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6-Ketocholestanol abolishes the effect of the most potent uncouplers of oxidative phosphorylation in mitochondria

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Abstract The effect of a keto-derivative of cholesterol, namely, 6-ketocholestanol (5α-cholestan-3β-ol-6-one; kCh) on the uncoupling of oxidation and phosphorylation by various uncouplers was studied in rat heart mitochondria. kCh was found to completely abolish the uncoupling effect (the increase in the respiration rate under the respiratory control conditions and the decrease in the membrane potential) caused of FCCP, CCCP and SF6847 and partially by TTFB at low concentrations of uncouplers. It was without effect on the uncoupling by PCP, DNP and palmitate. Carboxyatractylate, a specific inhibitor of the ADP/ATP-antiporter, was shown to almost completely abolish the uncoupling induced by palmitate and partially by low concentration of TTFB, PCP and DNP. Effects of high concentrations of all these uncouplers as well as of any concentrations of gramicidin proved to be kCh- and carboxyatractilate-insensitive. The data are discussed in terms of the hypothesis on the protein-mediated mechanism of the protonophorous uncoupling.

Key words: Uncoupling; 6-Ketocholestanol; Mitochondrion; Carboxyatractylate

1. Introduction

In bioenergetic studies, protonophorous uncouplers are, probably, the most frequently used agents. The great majority of uncouplers represent rather hydrophobic weak acids. The negative charge of their anionic form is strongly delocalized which decreases hydratation and, hence, makes the phospholipid bilayer permeable to the anionic form of these compounds. The mechanisms of proton transfer by uncouplers were extensively studied in experiments with model phospholipid membranes (see [1] for review). These studies revealed, in particular, the significance of electrical potentials to the efficiency of proton transfer by various uncouplers [2–4].

At least three distinct electrical potentials can be defined in lipid bilayers i.e. (i) surface potential, (ii) transmembrane potential and (iii) so-called dipole potential [5–7]. Although the nature of the dipole potential has not been clearly identified [8,9], it is believed to be responsible for the observed permeability differences of bilayers to hydrophobic cations and anions [3,4,6,10,11]. A number of compounds are known to modify the dipole potential. For example, phloretin was shown to reduce the magnitude of the dipole potential [7,12,13]. This compound was also shown to decrease the uncoupling efficiency of FCCP in rat liver mitochondria [14]. Recently, a new agent has been introduced, namely 6-ketocholestanol (5α-cholestan-3β-ol-6-one; kCh), the cholesterol derivative containing a keto group, which increases the membrane dipole potential, thus increasing the binding and the translocation rate of hydrophobic anions [11,15].

Here we report the evidence that 6-ketocholestanol abolishes the action of the most potent uncouplers on rat heart mitochondria.

2. Materials and methods

Mitochondria were isolated from rat heart muscle. Cooled muscles, previously purified from fat and tendons, were minced and passed through a stainless steel press with holes about 1 mm in diameter. The tissue was then homogenized for 3 min with a Teflon pestle in a glass (Pyrex) homogenizer, the tissue : isolation mixture ratio being 1 : 8. After the first centrifugation (10 min, 600 × *g*), the supernatant was decanted and filtered through gauze, then centrifuged (10 min, 12,000 × *g*). The sediment was suspended in 1 ml isolation mixture (250 mM sucrose, 10 mM MOPS, 1 mM EDTA, pH 7.4) supplemented with BSA (3 mg/ml). Then the mixture without BSA was added. The final mitochondrial precipitate (10 min, 12,000 × *g*) was suspended in the isolation medium with BSA. The mitochondrial suspension (70–90 mg/ml) was stored on ice.

In the majority of the experiments, the incubation mixture contained 250 mM sucrose, 10 mM MOPS, 2 mM KH_2PO_4, BSA (0.2 mg/ml), oligomycin (2 μg/ml) and 0.5 mM EGTA, pH 7.4. Glutamate (5 mM) and malate (5 mM) or 5 mM ascorbate and 1 μM PMS were used as oxidation substrates. In the latter case, the medium contained 2 μM rotenone.

Oxygen consumption was recorded with a Clark-type oxygen electrode and an LP-7E polarograph. The concentration of mitochondrial protein was 0.8–1.2 mg/ml. Temperature, 26°C.

The $\Delta\Psi$ changes were estimated using safranin O [16–18]. The difference of absorbances at 555 nm and at 523 nm was recorded; the $\Delta\Psi$ decrease led to an increasing absorbance difference. The concentration of mitochondrial protein was 0.7–0.9 mg/ml.

