Effect of 6-ketocholestanol on FCCP- and DNP-induced uncoupling in plant mitochondria

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

Dipartimento di Biologia ed Economia Agro-industriale, Sezione di Biologia Vegetale, Università di Udine; via Cotonificio, 108, I-33100 Udine, Italia

Received 6 April 1995

Abstract Effect of 6-ketocholestanol on FCCP-induced and DNP-induced uncoupling in beef liver and pea stem mitochondria was studied, under experimental conditions at which this steroid abolished the effect of low concentrations of FCCP and other most potent uncouplers in rat mitochondria [Starkov et al. (1994) FEBS Lett., 355, 305–308]. It is shown that, in both types of mitochondria, 6-ketocholestanol prevents or reverses the uncoupling induced by low concentrations of FCCP, but not that caused by high concentrations of FCCP or by any concentration of DNP. Progesterone and male sex hormones, showing recoupling capability in animal mitochondria, appear to be ineffective in the plant system. Cholesterol does not recouple in both animal and plant mitochondria. Plant steroids, such as β-sitosterol and stigmasterol, are also without effect.

Key words: Uncoupler; FCCP; DNP; 6-ketocholestanol; Steroid hormone; Plant mitochondria

1. Introduction

Recently it was found that ketocholestanol (kCh) abolishes the uncoupling effect induced by low concentrations of the most potent uncouplers, like FCCP, in rat heart and liver mitochondria, while the uncoupling caused by DNP, pentachlorophenol and palmitic acid is unaffected [1–3]. On the basis of these and other results and similarities in chemical structure between kCh and steroid hormones, the hypothesis that kCh, steroid hormones and FCCP-like uncouplers interact with a protein which mediates uncoupling induced by low concentrations of FCCP [2,3] was developed, just as thermogenin in brown fat mitochondria [4] or ATP/ADP antiporter in muscle and liver mitochondria mediate uncoupling by palmitate [5].

Pea stem and sunflower hypocotyl mitochondria were found to be similar with mammalian mitochondria in the participation of ATP/ADP antiporter in free fatty acid-induced uncoupling [6,7]. Therefore, it was interesting to study if the FCCP-induced uncoupling in these mitochondria was also sensitive to kCh and steroid hormones.

In the present paper the effects of kCh, some steroid hormones and plant steroids on FCCP- and DNP-induced uncoupling in mitochondria isolated from pea stem and bovine liver were studied.

2. Materials and methods

Pea mitochondria were isolated from etiolated stems (*Pisum sativum* L., cv. Alaska). Eighty grams of stems were cut and homogenized by a mortar with pestle in 100 ml homogenization medium: 0.4 M sucrose, 5 mM EGTA, 25 mM potassium metabisulphite, BSA (1 mg/ml) and 20 mM HEPES-Tris (pH 7.6). The homogenate was then filtered through eight gauze layers, debris were again homogenized in 100 ml of the homogenization medium and filtered once more. The filtrate was centrifuged at 28,000 × *g* for 5 min by a Sorvall RC-5B centrifuge, at 4°C. The pellet was resuspended in 120 ml homogenization medium by a Potter homogenizer. This fraction was centrifuged at 2,500 × *g* for 3 min and the supernatant centrifuged at 28,000 × *g* for 5 min. The pellet (mitochondrial fraction) was suspended in 1 ml of 0.4 M sucrose and 20 mM HEPES-Tris (pH 7.5). The final mitochondrial suspension contained about 10 mg protein per 1 ml and was stored on ice.

Pieces of *beef liver* were stored in ice-cold 0.25 M sucrose for about 30 min. Then, the pieces were minced with scissors and homogenized by a Potter homogenizer in a medium containing 0.25 M sucrose, 1 mM EGTA, 5 mM HEPES-Tris (tissue/medium ratio was about 1:7). The homogenate was filtered through two gauze layers. After first centrifugation (10 min at 800 × *g*), the supernatant was centrifuged for 10 min at 10,000 × *g*. The mitochondrial suspension (about 20 mg protein/ml) was stored on ice.

Oxygen consumption was recorded as described in [6].

To estimate the $\Delta\Psi$ changes, safranin O was used [8–10]. In the majority of experiments, 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.

