On the mechanism by which 6-ketocholestanol protects mitochondria against uncoupling-induced Ca^{2+} efflux

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Abstract This work shows that 6-ketocholestanol (kCh) inhibits the effect of carbonyl cyanide-*m*-chlorophenyl hydrazone (CCP) on mitochondrial Ca^{2+} efflux. Such an effect proved to be caused by diminution of membrane fluidity, therefore, it is affected by the incubation temperature. Furthermore, kCh reversed CCP-induced Ca^{2+} efflux depending on the accumulation of phosphate. It is also shown that kCh enhances the effect of carboxyatractyloside on membrane permeability transition.

Key words: Mitochondrial Ca^{2+}; Calcium; Mitochondrion (kidney); Permeability transition; 6-Ketocholestanol; Mitochondrial membrane

1. Introduction

Uncouplers abolish transmembrane potential by acting as protonophores [1]. As a result of the uncoupling, energy-linked functions, such as Ca^{2+} retention, become depressed [2]. The property of a molecule to act as uncoupler mainly depends on its pK and on its ability to be solubilized in the lipid milieu of the inner mitochondrial membrane [1]. Franklin and Cafiso [3] have shown that the incorporation of 6-ketocholestanol (kCh) to vesicles, formed by phosphatidyl choline, produces an increase in the binding and transport of hydrophobic ions by increasing the membrane dipole potential. Starkov et al. [4] found that kCh decreased the effect of uncouplers in heart mitochondria: kCh inhibited the collapse of membrane potential induced by CCP, FCCP and SF6847. Similarly, Vianello et al. [5] described that kCh prevented or reversed the effect of uncouplers in plant mitochondria. However, in a recent work, Starkov et al. [6] established that in in phospholipid planar membranes kCh stimulated, instead of inhibiting, the uncoupling effect of FCCP. The present study was designed to explore the effect of kCh on mitochondrial Ca^{2+} release induced by CCP, carboxyatractyloside (CAT) and menadione (MD). The results show that kCh inhibited Ca^{2+}-releasing action of CCP, depending on the incubation temperature. Furthermore, kCh reverted the effect of the uncoupler, through a mechanism which depended on the accumulation of phosphate. In addition, it was found that kCh increased the effect of CAT on mitochondrial Ca^{2+} efflux and slightly inhibited the effect of MD on matrix Ca^{2+} content.

2. Materials and methods

Mitochondria from rat kidney were prepared by homogenization of the tissue in 0.25 M sucrose-1 mM EDTA pH 7.3 as described [7]. Calcium movement was followed in a double-beam spectrophotometer at 675–685 nm, using the dye Arsenazo III [8]. The incubation mixtures essentially contained 125 mM KCl; 10 mM succinate; 10 mM HEPES; 2 mM phosphate; 50 μM $CaCl_2$; 75 μM Arsenazo III; 200 μM ADP; 5 μg rotenone; and 5 μg oligomycin; the media were adjusted to pH 7.3 with KOH. Final volume 3 ml. Mitochondrial swelling was analysed spectrophotometrically, at 540 nm, by incubation of mitochondria in the same medium described above, except that Arsenazo III was not added. Fluorescence polarization was measured in mitochondria (2 mg protein) incubated in 2 ml of media containing 0.25 M sucrose adjusted to pH 7.3 with Tris base. In addition, the media contained 1 μM 1,6-diphenyl-1,3,5-hexatriene (DPH) solubilized in dimethylformamide. The mixtures were incubated at 25°C during 30 min. After this time, the samples were incubated during 15 min with increasing concentrations of kCh. Each sample was subjected to polarization analysis at 25°C in a fluorometer; the excitation wavelength was 340 nm and the emission was 417 nm [9]. ADP exchange reaction was performed at 25°C by using 20 μM [^{14}C]ADP (sp. act. 4000 cpm/nmol); the media contained 125 mM KCl; 10 mM HEPES pH 7.3, 5 μg rotenone, and 5 μg oligomycin; final volume 1 ml. After 1 min of incubation, an aliquot of 0.2 ml was filtered through a Millipore filter of 0.45 μm pore diameter [10]. Determination of the critical micelle concentration of kCh was estimated by following the fluorescence increase of 8-anilinenaphthalene sulfonic acid (ANS) at excitation and emission wavelengths of 360 and 475 nm, respectively. Other experimental conditions were as described in the corresponding legends to the figures. Protein was determined by the biuret method [11].

