced uncoupling of pea and sunflower mitochondria [6,7] and for the presence of a thermogenin-like protein in potato mitochondria [8] have been recently reported. It was also shown that 6-ketocholestanol reverses uncoupling by a low amount of FCCP in plant mitochondria [9].

In animal mitochondria, one of the uncoupling mechanisms consists of the induction of the Ca^{2+}-dependent and cyclosporin A-sensitive non-specific permeability of the inner mitochondrial membrane for low molecular weight substances. This mitochondrial permeability transition is modulated by many factors and accompanied by high amplitude swelling (for reviews see [10,11]).

It remains obscure whether cyclosporin A-sensitive permeability transition pore is inherent in plant mitochondria. In the present paper, data will be reported suggesting occurrence of a cyclosporin A-sensitive pore in pea stem mitochondria.

2. Materials and methods

Pea mitochondria were isolated in cold box from etiolated stems of *Pisum sativum* L., cv. Alaska. 80 g stems were grinded with pestle in a mortar in 120 ml medium containing 0.4 M sucrose, 5 mM EGTA, BSA (1 mg/ml), 1 mM DTE and 20 mM HEPES-Tris (pH 7.6). The homogenate was then filtered through eight-gauze layers. Debris was once more homogenized in 100 ml of the medium and filtered. The filtrate was centrifugated at 28,000 × *g* for 5 min by a Sorvall RC-5B centrifuge, 4°C. The pellet was resuspended in 120 ml homogenization medium by a Potter homogenizer. This fraction was centrifugated at 2,500 × *g* for 3 min and the supernatant was centrifuged at 28,000 × *g* for 5 min. The pellet (mitochondrial fraction) was suspended in 2 ml resuspending medium containing 0.4 M sucrose, 20 mM HEPES-Tris (pH 7.5) and BSA (2 mg/ml), then 25 ml of similar medium without BSA was added and the suspension was centrifuged at 28,000 × *g* for 5 min. The pellet was suspended in 1 ml of resuspended medium, the suspension contained about 6 mg protein per 1 ml and was stored on ice. In some experiments, 25 mM potassium metabisulphite was added to the isolation medium instead of 1 mM DTE.

To estimate the $\Delta\psi$ changes, safranine O was used [12]. The difference in absorbance between 523 nm and 555 nm (ΔA) was recorded (in optical density units), at room temperature, by a double beam–double wave Perkin-Elmer spectrophotometer, model 356.

To study high amplitude swelling, the light scattering at 90° angle was recorded, at room temperature, at 620 nm by Perkin-Elmer fluorescence spectrometer, model LS-3.

Oxygen consumption was monitored, at room temperature, by a Clark-type oxygen electrode (YSI, model 4004).

The mitochondrial protein was determined by the Bradford method, using the Biorad protein assay.

Reagents. sucrose, rotenone, EGTA, HEPES, Tris, oligomycin, safranin O, fatty acid-free BSA, potassium metabisulphite, FCCP, DTE, dibucain, BHT, palmitic acid and CAtr were from Sigma (USA). Cyclosporin A was a gift from Sandoz

3. Results and discussion

The addition of 100 nM FCCP to pea mitochondria, supplemented with oligomycin, $CaCl_2$, palmitate and CAtr induced, after some lag phase, a progressing decrease in $\Delta\psi$ generated by succinate oxidation in the presence of rotenone (Fig. 1, trace A). Cyclosporin A, added just after mitochondria, caused a pronounced increase in the lag phase (Fig. 1, trace B). In most experiments the final cyclosporin A concentration was 0.2 μM. Some cyclosporin A effects were also observed at 0.1 μM and 0.05 μM (mitochondrial protein concentration being equal, i.e. 0.25 mg/ml). Cyclosporin A failed to reverse the $\Delta\psi$ drop when added after the lag phase (Fig. 1, trace C).

It should be mentioned that addition of cyclosporin A just after the mitochondria resulted in a $\Delta\psi$ increase. Such an effect

*Corresponding author. Fax: (39) (432) 558784.

**Permanent address: A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia.

Abbreviations. $\Delta\psi$, transmembrane electrical potential difference; BSA, bovine serum albumin; CsA, cyclosporin A; BHT, butylated hydroxytoluene; DTE, dithioerythritol; CAtr, carboxyatractylate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid, HEPES, *N*-(2-hydroxyethylpiperazine-*N'*-[2-ethanesulfonic acid]); TRIS, tris-(hydroxymethyl)aminomethane.