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

Reagents: 6-ketocholestanol, sucrose, rotenone, DNP, EGTA, HEPES, Tris, oligomycin, safranin O, fatty acid-free BSA, potassium metabisulphite, FCCP, PMS, TMPD, stigmasterol, β-sitosterol, cholesterol and progesterone were from Sigma (USA). Cholesterol, β-sitosterol, progesterone, stigmasterol and kCh were dissolved in absolute ethanol to give 8 mM stock solutions.

3. Results and discussion

The additions of 20–60 μM kCh to beef liver mitochondria reversed (Fig. 1A) or prevented (Fig. 1B) the 20 nM FCCP-induced decrease in $\Delta\Psi$. Addition of 20 μl ethanol (the amount of ethanol used to dissolve 80 μM kCh) exerted no effect on $\Delta\Psi$ (not shown). High FCCP concentrations added after kCh caused uncoupling. Under the same conditions, kCh did not increase the $\Delta\Psi$ level lowered by DNP (Fig. 1C). Thus in beef mitochondria, kCh seems to act in the same way as it was previously shown in rat mitochondria [1,3].

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

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

Abbreviations: $\Delta\Psi$, transmembrane electrical potential difference; BSA, bovine serum albumin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DNP, 2,4-*p*-dinitrophenol; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*, *N*, *N'*, *N'*,-tetraacetic acid; HEPES, *N*-(2-hydroxyethylpiperazine-*N'*-[2-ethanesulfon ic acid]) PMS, phenazine methosulphate; Tris, tris(hydroxymethyl)aminomethane; kCh, 6-ketocholestanol (5α-cholestan-3β-ol-6-one); TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

SSDI 0014-5793(95)00431-9

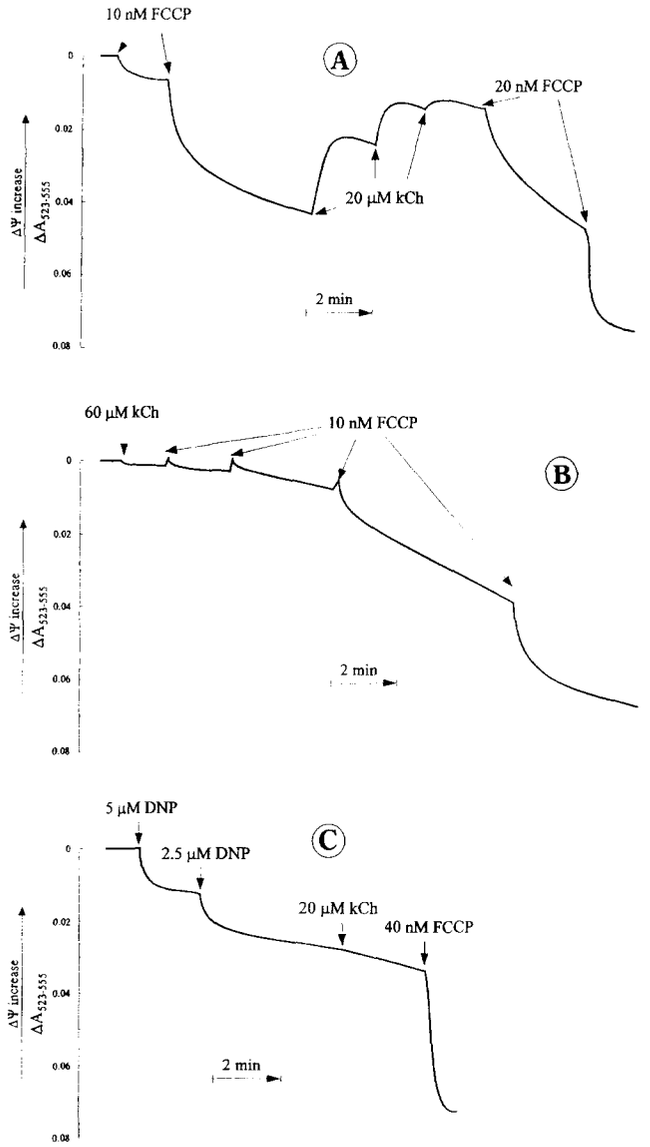

Fig. 1. Effect of 6-ketocholestanol and uncouplers on the membrane potential in beef liver mitochondria. Incubation medium: 7 μM safranin O, 0.25 M sucrose, BSA (0.2 mg/ml), 0.5 mM EGTA, 3 mM Na^+/K^+ phosphate, oligomycin (1 μg/ml), 1 μM rotenone and 0.5 mM ascorbate, 1 μM PMS and 5 mM HEPES-Tris (pH 7.4), mitochondria (0.5 mg protein/ml).