3. Results

The ability of kCh to block the releasing effect of CCP on mitochondrial Ca^{2+} content is shown in Fig. 1A. As indicated, in the absence of kCh, 0.5 μM CCP promotes the efflux of 60 nmol/mg protein of the accumulated Ca^{2+}. However, after the addition of 150 μM kCh to the incubation medium, a higher concentration (1.5 μM) of the uncoupler was required to discharge a similar amount of matrix Ca^{2+}, but at a lower rate. Fig. 1B shows the protective effect of increasing concentrations of kCh on the efficiency of 1.5 μM CCP to release Ca^{2+}. As observed, by varying the concentrations of kCh from 25 to 200 μM, a progressive inhibition on the CCP-induced Ca^{2+} efflux process was attained. Fig. 1C shows that 150 μM kCh, when added during the Ca^{2+} efflux phase, promoted a massive accumulation of the cation that had been previously released by 1.5 μM CCP. It should be noted that the incubation medium contained 6 mM phosphate (P_i). When the medium contained

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Abbreviations: kCh, 6-ketocholestanol; CCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; CAT, carboxyatractyloside; P_i, inorganic phosphate; MD, menadione; DPH, 1,6-diphenyl-1,3,5-hexatriene; ANS, 8-aniline-naphthalene sulfonic acid.

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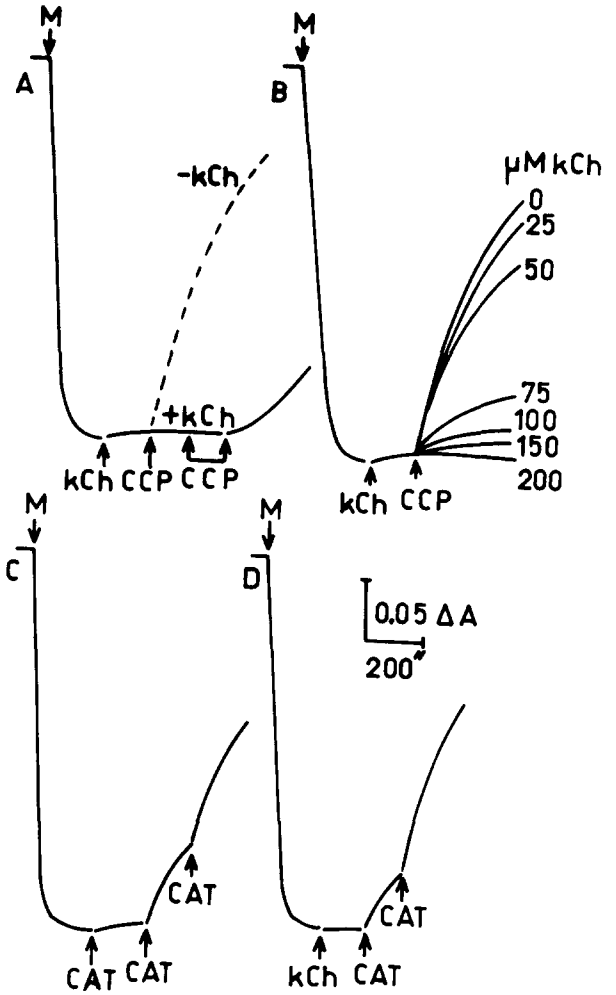