Palmitic acid, FCCP, CCCP, TTFB, SF6847, kCh, rotenone and oligomycin were dissolved in twice distilled ethanol.

Oligomycin, MOPS, palmitic acid, glutamate, CAtr, CCCP, kCh and fatty acid-free BSA were from Sigma; EDTA, EGTA, rotenone, and DNP were from Serva; malate was from Calbiochem; FCCP was from Boehringer.

3. Results

The respiration of rat heart mitochondria oxidizing gluta-

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Abbreviations: $\Delta\Psi$, transmembrane electrical potential difference; BSA, bovine serum albumin; cAtr, carboxyatractylate; CCCP, carbonylcyanide-3-chlorophenylhydrazone; Ch, cholesterol; DNP, 2,4-dinitrophenol; EGTA, ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; kCh, 6-ketocholestanol (5α-Cholestan-3β-ol-6-one); MOPS, morpholinopropane sulphonate; PMS, phenazine methosulphate; TPP^+, tetraphenyl phosphonium; TTFB, tetrachlorotrifluoromethylbenzimidazole.

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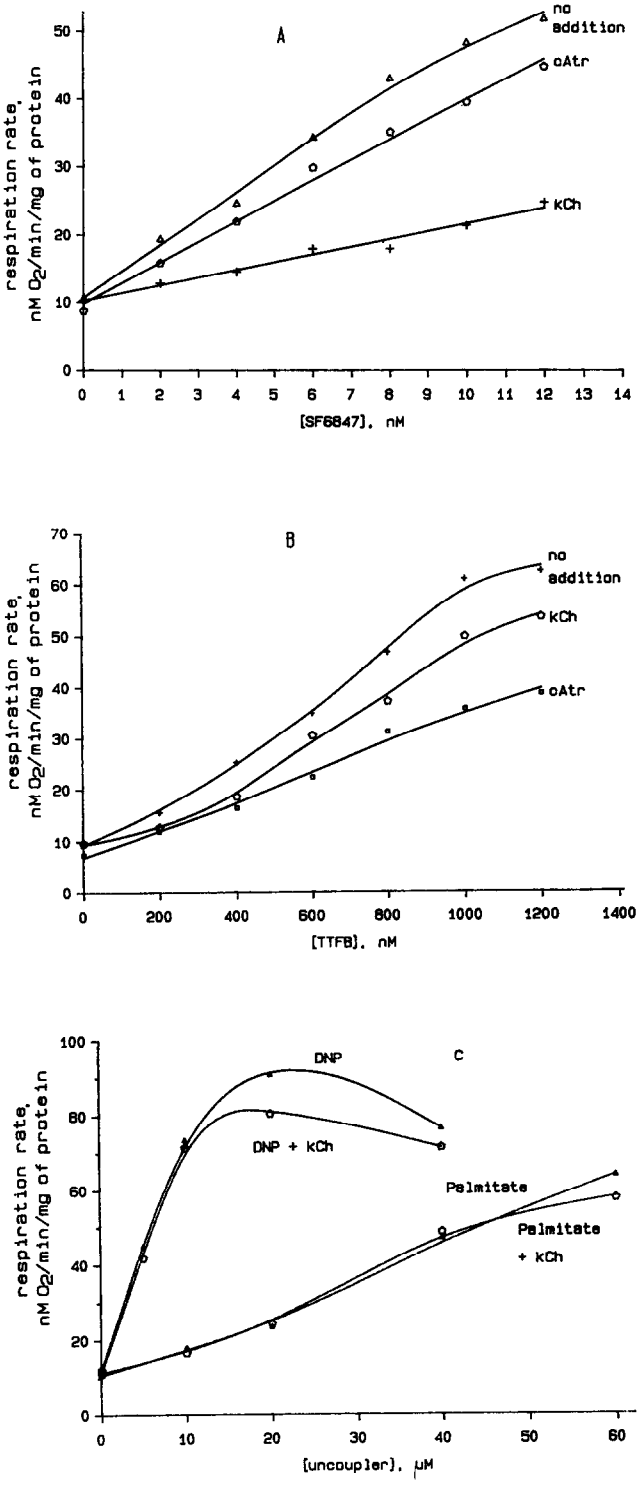

Fig. 1. Effects of cAtr and kCh on mitochondrial respiration stimulated by various uncouplers. The incubation mixture (see section 2) was supplemented with 5 mM glutamate and 5 mM malate. kCh and cAtr were added before mitochondria. Additions: 2 μM cAtr, 80 μM kCh.

mate and malate in the presence of oligomycin was measured at various concentrations of uncouplers with or without 6-ketocholestanol (kCh). Fig. 1A shows that kCh strongly inhibits respiration released by adding SF6847, whereas carboxyatractylate exerts only a very slight effect. Similar data were also obtained with FCCP and CCCP (not shown). When mitochondrial respiration is stimulated by TTFB, the effect of kCh is small and that of carboxyatractylate is rather strong (Fig. 1B). The increase in the respiration rate induced by DNP or by palmitate is, in fact, insensitive to kCh (Fig. 1C) but inhibited by carboxyatractylate, the inhibition being especially strong with palmitate (not shown).