In experiments with pea mitochondria TMPD was used instead of PMS to have a more stable level of the $\Delta\Psi$. The results of the experiments with kCh proved to be similar to those obtained with beef mitochondria. A low concentration of FCCP (20 nM) decreased $\Delta\Psi$, while 40 μM kCh, being added before or after FCCP, induced a recoupling effect (Fig. 2A,B). If 100 nM FCCP was added before other reagents, the following additions of oligomycin (1 μg/ml), 1 μM rotenone, 1 mM ascorbate, 10 μM TMPD and 40 μM kCh did not affect $\Delta\Psi$ (not shown). Additions of 40 μM cholesterol (Fig. 2C), progesterone (Fig. 2D), testosterone, dihydrotestosterone, β-sitosterol and stigmasterol (not shown) did not restore $\Delta\Psi$ collapsed by 20 nM FCCP. In fact, these steroids potentiated rather than to suppress the uncoupling induced by low FCCP, both the presence or absence of BSA (0.5 mg/ml). In the same experiments, stimulation of NADH oxidation by 20 nM FCCP was partially inhibited by 40 μM kCh, but the subsequent additon of a high concentration of FCCP (200 nM) again increased this oxidation (not shown). DNP-induced uncoupling proved to be kCh-resistant (Fig. 2E).

The presented results demonstrate that recoupling effect of kCh on FCCP-induced uncoupling is not specific for mammalian mitochondria being also inherent to plant mitochondria. The difference between mitochondria from the two sources concerns steroid hormones. Indeed, progesterone, testosterone and dihydrotestosterone showed recoupling effect in experiments with animal mitochondria [3], but not in pea mitochondria. Some plant steroids were also ineffective.

Acknowledgements: We thank Prof. V.P. Skulachev, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, for suggestions and the critical reading of the manuscript. This study was supported in part by a grant N MGQOO from International Science Foundation and by M.A.F. (Italy), Special Program 'Genetic Resistance of Crops to Biotic and Abiotic Stress'.

References

[1] Starkov, A.A., Dedukhova, V.I., and Skulachev, V.P. (1994) FEBS Lett. 355, 305–308.
[2] Skulachev, V.P. (1994) Biokhimiya (Moscow) 59, 910–912
[3] Starkov, A.A., Dedukhova, V.I., Bloch, D.A., Severina, I.I. and Skulachev, V.P. (1995) Thirty Years of Progress in Mitochondrial Bioenergetics and Molecular Biology (Palmer, F., Papa, S., Saccone, C. and Gadaleta, M.N. Eds.) Elsevier, Amsterdam.
[4] Heaton, G.M. and Nicholls, D.G. (1976) Eur. J. Biochem. 67, 511–517.
[5] 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.
[6] Vianello, A., Petrussa, E. and Macrì, F. (1994) FEBS Lett. 347, 239–242.
[7] Macrì, F., Vianello, A., Petrussa, E. and Mokhova, E.N. (1994) Biochem. Mol. Biol. Intern. 34, 217–224.
[8] Akerman, K.E.O. and Wikstrom, M.K.F. (1976) FEBS Lett. 68, 191–197.
[9] Zanotti, A. and Azzone, G.F. (1980) Arch. Biochem. Biophys. 201, 255–265.
[10] Harris, E.J. and Baum, H. (1980) Biochem. J. 192, 551–557.

