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Inhibition of respiratory complex I by 6-ketocholestanol: Relevance to recoupling action in mitochondria

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ABSTRACT

6-Ketocholestanol (kCh) is known as a mitochondrial recoupler, i.e. it abolishes uncoupling of mitochondria by such potent agents as carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and 3,5-di(tert-butyl)-4-hydroxybenzylidenemalononitril (SF6847) [Starkov et al., 1997]. Here, we report data on the kCh-induced inhibition of both NADH-oxidase and NADH-ubiquinone oxidoreductase activities of the respiratory complex I in bovine heart submitochondrial particles (SMP). Based on the absence of such inhibition with hexaammineruthenium (III) (HAR) as the complex I electron acceptor, the kCh effect could be associated with the ubiquinone-binding centre of this respiratory enzyme. In isolated rat liver mitochondria (RLM), kCh inhibited oxygen consumption with the glutamate/malate, substrates of NAD-linked dehydrogenases, while no inhibition of RLM respiration was observed with succinate, in agreement with the absence of the kCh effect on the succinate oxidase activity in SMP. Three kCh analogs (cholesterol, 6α-hydroxycholesterol, and 5α,6α-epoxycholesterol) exhibited no effect on the NADH oxidase activities in both SMP and RLM. Importantly, the kCh analogs were ineffective in the recoupling of RLM treated with CCCP or SF6847. Therefore, interaction of kCh with the complex I may be involved in the kCh-mediated mitochondrial recoupling.

1. Introduction

The respiratory electron transport chain of mitochondria comprising a set of enzymes, located in the inner mitochondrial membrane (IMM), catalyzes oxidation of various substrates coupled to transfer of hydrogen ions from the mitochondrial matrix to the intermembrane space, which leads to formation of an electrochemical potential difference of hydrogen ions (proton motive force, pmf) across IMM. The pmf is spent for synthesis of ATP, the main energy-carrying compound in living cells.

The so-called uncouplers of oxidative phosphorylation are known to prevent ATP synthesis by causing pmf dissipation via increasing the IMM proton permeability, which manifests itself in the pronounced stimulation of mitochondrial respiration. The mechanism of operation of these small hydrophobic molecules has not been fully elucidated: namely, in certain cases the action of uncouplers cannot be explained solely by their ability to shuttle protons across the lipid part of IMM, thereby dropping the pmf. Alternatively, interaction of uncouplers with certain proteins in IMM has been discussed in the literature [1–9]. In particular, observations on mitochondrial recoupling, i.e., discovery of various compounds causing relief from the uncoupling, have boosted interest to reconsidering the mechanism of the uncoupling action [10–16]. Of the recoupling agents, the 6-ketocholestanol (kCh) effect on mitochondria, first described by Starkov and colleagues in 1994 [17], seems to be the most mysterious. E.g., kCh does not exhibit the recoupling action with all the uncouplers, showing it with some of them (CCCP, FCCP, SF6847, TTFB), but not with others (e.g., 2,4-dinitrophenol (DNP) or pentachlorophenol) [17]. Generally, the recoupling action of kCh was assumed to originate from its effect on the physicochemical properties of the IMM lipid part, such as dipole potential and microviscosity [12,18]. On the other hand, the kCh-induced recoupling

Abbreviations: RLM, rat liver mitochondria; SMP, submitochondrial particles; IMM, inner mitochondrial membrane; pmf, proton motive force; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; SF6847, 3,5-di(tert-butyl)-4-hydroxybenzylidenemalononitril; DNP, 2,4-dinitrophenol; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; HAR, hexaammineruthenium (III); kCh, 6-ketocholestanol; Ch, cholesterol; 6α-OHC, 6α-hydroxycholesterol; 5α,6α-EC, 5α,6α-epoxycholesterol; ACMA, 9-amino-6-chloro-2-methoxyacridine.

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was considered to indicate the involvement of mitochondrial proteins in the uncoupling action [13,19]. Despite this challenge for the basics of bioenergetics and rather wide use of kCh as a recoupling agent [20–24], its impact on the activity of respiratory enzymes has not been studied in detail.

We believe that mitochondrial complex I (proton translocating NADH:ubiquinone oxidoreductase), the largest respiratory enzyme containing 45 subunits with a total molecular weight of about 1000 kDa, is a likely candidate for interaction with kCh. The NADH-binding center and all redox components of complex I (tightly bound FMN and several iron-sulfur centers) are located in the peripheral domain of the enzyme, protruding into the mitochondrial matrix [25,26]. At the interface between the peripheral and membrane domains, there is a ubiquinone-binding center in the form of a long channel, the width of which changes during the transition of the enzyme from the open to closed conformation. Both forms of the enzyme are assumed to be intermediates of the catalytic cycle, because they are detected under active turnover conditions [27]. In the open state, the primary binding of ubiquinone occurs. NADH oxidation and subsequent electron transfer through a chain of iron-sulfur clusters to the terminal FeS center N2 leads to significant structural rearrangements of subunits adjacent to the ubiquinone-binding channel, making it narrower and isolating the bound ubiquinone from the bulk solvent [27]. The enzyme goes to the closed state, in which the ubiquinone is reduced by the N2 center. This event is a key moment in the coupling of the redox reaction with the subsequent transmembrane transfer of four protons from the mitochondrial matrix to the intermembrane space and pmf creation. The proton pumping is carried out by concerted operation of three antiporter-like subunits ND2, ND4 and ND5 (according to the classification for the bovine heart enzyme), located in the membrane domain of the enzyme [28–30]. The central axis of charged amino acid residues located in the membrane domain connects the antiporter subunits and provides long-distance communication between them. During the NADH:ubiquinone oxidoreductase reaction, no significant structural changes of antiporter subunits are detected, therefore, the hypothetical mechanism of coupling of the redox reaction with vector proton transfer currently includes conformational rearrangements in the ubiquinone-binding center region, leading to the electrostatically driven relay transfer of protons between the charged residues of the central axis of the membrane domain and the ejection of four protons into the intermembrane space [27,29]. The study of hydration patterns in antiporter subunits, as well as the results obtained by the method of molecular dynamic simulations, show that only in the ND5 subunit there are full proton input pathway from the matrix side and proton output pathway into the intermembrane space. The ND4 subunit has only an input half-channel for protons from the mitochondrial matrix leading to the central axis, but there is no visible connection of this way with the intermembrane space. Apparently, all four vector protons are ejected into the intermembrane space through the proton channel formed in the ND5 subunit [27]. A wide range of hydrophobic compounds are known to inhibit the NADH:ubiquinone-oxidoreductase reaction catalyzed by the complex I [31]. According to cryo-electron microscopy data, many of these compounds are found in the ubiquinone-binding channel [28–30,32,33].

