Further studies on the recoupling effect of 6-ketocholestanol upon oxidative phosphorylation in uncoupled liver mitochondria

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Abstract The effect of 6-ketocholestanol was studied on CCCP-induced uncoupling in liver mitochondria, submitochondrial particles and cytochrome oxidase proteoliposomes. It was found that 6-ketocholestanol prevents and reverses uncoupling induced by nM concentrations of CCCP on the three systems assayed. As it was reported on kidney mitochondrial membranes [Chávez et al. (1996) FEBS Lett. 379, 305–308], the recoupling effect caused by 6-ketocholestanol on submitochondrial particles and proteoliposomes could be due to a diminution of membrane fluidity.

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Key words: Liver mitochondria; Submitochondrial particle; 6-Ketocholestanol; Uncoupling; Cytochrome-oxidase-proteoliposome

1. Introduction

The protonophores are uncouplers of the oxidative phosphorylation because they dissipate the electrochemical gradient of protons generated through the mitochondrial membrane. Many uncouplers are weak hydrophobic acids that shuttle across the phospholipid bilayers in a negatively charged form acting as carriers for protons. [1,2]. This process may be controlled by the internal dipole potential that arises from dipolar groups in the membrane interface [3]. At the same time, several agents can modify it, resulting in various changes in the binding and translocation rates of hydrophobic ions. Simon et al. [4] and Franklin and Cafiso [5], demonstrated that the incorporation of 6-kch into phosphatidylcholine bilayers increased the magnitude of the membrane dipole potential, while increasing the binding and translocation rate of hydrophobic anions. In accordance with this, Starkov et al. [6] observed, in planar bilayer phospholipid, that the effect of FCCP as protonophore was stimulated by 6-kch, but on the contrary, they found that in rat liver mitochondria, 6-kch inhibited instead of potentiating, the uncoupling effect of low concentrations of several protonophores, such as CCCP, FCCP and SF6847. The same coupling effect of 6-kch was observed in rat liver mitochondria ATPase [7] and in plant mitochondria [8,9] in the presence of uncouplers. In recent work, Chávez et al. [10], demonstrated the inhibitory effect of 6-kch on kidney mitochondria Ca^{2+} efflux induced by CCCP, which proved to be caused by a diminution of the fluidity of the membrane, and depended on the accumulation of phosphate. Thus, the aim of this work was to make further studies on the effect of 6-kch upon oxidative phosphorylation in rat liver mitochondria, and its behavior on an artificial system of cytochrome oxidase proteoliposomes and the uncoupler CCCP, in order to clarify its mechanism of action.

In the present report we demonstrate that at increasing concentrations 6-kch recouples the oxidative phosphorylation in rat liver mitochondria, uncoupled with CCCP, showing a concentration-dependent fashion. In SMP, 6-kch protected and reverted the stimulatory effect of the uncoupler on oxygen consumption. The polarization of fluorescence in SMP membranes containing 6-kch increased as compared to controls at the same temperature, showing a progressive increment as the temperature was lowered. In cytochrome oxidase proteoliposomes, 6-kch protected and reverted the inhibition of the $\Delta\psi$ induced by nM concentrations of CCCP. These proteoliposomes showed an increment in fluorescence polarization dependent on the 6-kch concentration. This suggests that the modification of the lipidic environment of the membranes, could explain, along with other mechanisms, the recoupling effect of 6-kch.