Fig. 1. The protective effect of kCh on CCP-induced mitochondrial Ca^{2+} release and the effect of phosphate on the reversion by kCh of CCP-induced Ca^{2+} release. 2 mg of mitochondrial protein were added to incubation media containing the reagents described in section 2. In trace A, where indicated, 150 μM kCh and 0.5 μM CCP were added. In trace B, the additions were 1 μM CCP and the indicated concentrations of kCh. It should be noted that the media contained 2 mM phosphate. In trace C, as indicated, the media contained 6 mM phosphate and, where indicated, the additions were as follows: 1 μM CCP and 150 μM kCh. In trace D, the media contained 2 mM phosphate and the additions were 1 μM CCP, 150 μM kCh and 2 mM phosphate. Volume 3 ml. The incubation temperature was maintained at 25°C.

2 mM P_i (Fig. 1D), kCh was unable to reverse the effect of CCP. Interestingly, further additions of P_i, to reach a concentration of 6 mM, did not re-established Ca^{2+} efflux. The latter would imply that kCh inhibited P_i transport; however, when the transport of phosphate was analysed by swelling experiments [12], no inhibition by kCh was observed (not shown).

The amphiphilic molecule of kCh might be susceptible of forming micelles which would be able to sequester CCP; thus, such possibility was examined. The determination of the critical micelle concentration for sodium cholate, sodium deoxycholate and Triton X-100, following the increase in ANS fluorescence gave values similar to those reported, i.e. 8.05 mM, 2.29 mM and 0.13 mM, respectively [13]. However, kCh or cholesterol, which as it is well-established does not form micelles, did not produce a shift in ANS fluorescence, but rather increased it quasi-linearly. In addition, the ANS fluorescence of Triton X-100 micelles was increased 5–6-fold by kCh, suggesting an increase in their hydrophobicity.

It is known that, besides its inhibitory action on ADP/ATP translocase, CAT induces a non-specific increase in membrane permeability [14]. The hyperpermeable state is mainly characterized by efflux of Ca^{2+} [15,16]. The effect of kCh on CAT-induced permeability transition is illustrated in Fig. 2. Trace A shows that 0.225 μM CAT brought about the efflux of 56 nmol matrix Ca^{2+}/mg protein. Interestingly, Fig. 2B shows that after the addition of 150 μM kCh, a lower concentration of CAT (0.15 μM) was required to promote the efflux of a similar amount of Ca^{2+}. It would appear that kCh increased the sensitivity of the adenine nucleotide translocase to CAT. However, 150 μM kCh did not improve the inhibitory effect of CAT on the ADP exchange reaction (not shown).

The physical properties of membranes, i.e. fluidity, can be changed by introducing cholesterol [17]. Taking into account that kCh is a cholesterol analogue, presumably its inhibitory effect on the uncoupling by CCP might be accomplished by diminution of membrane fluidity. This possibility was analysed evaluating the influence of kCh on the motion of the fluorescent probe DPH across the mitochondrial membrane. The results in Fig. 3 indicate that, indeed, at increasing concentrations of kCh a gradual diminution of the freedom in the mobility of the probe in mitochondrial membrane is observed.

To further examine the effect of kCh on membrane fluidity, we determined the influence of the incubation temperature on the inhibition by kCh of CCP-induced Ca^{2+} release. Fig. 4 shows that, indeed, at 37°C, when membrane fluidity was higher [18] the protective effect of kCh was abolished.