The present study revealed an inhibitory effect of kCh on NADH-oxidase and NADH-ubiquinone oxidoreductase activities of the respiratory complex I in bovine heart submitochondrial particles (SMP). In line with these data, kCh also suppressed the complex I-mediated respiration of isolated rat liver mitochondria. The inability of a series of kCh analogs to inhibit the NADH oxidase activities in both SMP and RLM along with complete inefficacy of these analogs in the recoupling of CCCP-treated RLM enabled us to suggest the involvement of the complex I in the mechanism of the kCh-induced mitochondrial recoupling.

2. Materials and methods

2.1. Chemicals

Most chemicals, including CCCP, DNP, tyrphostin A9 (3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile, SF6847), rotenone, and safranine O were from Sigma.

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from rat liver by using differential centrifugation [34], according to a slightly modified procedure previously described [35]. The animals were handled and experiments were performed in accordance with the international guidelines for animal care and use and the Institutional Ethics Committee of A.N. Belozersky Institute of Physico-Chemical Biology at the Lomonosov Moscow State University approved them (protocol #3 on February 12, 2018).

2.3. Preparation of submitochondrial particles from bovine heart

Bovine heart inside-out SMP were prepared, activated, and coupled by treatment with oligomycin (0.5 μg/mg of SMP protein), as described in [36].

2.4. Mitochondrial respiration

The respiration of isolated rat liver mitochondria was measured at the mitochondrial protein concentration of 0.8 mg/ml by using a Clark-type oxygen electrode (Strathkelvin Instruments, UK), as described previously [35]. The ADP/O ratio was calculated as in [37].

2.5. Membrane potential (ΔΨ) measurement in isolated mitochondria

The mitochondrial membrane potential (ΔΨ) was evaluated from the difference in the absorbance at 555 and 523 nm (ΔA) of the safranine O dye [38] measured with an Aminco DW-2000 spectrophotometer, as described previously [35]. Mitochondria were incubated in the medium containing 250 mM sucrose, 5 mM MOPS, 0.5 mM KH_2PO_4, 1 mM EGTA, 2 μM rotenone, 5 mM succinate (pH 7.4), 1 μg/ml oligomycin, and 15 μM safranine O at the mitochondrial protein content of 0.7–0.9 mg protein/ml.

2.6. NADH oxidase or NADH-ubiquinone reductase activities of SMP

NADH oxidase activity of SMP was measured at 340 nm in the standard assay mixture composed of 0.25 M sucrose, 50 mM Tris/HCl (pH 8.0), 0.2 mM EDTA at 30 °C supplemented by 100 μM NADH or 100 μM NADH and 100 μM ubiquinone Q_1 (2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone) in the presence of 1.6 μM myxothiazol. The reaction was initiated by the addition of SMP (10 μg of protein per ml). Q1 reduction was inhibited after the addition of 5 μM rotenone.

2.7. NADH:hexaammineruthenium III (HAR) reductase activity of SMP

NADH:HAR reductase activity of SMP was measured at 340 nm in the standard assay mixture at 30 °C supplemented by 100 μM NADH, 0.5 mM HAR and 5 μM rotenone. The reaction was initiated by the addition of SMP (5 μg of protein per ml).

2.8. Succinate oxidase activity of SMP

Succinate oxidase activity was measured photometrically by following fumarate formation (increase of absorption at 278 nm, $\varepsilon^{278}_{mM} = 0.3$, [39]) in the standard assay mixture containing 5 mM potassium succinate. Protein content was determined by the Biuret assay. The experimental details are indicated in the legends to the Figures.

