Volume 315, number 3, 233–236 FEBS 11975 January 1993

Cyclosporin A suppression of uncoupling in liver mitochondria of ground squirrel during arousal from hibernation

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Received 2 November 1992; revised version received 13 November 1992

Energy coupling parameters were studied in liver mitochondria of ground squirrel during arousal from hibernation. It was found that such mitochondria become uncoupled during incubation with phosphate in a salt medium. The uncoupling was revealed by respiration rate increase and membrane potential decrease in the presence of oligomycin. Both effects were reversed by addition of cyclosporin A. Under the same in vitro conditions, mitochondria from aroused (active) animals showed no uncoupling but could be uncoupled by addition of palmitate in the cyclosporin A-sensitive fashion. It is proposed that formation of cyclosporin A-sensitive pores can be involved in urgent heat production in arousing hibernators.

Thermoregulatory uncoupling; Cyclosporin A; Fatty acid; Hibernation

1. INTRODUCTION

Uncoupling of oxidative phosphorylation is known to be a mechanism of cold-induced urgent heat production in warm-blooded animals [1–3]. In some cases the uncoupling is mediated by an increase in the level of free fatty acids [2–5]. The mechanism of uncoupling by FFA was shown to consist of an increase in the H^+ permeability of the inner mitochondrial membrane catalyzed in brown fat by a special FFA-activated protein, thermogenin [2], and in other tissues by the ATP/ADP antiporter, a protein strongly resembling thermogenin in many essential features [3,6,7]. It was suggested that both proteins facilitate FFA anion transport across the membrane [3,8]. Such a function was assumed to be (i) the only function of thermogenin, and (ii) a supplementary function of the ATP/ADP antiporter, which is very specific for hydrophilic anions, i.e. ADP^{4-} and ADP^{3-}, and seems to be rather non-specific to monovalent monopolar hydrophobic anions [3,6,8]. The FFA-induced uncoupling mediated by the ATP/ADP antiporter is specifically suppressed by the antiporter inhibitor CAtr.

There is one more effect resulting in uncoupling which seems to be mediated by FFA and the ATP/ADP antiporter. It was found that in the inner mitochondrial membrane FFA facilitates formation of a Ca^{2+}-induced non-specific pore permeable not only for H^+ but also for other low-molecular weight solutes. The pore formation is strongly stimulated by CAtr and completely inhibited by CSA. The possible physiological significance of the pore remains obscure [9–12].

It was shown in our group that arousal of hibernating ground squirrels is accompanied by at least two metabolic effects favorable for heat production: (i) activation of the respiratory electron transport chain between cytochromes *b* and c_1, which is inhibited in mitochondria of hibernating animals [13]; and (ii) CAtr-inhibited uncoupling [14]. We recently reported that arousal causes significant increase in the FFA level in mitochondria [5].

In this paper we show that one more mechanism of uncoupling develops in the liver mitochondria of arousing ground squirrels. In contrast to the one mentioned above, it is stimulated by CAtr and abolished by CSA. This is, in fact, the first indication that the CSA-sensitive pore is functionally important under physiological conditions.

2. MATERIALS AND METHODS

Ground squirrels *Citellus undulatus* from Yakutia (North-East Siberia) were studied. Hibernating and aroused ground squirrels were kept at 4°C. Aroused animals were kept for 1.5–2 months at 20°C.

Body temperature was measured in the region of the heart.

Mitochondria were isolated from liver in medium containing 0.3 M sucrose and 10 mM Tris-HCl (pH 7.4). Homogenate was centrifuged for 10 min at 600 × *g*, and the resulting supernatant was centrifuged for 15 min at 10,000 × *g*. Mitochondria were suspended in the isolation medium to 70–100 mg protein/ml (for details, see [14]).

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Abbreviations: BSA, bovine serum albumin; CSA, cyclosporin A; CAtr, carboxyatractylate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; FFA, free fatty acids; DNP, 2,4-dinitrophenol; TPP^+, tetraphenyl phosphonium cation.