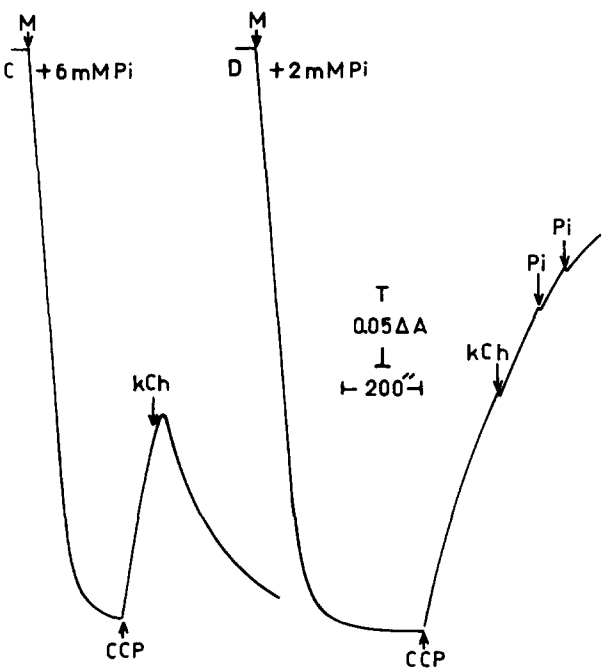

Fig. 2. The improving effect of CAT on Ca^{2+} release, induced by kCh. Experimental conditions as described in section 2. Where indicated, 150 μM kCh was added and subsequent additions of CAT (0.075 μM each time) were made. The media contained 2 mM phosphate. Final volume 3 ml. Incubation temperature 25°C.

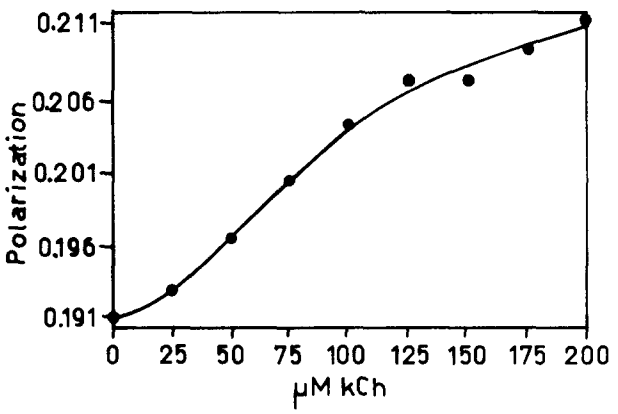

Fig. 3. The effect of kCh on the mobility of the fluorescent probe DPH. Experimental conditions were as described in section 2. Fluorescence polarization (P), was calculated according to the equation

$$P = \frac{I_v - G.I_H}{I_v + G.I_H}$$

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 positions, respectively, and

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

measured with the excitation polarized in the horizontal position. The curve fitting to the Monod, Wyman and Changeaux equation:

$$v = \frac{V_{max} S/K_s (1 + s/K_s)^{n-1}}{L + (1 + S/K_s)^n}$$

was made assuming that the value of polarization fluorescence at 0.191 was zero. The solid line represents the best fitting of the experimental points; the values of the parameters of the theoretical curve were V_{max} (maximal fluorescence polarization) = 0.217; K_s = 2.47;; n = 1.87; L = 997.8.

Therefore, it can be concluded that kCh inhibits the mobility of the uncoupler through the lipid phase of the membrane. To support this assumption we decided to investigate the effect of kCh on the action of MD on mitochondrial Ca^{2+} release. MD is a hydrophobic molecule which discharges mitochondrial Ca^{2+} by oxidizing membrane thiols [19], an effect not associated with the mobility of the reagent in the hydrophobic phase of the membrane. In agreement with the hypothesis of this study, 150 μM kCh only slightly inhibited MD-induced Ca^{2+} release, i.e. 10% (not shown).

4. Discussion

This study shows that kCh exerts a protective effect on mitochondria by decreasing their sensitivity to CCP. In consequence, a higher concentration of the uncoupler was required to promote matrix Ca^{2+} efflux. This effect of kCh might have been expected, considering that this reagent inhibits the collapse of membrane potential induced by uncouplers [4,5]. Nevertheless, this work establishes important points that merit attention: (i) the protective effect of kCh on CCP-induced Ca^{2+} release shows to be temperature-dependent; (ii) kCh decreases membrane fluidity; (iii) kCh reverses the effect of CCP through a mechanism which depends on the phosphate concentration; and (iv) kCh stimulates CAT-induced non-specific release of matrix Ca^{2+}, but it does not affect MD-induced Ca^{2+} release.

