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6-Ketocholestanol is a recoupler for mitochondria, chromatophores and cytochrome oxidase proteoliposomes

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Abstract

The effect of 6-ketocholestanol (kCh) on various natural and reconstituted membrane systems has been studied. 6-ketocholestanol (5α-Cholestan-3β-ol-6-one), a compound increasing the membrane dipole potential, completely prevents or reverses the uncoupling action of low concentrations of the most potent artificial protonophore SF6847. This effect can be shown in the rat liver and heart muscle mitochondria, in the intact lymphocytes, in the *Rhodobacter sphaeroides* chromatophores, and in proteoliposomes with the heart muscle or *Rh. sphaeroides* cytochrome oxidase. The recoupling effect of kCh disappears within a few minutes after the kCh addition and cannot be observed at all at high SF6847 concentrations. Almost complete recoupling is also shown with FCCP, CCCP, CCP and platanetin. With 2,4-dinitrophenol, fatty acids and gramicidin, kCh is ineffective. With TTFB, PCP, dicoumarol, and zearalenone, low kCh concentrations are ineffective, whereas its high concentrations recouple but partially. The kCh recoupling is more pronounced in mitochondria, lymphocytes and proteoliposomes than in chromatophores. On the other hand, mitochondria, lymphocytes and proteoliposomes are much more sensitive to SF6847 than chromatophores. A measurable lowering of the electric resistance of a planar bilayer phospholipid membrane (BLM) are shown to occur at SF6847 concentrations which are even higher than in chromatophores. In BLMs, kCh not only fails to reverse the effect of SF6847, but even enhances the conductivity increase caused by this uncoupler. It is assumed that action of low concentrations of the SF6847-like uncouplers on coupling membranes involves cytochrome oxidase and perhaps some other membrane protein(s) as well. This involvement is inhibited by the asymmetric increase in the membrane dipole potential, caused by incorporation of kCh to the outer leaflet of the membrane.

Keywords: Uncoupler; Recoupler; 6-Ketocholestanol; Mitochondrion; Chromatophore; Cytochrome oxidase

Abbreviations: $\Delta\Psi$, transmembrane electric potential difference; BLM, bilayer planar phospholipid membrane; BSA, bovine serum albumin; cAtr, carboxyatractylate; CCP, carbonylcyanide phenylhydrazone; 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; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanin iodide; kCh, 6-ketocholestanol (5α-Cholestan-3β-ol-6-one); MOPS, morpholinopropane sulphonate; PCP, pentachlorophenol; SF6847, 3,5-di(tret-butyl)-4-hydroxybenzylidenemalononitrile; TTFB, tetrachlorotrifluoromethylbenzimidazole.

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1. Introduction

According to the Mitchell's chemiosmotic theory [1,2], uncouplers of the oxidative and photosynthetic phosphorylations operate as protonophores traversing the hydrophobic membrane region in their protonated and deprotonated forms. In line with this point of view, it was found that the H^+-conductance of planar bilayer phospholipid membrane (BLM) is strongly increased by various uncouplers [3–7]. This observation seemed to indicate that proteins are not involved in uncoupling. Such an assumption was, however, shaken by the discovery that uncoupling by free fatty acids is mediated by a specific protein, namely, by thermogenin in brown fat [8–11] and by ATP/ADP-antiporter in other animal tissues [12–15]. In the latter case, it was found that the antiporter inhibitor carboxyatractylate (cAtr) almost completely suppresses uncoupling of heart muscle mitochondria by low concentrations of fatty acids [14,15]. In the same studies, uncoupling by phenylhydrazone derivatives (FCCP and CCCP) was shown to be cAtr-resistant at all concentrations of these protonophores. As to dinitrophenol (DNP), its uncoupling effect was suppressed by cAtr, but only partially [14]. Thus, involvement of protein(s) in uncoupling allows the system to be recoupled when the activity of the protein in question is arrested by an inhibitor. This phenomenon has been defined as 'recoupling' [16]. We recently reported [17] that uncoupling of the heart mitochondria by low concentrations of FCCP, CCCP and the most potent uncoupler SF6847 can be completely prevented or reversed by 6-ketocholestanol, a compound which increases the membrane dipole potential [18,19], whereas phloretin, which decreases this potential [20–22], was ineffective. The analogous effect of kCh with plant mitochondria was subsequently reported by Vianello et al. [23]. In the present paper we report results of the study on the mechanism of this effect, using several experimental systems – liver and heart muscle mitochondria, intact lymphocytes, *Rhodobacter sphaeroides* chromatophores, proteoliposomes containing the beef heart or *Rh. sphaeroides* cytochrome oxidase, and BLMs. In all the systems but BLMs, kCh was active as recoupler with respect to SF6847, FCCP, CCCP and CCP but not with respect to fatty acids, DNP and gramicidin. The effect of kCh disappeared in time. In BLMs and chromatophores, the efficiency of uncouplers was found to be much lower than in the other systems studied. The mechanism of action of uncouplers and recouplers and its possible physiological significance are discussed.

2. Materials and methods

2.1. Mitochondria

The heart mitochondria were isolated from the rat heart muscle. Cooled muscles, previously separated 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 medium (250 mM sucrose, 10 mM MOPS, 1 mM EDTA, pH 7.4) ratio being 1:8. After the first centrifugation (10 min, $600 \times g$), the supernatant was decanted and filtered through gauze, then centrifuged again (10 min, $12\,000 \times g$). The sediment was suspended in 1 ml isolation medium supplemented with BSA (3 $mg \times ml^{-1}$). Then the medium without BSA was added. The final mitochondrial precipitate (10 min, $12\,000 \times g$) was suspended in the isolation medium with BSA. The mitochondrial suspension (70–90 $mg \times ml^{-1}$) was stored on ice.

The liver mitochondria were isolated essentially as those from the heart muscle, but the tissue was homogenized for 30 s. The isolation medium for the liver mitochondria contained 1 mM EGTA instead of EDTA.

2.2. Thymocytes

Thymuses were obtained from male Sprague–Dawley rats. Thymocytes were gently dispersed, filtered and washed twice in Hanks' balanced salt solution (HBSS, Sigma) without Ca^{2+}, supplemented with 10 mM glucose and 10 mM Hepes (pH 7.4), at room temperature. Cell suspension (approx. 10^9 cells/ml) in the solution of the same composition was stored on ice. Viability, determined by Trypan Blue exclusion, was higher than 90% and did not change during 4–5 h of storage.

2.3. Chromatophores

Cells of *Rhodobacter sphaeroides* 2R (the purple wild-type strain, Moscow State Univ.) were grown phototrophically on the Sistrom's minimal medium supplemented with casamino acids and vitamins [24], as described [25]. Cells were harvested at the late logarithmic growth phase, washed twice and stored at −20°C. Chromatophores were isolated using a French press cell disruption procedure [26]. Chromatophores were suspended in 20 mM Hepes (pH 7.5), 50 mM KCl, 2 mM $MgSO_4$, and stored at −20°C. The bacteriochlorophyll concentration in the chromatophore suspensions was estimated using $e_{375} = 100$ mM^{-1} cm^{-1}.