2. Materials and methods

Mitochondria were isolated from rat liver by homogenization of the tissue in 0.25 M sucrose, 10 mM HEPES and 1 mM EGTA adjusted to pH 7.4 (IM), as previously described [11]. Before the last centrifugation, the mitochondrial pellet was suspended in 1 ml of IM supplemented with fatty acid-free BSA (3 mg/ml) and incubated 10 min at 4°C. Then 40 ml of IM were added and the mixture was centrifuged 10 min at $12\,000 \times g$. The final mitochondrial pellet was suspended in IM and diluted to a protein concentration of about 50 mg/ml. The mitochondria used for oxygen consumption measurements were prepared daily and stored at −70°C for the other experiments. Protein was determined by the method of Lowry et al. [12]. Submitochondrial particles (SMP) were obtained by sonication of a suspension of thawed out rat liver mitochondria as described [13]. Fluorescence polarization was measured in SMP (1 mg protein/ml of IM) as reported in [10]. Oxygen consumption was recorded with a Clark-type oxygen electrode. The beef heart cytochrome oxidase was isolated as indicated in [14]. The cytochrome oxidase proteoliposomes were prepared as described [15]. Briefly, 40 mg/ml azolectin (Sigma type IIS) was dispersed in 50 mM potassium phosphate buffer adjusted to pH 7.2, 1 mM $MgSO_4$, 200 mM sucrose and 1.5% cholate. Cytochrome oxidase was added (17 μg/ml) and dialyzed against 500 volumes of 50 mM potassium phosphate buffer, 1 mM $MgSO_4$ an 200 mM sucrose for 4 h. The dialysis was repeated for 4 h and then overnight. The reaction mixture was prepared with 50 μl of cytochrome oxidase-proteoliposomes, 2 ml of phosphate buffer pH 7.0 and the fluorescence indicator, (0.5 μM) of dithiocarbocyanine 3 (DiSC3). The $\Delta\psi$ was generated by adding the electron donor system 5 mM ascorbate-0.1 mM TMPD-7.5 μg/ml cytochrome c and the changes were followed, after the addition of 6-kch, by the quenching of the fluorescence of the

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Abbreviations: 6-kch, 6-ketocholestanol (5α-cholestan-3β-ol-6-one); $\Delta\psi$, transmembrane electrical potential difference; CCCP, carbonylcyanide-3-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DPH, 1,6-diphenyl-1,3,5-hexatrieno; SMP, submitochondrial particles; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylendiamine dihydrochloride

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indicator at 540–580 nm. Cytochrome oxidase proteoliposomes for fluorescence polarization measurement were prepared as indicated above, except that the sample, prepared with 50 μl of proteoliposomes in 2 ml of 50 mM phosphate buffer pH 7.2 and 1 μM of the probe DPH, was incubated for 90 min in ice before being subjected to polarization analysis at 25°C. After the control reading, 6-kch was added. Calculations were carried out as indicated in [10]. All other experimental conditions are described in the corresponding figure legends.

3. Results and discussion

The results shown in Fig. 1 demonstrate the effect of 6-kch on the uncoupled state 4 respiration of the mitochondria induced by 100 nM CCCP. The addition of increasing concentrations of 6-kch (25–150 μM) reversed the stimulation induced by the uncoupler, diminishing oxygen consumption, which was correlated to the increasing concentrations of 6-kch, indicating an inhibition of the CCCP uncoupling effect. It should be mentioned that the respiratory rate of the state 4 of the control was 8.5 natg O min^{-1} mg^{-1}. As the oxidative phosphorylation is tightly coupled to the electron transport chain, mitochondria in the same preparation were stimulated with ADP, after each addition of 6-kch (Table 1). The respiratory control improved from 1.8, that corresponded to about 78% uncoupling with 100 nM CCCP, to 6.2 which was 68% recoupling with 150 μM 6-kch. The ADP/O ratio was restored in a similar fashion, from 1.4 to 2.3 that corresponded to the control. The modification of the oxygen consumption generated by the influence of 6-kch was larger in the state 4 respiration; the change noted on state 3 was less extensive. No change was detected in the above mentioned parameters, in mitochondrial control preparations, after adding the same increasing concentrations of 6-kch (data not shown). These results show that 6-kch strongly inhibits state 4 of the respiration, supported by malate-glutamate, stimulated by CCCP, and it is noteworthy that these mitochondria are capable of responding to the subsequent stimulation by ADP, showing a recoupling of almost 70% of oxidative phosphorylation with the maximum concentration of 6-kch. These data are in accordance with the reports of Starkov et al. [6], and Vianello et al. [8], about the coupling effect of 6-kch on beef heart and liver mitochondria treated with nanomolar concentrations of CCCP; and with that of Chávez et al. [10] concerning the protective action of 6-kch on Ca^{2+} efflux produced by CCCP in rat kidney mitochondria. When oxygen consumption was measured in SMP, in the presence of oligomycin, they showed that 6-kch reverted and protected from the stimulation of the respiration by CCCP (Fig. 2a and b, respec-

Table 1
Effect of 6-kch on the oxidative phosphorylation in rat liver mitochondria