Recently, it has been reported that kCh increases the magnitude of the membrane dipole potential, thus, stimulating the binding of hydrophobic anions [2]. In contrast, Starkov et al. [4] and Vianello et al. [5] found that kCh inhibits the effect of uncouplers; therefore, they propose that kCh blocks the binding of protonophores to a membrane protein responsible for the uncoupling effect. In agreement to the proposal of these authors, the findings in this work suggest the possibility that by decreasing membrane fluidity kCh restrains the mobility of the uncoupler in such a way that this reagent would not interact with the above cited protein or that the diminution in membrane fluidity would arrest the activity of the complex protein-CCP. The assumption that kCh diminishes membrane fluidity is based on the observations that kCh restrained the movement of DPH across the lipid milieu of mitochondrial membrane (Fig. 3) and that kCh at 37°C, when membrane fluidity was higher, was unable to inhibit the effect of the uncoupler CCP.

On the other hand, in this work it is shown that kCh increases the effect of CAT on membrane permeability transition (Fig. 2). This result appears to be in agreement with the observation that kCh increases the binding of hydrophobic anions to membranes [3]. Interestingly, this effect was not reflected on an improvement of the CAT-induced inhibition on ADP exchange. It should be noted that the binding site for CAT is located in the cytosol side of the ADP/ATP translocase [20]. Finally, the phosphate requirement for the effect of kCh can

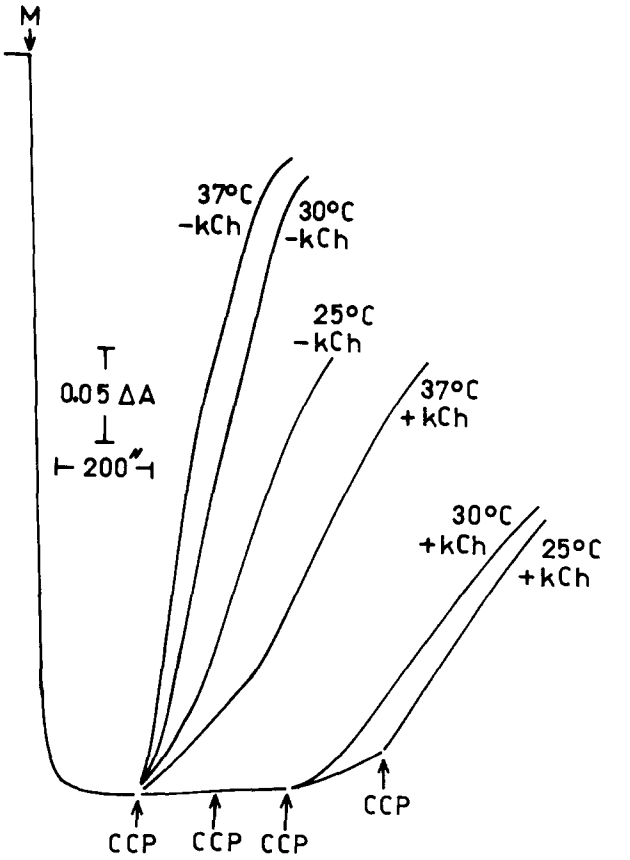

Fig. 4. The effect of the incubation temperature on the protective action of kCh on CCP-induced Ca^{2+} release. Experimental conditions as indicated in section 2, except for the incubation temperatures that were as indicated at the side of the tracing. The additions were: 150 μM kCh and 0.5 μM CCP.

be rationalized, considering that P_i is a necessary component for the electrophoretic uptake of Ca^{2+} [21]; in addition, the increased phosphate accumulation conduces to the formation of insoluble Ca-phosphate deposits [22] that stimulate Ca^{2+} retention.

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