2.4. Proteoliposomes

The beef heart cytochrome oxidase was isolated as described in refs. [27,28]. The cytochrome oxidase proteoliposomes were prepared by the cholate-dialysis method [29,30]. Asolectin (Sigma, type IIS) was dispersed to concentration of 40 $mg \times ml^{-1}$ in 50 mM potassium phosphate buffer (pH 7.4), 2 mM $MgSO_4$ and 1.5% cholate. The mixture was bubbled with argon and then sonicated 5 times for 30 s in an ultrasonic USDG-2T disintegrator (44 kHz, 0.4A). Cytochrome oxidase was added to 3 mM concentration and the mixture was dialysed against 500 volumes of 50 mM potassium phosphate with 2 mM $MgSO_4$ for 2 h. Dialysis was repeated for 2 h and then overnight. The *Rhodobacter sphaeroides* cytochrome oxidase proteoliposomes were kindly provided by Dr. A. Konstantinov.

2.5. Planar phospholipid bilayer membranes

To make BLMs, an asolectin solution in decane (50 $mg \times ml^{-1}$) was used. A BLM was formed in a 1-mm aperture in a Teflon septum separating the chamber into two compartments. Two Ag/AgCl electrodes were put into the solution of 250 mM sucrose, 10 mM MOPS/Tris (pH 7.4) and 1 mM EGTA, one on each side of the septum. The BLM conductance was measured with a VA-J-51 electrometer [31].

2.6. Measurements

In the majority of the experiments with mitochondria, the incubation mixture contained 250 mM sucrose, 10 mM MOPS/Tris (pH 7.4), oligomycin (2 $\mu g \times ml^{-1}$), 2 μM rotenone, 5 mM succinate and 1 mM EGTA. The oxygen consumption was measured at 26°C with a Clark-type oxygen electrode. The ATPase reaction in mitochondria was measured spectrophotometrically with the pH probe Phenol Red, employing the 557–620 nm wavelength pair [32]. The incubation medium contained 150 mM sucrose, 75 mM KCl, 0.1 mM EDTA, 5 mM Tris-HCl (pH 8.0), and 5 mM succinate. The incubation medium was supplemented with 8 mM K_2SO_3 in order to prevent the ATPase inhibition by ADP [33]. In the aerobic experiments, the incubation medium was supplemented with 0.5 mM ATP, the reaction was started by addition of mitochondria. For the anaerobic experiments, ATP was added when O_2 was exhausted by the respiring mitochondria.

The $\Delta\Psi$ changes in mitochondria and proteoliposomes were estimated with safranin O [34,35] using wavelength pair 523–555 nm. The concentration of mitochondrial protein (0.4–0.6 $mg \times ml^{-1}$) and that of safranin O (8–12 μM) were adjusted to keep the dye/protein ratio 20:1 (nM of dye/mg of protein). The safranin O response was calibrated with K^+-valinomycin according to [36]. In all the reported experiments with mitochondria, the initial $\Delta\Psi$ was about 178 ± 15 mV. It should be noted that the safranin O response linearly depends on $\Delta\Psi$ in the region 50–170 mV, as reported earlier [34–36]. In most of the experiments, the $\Delta\Psi$ changes were expressed as % of maximum value observed before addition of uncouplers.

A fluorescent potential-sensitive dye JC-1 was used to follow the mitochondrial membrane potential in the rat thymocytes. This lipophilic cation was shown to be accumulated by mitochondria in various cell lines. It forms J-aggregates at high $\Delta\Psi$ values [37,38]. This process caused large increase in orange fluorescence (emission at 590 nM) that was detectable both with fluorescent microscopy and with fluorimetric measurement in cell suspension. Recently the mitochondrial $\Delta\Psi$ level was measured with JC-1 in lymphocytes using flow-cytometric technique [39,40]. The preferentially mitochondrial localization of the

dye in lymphocytes was confirmed with confocal microscopy [40]. For the $\Delta\Psi$ measurements, thymocytes were diluted to 2×10^7 cells/ml at 37°C by solution of the same composition as that used for washing and suspending the cell (see above) and incubated at stirring with 3×10^{-7} M JC-1 added from stock solution in DMSO. Fluorescence was measured at the 490 nm excitation and the 593 nm emission with a Perkin-Elmer spectrofluorimeter. During first 10 min, fluorescence slowly increased and reached a plateau that remained stable for at least 10 min more. This level was assumed as 100%.

The $\Delta\Psi$ changes in chromatophores were measured by monitoring the carotenoid absorbance band shift [41,42]. The kinetics or steady-state level of carotenoid absorbance at 523 nm were recorded using a home-made single-beam spectrophotometer equipped with a magnetic stirrer. Measurements were performed in a cuvette with 1 cm optical path length. For single excitation, we used fast actinic light flashes of 95% saturation from a Q-switched Nd-YAG laser equipped with a frequency-doubling module (532 nm, 20 mJ/cm^2/flash, 20 ns halfwidth). For the continuous excitation, the actinic illumination was from a halogen lamp (200 W), shielded with a KC-14 red cut-off optical glass filter.

2.7. Chemicals

Palmitic acid, FCCP, CCCP, CCP, TTFB, SF6847, kCh, rotenone and oligomycin were dissolved in twice distilled ethanol. Oligomycin, MOPS, CCCP, kCh and fatty acid-free BSA were from Sigma; EDTA, EGTA, rotenon, and DNP were from Serva; FCCP was from Boehringer; platanetin and zearalenone were gifts of Professor P. Ravanel and Professors F. Macri and A. Vianello, respectively.

3. Results

3.1. Recoupling in the respiring mitochondria

In agreement with data previously obtained on the heart mitochondria [17], addition of kCh to the liver mitochondria uncoupled by low concentrations of SF6847 resulted in recoupling, i.e., almost complete inhibition of the uncoupler-stimulated respiration (Fig.