Fig. 2A shows that kCh completely restores the membrane potential of rat heart mitochondria strongly lowered by 8 nM SF6847, while the addition of cholesterol is without effect. On the other hand, kCh is ineffective when the membrane potential is lowered by PCP. In this case carboxyatractylate partially restores the potential (Fig. 2B).

The effect of different concentrations of kCh on the membrane potential decrease induced by SF6847, FCCP and TTFB is shown in Fig. 3A–C. It is seen that kCh is equally efficient in completely abolishing the $\Delta\Psi$ decrease induced by low concentrations of FCCP and SF6847 (Fig. 3A and B) whereas it has only a slight effect on that induced by TTFB (Fig. 3C). kCh fails to abolish the effect of high concentrations of SF6847, FCCP and TTFB (Fig. 3) and of any concentrations of PCP, DNP, palmitate and gramicidin (not shown).

4. Discussion

It is known that 6-ketocholestanol (kCh) increases both the translocation rate and the binding of hydrophobic anions in the

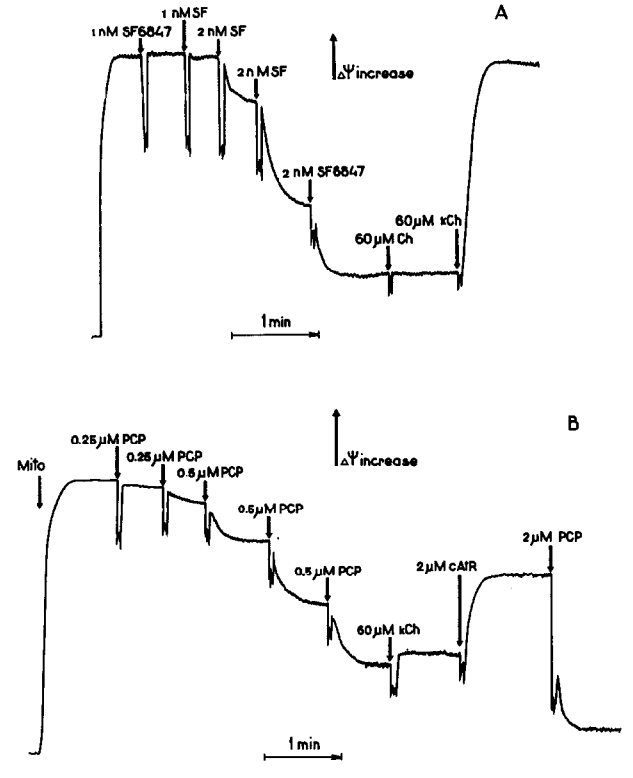

Fig. 2. Dynamics of the mitochondrial membrane potential ($\Delta\Psi$) measured with safranin O: effects of uncouplers, kCh and cAtr. The incubation mixture (see section 2) was supplemented with 7 μM safranin O; 5 mM ascorbate and 1 μM PMS were used as oxidation substrates. Mito, rat heart mitochondria (0.7 mg/ml).