SSDI 0014-5793(95)00897-7

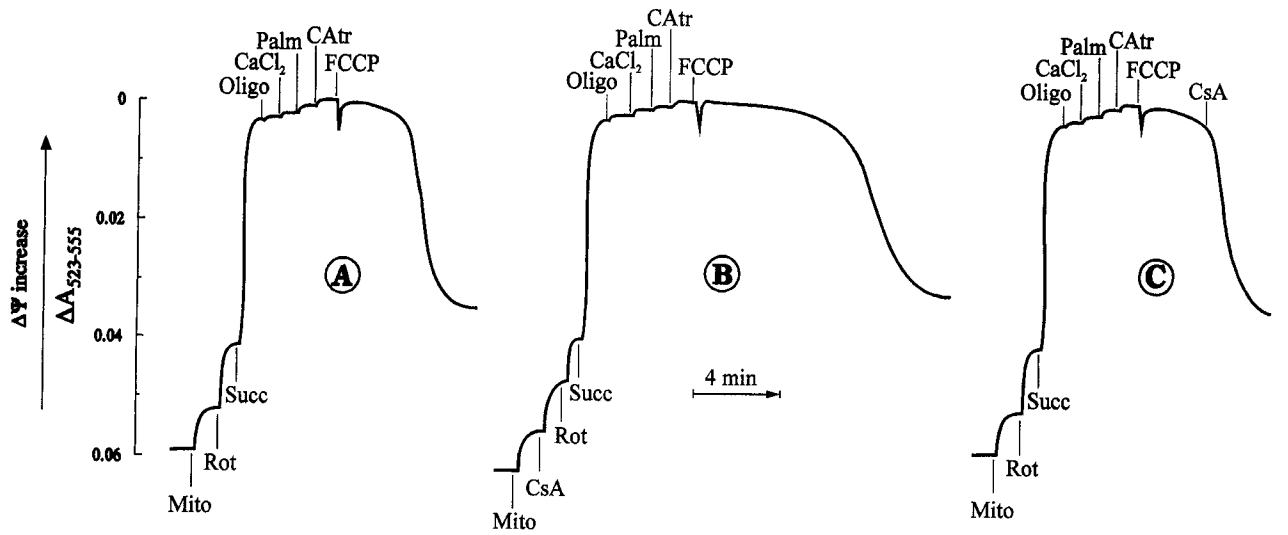

Fig. 1. Effect of cyclosporin A on the FCCP-induced decrease in $\Delta\psi$ generated by succinate oxidation. Mitochondria were isolated in DTE-supplemented medium and washed in medium without EDTA (see section 2). The incubation medium contained 5 μM safranin O, 0.4 M sucrose, BSA (0.2 mg/ml), 3 mM phosphate, and 5 mM HEPES-Tris (pH 7.5), and mitochondria (0.25 mg protein/ml). Additions: CsA, 0.2 μM cyclosporin A; Succ, 5 mM succinate; Oligo, oligomycin (1 μg/ml); Rot, 1 μM rotenone; $CaCl_2$, 10 μM $CaCl_2$, Palm, 10 μM palmitic acid, CAtr, 0.5 μM carboxyatractylate; FCCP, 100 nM FCCP.

disappeared if the oxidation substrates were already present (Fig. 1–3).

The rapid $\Delta\psi$ decrease was not accompanied or followed by high amplitude swelling (not shown).

A similar cyclosporin A effect could be observed using the incubation medium supplemented with 0.5 mM EGTA and 1.5 mM $MgCl_2$. However, CsA exerted a small opposite effect (some decrease in the lag phase) if the incubation medium was supplemented with EGTA without $MgCl_2$ (not shown).

Similar, but less pronounced responses were observed when $\Delta\psi$ was generated by NADH oxidation in the presence of rotenone. FCCP-induced $\Delta\psi$ collapse was accompanied by a stimulation of oxygen consumption (not shown).

$\Delta\psi$ generated under similar condition but in the presence of ADP was found to collapse after CAtr addition and again cyclosporin A increased the lag phase (Fig. 2).

Fatty acids have been shown to potentiate the Ca^{2+}-induced mitochondrial permeability transition [13–15]. Indeed, in their presence, a lower Ca^{2+} concentration to induce mitochondrial permeability transition is needed [13]. In our experiments, palmitate was added to use a low Ca^{2+} concentration and thus to minimize a possible competition between Ca^{2+} and safranin O for mitochondrial binding sites [12].

In similar experiments with rat liver mitochondria, addition in incubation of dibucain, or BHT, or EGTA was shown to prevent the CAtr-induced uncoupling [14]. BHT appears to be a CsA synergist [16].

Fig. 3 shows that in our case BHT per se was of little effect on the lag phase and its increase by CsA. Similarly, addition of 100 μM–200 μM dibucain to pea mitochondria did not increase the lag phase preceding the drop of the $\Delta\psi$ (not shown).