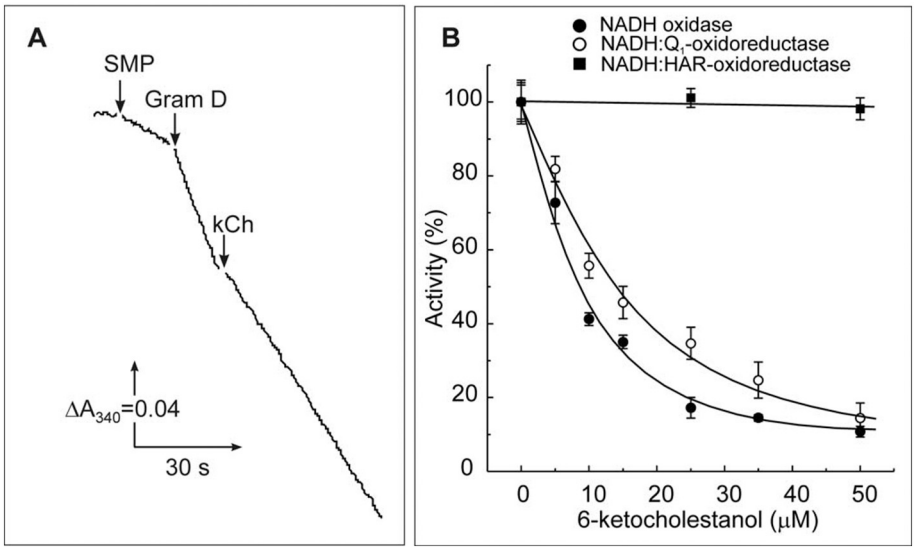

Fig. 1. Effect of kCh on the NADH-oxidizing activity of submitochondrial particles (SMP). A. The activity was assayed via the NADH absorption at 340 nm in the medium containing 0.25 M sucrose, 50 mM Tris-Cl (pH 8.0), 0.2 mM EDTA and 100 μM NADH. The reaction was initiated by the addition of coupled SMP (30 μg/ml) with subsequent addition of gramicidin D (Gram D, 0.05 μg/ml) or kCh (7 μM). B. Dose dependence of the effect of kCh on NADH oxidase (filled circles), NADH:Q_1-oxidoreductase (open circles), or NADH:HAR-oxidoreductase activities of SMP.

Table 1
Effect of kCh analogs on NADH and succinate oxidation and ATPase activity in SMP.

	Activity (μmol/min per mg of SMP protein)				
	NADH oxidase	NADH:Q_1-oxidoreductase	NADH:HAR-oxidoreductase	Succinate oxidase	ATPase
Control	1.38 ± 0.07 (100 %)	0.46 ± 0.04 (100 %)	3.37 ± 0.15 (100 %)	0.75 + 0.05 (100 %)	1.85 ± 0.15 (100 %)
kCh	0.15 ± 0.02 (10 %)	0.07 ± 0.02 (15 %)	3.30 ± 0.10 (98 %)	0.62 ± 0.06 (83 %)	0.84 ± 0.10 (45 %)
6α-OHC	1.49 ± 0.06 (108 %)	0.55 ± 0.05 (120 %)	3.33 ± 0.18 (99 %)	0.73 ± 0.06 (97 %)	1.81 ± 0.14 (98 %)
5α,6α-EC	1.54 ± 0.05 % (112 %)	0.45 ± 0.04 (98 %)	3.50 ± 0.2 (104 %)	0.76 ± 0.06 (99 %)	1.81 ± 0.15 (98 %)
Cholesterol	1.36 ± 0.05 (99 %)	0.41 ± 0.04 (89 %)	3.11 ± 0.20 (93 %)	0.78 ± 0.06 (96 %)	1.42 ± 0.13 (77 %)

All experiments were performed in triplicate.
Concentration of kCh and its analogs – 50 μM.

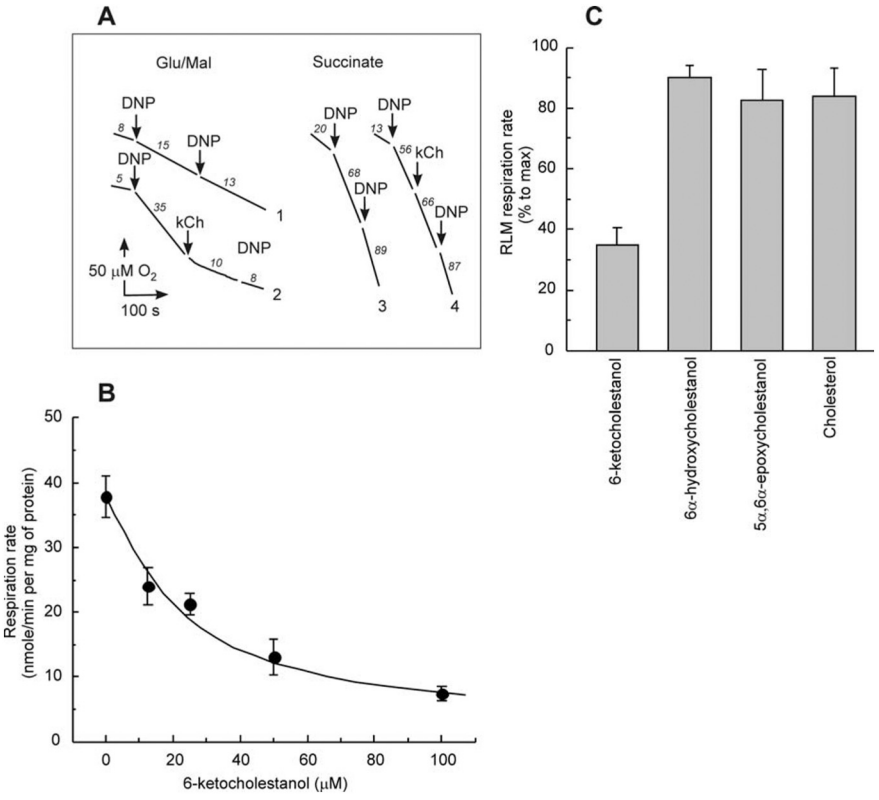

Fig. 2. A. Effect of kCh (50 μM) on the stimulation of glutamate/malate- (curves 1, 2) or succinate- (curves 3, 4) driven respiration of rat liver mitochondria (RLM) by DNP (20 μM). Curves 1 and 3 differ from curves 2 and 4 by the presence of kCh in the incubation medium. 40 μM of DNP was added at the end of each experiment. B. Dose dependence of the inhibition of respiration by kCh with glutamate/malate as a substrate. Shown are Mean ± S.D. ($n = 4$). C. Effect of 20 μM of kCh, 6α-OHC, 5α,6α-EC, and Ch on the DNP-mediated respiration of RLM. For experimental conditions, see Materials and methods.