6-kch (μM)	+100 nM CCCP		R.C.	ADP/O	Recoupling (%)
	State 3[a]	State 4[a]			
0	73.0	39.5	1.8[b]	1.4	–
25	58.0	24.0	2.4	1.6	9.3
50	58.0	20.0	2.4	1.8	17.3
75	48.6	13.7	3.5	2.0	26.6
100	53.0	10.0	5.3	2.2	54.6
125	61.0	10.5	5.8	2.0	62.4
150	56.0	9.0	6.2	2.3	68.7
Control[c]	70.0	8.5	8.2	2.3	–

[a]natg O min^{-1} mg^{-1} after stimulating with 125 μM ADP.
[b]Taken as 100% uncoupling.
[c]Without CCCP.
The oxygen consumption was carried out in the same conditions as indicated in the legend of Fig. 1.

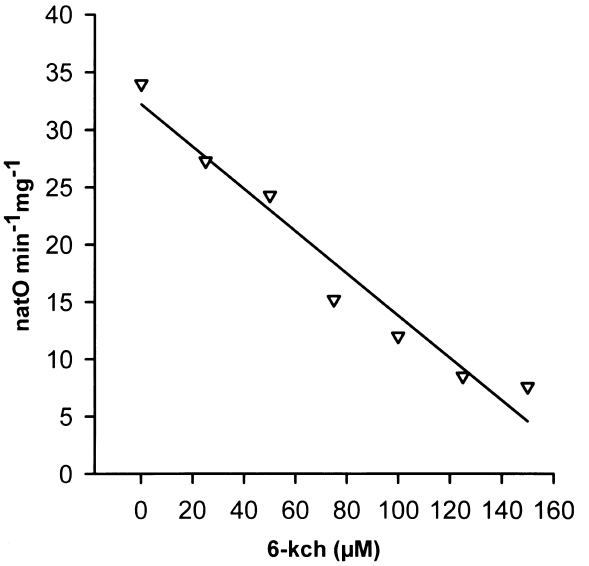


Fig. 1. Recoupling effect of 6-kch on state 4 respiration stimulated by CCCP. Oxygen consumption was measured in 0.8 mg protein/ml incubated with 125 mM KCl, 10 mM HEPES, 10 mM KH_2PO_4, 5 mM malate, 5 mM glutamate adjusted to pH 7.3 with KOH. Final volume: 2 ml. The CCCP concentration was 100 nM in all the determinations. Temperature: 27°C. The data were adjusted by linear regression (correlation coefficient $r = 0.9550$).

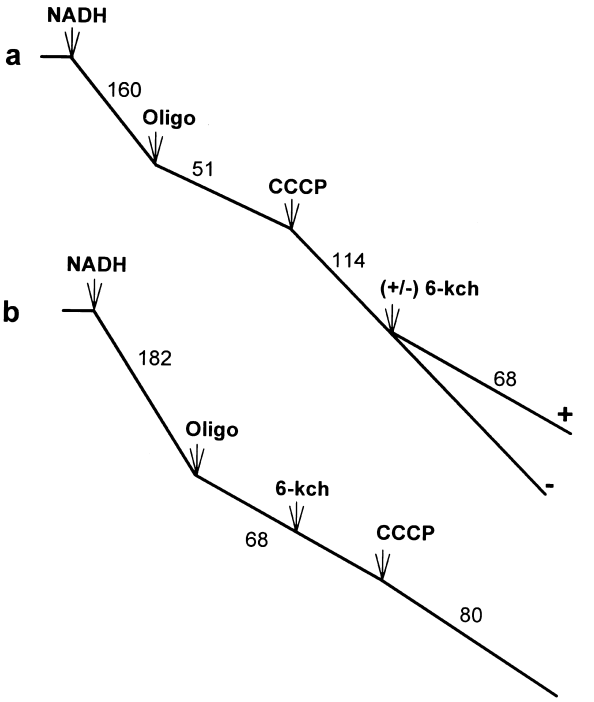


Fig. 2. The effect of 6-kch on oxygen consumption by SMP stimulated by CCCP. Submitochondrial particles (0.5 mg protein/ml) were incubated with 125 mM KCl, 1 mM EDTA adjusted to pH 7.2 with Tris. Final volume: 2 ml. When indicated 2 mM NADH, 5 μg oligomycin, 400 nM CCCP and 150 μM 6-kch were added. Temperature: 27°C. Values in natg O min^{-1} mg^{-1}.