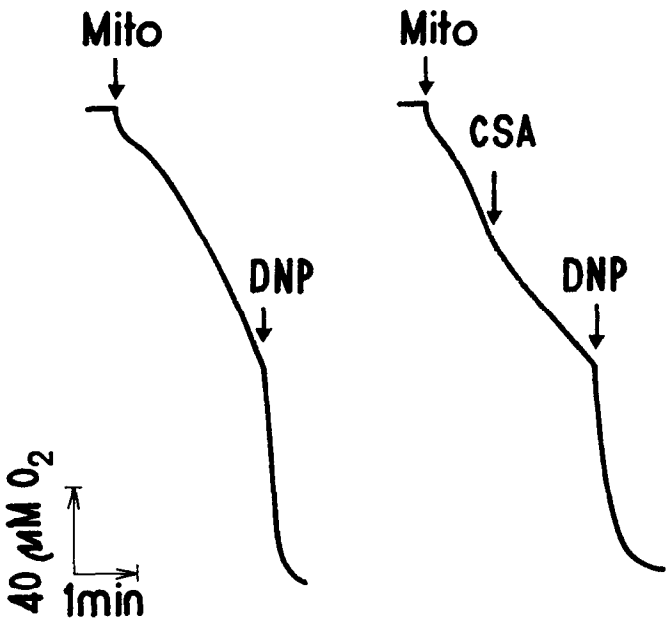

Fig. 1. Effect of CSA on respiration of liver mitochondria from aroused ground squirrel. For incubation mixture, see section 2. Additions: mitochondria, 2.9 mg protein · ml^{-1}; cyclosporin A, 1 $\mu g \cdot ml^{-1}$; 4×10^{-5} M DNP.

Respiratory rate was measured polarographically with a Clark-type oxygen electrode.

Membrane potentials were recorded using the TPP^+ probe [15]. The concentration of the probe was measured with a TPP^+-sensitive electrode [16].

The incubation medium contained 0.125 M KCl, 5 mM KH_2PO_4, 4 μM cytochrome *c*, 10 mM Tris-HCl (pH 7.4), 4 mM succinate, 3 μM rotenone oligomycin (3 μg/ml), mitochondria (2.5–3.5 mg protein/ml); the temperature was 27°C. Where indicated, 2 μM CAtr, 1 μg/ml CSA, 0.2% BSA, 100 μM ADP, 40 μM DNP, and 20 μM or 40 μM palmitate were added.

Oligomycin, palmitic acid, succinate, rotenone, CAtr, Tris, and fatty acid-free BSA were from Sigma; DNP was from Serva.

3. RESULTS

Fig. 1 shows the kinetics of oxygen consumption by liver mitochondria isolated from aroused ground squirrels. Oxidation of succinate in the presence of oligomycin was measured. One can see that a spontaneous increase in the respiration rate develops during the first 3 min of incubation. This acceleration of oxygen consumption could be interrupted by CSA. Further addition of DNP caused maximal stimulation of respiration in samples both with and without CSA. These effects were studied at various stages of the body temperature increase (Table I). It is seen that CSA inhibited respiration of the aroused animals at any studied stage of arousal. For active (aroused) ground squirrels kept at room temperature, CSA was without effect. In fact, CSA abolished additional oxygen consumption observed in arousing as compared with aroused ground squirrels.