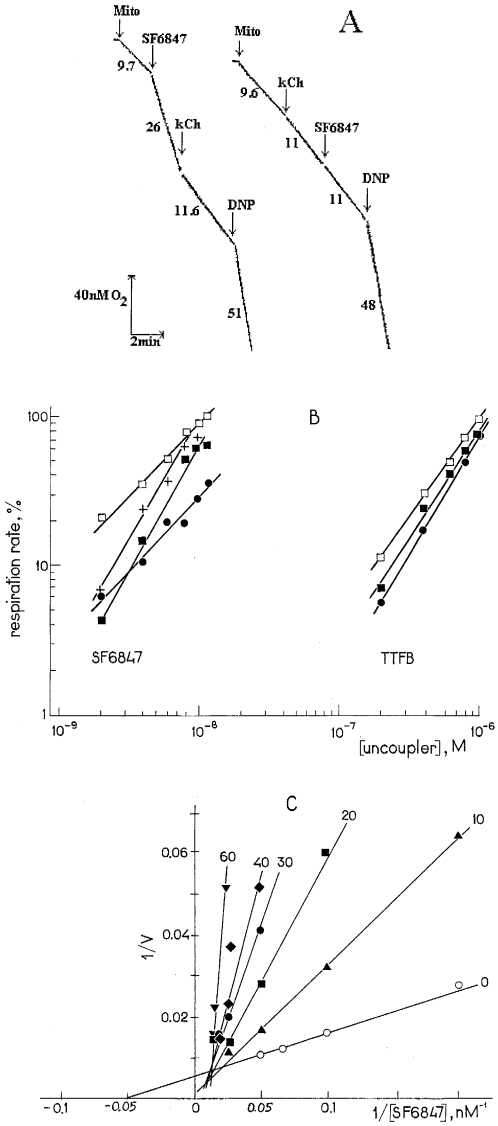

Fig. 1. Effect of 6-ketocholestanol on the mitochondrial respiration stimulated by SF6847. The incubation mixture (see Section 2) was supplemented with BSA (0.2 mg$\times$ml^{-1}). A, additions: Mito, the rat liver mitochondria (1.2 mg protein$\times$ml^{-1}); SF6847, 80 nM; kCh, 75 μM; DNP, 20 μM. B, the rat heart mitochondria (0.9 mg protein$\times$ml^{-1}); BSA and rotenone were excluded, 5 mM glutamate +5 mM malate substituted for succinate. Open squares, no kCh; crosses, 20 μM kCh; closed squares, 40 μM kCh; closed circles, 80 μM kCh. C, respiration rates in the presence of different concentrations of kCh are plotted versus SF6847 concentrations in Lineweaver-Burk coordinates. V, rate of respiration stimulated by SF6847 (respiration rate without SF6847 is subtracted), nM $O_2 \times \min^{-1} \times mg^{-1}$ protein. Conditions as in Fig. 1A, but BSA was excluded. Numbers near the curves, the kCh concentrations (μM).

1) and increase in $\Delta\Psi$ up to level observed before the uncoupler addition (Fig. 2). Fig. 1B shows that in the rat heart mitochondria, the respiration rate is a linear function of the uncoupler concentration both in the presence and in the absence of kCh. However, for SF6847 the slope of the curves depends on the kCh concentrations, being close to 1 in absence of kCh and equal to 2 at 20–40 μM kCh. Higher concentrations of kCh return the slope to the initial value. For TTFB, the slope of the curve is close to 2 and is unaffected by 40 μM kCh. The same relationships were observed in the liver mitochondria (not shown). Fig. 1C shows the Lineweaver-Burk plot of respiration rate of the rat liver mitochondria versus the SF6847 concentrations in the presence of different amounts of kCh. The relationships revealed, i.e., the crossing of lines in the upper right part of the figure, point to a complex character of the kCh action which should include, besides the major kCh inhibitory effect on the uncoupled respiration (recoupling), also some facilitation of the uncoupling process. Such a facilitation was really observed in experiments on chromatophores incubated with kCh for a long time as well as in BLM where the kCh recoupling was absent (see below).

6-Ketocholestanol proved to be effective in both preventing (Fig. 1A, Fig. 2B) and reversing (Fig. 1A, Fig. 2A) the SF6847-induced uncoupling. In both cases, subsequent addition of gramicidin completely discharged $\Delta\Psi$ (Fig. 2A,B). Higher SF6847 concentrations could also abolish the kCh recoupling effect (Fig. 2C). In fact, the dependence of degree of the $\Delta\Psi$ decrease upon the SF6847 concentration became much steeper when kCh was present (Fig. 2C), just as was observed with the respiration rate (Fig. 1B). This was also the case for FCCP (Fig. 2C) and CCCP (not shown) but not for TTFB (Fig. 1B, Fig. 2C). In the case of TTFB, the above dependence in the absence of kCh was already as steep as for SF6847 (or FCCP) with 40 μM kCh so the kCh addition was almost without influence (Fig. 2C). Further experiments, however, showed that partial recoupling can be observed even with TTFB if much higher kCh concentration was used. Relationships similar to those with TTFB were found to be inherent in PCP, dicoumarol and zearalenone. In the case of gramicidin, DNP and palmitate, recoupling effect of 200 μM kCh was absent or $< 15\%$ (Table 1).

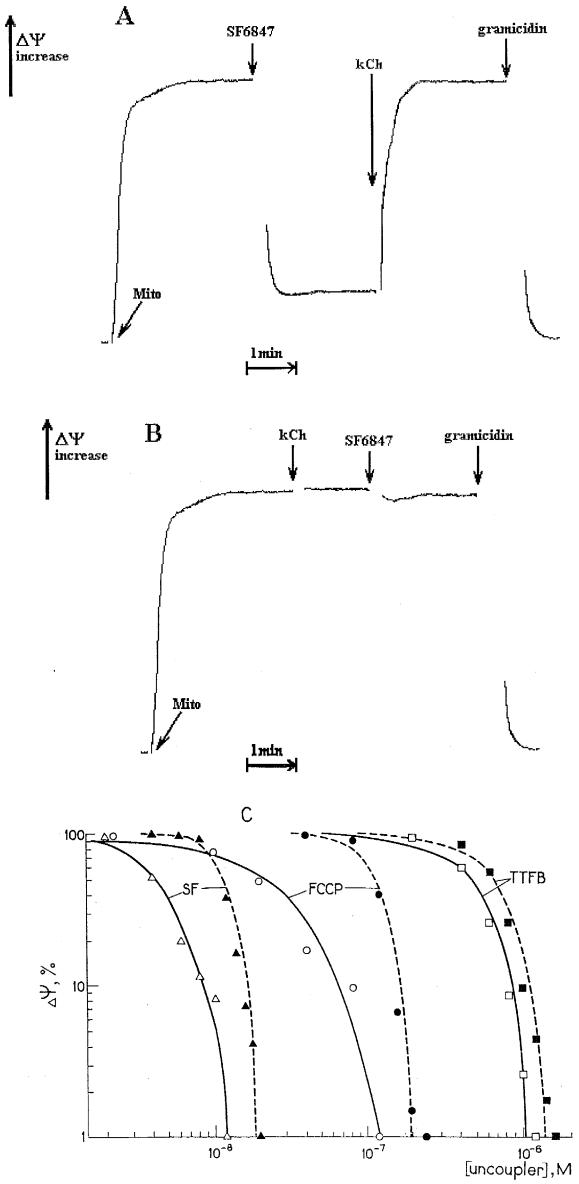

Fig. 2. Effects of SF6847 and kCh on the mitochondrial membrane potential ($\Delta\Psi$) measured with safranin O. The incubation mixture (see Section 2) was supplemented with 8 μM safranin O and 0.2 mg$\times$ml^{-1} BSA. A,B, the effects of SF6847 and kCh on dynamics of $\Delta\Psi$ in rat liver mitochondria. Additions: Mito, the rat liver mitochondria (0.4 mg protein$\times$ml^{-1}); SF6847, 30 nM; kCh, 80 μM; gramicidin, 2 μg$\times$ml^{-1}. C, effect of kCh on $\Delta\Psi$ in the presence of three different uncouplers in the heart muscle mitochondria. The incubation mixture was as in Fig. 1B. Solid line, no kCh; dashed line, 40 μM kCh was added to mitochondrial suspension 1.5 min before the addition of the uncoupler; triangles, SF6847; circles, FCCP; squares, TTFB.