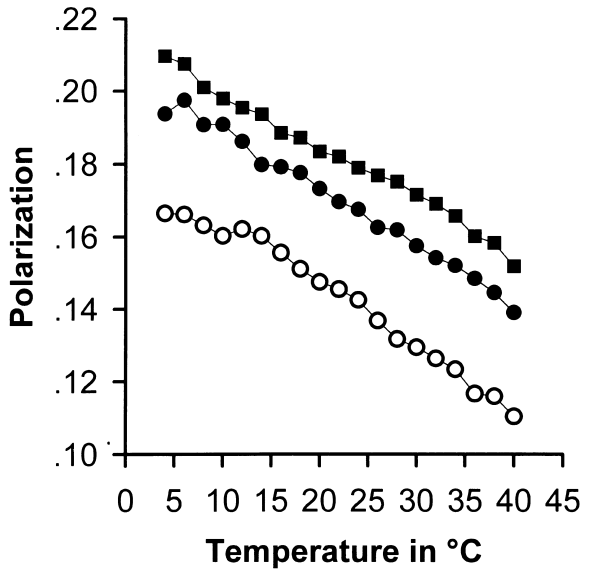


Fig. 3. Temperature dependence of the mobility of the fluorescent probe DPH embedded in SMP. Fluorescence polarization was measured in submitochondrial particles (1 mg protein/ml) incubated in 2 ml of media containing 0.25 sucrose adjusted to pH 7.3 with Tris. As a probe, 1 μM DPH dissolved in dimethylformamide was used. The mixture was incubated at 25°C for 30 min. After this time 6-kch was added and incubated for 10 min. Control (○), +100 μM 6-kch (●), +200 μM 6-kch (■). Excitation wavelength 340 nm, emission 417 nm. Fluorescence polarization (P) was calculated according to the equation:

$$P = \frac{I_v - GI_H}{I_v + GI_H}$$

where I_v and I_h are the relative intensities measured at an angle of 90° to the incident beam with the emission polarized in the vertical and horizontal position, respectively, and

$$G = \frac{I_v}{I_H}$$

measured with the excitation polarized in the horizontal position. ←

tively). It was observed that a concentration of 150 μM 6-kch reverted 66% and protected 82% from the stimulating effect of the uncoupler.

Assuming that 6-kch causes diminution of the fluidity of the mitochondrial membrane [10], we examined this effect upon SMP by measuring the fluorescence polarization, varying the temperature from 4 to 40°C, in order to investigate the action of 6-kch on lipids of biological membranes. Fig. 3 shows that addition of 100 and 200 μM 6-kch induced a noticeable increment in the fluorescence polarization, in relation to the control, at all the temperatures examined. Such increment shows an inverse relationship to the temperature, indicating that the mobility of the probe was restricted by decreasing membrane fluidity. These data suggest that 6-kch modifies the lipidic milieu of the membrane. The change in membrane fluidity by 6-kch in kidney mitochondrial membranes, detected by fluorescence polarization, was reported by Chávez et al. [10], demonstrating also that its protective effect on Ca^{2+} efflux, induced by CCCP and observed at 25°C was lost when the incubation was made at 37°C. The same relationship between the increment of polarization and diminution of the fluidity was reported by Shinitsky and Inbar [16] and Pang and Miller [17], in natural membranes and in model systems.