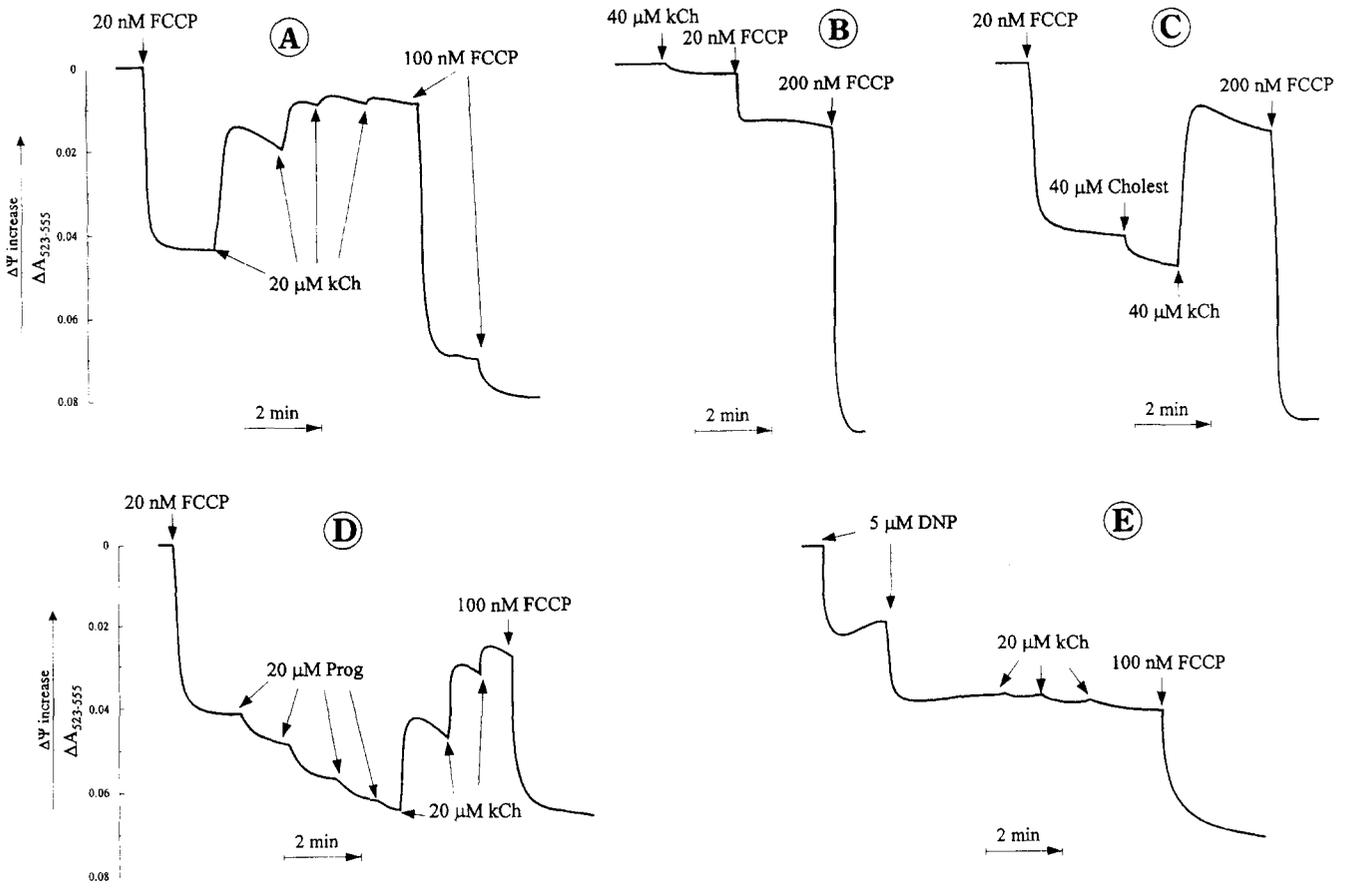

Fig. 2. Effect of 6-ketocholestanol, progesterone and uncouplers on the membrane potential in pea stem mitochondria. Incubation medium: 7 μM safranin O, 0.25 M sucrose, BSA (0.2 mg/ml), 0.5 mM EGTA, 3 mM Na^+/K^+ phosphate, oligomycin (1 μg/ml), 1 μM rotenone and 1 mM ascorbate, 10 μM TMPD and 5 mM HEPES-Tris (pH 7.4), mitochondria (0.5 mg protein/ml).