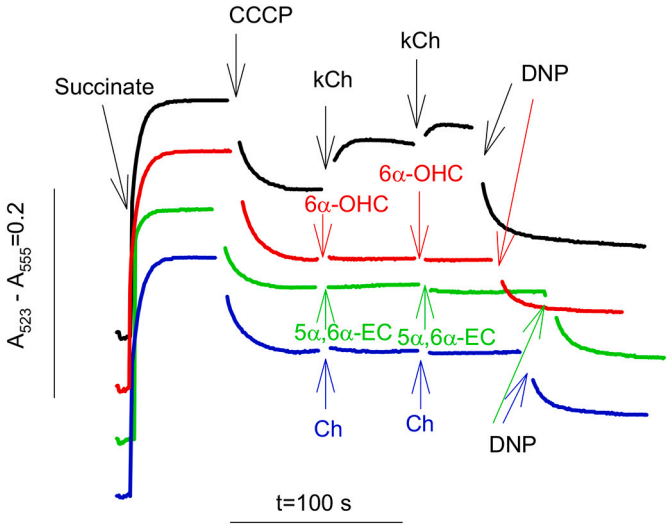

Fig. 3. Effect of kCh analogs on the uncoupling activity of CCCP (100 nM) in rat liver mitochondria (RLM). Arrows mark two successive additions of the analogs of 25 μM. 40 μM of DNP was added at the end of each experiment. Substrate: succinate. The membrane potential of mitochondria was estimated from changes in the absorbance of the potential-sensitive dye safranine O (15 μM) at 555 nm and 523 nm. Ch, cholesterol; 6α-OHC, 6α-hydroxycholesterol; 5α,6α-EC, 5α,6α-epoxycholesterol; kCh, 6-ketocholestanol. For other conditions, see Materials and methods.

2.9. ATPase activity of SMP

ATPase activity was evaluated from the initial rates of proton release detected as phenol red response at 557/618 nm [40] in the reaction mixture comprising 0.25 M sucrose, 100 mM KCl, 5 mM Hepes, 0.1 mM EDTA (potassium salts, pH 7.4), 2 mM $MgCl_2$, 2 mM ATP and 30 μM phenol red. In these experiments SMP were activated and coupled by treatment with oligomycin (0.15 μg/mg of SMP protein), as described in [36].

2.10. Generation of pH gradient (ΔpH) in submitochondrial particles

Generation of the transmembrane pH gradient in response to NADH addition was measured by following the changes in fluorescence of 9-amino-6-chloro-2-methoxyacridine (ACMA) with a Spectrofluorometer Fluorat-02-Panorama - Lumex Instruments (410 nm emission, 480 nm excitation), as described in [41]. The buffer solution (pH 7.4) contained 10 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 0.5 μg/ml ACMA.

3. Results

To monitor respiratory electron transfer in bovine heart SMP, we measured oxidation of the added NADH by its absorbance at 340 nm. As seen in Fig. 1A, the addition of 7 μM kCh to SMP, that were completely deenergized by ion channel-forming gramicidin *D* (known to be insensitive to the recoupling by kCh [12]), caused immediate deceleration of NADH oxidation, thereby showing inhibition of the electron transfer. Earlier, the kCh-induced suppression of the CCCP-stimulated oxygen consumption by SMP upon NADH oxidation was observed in [42]. Fig. 1B illustrates dose dependences of the kCh inhibitory effect on both NADH-oxidase (closed circles) and NADH-ubiquinone oxidoreductase (open circles) activities of SMP. An equilibrium constant of the inhibition by kCh was 9 μM for NADH oxidase and 14 μM for NADH-ubiquinone oxidoreductase. By contrast, kCh did not affect the rate of NADH oxidation by SMP in the presence of artificial electron acceptor HAR, closed squares. The rate of succinate oxidation by SMP was also only slightly sensitive to kCh (Table 1). These data suggest an