Skulachev [18] and Andreyev et al. [19], demonstrated that

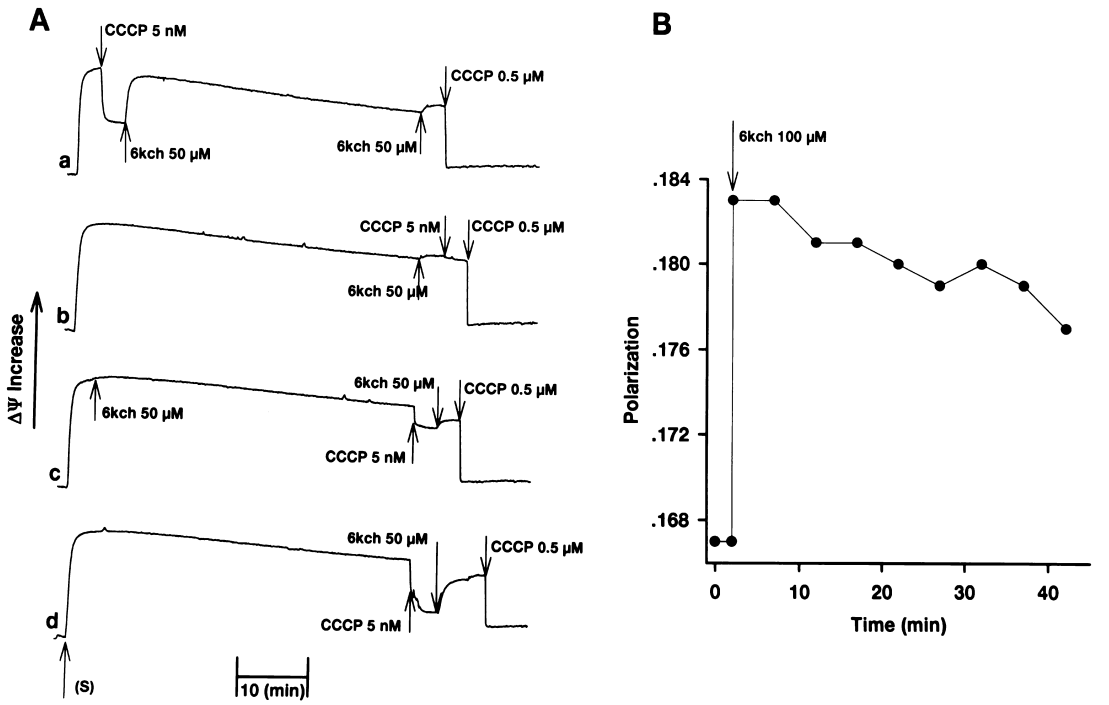


Fig. 4. Effects of 6-kch on collaps of membrane potential by CCCP, and in fluorescence polarization in cytochrome oxidase proteoliposomes. (A) Protection (traces b and c) and reversion (traces a and d) by 6-kch, of the collapse of membrane potential induced by CCCP, additions as indicated. (B) Time course of fluorescence polarization of cytochrome oxidase proteoliposomes supplemented with 6-kch. Reaction conditions as indicated in Section 2.

fatty acids act as natural uncouplers of oxidative phosphorylation in mitochondria, a process mediated by the ATP/ADP antiporter; but this process does not operate in model systems such as cytochrome oxidase-proteoliposomes. In contrast, they found that artificial protonophores such as FCCP or CCCP in low concentrations, which increase conductance of planar phospholipid bilayers and liposomes, are efficient uncouplers in proteoliposomes, since here cytochrome oxidase may be taking part in the uncoupling process [21]. Considering the above, we studied the effect of 6-kch on a reconstituted system with cytochrome oxidase and soybean phospholipids. In Fig. 4A it is shown that 6-kch increases the potential supported by ascorbate oxidation, after the collapse of the potential induced by low CCCP concentrations (traces a and d); 6-kch also protects, completely or in part, from the uncoupling effect (traces b and c), of following additions of CCCP. The recoupling effect of 6-kch diminished in time, even though in our preparation, this change was not as extensive as that reported by Starkov et al. [21]. In parallel measurements of fluorescence polarization of cytochrome oxidase proteoliposomes with 100 μM 6-kch added (Fig. 4B) was found that polarization decayed slowly in time. When the fluorescence polarization was measured in the same preparation of proteoliposomes with increasing concentrations of 6-kch added, a concentration-response effect was found, similar to that observed in mitochondria and SMP preparations and in liposomes void of cytochrome oxidase (data not shown).

The results of the latter experiments suggest that 6-kch is acting through the lipid milieu in protein-free reconstituted system, and in cytochrome oxidase-proteoliposomes.

In conclusion, 6-kch showed that the recoupling effect observed in mitochondria, SMP and in cytochrome oxidase-proteoliposomes is mainly due to modifications in the lipidic environment. This consideration does not exclude the possibility that in biological membranes the lipids, modified by an effector such as 6-kch or other steroids as Starkov et al. [20] have demonstrated, could exert modifications upon some proteins that may mediate the action of some uncouplers.

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