Preincubation of mitochondria with kCh for several minutes was found to lower the recouping effect. This phenomenon was especially clear in chromatophores and proteoliposomes where it was studied in detail (see below).

In Fig. 2, $\Delta\Psi$ was measured with safranin O. Quite similar results were obtained when we used tetraphenyl phosphonium to monitor $\Delta\Psi$ (not shown). Cholesterol proved to be absolutely ineffective as a recoupler (see also Ref. [17]), whereas its oxidized derivative (3-one instead of 3-ol) was of similar efficiency as kCh (not shown).

3.2. Recoupling in ATP-hydrolyzing mitochondria

The above relationships shown with the respiration-energized mitochondria also proved to be inherent in the mitochondria energized by hydrolysis of ATP. As shown in Fig. 3A,B, addition of kCh after ATP and SF6847 inhibits the uncoupler-stimulated ATP hydrolysis and increases the $\Delta\Psi$ level. The recoupling activity of kCh, added before ATP, was found to decrease with increasing the time interval between the kCh and ATP additions. A pronounced recoupling effect was observed when this interval was 2 min, whereas recoupling was small when the interval was 20 min (Fig. 3C).

Table 1
The kCh effect on the $\Delta\Psi$ decrease by uncouplers in the rat liver mitochondria

Uncoupler		kCh	
Name	$C_{1/2}$, M	Maximal recoupling, %	$C_{1/2}$, M
SF6847	3.6×10^{-8}	100	3×10^{-5}
FCCP	1.8×10^{-7}	95	3×10^{-5}
CCP	2×10^{-7}	90	6×10^{-5}
CCCP	2×10^{-7}	90	6×10^{-5}
TTFB	1.3×10^{-6}	80	1×10^{-4}
PCP	6×10^{-6}	50	1.4×10^{-4}
Platanetin	1.1×10^{-5}	90	8×10^{-5}
2,4-DNP	1.4×10^{-5}	0	–
Dicoumarol	1.4×10^{-5}	55	1.4×10^{-4}
Palmitate	3×10^{-5}	<15	7.5×10^{-5}
Zearalenone	7×10^{-5}	50	1.1×10^{-4}
Gramicidin D	0.005 μg/ml	0	–

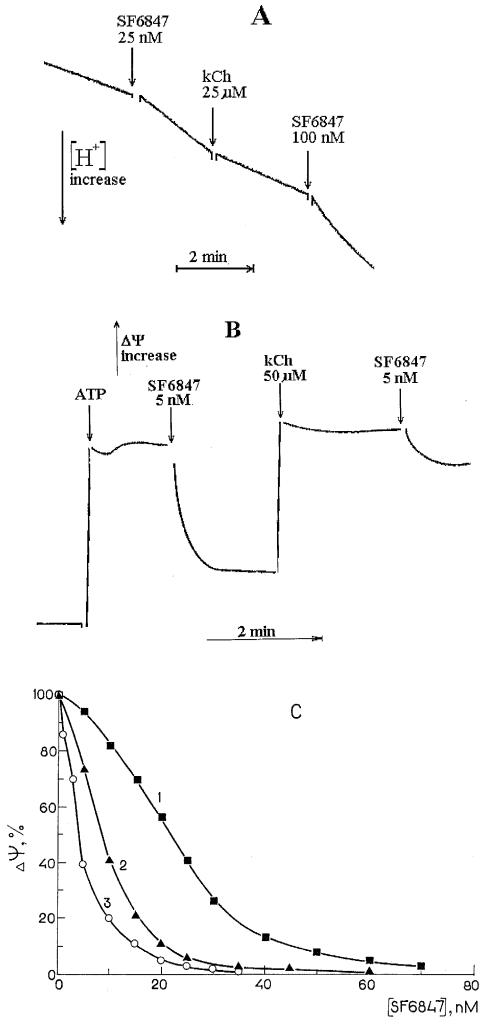

Fig. 3. Recoupling by 6-ketocholestanol of rat liver mitochondria hydrolyzing ATP in the presence of SF6847. A, the ATP hydrolysis rate; B,C, the ATPase-supported $\Delta\Psi$. The incubation mixture: A, 150 mM sucrose, 75 mM KCl, 0.1 mM EDTA, 5 mM succinate, 8 mM K_2SO_3, 5 mM Tris-HCl (pH 8.0), the rat liver mitochondria (0.8 mg protein$\times$ml^{-1}), aerobiosis; B,C, 250 mM sucrose, 2 mM EGTA, 5 mM succinate, 2 mM rotenone, 10 mM MOPS (pH 7.4), the rat liver mitochondria (0.8 mg protein$\times$ml^{-1}), anaerobiosis. In C, curves 1 and 2, 50 μM kCh was added 2 and 20 min before ATP, respectively; curve 3, without kCh.

3.3. Recoupling in thymocytes

Mitochondrial $\Delta\Psi$ was monitored in rat thymocytes using JC-1 as fluorescent probe. Effect of small concentrations of SF6847 or FCCP, causing 50% $\Delta\Psi$ decrease drop, was found to be reversed by subsequent addition of 4×10^{-5} M kCh. Higher uncoupler

Table 2
Recoupling effect of kCh on thymocytes

Uncoupler or ionophore	Mitochondrial $\Delta\Psi$ (JC-1 fluorescence, %)	
	Without kCh	With 4×10^{-5} M kCh
None	100	103
2×10^{-9} M SF6847	53	105
2×10^{-7} M SF6847	0	7
2.5×10^{-8} M FCCP	57	98
2×10^{-6} M FCCP	0	0
3×10^{-5} M DNP	50	53
1.5×10^{-8} M valinomycin	48	50

concentrations abolished the recoupling effect of kCh (Table 2). Similar relationships were revealed when kCh was added before uncoupler (not shown). The $\Delta\Psi$ decrease caused by DNP or valinomycin proved to be kCh-resistant even at low concentrations of these agents (Table 2).

3.4. Effects of kCh in Rh. sphaeroides chromatophores

In *Rh. sphaeroides* chromatophores, the recoupling action of kCh was demonstrated when $\Delta\Psi$, generated by photosynthetic electron transfer, was measured by means of the carotenoid spectral shift. Fig. 4A shows that addition of kCh after SF6847 reverses the uncoupler-induced increase in the $\Delta\Psi$ dissipation rate constant (τ), measured after a short laser flash. Just as in the case of respiration and ATP hydrolysis in mitochondria, the recoupling effect was transient. In fact, kCh induced some increase (rather than a decrease) in the $\Delta\Psi$ dissipation rate measured 15 min after kCh addition (Fig. 4A). The decay of recoupling effect was slowed down at lower temperature (Fig. 4B). Repeated kCh addition resulted in a new recoupling cycle (Fig. 4A,B). Under continuous illumination, kCh decreased or increased the $\Delta\Psi$ sensitivity to SF6847 when kCh was added 40 s after SF6847 or 15 min before SF6847, respectively (Fig. 4C).

