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Inhibition of uncoupled respiration in tumor cells

A possible role of mitochondrial Ca^{2+} efflux

Vladimir L. Gabai

Medical Radiology Research Center, Russian Academy of Medical Sciences, Obninsk 249020, Russian Federation

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Uncouplers CCCP (2–4 μM) or DNP (200–400 μM) when added to EL-4 thymoma or Ehrlich carcinoma ascites cells initially stimulated endogenous respiration about 2-fold but then inhibited it to a first-order rate 20–25% of controls. This inhibition was accelerated by intracellular acidification or by A23187, a Ca^{2+}/H^+-antiporter (i.e. when mitochondrial Ca^{2+} efflux was stimulated) whereas Ruthenium red, an inhibitor of uniporter-driven Ca^{2+} efflux, significantly slowed down the effect of uncouplers. The respiratory inhibition was associated with NAD(P)H oxidation and was partially reversed by exogenous substrates (glutamine or glucose). In the permeabilized cells, endogenous and glutamine-supported respiration was inhibited by EGTA, while succinate-supported respiration was Ca^{2+} independent. It is suggested that mitochondrial Ca^{2+} is necessary for NADH-dependent respiration of tumor cells, and uncouplers inhibit it by activation of mitochondrial Ca^{2+} efflux.

Mitochondrial Ca^{2+}; Respiration; Uncoupler; Ascites tumor cell

1. INTRODUCTION

Mitochondrial Ca^{2+} transport has been intensively studied but its role in cellular physiology is still not understood. Two main possibilities are discussed here: (i) protection of a cell from injurious effect of Ca^{2+} overload; and (ii) regulation of cellular respiration by Ca^{2+}_m via activity of key NADH-generating enzymes of Krebs cycle (pyruvate-, isocitrate- and α-ketoglutarate-dehydrogenases) [1–3]. In isolated mitochondria, there is much evidence supporting the above mechanisms but no clear evidence has been obtained in whole cells. Recently, for example, the Ca^{2+}_m level was monitored under cell stimulation with exogenous ATP. A short-term increase in Ca^{2+}_m was observed which was prevented in uncoupler-treated cells; however, respiratory stimulation was not reported [4].

Mitochondria of tumor cells have a higher Ca^{2+}-accumulating capacity and are more resistant to Ca^{2+} overload than that of normal cells [5–7]. In EAC mitochondria, inhibition of ADP-stimulated respiration by Ca^{2+} was reported; this inhibition was reversed by uncouplers and the suppression of H^+-ATPase or ATP/ADP-translocase was suggested to be responsible for this effect of Ca^{2+} [6,8]. When hepatoma and liver NAD^+-dependent isocitrate and α-ketoglutarate dehydrogenases were studied, much higher inhibition of isocitrate dehydrogenase activity at subsaturating $[Ca^{2+}]$ was observed in hepatoma mitochondrial extracts compared with rat liver ones [9]. However, the significance of these findings for tumor cell energy metabolism has not been evaluated.

In this report, dependence of respiration on Ca^{2+} was studied in tumor cells and the results obtained indicate that efflux of Ca^{2+}_m inhibits NADH-dependent respiration in them.

2. MATERIALS AND METHODS

The EL-4 ascites thymoma and EAC were grown as described previously [10]. The isolated cells were washed by HBSS with 20 mM HEPES buffer (pH 7.4) and resuspended in the same medium. Their viabilities estimated by Trypan blue staining were 95%.

Cell respiration was monitored by a Clark oxygen electrode at 37°C in Ca^{2+}-free HBSS with 5 mM phosphate buffer at different pH (6.0–7.3). Permeabilization of the cells was performed by 50–80 μM of digitonin in the medium containing: KCl 120 mM, NaCl 10 mM, K_2HPO_4 1 mM, $NaHCO_3$ 5 mM, HEPES 10 mM (pH 7.1).

Intracellular pH was determined with 12 μM of fluorescein diacetate and $[Ca^{2+}]_i$ was evaluated with Quin-2AM (30 μM) as described previously [10,11].

Cellular NAD(P)H fluorescence was measured at λ_{ex} = 340 nm and λ_{em} = 460 nm.

CCCP, HEPES, fluorescein diacetate and RR were from Sigma; rotenone, DNP and glutamine were from Serva; oligomycin was from Reanal; Quin-2AM was from Calbiochem; all other reagents were of analytical grade.

Correspondence address: V.L. Gabai, Medical Radiology Res. Ctr., Russian Academy of Medical Sci., 4 Korolev's st., Obninsk 249020, Russian Federation. Fax: (7) (095) 255-2486.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; EAC, Ehrlich ascites carcinoma; HBSS, Hank's balanced salt solution; $[Ca^{2+}]_i$, intracellular free Ca^{2+}; Ca^{2+}_m, intramitochondrial Ca^{2+}; pH_i, intracellular pH; RR, Ruthenium red.