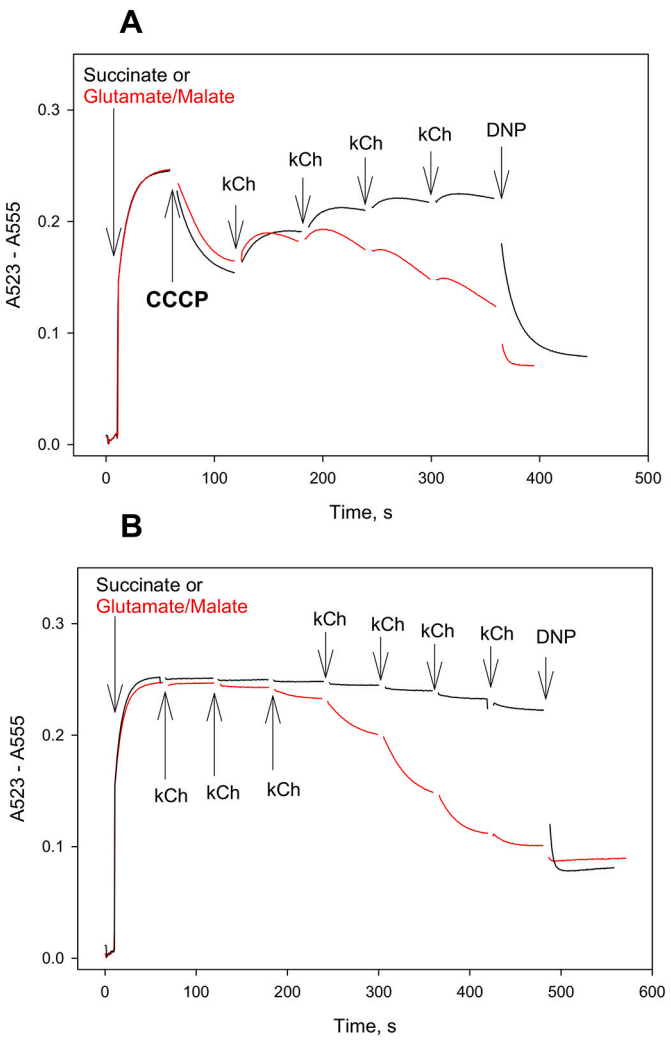

Fig. 4. Comparison of the kCh effect on the membrane potential of RLM generated in the presence of succinate (black curves) or glutamate/malate (red curves) with (A) and without (B) CCCP. 50 μM of DNP was added at the end of each experiment. The membrane potential of mitochondria was estimated from changes in the absorbance of the potential-sensitive dye safranine O (15 μM) at 555 nm and 523 nm. Each arrow marks the addition of 20 μM (A) or 50 μM (B) of kCh. CCCP concentration in panel A was 100 nM (black curve) or 50 nM (red curve). For other conditions, see Materials and methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

involvement of the complex I ubiquinone-binding center in the inhibition of respiratory electron transport by kCh. No inhibition of the electron transfer in SMP was observed with kCh analogs, such as cholesterol (Ch), 6α-hydroxycholesterol (6α-OHC), and 5α,6α-epoxycholesterol (5α,6α-EC) (Table 1). Of note, kCh partially (by 50 %) suppressed the ATPase activity of SMP, while all the three analogs were ineffective (Table 1).

In line with the data on SMP, the addition of kCh to isolated rat liver mitochondria (RLM), that were fully uncoupled by dinitrophenol (DNP), inhibited oxygen consumption when using glutamate/malate as respiratory substrates (Fig. 2A, curves 1 and 2) but was of no effect with succinate as a substrate (Fig. 2A, curves 3 and 4). Curves 1 and 3 differ from curves 2 and 4 in Fig. 2A by the presence of kCh in the incubation medium. The dependence of the glutamate/malate driven respiration on the kCh concentration is shown in Fig. 2B. Here, DNP was chosen as an uncoupler because of its insensitivity to the recoupling action of kCh [12,17]. The three analogs of kCh, Ch, 6α-OHC and 5α,6α-EC, did not

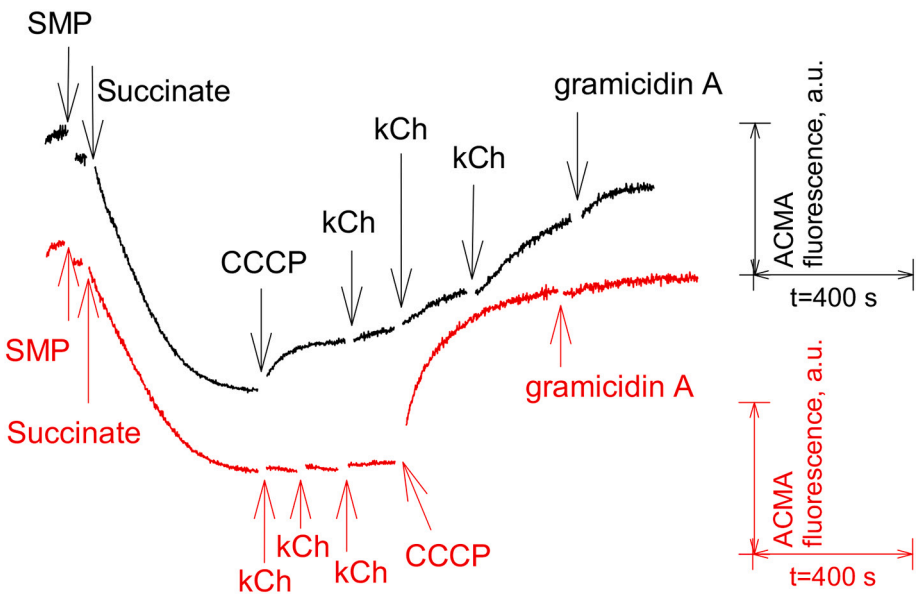

Fig. 5. Effect of kCh and CCCP on the pH gradient across the membranes of submitochondrial particles (SMP), as measured by ACMA (0.5 μg/ml) fluorescence. CCCP concentration was 100 nM, three successive additions of kCh were 1 μM, 2 μM, and 7 μM. Shown are traces of fluorescence at 480 nm (excited at 410 nm) in the medium containing 100 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4. In the traces, 1.5 mM succinate was supplemented at $t = 120$ s. 1 μg/ml of gramicidin A was added at the end of each trace. Protein concentration was 25 μg/ml.

affect the respiratory rate of RLM with glutamate/malate (Fig. 2C).

To test recoupling properties of the kCh analogs, we measured membrane potential in isolated RLM that were energized with succinate or glutamate/malate and uncoupled by CCCP or SF6847. The absorbance changes of the voltage-dependent cationic dye safranine O were used for these measurements. In contrast to the high recoupling potency of kCh, all the three analogs were completely ineffective both with CCCP (Fig. 3) and SF6847 (data not shown). Earlier similar observation was made by [12,17] with Ch compared to kCh. In case of glutamate/malate-incubated RLM, the kCh-induced inhibition of the complex I-mediated electron transfer superimposed on the kCh-induced recoupling, which manifested itself in a transient elevation of the membrane potential dropped by CCCP, with its subsequent decrease due to the inhibition (Fig. 4A). As seen in Fig. 4B, in the absence of any uncouplers, kCh provoked a pronounced decrease in the membrane potential of RLM with glutamate/malate, but not with succinate, which was in agreement with the data on the inhibition of RLM respiration (Fig. 2).