3.5. Recoupling in cytochrome oxidase proteoliposomes

The next series of experiments revealed that the recoupling effect of kCh can be shown in reconstituted systems, i.e., in liposomes containing cytochrome oxidase from the beef heart muscle or from *Rh. sphaeroides*. Fig. 5A shows a typical $\Delta\Psi$ record in the beef heart cytochrome oxidase proteoliposomes. Again, as in the case of other systems

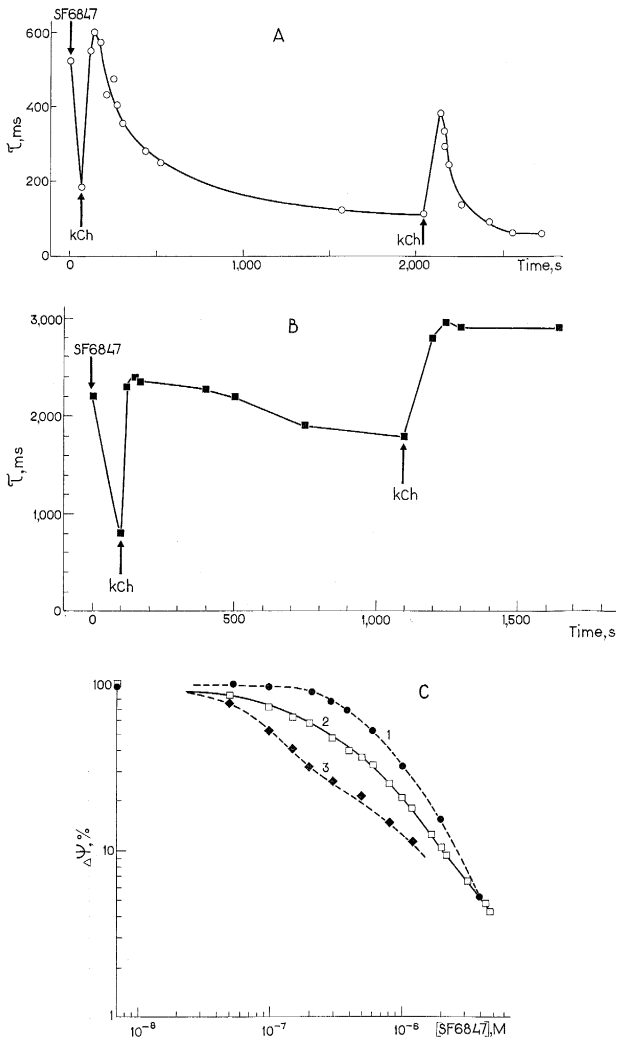

Fig. 4. The 6 − ketocholestanol recoupling effect in the *Rhodobacter sphaeroides* chromatophores. The incubation mixture contained 250 mM sucrose, 10 mM MOPS (pH 7.2), 1 mM EGTA, oligomycin (8 $\mu g\times ml^{-1}$), 2 mM ascorbate and 10 μM TMPD ($E_h = +180$ mV), chromatophores (18 μM bacteriochlorophyll). A,B, the single flash-induced carotenoid shift was measured and the time constant, τ, of the initial $\Delta\Psi$ decay rate was calculated; A, $t = 25$°C; B, $t = 10$°C. C, the magnitude of the carotenoid absorbance changes was measured under continuous illumination (see Section 2), $t = 25$°C; curve 1, kCh was added 40 s after the SF6847 addition; curve 2, no kCh; curve 3, chromatophores were preincubated with kCh for 15 min before SF6847 addition. Additions, 200 nM SF6847, 50 μM kCh.

studied, kCh recoupled at low SF6847 concentrations, whereas excess of the uncoupler reversed the kCh recoupling effect on $\Delta\Psi$. The $\Delta\Psi$ increase was accompanied by inhibition of the uncoupler-stimulated respiration (Fig. 5B). Recoupling decayed in min time-scale (Fig. 5C,D). When added before SF6847, kCh caused some increase in the $\Delta\Psi$ level which was completely abolished by the subsequent addition of a high concentration of SF6847 or gramicidin. It should be stressed that such an effect was