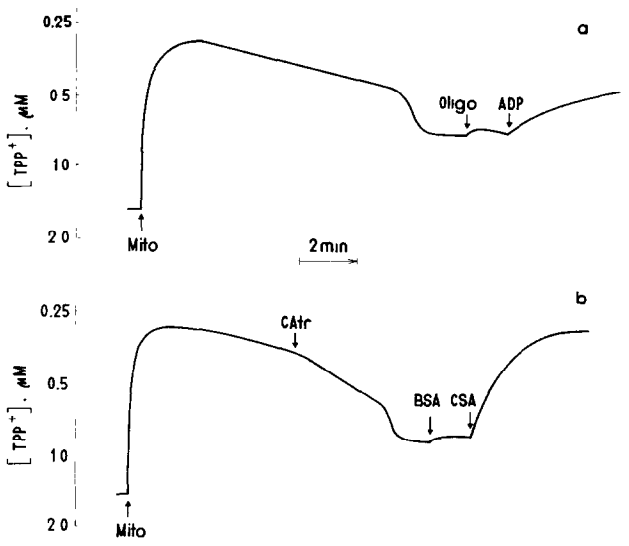

Fig. 2. Membrane potential in liver mitochondria from aroused ground squirrel. For incubation mixture, see section 2. Additions: mitochondria, 2.8 mg protein · ml^{-1}; oligomycin, 1 $\mu g \cdot ml^{-1}$; 1×10^{-4} M ADP; 0.2% BSA; cyclosporin A, 1 $\mu g \cdot ml^{-1}$

Addition of DNP, which allows the maximal capacity of the respiratory chain to be demonstrated, showed an increase in the succinate oxidation rate in the arousing animals when their body temperature increased to 25°C. As shown previously, this effect is due to activation of the cytochrome $b \rightarrow c_1$ electron transfer, which is strongly inhibited in the hibernating ground squirrels [13].

As shown in Fig. 2, the membrane potential measured with the TPP^+ probe was unstable in mitochondria from the arousing animals, decreasing during the incubation. This decrease was stimulated by CAtr. Subsequent addition of ADP, BSA, and CSA increased the $\Delta\Psi$ level, the CSA effect being especially strong. In the aroused

Table I

Effect of cyclosporin A on the respiration rate in liver mitochondria of arousing and aroused ground squirrels

Animals	Body temperature (°C)	Number of animals	Respiration rate (ngatom O/min per mg protein)		
			Without additions	CSA	CSA, DNP
Arousing	15	3	34	18	33
	17	3	38	23	75
	23	1	52	25	78
	25	1	55	26	107
	27	1	49	26	108
Aroused	37	8	26 ± 3	25 ± 4	108 ± 11

For experimental conditions, see section 2. Additions of DNP (40 μM) and CSA (1 μg/ml) were made before mitochondria were added. Respiration rate was measured 1 min after addition of mitochondria.