In further experiments, we examined an effect of kCh on the membrane potential of bovine heart SMP, as measured by the fluorescence of the potential-sensitive anionic dye ACMA. The addition of kCh after a partial drop of the SMP membrane potential, caused by CCCP, did not lead to the recoupling, as found in RLM (black curve in Fig. 5), but brought about an additional decrease in the membrane potential. Of note, no decrease in the membrane potential occurred upon the addition of kCh without CCCP in case of succinate oxidation (red curve in Fig. 5), in contrast to a substantial drop of the membrane potential caused by kCh in the similar experiments with NADH oxidation, which was associated with the inhibition of the respiratory complex I (the data not shown).

4. Discussion

The present study for the first time revealed the inhibitory effect of kCh on the respiratory complex I activities in both isolated mitochondria and inverted SMP (Figs. 1A, B, 2A, B). In particular, kCh selectively inhibits the reduction of ubiquinone and does not affect the NADH-binding center of the complex I, since kCh suppressed NADH-oxidase and NADH:Q1-reductase activity, but not the oxidation of NADH by an artificial electron acceptor HAR (Fig. 1). Complexes II, III and IV of the respiratory chain are not involved in the inhibitory effect, because kCh did not inhibit the succinate oxidase activity of mitochondria and SMP. The inhibitory effect appeared to be specific for the structure of kCh, in particular it requires the presence of a carbonyl group at the position 6. The three kCh analogs lacking this keto moiety failed to suppress the complex I activity (Fig. 2C and Table 1). Importantly, all these analogs, in contrast to kCh, were unable to recouple mitochondria, i.e. to remove the uncoupling effect of both CCCP (Fig. 3) and SF6847 (data not shown). Based on these findings, it can be assumed that i) the protonophoric action of the conventional mitochondrial uncouplers could be at least partially mediated by the respiratory complex I and/or other proteins of the mitochondrial respiratory chain, and ii) therefore the complex I and/or other membrane proteins could participate in the kCh-induced mitochondrial recoupling. These assumptions are supported by previous data on the partial suppression of the protonophoric activity of some uncouplers by inhibitors of certain mitochondrial enzymes [10,11,13,16,17,35,43,44].

It can be hypothesized that CCCP increases the proton permeability of the inner membrane of intact mitochondria in two ways. Being a hydrophobic molecule and having weakly acidic properties, CCCP in relatively high concentrations (1 μM) is able to translocate protons directly through the lipid bilayer of IMM, thus completely dissipating the membrane potential. Another mechanism of the uncoupling, involving the interaction of CCCP with the membrane domain of complex I, can be traced only at low concentrations of CCCP. In the absence of active turnover, complex I is in an open conformation, and proton exchange is possible only between the mitochondrial matrix and the central axis through hydrated half-channels of ND4 and ND5 subunits (see the scheme in Fig. 2 in Ref. [27] illustrating the coupling mechanism of mammalian respiratory complex I proposed by D. Kampjut and L. Sazanov). When pmf is created on IMM by succinate oxidation, this path by itself does not lead to uncoupling, but CCCP is able to deliver protons from the intermembrane space through the hydrophobic region of the enzyme to the charged residues of antiporter subunits localized in the central axis and involved in proton pumping. Then protons can be ejected through half-channels of ND4 and ND5 subunits into the mitochondrial matrix, providing the mitochondrial uncoupling. Upon interaction with complex I, kCh may block this CCCP-mediated proton translocation, thereby causing the mitochondrial recoupling. Of note, in the structure of complex I there are three binding sites of rotenone, a specific enzyme inhibitor. Two binding sites are located in the center and in the depth of the ubiquinone-binding channel, and the third site is found in the antiporter subunit ND4 in close proximity to the Lys206 residue involved in proton pumping [29]. It can be hypothesized that kCh, like rotenone, can also bind in the membrane domain of complex I

and prevent the direct interaction of CCCP with proton-pumping antiporter residues. The recoupling effect of kCh can be detected only at low concentrations of CCCP. With an increase in CCCP concentration, the CCCP-mediated direct proton transport through the lipid bilayer becomes predominant, which leads to kCh-insensitive uncoupling of mitochondria. The proton channels, that are accessible for the action of CCCP in mitochondria, become inaccessible for this uncoupler in the "inverted" membranes of SMP, due to the opposite orientation of the complex I with respect to that in RLM. As a result, there is no proton leakage through this protein, and there is also no recoupling effect of kCh. It is known that CCCP is much less effective uncoupler with SMP, than with RLM, i.e., uncoupling of SMP requires significantly higher, compared to RLM, concentrations of CCCP [14,42], apparently because the uncoupler transports protons only through a lipid bilayer in SMP, but not in RLM. Moreover, the addition of kCh to SMP after CCCP even leads to an additional decrease in the membrane potential which can be explained by the well-known ability of kCh to increase the dipole potential of lipid membranes, thereby stimulating translocation of anion carriers. Earlier, the kCh-provoked enhancement of the uncoupler-induced proton conductance was actually observed on planar BLM with the anionic protonophore SF6847 [12].

In conclusion, it should be stated that the main result of the present study consists in the observation of the inhibitory effect of kCh on the electron transfer reactions catalyzed by the respiratory complex I, whereas the relation of this inhibition to the recoupling activity of kCh remains hypothetical, albeit quite plausible.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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References