3. RESULTS

Initially the effect of $[Ca^{2+}]_i$ elevation on the endogenous respiration of EL-4 tumor cells was studied. Addition of ATP (0.1–1 mM), which induces intracellular Ca^{2+} mobilization [4,12], rapidly increased $[Ca^{2+}]_i$ about 4-fold (from 64 ± 10 to 253 ± 48 nM), but no respiratory activation was observed (data not shown). Other ways of increasing $[Ca^{2+}]_i$, e.g. by inhibition of Ca^{2+}-ATPases with vanadate (100 μM) and quercetin (100 μM) [1], also did not activate cellular respiration, but rather slightly (20-25%) inhibited it. These results indicate that an increase in $[Ca^{2+}]_i$ plays no significant role in the activation of EL-4 tumor cell respiration. On the contrary, oligomycin (1 μg/ml), an inhibitor of mitochondrial H^+-ATPase, suppressed endogenous respiration in EL-4 and EAC to 15–20% of controls while uncouplers (2 μM of CCCP and 100 μM of DNP) stimulated it to 220 ± 10%; this shows that the inner mitochondrial membrane proton permeability is the main controlling factor of tumor cell respiration.

However, an unusual effect was found after more prolonged incubation with the uncouplers: the respiratory activation changed to strong respiratory inhibition; this inhibition was partly reversed (after lag-phase) by main respiratory substrates of tumor cells, glutamine and glucose (Fig. 1a). This effect was not due to endogenous substrates (usually fatty acids [5]) exhaustion, since without uncouplers the cells could maintain their initial respiratory rate for more than 1 h (not shown),

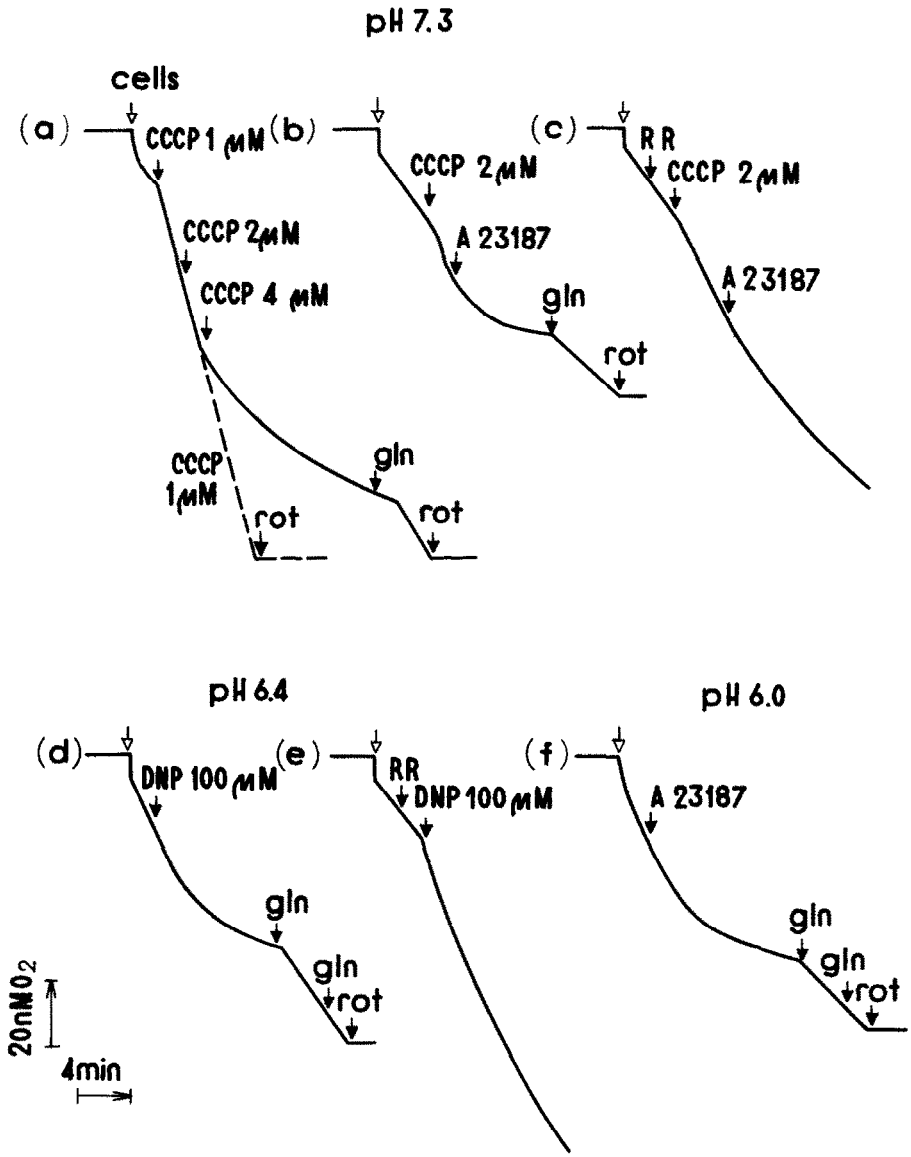

Fig. 1. Effect of uncouplers on EL-4 tumor cell respiration under different conditions. (a–c) pH 7.3; (d,e) pH 6.4; (f) pH 6.0. Rotenone = 2 μM; A23187 = 10 μM; Ruthenium red = 5 μM; glucose (glc) = 1 mM; glutamine (gln) = 1 mM. Approx. 2×10^7 cells/ml was added in each case (↓). Initial respiratory rates at different pH's were 0.6–0.7 nM O_2/min · 10^6 $cells^{-1}$. Ca^{2+}-free HBSS (+ 1 mM of EGTA) was used with A23187.