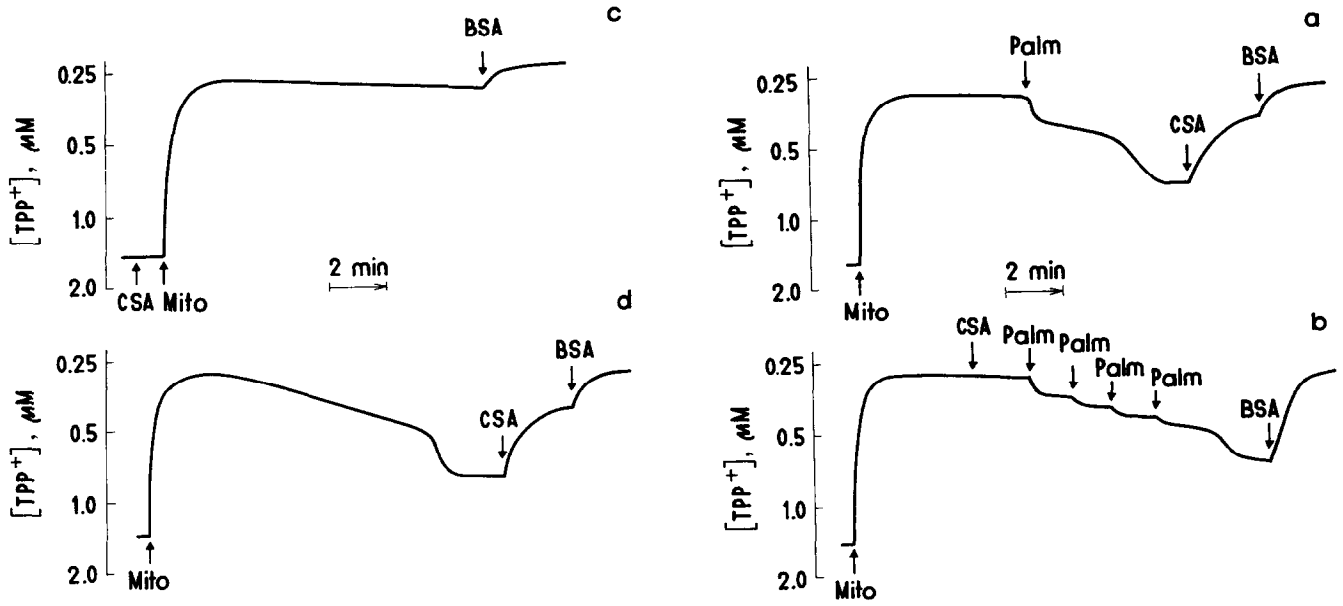

Fig. 3. Membrane potential in liver mitochondria from aroused (active) ground squirrel. Incubation mixture and additions as in Fig. 2. (a,b) Each palmitate addition was equal to 4×10^{-5} M. (c,d) Incubation mixture was supplemented with 2×10^{-5} M palmitate.

(active) ground squirrels (Fig. 3), the $\Delta\Psi$ level was stable but could be decreased by addition of palmitate. In Fig. 2 it is shown that the palmitate-induced decrease in $\Delta\Psi$ was composed of fast and slow components, the latter being specifically reversed by CSA. The addition of BSA after CSA completely reversed the palmitate-induced $\Delta\Psi$ decrease. In the presence of CSA, a much higher palmitate concentration was required to cause the slow phase of the $\Delta\Psi$ decrease. In the same experiments, this addition of palmitate was found to stimulate respiration of oligomycin-treated mitochondria in a CSA-sensitive fashion (Table II).

All the above-mentioned CSA-sensitive effects were completely abolished by EGTA (not shown).

4. DISCUSSION

The data reported in this paper suggest that arousal of ground squirrels after hibernation increases the probability of opening of the CSA-sensitive pore. The following features inherent in formation of such a pore were observed: (i) CSA-sensitive acceleration of State 4 respiration and $\Delta\Psi$ decrease during incubation of mitochondria with phosphate; (ii) stimulation of effect (i) by CAtr; (iii) its inhibition by EGTA.

In mitochondria from the active (aroused) animals, CSA-sensitive uncoupling does not occur.

In this context, it should be mentioned that some years ago it was found in this group that the cold exposure of rats was accompanied by some effects similar to those described in this paper [17]. Unfortunately, the action of CSA was not disclosed yet when this work was done.

Summarizing the above results and those obtained previously in our group [14], we may conclude that two mechanisms of uncoupling may be actuated in liver mitochondria of arousing animals; i.e. (i) uncoupling inhibited by CAtr, and (ii) uncoupling inhibited by CSA and stimulated by CAtr. The former mechanism is most probably mediated by the ATP/ADP antiporter-catalyzed circulation of FFA anions and protonated FFA, resulting in the same increase in the specific H^+ conductance. This way of the additional heat production may be effective in those tissues where the ATP/ADP antiporter concentration is high, such as in muscles. In liver this concentration is relatively low [7] so that activation of the stronger uncoupling mechanism, i.e. pore formation, may be necessary. Again, as in the former mechanism, FFA may be a mediator of uncoupling since (a) mitochondrial FFA content increases during arousal of the hibernating animal [5], and (b) addition of palmitate to the mitochondria of aroused animals induces the CSA-sensitive uncoupling (see Fig. 3). An