All the above results were obtained when mitochondria were isolated in DTE-supplemented medium without metabisulphite (see section 2). If mitochondria were isolated in metabisulphite-

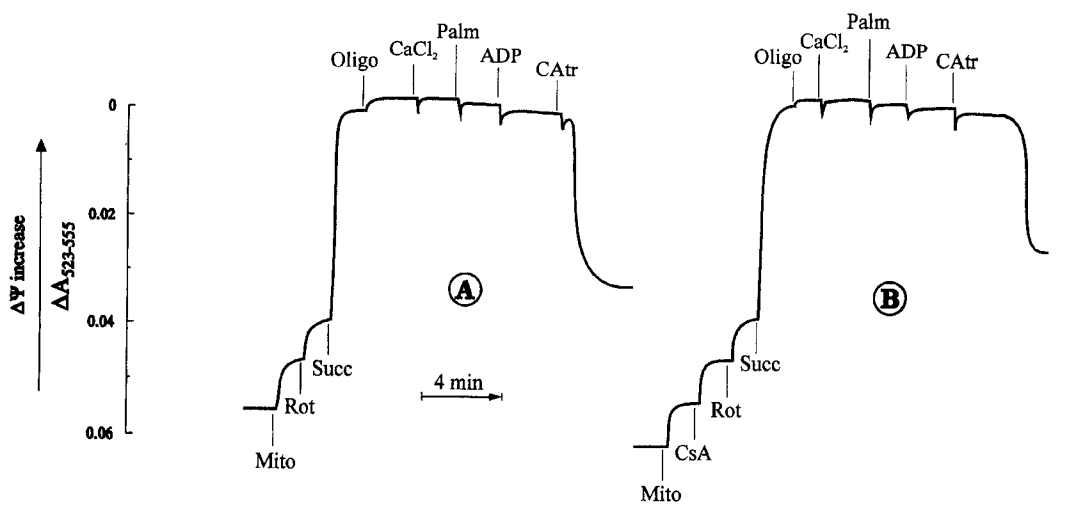

Fig. 2. Effect of cyclosporin A on the CAtr-induced decrease in $\Delta\psi$ generated by succinate oxidation. Experimental conditions and additions were as in Fig. 1. Other additions: ADP, 100 μM ADP.

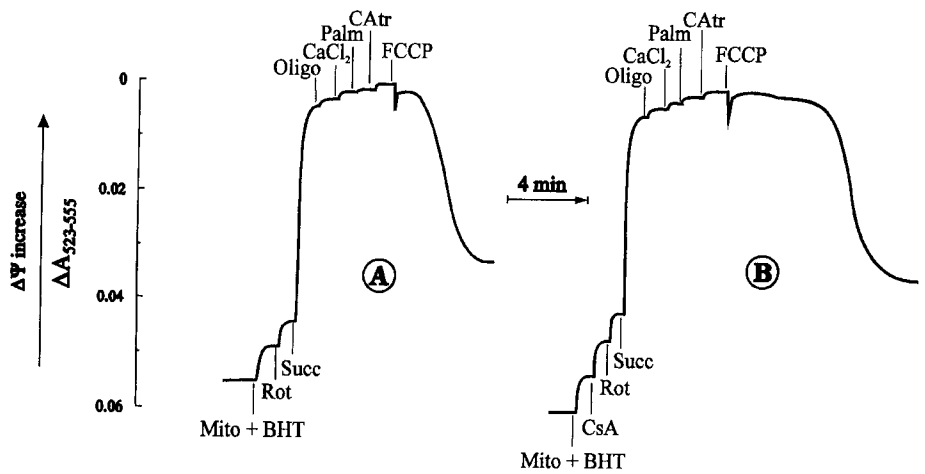

Fig. 3. Effect of BHT and cyclosporin A on the FCCP-induced decrease in $\Delta\psi$ generated by succinate oxidation. Experimental conditions and additions were as in Fig. 1. Other additions BHT, 100 μM BHT.

supplemented medium, cyclosporin A did not affect the $\Delta\psi$ kinetics.

Ca^{2+} uptake is the first step of mitochondrial permeability transition; for opening the pore in the presence of Mg^{2+}, the higher Ca^{2+} concentration should be added [17]. On the other hand, addition of EGTA to Ca^{2+}-loaded mitochondria promotes mitochondrial permeability transition [11], as in this case EGTA prevents Ca^{2+} efflux from mitochondria [18].