[1] E.C. Weinbach, J. Garbus, Protein as the mitochondrial site for action of uncoupling phenols, Science 145 (1964) 824–826.
[2] W.G. Hanstein, Y. Hatefi, Characterization and localization of mitochondrial uncoupler binding sites with an uncoupler capable of photoaffinity labeling, J. Biol. Chem. 249 (1974) 1356–1362.
[3] Y. Hatefi, Energy conservation and uncoupling in mitochondria, J. Supramol. Struct. 3 (1975) 201–213.
[4] G.W. Cyboron, R.L. Dryer, Uncoupling of hamster brown adipose and liver mitochondria by 2-azido-4-nitrophenol and binding properties of the reagent, Arch. Biochem. Biophys. 179 (1977) 141–146.
[5] C.K.R. Kurup, D.R. Sanadi, Photoaffinity labeling of uncoupler binding sites on mitochondrial membrane, J. Bioenerg. Biomembr. 9 (1977) 1–15.
[6] N.V. Katre, D.F. Wilson, Interaction of uncouplers with the mitochondrial membrane: identification of the high affinity binding site, Arch. Biochem. Biophys. 191 (1978) 647–656.
[7] M. Bona, M. Antalik, Z. Gazova, A. Kuchar, V. Dadak, D. Podhradsky, Interaction of carbonyl cyanide 3-chlorophenylhydrazone with cytochrome c oxidase, Gen. Physiol. Biophys. 12 (1993) 533–542.
[8] A.Y. Andreyev, T.O. Bondareva, V.I. Dedukhova, E.N. Mokhova, V.P. Skulachev, L. M. Tsofina, N.I. Volkov, T.V. Vygodina, The ATP/ADP-antiporter is involved in the uncoupling effect of fatty acids on mitochondria, Eur. J. Biochem. 182 (1989) 585–592.
[9] P. Schonfeld, P. Jezek, E.A. Belyaeva, J. Borecky, V.S. Slyshenkov, M. R. Wieckowski, L. Wojtczak, Photomodification of mitochondrial proteins by azido fatty acids and its effect on mitochondrial energetics. Further evidence for the role of the ADP/ATP carrier in fatty-acid-mediated uncoupling, Eur. J. Biochem. 240 (1996) 387–393.
[10] A.Y. Andreyev, T.O. Bondareva, V.I. Dedukhova, E.N. Mokhova, V.P. Skulachev, N. I. Volkov, Carboxyatractylate inhibits the uncoupling effect of free fatty acids, FEBS Lett. 226 (1988) 265–269.
[11] P. Schonfeld, Does the function of adenine nucleotide translocase in fatty acid uncoupling depend on the type of mitochondria? FEBS Lett. 264 (1990) 246–248.
[12] A.A. Starkov, D.A. Bloch, B.V. Chernyak, V.I. Dedukhova, S.E. Mansurova, I. I. Severina, R.A. Simonyan, T.V. Vygodina, V.P. Skulachev, 6-ketocholestanol is a recoupler for mitochondria, chromatophores and cytochrome oxidase proteoliposomes, Biochim. Biophys. Acta 1318 (1997) 159–172.
[13] V.P. Skulachev, Uncoupling: new approaches to an old problem of bioenergetics, Biochim. Biophys. Acta 1363 (1998) 100–124.
[14] I.R. Iaubasarova, L.S. Khailova, A.M. Firsov, V.G. Grivennikova, R.S. Kirsanov, G. A. Korshunova, E.A. Kotova, Y.N. Antonenko, The mitochondria-targeted derivative of the classical uncoupler of oxidative phosphorylation carbonyl cyanide m-chlorophenylhydrazone is an effective mitochondrial recoupler, PLoS One 15 (2020), e0244499.
[15] L.S. Khailova, T.V. Vygodina, G.Y. Lomakina, E.A. Kotova, Y.N. Antonenko, Bicarbonate suppresses mitochondrial membrane depolarization induced by conventional uncouplers, Biochem. Biophys. Res. Commun. 530 (2020) 29–34.
[16] K. Zuna, O. Jovanovic, L.S. Khailova, S. Skulj, Z. Brkljaca, J. Kreiter, E.A. Kotova, M. Vazdar, Y.N. Antonenko, E.E. Pohl, Mitochondrial uncoupling proteins (UCP1-UCP3) and adenine nucleotide translocase (ANT1) enhance the protonophoric action of 2, 4-dinitrophenol in mitochondria and planar bilayer membranes, Biomolecules 11 (2021) 1178.
[17] A.A. Starkov, V.I. Dedukhova, V.P. Skulachev, 6-ketocholestanol abolishes the effect of the most potent uncouplers of oxidative phosphorylation in mitochondria, FEBS Lett. 355 (1994) 305–308.
[18] E. Chavez, R. Moreno-Sanchez, C. Zazueta, A. Cuellar, J. Ramirez, H. Reyes-Vivas, C. Bravo, S. Rodriguez-Enriquez, On the mechanism by which 6-ketocholestanol protects mitochondria against uncoupling-induced Ca2+ efflux, FEBS Lett. 379 (1996) 305–308.
[19] A.A. Starkov, "Mild" uncoupling of mitochondria, Biosci. Rep. 17 (1997) 273–279.
[20] H. Ligeret, S. Barthelemy, D.G. Bouchard, P.A. Carrupt, J.P. Tillement, S. Labidalle, D. Morin, Fluoride curcumin derivatives: new mitochondrial uncoupling agents, FEBS Lett. 569 (2004) 37–42.
[21] V.L. Dalla, A.N. Garcia-Argaez, A. Braga, M. Martinez-Vazquez, S. Grancara, P. Martinis, E. Agostinelli, A. Toninello, An eudesman derivative from Verbesina persicifolia D.C. as a natural mild uncoupler in liver mitochondria. A new potential anti-obesity agent? Curr. Pharm. Des. 20 (2014) 253–261.
[22] J.L. Figarola, J. Singhal, J.D. Tompkins, G.W. Rogers, C. Warden, D. Horne, A. D. Riggs, S. Awasthi, S.S. Singhal, SR4 uncouples mitochondrial oxidative phosphorylation, modulates AMP-dependent kinase (AMPK)-mammalian target of rapamycin (mTOR) signaling, and inhibits proliferation of HepG2 hepatocarcinoma cells, J. Biol. Chem. 290 (2015) 30321–30341.
[23] F.A. Urra, M. Cordova-Delgado, M. Lapier, A. Orellana-Manzano, L. Acevedo-Arevalo, H. Pessoa-Mahana, J.M. Gonzalez-Vivanco, M. Martinez-Cifuentes, O. Ramirez-Rodriguez, J.P. Millas-Vargas, B. Weiss-Lopez, M. Pavani, J. Ferreira, R. Araya-Maturana, Small structural changes on a hydroquinone scaffold determine the complex I inhibition or uncoupling of tumoral oxidative phosphorylation, Toxicol. Appl. Pharmacol. 291 (2016) 46–57.
[24] C. Ballmoos, O. Biner, T. Nilsson, P. Brzezinski, Mimicking respiratory phosphorylation using purified enzymes, Biochim. Biophys. Acta 2016 (1857) 321–331.
[25] J. Hirst, Mitochondrial complex I, Annu. Rev. Biochem. 82 (2013) 551–575.
[26] K. Parey, C. Wirth, J. Vonck, V. Zickermann, Respiratory complex I - structure, mechanism and evolution, Curr. Opin. Struct. Biol. 63 (2020) 1–9.
[27] D. Kampjut, L.A. Sazanov, Structure of respiratory complex I – an emerging blueprint for mechanism, Curr. Opin. Struct. Biol. 74 (2022), 102350.
[28] J. Gutierrez-Fernandez, K. Kaszuba, G.S. Minhas, R. Baradaran, M. Tambalo, D. T. Gallagher, L.A. Sazanov, Key role of quinone in the mechanism of respiratory complex I, Nat. Commun. 11 (2020) 4135.
[29] D. Kampjut, L.A. Sazanov, The coupling mechanism of mammalian respiratory complex I, Science 370 (2020), eabc4209.
[30] R. Baradaran, J.M. Berrisford, G.S. Minhas, L.A. Sazanov, Crystal structure of the entire respiratory complex I, Nature 494 (2013) 443–448.
[31] M.Degli Esposti, Inhibitors of NADH-ubiquinone reductase: an overview, Biochim. Biophys. Acta 1364 (1998) 222–235.
[32] H.R. Bridges, J.G. Fedor, J.N. Blaza, A. Di Luca, A. Jussupow, O.D. Jarman, J. J. Wright, A.-N.A. Agip, A.P. Gamiz-Hernandez, M.M. Roessler, V.R.I. Kaila, J. Hirst, Structure of inhibitor-bound mammalian complex I, Nat. Commun. 11 (2020) 5261.
[33] D.N. Grba, J.N. Blaza, H.R. Bridges, A.-N.A. Agip, Z. Yin, M. Murai, H. Miyoshi, J. Hirst, Cryo-electron microscopy reveals how acetogenins inhibit mitochondrial respiratory complex I, J. Biol. Chem. 298 (2022), 101602.
[34] D. Johnson, H. Lardy, Isolation of liver or kidney mitochondria, Methods Enzymol. 10 (1967) 94–96.
[35] Y.N. Antonenko, S.S. Denisov, L.S. Khailova, P.A. Nazarov, T.I. Rokitskaya, V. N. Tashlitsky, A.M. Firsov, G.A. Korshunova, E.A. Kotova, Alkyl-substituted phenylamino derivatives of 7-nitrobenz-2-oxa-1, 3-diazole as uncouplers of oxidative phosphorylation and antibacterial agents: involvement of membrane proteins in the uncoupling action, Biochim. Biophys. Acta Biomembr. 2017 (1859) 377–387.
[36] V.G. Grivennikova, G.V. Gladyshev, A.D. Vinogradov, Deactivation of mitochondrial NADH:ubiquinone oxidoreductase (respiratory complex I): extrinsically affecting factors, Biochim. Biophys. Acta Bioenerg. 1861 (2020), 148207.