Table II

Effect of cyclosporin A on palmitate-stimulated respiration of liver mitochondria of aroused (active) ground squirrels

Additions	Respiration rate (ng atom O/min per mg protein)	
	Without DNP	With DNP
Without addition	29 ± 3	118 ± 10
Palmitate	76 ± 6	128 ± 9
Palmitate, CSA	28 ± 4	117 ± 12

For experimental conditions, see section 2. Additions: palmitate (20 μM), CSA (1 μg/ml). Palmitate and CSA were added before mitochondria. Other conditions as in Table I.

interesting possibility exists in that the pore formation is a result of a membrane potential decrease caused by the FFA-mediated H^+ conductance increase. According to Bernardi [18], any lowering of the membrane potential is favorable for opening of the pore in liver mitochondria. This does not exclude, however, involvement of other intracellular mediators such as Ca^{2+}. This involvement seems probable within the framework of the concept that assumes that in adaptation to an extremal situation all possible adaptive mechanisms are actuated.

It should be stressed that in isolated mitochondria the CAtr-inhibited and the CSA-inhibited mechanisms are demonstrated in quite different incubation mixtures, i.e. in sucrose-EGTA medium in the former case and in KCl-phosphate medium in the latter case. It was found that the former medium is infavourable for the latter effect and vice versa.

To extrapolate the above observation to in vivo conditions, some in vivo or in situ measurements are required. Such measurements might include in vivo NMR of the high energy compounds and in situ studies of morphology of mitochondria. In this connection, one may mention that the [FFA] increase during arousal of ground squirrels was shown not only in isolated mitochondria but also in tissues [5].

REFERENCES

[1] Skulachev, V.P. and Maslov, S.P. (1960) Biokhimiya 25, 1058–1064 (Russ.).
[2] Nicholls, D.S. and Locke, R.M. (1984) Physiol. Rev. 64, 1064.
[3] Skulachev, V.P. (1988) Membrane Bioenergetics, Springer-Verlag, Berlin.
[4] Levachev, M.M., Mishukova, E.A., Sivkova, V.G. and Skulachev, V.P. (1965) Biokhimiya 30, 864–874 (Russ.).
[5] Brustovetsky, N.N., Egorova, M.V., Gnutov, D.Yu., Gogvadze, V.G., Mokhova, E.N. and Skulachev, V.P. (1992) FEBS Lett. 305, 15–17.
[6] 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.
[7] Schönfeld, P. (1990) FEBS Lett. 264, 246–248.
[8] Skulachev, V.P. (1991) FEBS Lett. 294, 158–162.
[9] Hunter, D.R., Haworth, R.A. and Southard, J.H. (1976) J. Biol. Chem. 152, 5069–5077.
[10] Crompton, M., Ellinger, H. and Costi, A. (1988) Biochem. J. 255, 357–360.
[11] Gunter, T.E. and Pfeiffer, D.R. (1990) Am. J. Physiol. 258, 755–787.
[12] Crompton, M., McGuinness, O. and Nazareth, W. (1992) Biochim. Biophys. Acta 1101, 214–217.
[13] Brustovetsky, N.N., Amerkanov, Z.G., Popova, E.Yu. and Konstantinov, A.A. (1990) FEBS Lett. 263, 73–76.
[14] Brustovetsky, N.N., Amerkanov, Z.G., Egorova, M.V., Mokhova, E.N. and Skulachev, V.P. (1990) FEBS Lett. 272, 190–192.
[15] Grinius, L.L., Jasaitis, A.A., Kadziauskas, J.P., Liberman, E.A., Skulachev, V.P., Topali, V.P. and Vladimirova, M.A. (1970) Biochim. Biophys. Acta 216, 1–12.
[16] Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membr. Biol. 49, 105–121.
[17] Zhigacheva, I.V. (1976) Biol. Nauki 10, 21–25 (Russ.).
[18] Bernardi, P. (1992) J. Biol. Chem. 267, 8854–8859.