The CsA-sensitive $\Delta\psi$ decrease, observed in pea mitochondria in the presence of 0.5 mM EGTA and 1.5 mM $MgCl_2$, suggests that for these mitochondria, a very low concentration of Ca^{2+} is sufficient for induction of the $\Delta\psi$ collapse. One of possible reasons of these relationships is an oxidation of SH-groups during isolation of pea mitochondria. The SH-group oxidation was shown to promote mitochondrial permeability transition [10,19]. The lack of the cyclosporin A effect in pea mitochondria isolated with metabisulphite, also seems to be explained by modification of SH-groups by this agent [20].

A lack of a high amplitude mitochondrial swelling, always accompanying the pore formation in animal mitochondria, may be explained by some specific feature of pea mitochondria. We found that decrease in incubation medium tonicity to 100 osmM exerted low effect on the light scattering of mitochondrial suspension (not shown). According to Gaynutdinov and cowerkers, at low Ca^{2+} concentration the CsA-sensitive pore seems to be permeable only for ions of small diameter [21].

The molecular mechanism of induction of mitochondrial permeability transition is still unknown. One of the attractive hypotheses suggests that a protein complex composed of the ATP/ADP-antiporter and the outer mitochondrial membrane porin may be involved in this process [22]. Our results on the CAtr effect (Figs. 2 and 3) are in line with these hypotheses.

Acknowledgements: This work was supported in part by C.N.R. (bilateral project, 1995) Italy. Dr. E.N. Mokhova was supported by a Fellowship of the University of Udine (Leg ge 19/91) and by Grant N MGQOO from the International Science Foundation.

References

[1] Heaton, G.M. and Nicholls, D.G. (1976) Eur. J. Biochem. 67, 511–517.
[2] Andreyev, A.Yu., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Tsofina, L.M., Volkov, N.I. and Vigodina, T.V. (1989) Eur J. Biochem. 182, 585–592.
[3] Starkov, A.A., Dedukhova, V.I. and Skulachev, V.P. (1994) FEBS Lett. 355, 305–308.
[4] Skulachev, V.P. (1994) Biokhimiya (Moscow) 59, 910–912.
[5] Starkov, A.A., Dedukhova, V.I., Bloch, D.A., Severina, I.I. and Skulachev, V.P. (1995) in: Thirty Years of Progress in Mitochondrial Bioenergetics and Molecular Biology (Palmer, F., Papa, S., Saccone, C. and Gadaleta, M.N., eds.) Elsevier, Amsterdam (accepted).
[6] Vianello, A., Petrussa, E. and Macri, F (1994) FEBS Lett. 347, 239–242.
[7] Macri, F., Vianello, A., Petrussa, E. and Mokhova, E.N. (1994) Biochem. Mol. Biol. Intern. 34, 217–224.
[8] Vercesi, A.E., Martins, I.S., Silva, M.A.P., Leite, H M.F., Cuccovia, I.M. and Chaimovich, H. (1995) Nature 375, 24.
[9] Vianello, A., Macri, F., Braidot, E. and Mokhova, E.N. (1995) FEBS Lett. 365, 7–9.
[10] Gunter, T.E. and Pfeiffer, D.R. (1990) Am. J. Physiol. 258, C755–C786.
[11] Petronilli, V., Nicolli, A., Costantini, P., Colonna, R. and Bernardi, P. (1994) Biochim. Biophys. Acta 1187, 255–259.
[12] Smith, J.C. (1990) Biochim. Biophys. Acta 1016, 1–28.
[13] Hunter, D.R., Haworth, R.A. and Southard, J.H. (1976) J. Biol. Chem. 251, 5069–5077.
[14] Andreyev, A.Yu., Dedukhova, V.I. and Mokhova, E.N. (1990) Biologicheskie Membrani 7, 480–486 (in Russian).
[15] Petronilli, V, Cola, C., Massary, S., Colonna, R. and Bernardi, P (1993) J. Biol. Chem. 268, 21939–21945.
[16] Broekemeier, K M. and Pfeiffer, D.R. (1989) Biochem. Biophys. Res. Commun. 163, 561–566.
[17] Haworth, R.A. and Hunter, D.R. (1979) Arch. Biochem. Biophys 195, 460–467.
[18] Leikin, Yu.N. and Gonsalves, M.P.P (1986) Dokladi Akademii Nauk SSSR 290, 1011–1014 (in Russian).
[19] Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S and Bernardi, P. (1994) J. Biol. Chem. 269, 16638–16642.
[20] Means, G.E. and Feeney, R.E. (1971) Chemical Modifications of Proteins, Holden-Day, San Francisco, pp. 152.
[21] Gainutdinov, M.Kh., Konov, V.V., Ishmukhamedov, R.N., Zakharova, T.N., Khalilova, M.A. and Safarov, K.S. (1992) Biochemistry (Moscow) 57, 1120–1126.
[22] Halestrap, A.P and Davidson, A.M. (1990) Biochem. J. 268, 153–160