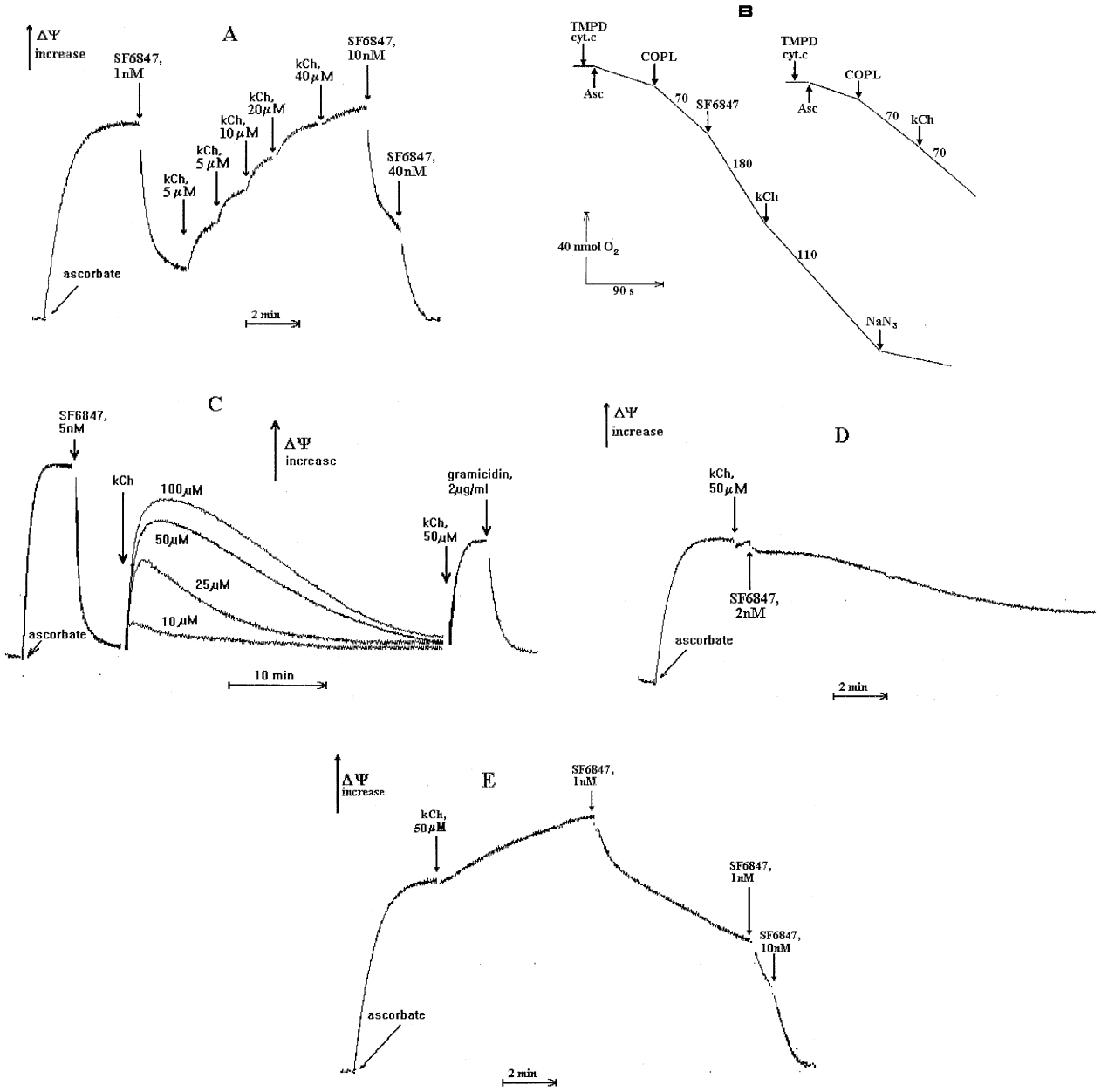

Fig. 5. Recoupling by kCh of the cytochrome oxidase proteoliposomes uncoupled by SF6847. The incubation mixture contained 250 mM sucrose, 2 mM EGTA, 5 mM MOPS (pH 7.4), 8 μM safranin O (A, C–E), 1 μM (A, D, E), 5 μM (C) or 30 μM (B) cytochrome *c*. Concentration of cytochrome oxidase proteoliposomes (COPL) was 8×10^{-3} mg protein $\times$ ml^{-1} (A,C,D,E) or 4×10^{-3} mg protein $\times$ ml^{-1} (B); $t = 26$°C. Additions, 5 mM ascorbate (A,C–E) or 3 mM ascorbate (B); 100 μM TMPD, 50 μM kCh, 1 mM NaN_3, 30 nM SF6847 (B). B, the cytochrome oxidase turnover numbers are indicated near the curves.

Table 3
Effects of SF6847, kCh, dihydrotestosterone (DTS) and estron on resistance of the asolectin planar bilayer membrane

Exp. N	Subsequent additions	R (Ohm)
1	–	2×10^{11}
	200 nM SF6847	3×10^{9}
	50 μM kCh	1×10^{9}
	50 μM DTS	5×10^{8}
	20 μM estron	3×10^{8}
2	–	2×10^{11}
	40 nM SF6847	5×10^{10}
	50 μM kCh	3×10^{10}

Incubation mixture, 150 mM KCl, 10 mM Tris-HCl, pH 7.2.

demonstrative only at low concentrations of cytochrome *c* (below 5 μM) when the respiration rate was small and the $\Delta\Psi$ level was not maximal (Fig. 5E). When the concentration of cytochrome *c* was sufficient to support a high level of the $\Delta\Psi$ generation (25 μM and above), the effect of kCh on $\Delta\Psi$ in the absence of SF6847 was not observed. Similar relationships were shown in proteoliposomes with cytochrome oxidase from *Rh. sphaeroides* (not shown).

3.6. Effect of kCh on BLMs

All attempts to show the recoupling effect of kCh on BLMs failed. Instead, kCh stimulated the drop of the BLM resistance induced by SF6847 or FCCP (Table 3). The time course of the BLM resistance in the presence of different SF6847 concentrations showed that a resistance decrease is always the first response to the addition of kCh on one or both sides of a BLM (time resolution, 30 s). Without uncouplers, kCh did not change the BLM resistance (not shown) (Table 4).

Summarizing the above data, we may conclude that recoupling by kCh is inherent in natural and reconstituted protein-lipid membranes being absent from BLM containing no proteins. A consequence of this conclusion is that in mitochondria, chromatophores and cytochrome oxidase proteoliposomes, uncoupling by SF6847, FCCP and CCCP is facilitated by protein component(s) and this effect is inhibited to some degree by kCh. This means that the above protonophores must be less effective in BLM than in protein-containing membranes.

In Fig. 6 the SF6847 sensitivity of BLMs is compared with that of various protein-containing membranes. According to this figure, the $\Delta\Psi$ levels in mitochondria and in cytochrome oxidase proteoliposomes as well as the BLM resistance are sensitive to low concentrations of the uncoupler. On the other hand, $\Delta\Psi$ in chromatophores is very much more resistant to SF6847. This result resembles the data of Miyoshi and Fujita [43] who showed that SF6847 uncouples in chloroplasts at concentrations two order of magnitude higher than in mitochondria. On the face of it, these results are paradoxical since there are lipid bilayer regions in the chromatophore and chloroplast membranes. Hence, their sensitivity to uncouplers should be at least as high as that of BLMs.

This problem was solved when we took into account that the concentration of SF6847 in the lipid phase must be much higher in the experiments with BLMs than that with the suspension of natural or reconstituted membrane vesicles. In fact, a BLM occupies such a small part of the experimental system that even small amounts of SF6847 added to the

Table 4
Three types of uncoupling effects in mitochondria

N	Uncouplers	Recouplers
1	Low concentrations of SF6847, FCCP, CCCP, CCP and platanetin	Low concentrations of kCh; testosterone, dihydrotestosterone, progesterone in the presence of BSA
2	Low concentrations of free fatty acids and DNP	In muscle and liver, carboxyatractylate, bongkrekic acid, ADP; in brown fat, ADP, GDP, ATP, GTP
3	Gramicidin, high concentrations of other uncouplers	Absent

Note: (i) Uncoupling by low concentrations of TTFB, dicoumarol, PCP and zearalenone can be partially reversed by high kCh concentrations; (ii) uncoupling by platanetin is DTS-resistant.