[37] B. Chance, G.R. Williams, A simple and rapid assay of oxidative phosphorylation, Nature 175 (1955) 1120–1121.
[38] K.E. Akerman, M.K. Wikstrom, Safranine as a probe of the mitochondrial membrane potential, FEBS Lett. 68 (1976) 191–197.
[39] M.V. Loskovich, V.G. Grivennikova, G. Cecchini, A.D. Vinogradov, Inhibitory effect of palmitate on the mitochondrial NADH:ubiquinone oxidoreductase (complex I) as related to the active-de-active enzyme transition, Biochem. J. 387 (2005) 677–683.
[40] B. Chance, M. Nishimura, Sensitive measurements of changes of hydrogen ion concentration, Methods Enzymol. 10 (1967) 641–650.
[41] G. Deleage, F. Penin, C. Godinot, D.C. Gautheron, Correlation between ATP hydrolysis, ATP synthesis, generation and utilization of delta pH in mitochondrial ATPase-ATP synthase, Biochim. Biophys. Acta 725 (1983) 464–471.
[42] A. Cuellar, J. Ramirez, V.M. Infante, E. Chavez, Further studies on the recoupling effect of 6-ketocholestanol upon oxidative phosphorylation in uncoupled liver mitochondria, FEBS Lett. 411 (1997) 365–368.
[43] V.N. Samartsev, A.V. Smirnov, I.P. Zeldi, O.V. Markova, E.N. Mokhova, V. P. Skulachev, Involvement of aspartate/glutamate antiporter in fatty acid-induced uncoupling of liver mitochondria, Biochim. Biophys. Acta 1319 (1997) 251–257.
[44] A.M. Firsov, L.B. Popova, L.S. Khailova, P.A. Nazarov, E.A. Kotova, Y. N. Antonenko, Protonophoric action of BAM15 on planar bilayers, liposomes, mitochondria, bacteria and neurons, Bioelectrochemistry 137 (2020), 107673.