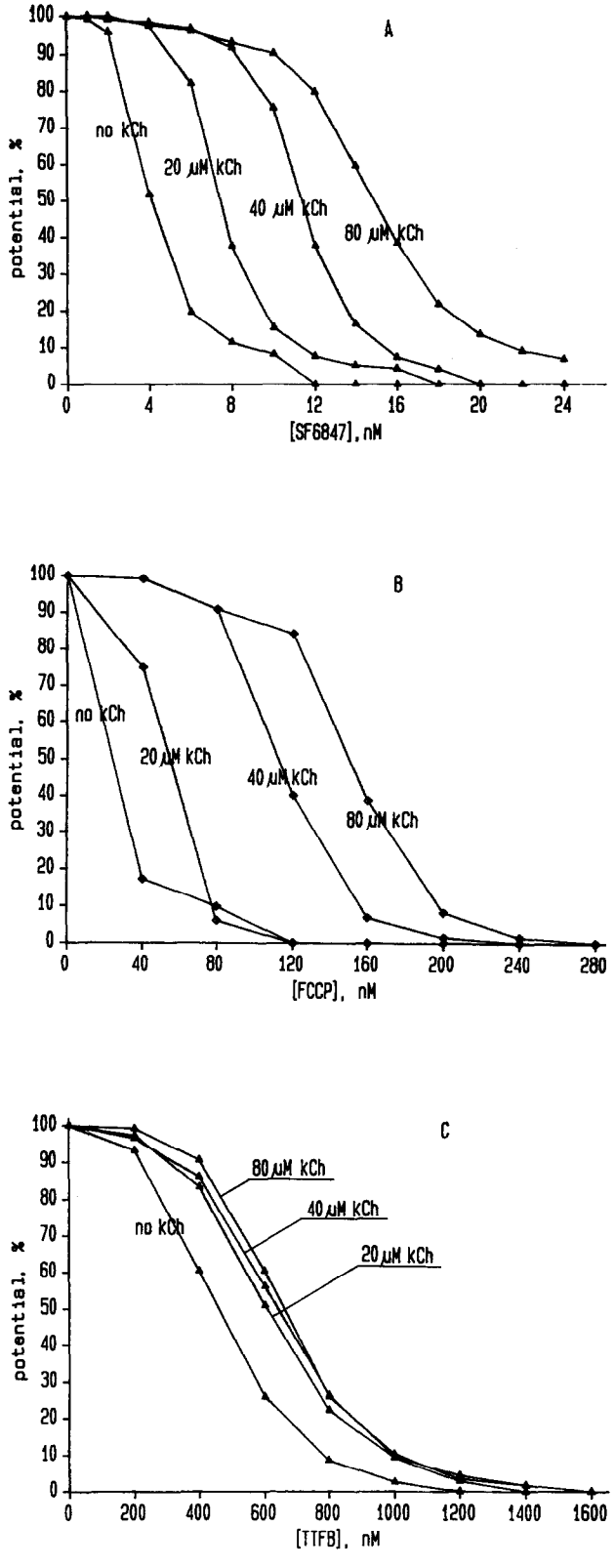

Fig. 3. Effects of different concentrations of 6-ketocholestanol on the uncoupler-induced $\Delta\Psi$ decrease in rat heart mitochondria. The amplitude of the absorption changes from the initial maximal level (after addition of mitochondria) to the final lowest level (when the oxygen in the incubation cuvette was exhausted) were taken as 100%. When present, kCh was added to incubation media before the addition of mitochondria. The incubation mixture was as in Fig. 2.

phosphatidylcholine liposomes [11]. Recently we have reproduced these data on planar bilayer phospholipid membranes composed of azolectin. In this system, kCh stimulates the effect of FCCP as a protonophore. We expected this compound to potentiate the uncoupling effect of protonophores in mitochondria. Instead, kCh was shown to abolish uncoupling by low concentrations of the most potent protonophores, i.e. SF6847, FCCP and CCCP.

It should be noted that kCh inhibits the action of uncouplers which were shown to cross the model phospholipid membrane in the form of the anion, A^- (FCCP, CCCP, SF6847), rather than of the dimer of anionic and protonated species, HA_2^- (TTFB, DNP, PCP). Interestingly, the uncoupling effects of TTFB, PCP and DNP were partially released by carboxyatractylate (the specific inhibitor of the ADP/ATP-antiporter) which almost completely abolished uncoupling by low concentrations of palmitate.

The effect of carboxyatractylate on the palmitate uncoupling, discovered in this group on mitochondria [19–23], has recently been confirmed by Brustovetsky and Klingenberg on proteoliposomes inlaid with the ATP/ADP-antiporter [24]. One may suggest that there are other, supposedly anion-translocating protein(s), mediating the carboxyatractylate-resistant action of SF6847, FCCP and CCCP. The effects of kCh can be explained assuming that it may somehow hinder the access of the uncoupler anions to their binding site on these protein(s).

It should be stressed, however, that the carboxyatractylate- or kCh-sensitive uncoupling is inherent in the action of *low* concentrations of uncouplers. At *high* concentrations, both inhibitors fail to abolish the uncoupling effect which is apparently directed at the phospholipid, rather than the protein, constituent of the mitochondrial membrane, as was originally proposed by Mitchell [25] and demonstrated in our [26] and Lehninger's [27] groups. In agreement with the above concept, the action of the channel-forming uncoupler, gramicidin, proved to be resistant to kCh as well as carboxyatractylate.

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