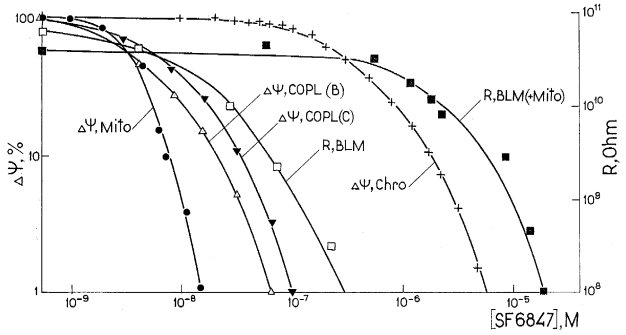

Fig. 6. Effects of SF6847 on mitochondria, cytochrome oxidase proteoliposomes, chromatophores and BLM. Conditions: for mitochondrial experiments, the incubation medium (see Section 2) was supplemented with 8 μM safranin O, concentration of the rat heart mitochondria was 0.7 mg protein$\times$ml^{-1}; for proteoliposome experiments, rotenone, succinate and oligomycin were omitted, whereas 5 mM ascorbate, 100 nM monensin, 200 nM TMPD and 0.1 μM cytochrome *c* were included, concentration of safranin O was 7 μM, that of proteoliposomes, 8×10^{-3} mg protein$\times$ml^{-1}; in experiments with chromatophores, the incubation medium contained 250 mM sucrose, 5 mM MOPS (pH 7.4), 2 mM ascorbate, 10 μM TMPD, 1 mM EGTA and oligomycin (8 μg$\times$ml^{-1}), the steady-state $\Delta\Psi$ level was measured as described in Section 2; experiments with BLM were performed as described in Section 2. Mito, the rat heart mitochondria; COPL(B), proteoliposomes with the bovine cytochrome oxidase; COPL(C), proteoliposomes with cytochrome oxidase from the *Rh. sphaeroides* chromatophores; Chro, chromatophores; R, BLM, electric resistance of BLM; R,BLM(+Mito), as R,BLM but rat liver mitochondria (0.7 mg protein$\times$ml^{-1}) were added to the electrolyte solutions separated by BLM.

water phase are sufficient to saturate this membrane. Equal SF6847 quantities added, say, to a mitochondrial suspension are immediately absorbed by the large amount of mitochondrial membranes, giving significantly lower uncoupler concentration in the water phase than in the case of the BLM. Since the uncoupler concentration in a membrane is equilibrated with that in water, this means that a concentration of SF6847 added to mitochondria must, in the simple case, be much less efficient than the same SF6847 concentration added to a BLM system. To equalize the acting concentrations of SF6847 in studies on mitochondria and BLMs, we supplemented the BLM-separated solutions by the same amounts of mitochondria as in experiments on measurements of mitochondrial respiration and $\Delta\Psi$. This strongly increased the concentrations of SF6847 required to cause measurable lowering of the BLM electric resistance (cf. curves 'R, BLM' and 'R, BLM(+Mito)' in Fig. 6). In fact, these concentrations proved to be higher than those causing uncoupling in any lipid-protein membranes tested, including chromatophores.

4. Discussion

Studies on the kCh recoupling clearly showed that this phenomenon could be demonstrated in quite different types of energy-transducing membranes, such as heart mitochondria [17], plant mitochondria [23], liver mitochondria, intact lymphocyte cells, bacterial chromatophores, proteoliposomes with animal or bacterial cytochrome oxidase (this paper). As an energy source, respiration or ATP hydrolysis (mitochondria and proteoliposomes) and light (chromatophores) could be used. Recoupling was shown by measuring the respiration rate (mitochondria and proteoliposomes) or the ATPase activity (mitochondria), by measuring $\Delta\Psi$ (by two methods, optically with safranin O or JC-1 and potentiometrically with a tetraphenyl phosphonium electrode) (lymphocytes, mitochondria and proteoliposomes), or by measuring the carotenoid shift (chromatophores). Recoupling was shown with different uncouplers, such as SF6847, FCCP, CCCP, CCP, platanetin and partially with TTFB, dicoumarol, PCP, zearalenone ([17,44] and this paper). Besides kCh, the 3-keto-derivative of cholesterol was shown to possess the recoupling activity, whereas cholesterol was ineffective ([17] and this paper). Thus, we may conclude that the kCh effect is related to a fundamental property of the mechanism of uncoupling by the most potent protonophores.

Obviously, we can exclude direct interaction of kCh and uncouplers (i.e., complex formation) since in this case the effect (a) could be seen also in BLMs, (b), should not disappear in time and (c) could depend on concentration of uncoupler rather than on the type of the coupling membrane. The last statement may be illustrated by the fact that, e.g., heart muscle mitochondria and chromatophores differ in the $C_{1/2}$ values for SF6847 uncoupling by almost two orders of magnitude, whereas the $C_{1/2}$ values for the kCh recoupling were the same. Special measurements

showed that kCh does not change the absorption spectrum of the SF6847 solution. Chaves et al. [45], who reproduced the kCh recoupling effect on the kidney mitochondria, have shown that the effective kCh concentrations are much lower than those required to form micelles. This excludes the possibility that kCh sequesters uncoupler by means of the micelle formation.

The absence of the kCh recoupling with BLMs indicates that protein(s) are involved in recoupling by kCh and, hence, in uncoupling by the SF6847-type protonophores. The involvement of proteins in uncoupling by fatty acids was already demonstrated by direct experiments. The proteins in question are thermogenin in brown fat [11–13], the ATP/ADP-antiporter [12–15,46] and cytochrome oxidase [47,48] in animal tissues other than brown fat. In our group, it was found that the uncoupling by low concentrations of DNP is partially mediated by the ATP/ADP-antiporter, as indicated by a recoupling effect of carboxyatractylate (cAtr) [14]. The cAtr recoupling was also demonstrated with PCP and TTFB but was not so pronounced as with DNP and especially with fatty acids [17].

Involvement of a carrier in effect of DNP, PCP and TTFB on mitochondria was postulated by Yaguzhinsky et al. [49]. With all the cAtr-sensitive uncouplers, kCh proved to be of low efficiency. In fact, kCh could not influence the fatty acid- and DNP-induced uncoupling and only partially decreased, under high concentrations, uncoupling by low amounts of PCP and TTFB.

It should be stressed that uncoupling by high concentrations of any uncoupler tested, as well as by any concentration of gramicidin was resistant to both kCh and cAtr (Table 1). Such uncoupling can be explained in terms of the classical Mitchellian theory [1,2] and can be readily reproduced with BLMs [3–7]. The apparent correlation of the absolute values of efficiencies of low concentrations of different protonophores in mitochondria and BLMs [4–6] is, most probably, a result of superposition of two oppositely-directed effects. On the one hand, a BLM is, in fact, much less sensitive to uncouplers due to the absence of uncoupling- facilitating proteins. On the other hand, the uncoupler/lipid phase volume ratio is much higher in the BLM experiments than in the mitochondrial experiments, a fact which must increase the apparent sensitivity of the BLM to uncoupler (Fig. 6).

According to our hypothesis [12,13], thermogenin and ATP/ADP-antiporter mediate uncoupling by fatty acids and some other protonophores by facilitating the transport of anionic form of protonophores. Recently this hypothesis has received experimental support from Garlid and co-workers [11,50]. Mechanism of uncoupling by low concentrations of SF6847, FCCP and CCCP is apparently different from that of fatty acids because it is not sensitive to cAtr or purine nucleotides suppressing the antiporter or thermogenin-mediated uncoupling. Instead, this uncoupling is sensitive to kCh, which appears to be ineffective in the case of protonophores interacting with antiporter or thermogenin.

It is known that kCh increases the membrane dipole potential, thus enhancing the binding and translocation rate of lipophilic anions [18,51]. This may explain stimulation by kCh of the SF6847-induced decrease in the BLM resistance (Table 3) and stimulation of the SF6847 uncoupling in chromatophores when they were incubated with kCh for a long time (Fig. 4A,C).

On first sight, the immediate action of kCh on mitochondria, proteoliposomes and chromatophores was just opposite, i.e., the attenuation and even complete arrest of the SF6847 uncoupling, if the SF6847 concentration was not too high. Such recoupling, however, disappeared in time after kCh addition. It is important to note that the decay of the kCh recoupling effect in chromatophores is strongly slowed down by decreasing the temperature from 25°C to 10°C (Fig. 4B). This may be accounted for by a decrease in rate of the flip-flop of kCh in the chromatophore membrane, due to lipid phase transition ('freezing') of the membrane when the temperature is decreased to 10°C. It should also be noted that low kCh concentrations inhibit the action of those uncouplers that have been shown to cross protein-containing membranes in the form of anions, A^- (FCCP, CCCP, SF6847), rather than of dimers composed of an anionic and a protonated species, HA^-_2(TTFB, DNP, PCP) [52].

A possible explanation of the kCh recoupling effect is the following. Being added to mitochondria, proteoliposomes or chromatophores, kCh incorporates first into the outer leaflet of their membranes,

thus inducing a strong asymmetrical increase in the membrane dipole potential. Apparently such an event decelerates the protein-mediated movement of the uncoupler anion in the membrane. This immediate effect of kCh disappears in time due to the flip-flop of kCh in the membrane, resulting in occupation of kCh of both leaflets and, hence, in disappearance of the dipole potential asymmetry. Only after this, a kCh effect favorable for uncoupling (i.e., increase in the uncoupler anion binding and the translocation rate) can be seen.

To explain why small amounts of kCh inhibit uncoupling by SF6847, being inefficient with TTFB, we can assume that dipole asymmetry inhibits, for some unknown reason, the protein-mediated translocation of the uncoupler anion monomer (A^-) rather than that of a complex of A^- with protonated uncoupler (HA^-_2). This assumption accounts for the fact that moderate kCh concentrations increase from 1 to 2 the slope of dependence of the mitochondrial respiration rate on the uncoupler concentration (Fig. 1B). The observed back-shift of the slope from 2 to 1 at high kCh concentrations is also compatible with the above explanation, taking into account that kCh action is, in fact, determined by the superposition of two oppositely-directed processes, i.e., an inhibition of a protein-mediated transport of A^- and a increase in binding and translocation rate of A^- in the phospholipid bilayer.

It should be stressed that even in the presence of kCh, the SF6847-type uncouplers are more efficient in mitochondria and cytochrome oxidase proteoliposomes than in BLM (cf. Fig. 2C and Fig. 6). Apparently, cytochrome oxidase and, perhaps, also some other protein(s), are still involved, to some degree, in uncoupling in spite of the kCh addition. The protein(s) in question should be much more efficient in the mitochondrial membrane than in chromatophores since chromatophores are much less sensitive to SF6847 than mitochondria. As to the cytochrome oxidase proteoliposomes, they were slightly less sensitive than mitochondria (see Fig. 6). A similar range of efficiencies was shown for kCh as a recoupler (mitochondria > proteoliposomes > chromatophores, cf. Fig. 2C, Fig. 4C and Fig. 5A).

Summarizing the above results, the following scheme of uncoupling by SF6847 in mitochondria can be proposed.

A. In the absence of kCh or in the presence of kCh on both sides of the membrane:

$$A^-_{out} + H^+_{out} \rightarrow AH_{out} \quad (1)$$

$$AH_{out} \rightarrow AH_{in} \quad (2)$$

$$AH_{in} \rightarrow A^-_{in} + H^+_{in} \quad (3)$$

$$A^-_{in} + Pr \rightarrow (A^-_{in}Pr) \quad (4)$$

$$(A^-_{in}Pr) \rightarrow (A^-_{out}Pr) \quad (5)$$

$$(A^-_{out}Pr) \rightarrow A^-_{out} + Pr \quad (6)$$

where Pr is for protein(s) facilitating translocation of the uncoupler anion (A^-).

B. In the presence of kCh on one side (reactions 4–6 are inhibited): (1)–(3) as above, then

$$A^-_{in} + AH_{in} \rightarrow A_2H^-_{in} \quad (7)$$

$$A_2H^-_{in} + Pr \rightarrow (A_2H^-_{out}Pr) \quad (8)$$

$$(A_2H^-_{out}Pr) \rightarrow 2A^-_{out} + Pr + H^+ \quad (9)$$

In the case of TTFB the B-type mechanism is faster than the A-type one even if moderate concentrations of kCh are added. It is inhibited only at high kCh concentrations.

For fatty acids and DNP, the role of the uncoupling-facilitating protein Pr is performed mainly by the ATP/ADP-antiporter. For the SF-type, (i) other protein(s) are involved or (ii) the antiporter translocates uncouplers in a cAtr-insensitive fashion. In any case, cytochrome oxidase can also take part since in the cytochrome oxidase proteoliposomes, kCh is operative and the efficiency of SF6847 is much higher than in BLM.

The *Rh. sphaeroides* chromatophores are interesting in that (a) they still respond to kCh (although much more weakly than mitochondria or proteoliposomes with the *Rh. sphaeroides* cytochrome oxidase), and (b) the SF6847 efficiency is much lower than in mitochondria and proteoliposomes but still slightly higher than in BLMs (see Figs. 5 and 6).

The *Rh. sphaeroides* used as a source of chromatophores was growing anaerobically at high illumination, i.e., under conditions when the amount of cytochrome oxidase in the chromatophore membrane is rather low. Apparently other chromatophore proteins are much less active in facilitation of the SF6847 uncoupling. This means that the facilitation of uncoupling by SF6847 is hardly a property inherent in all the membrane proteins.

On the face of it, kCh recoupling of the SF6847-treated mitochondria looks like an interplay of two artificial modifiers of mitochondrial functions. However, it should be stressed that not only 6-keto-cholestanol, but also its 3-keto analog, a natural product of the oxidation of cholesterol in animal tissues, proved effective as recoupler, the effect being similar to that of kCh. Some steroid hormones possessing keto group in position 3, i.e., testosterone, dihydrotestosterone and progesterone, were shown to recouple SF6847-treated mitochondria [53,54].

As for the SF-type uncouplers, they might be analogs of some naturally occurring regulators of oxidative phosphorylation, just as DNP and other cAtr-sensitive artificial uncouplers mimic, in fact, the action of natural uncouplers, fatty acids. In this respect, we can mention thyroid hormones. Hydrophobic part of these hormones is structurally similar to SF6847 (for discussion, see Refs. [16,54,55]). In the accompanying paper [54], the data will be described showing some specific relationships of uncoupling and recoupling effects of thyroid and steroid hormones, respectively. Moreover, it is noteworthy that kCh affects uncoupling by platanetin, the natural uncoupler found in a plant [56,57] and by zearalenone, another natural uncoupler regulating development of plants [58] (about the kCh-induced recoupling in the plant mitochondria, see Refs. [23,44]).

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