Table I

Effect of pH, A23187, Ruthenium red and oligomycin on first-order rate constant (k) of endogenous respiration of EL-4 and EAC cells in the presence of uncouplers

pH_{out}	pH_i EL-4	Compounds	k (min^{-1}) × 10^{-3}	
			EL-4	EAC
7.3	6.96 ± 0.15	CCCP 2 μM	45.0	172
		+ A23187	31.0	92.4
		RR + CCCP + A23187	78.6	–
		CCCP 4 μM	27.4	128
		oligomycin + CCCP	56.0	–
		DNP 200 μM	25.4	–
		+ A23187	17.7	–
6.4	6.56 ± 0.07	DNP 100 μM	20.9	57.5
		RR + DNP	47.4	–
		DNP 200 μM	13.2	–
6.0	6.26 ± 0.10	CCCP 1 μM	11.9	–
		A23187	14.8	43.3

A23187 = 10 μM; RR = 5 μM; oligomycin = 2.5 μg/ml. The rate constants were calculated for 10^7 cells. pH_i was measured as described in section 2.

and low concentration of uncouplers (1 μM of CCCP or 50 μM of DNP) stimulated respiration without subsequent inhibition (Fig. 1a). We suggested that this respiratory inhibition may depend on Ca^{2+}_m efflux, a well-known effect of uncouplers on mitochondria [2,7,13]. Indeed, the uncouplers increased $[Ca^{2+}]_i$ in EL-4 and EAC cells about 3-fold after 15–30 min of incubation (not shown). Moreover, activation of this efflux by $Ca^{2+}/2H^+$-antiporter (A23187) [2,7] in Ca^{2+}-free medium significantly accelerated the respiratory inhibition, while Ruthenium red (5 μM), an inhibitor of uniporter-driven Ca^{2+} efflux [2,7] greatly slowed down the effect of uncouplers (Fig. 1b,c). The effect of protonophores was also significantly accelerated after intracellular acidification (Fig. 1d; Table I); in this case, RR also suppressed the effect (Fig. 1e). At low pH (6.0) A23187 alone had high inhibitory effect on respiration which was partially reversed by exogenous substrates (glutamine or glucose) (Fig. 1f).

The kinetics of oxygen consumption by the tumor cells in the presence of uncouplers is zero-order process at low concentration of them but became first-order at high concentrations indicating substrate limitation; after exogenous substrates additions, the respiration increased and again became zero-order process (Fig. 1). The first-order rate constants (k) of the respiration for EL-4 and EAC under different conditions are shown in Table I. As one can see, EAC had much higher k, i.e. their respiration was inhibited by protonophores not so rapidly as in EL-4 cells. However, as in EL-4 cells, intracellular acidification and A23187 decreased this rate (Table I). Besides RR, the respiratory inhibition by uncouplers was also decelerated by oligomycin, probably since it increased mitochondrial membrane potential and Ca^{2+} accumulating capacity. Both these agents were effective only when added before protonophores (not shown).

In Table II, the effect of CCCP and A23187 on the stationary respiratory rate in EL-4 cells is shown. The lowest respiration was observed with endogenous substrates when both CCCP and A23187 were added. A23187 alone inhibited both endogenous and glutamine-supported respiration while no respiratory inhibi-

Table II

Effect of A23187 and CCCP on the stationary respiratory rates of EL-4 cells with different substrates

Substrates	Respiratory rate (% of control)		
	A23187	CCCP	A23187 + CCCP
Endogenous	65 ± 4*	27 ± 3*	21 ± 5*
Glucose	96 ± 8	86 ± 9	60 ± 6*
Glutamine	80 ± 7*	101 ± 5	82 ± 6*

The data shown are means ± S.E.M. of 3–8 experiments. *$P < 0.05$ comparing with control. CCCP = 2 μM; A23187 = 10 μM; glucose and glutamine = 1 mM; pH 7.3. The initial rates of respiration with either substrates were identical and taken as 100%.

tion was observed with glucose. CCCP alone inhibited endogenous respiration more than 3-fold but no inhibition was observed with glucose or glutamine. However, A23187 + CCCP resulted in respiratory inhibition with all substrates studied (Table II). The similar results was obtained with EAC cells (not shown).

The above results clearly indicate that substrate availability is an important factor of the respiratory inhibition by uncouplers. However, since a direct effect of uncouplers on the respiratory chain cannot be excluded, we compared their action with that of rotenone, an inhibitor of NADH-dehydrogenase, on cellular NAD(P)H level. As shown in Table III, CCCP, unlike rotenone, did not increase NAD(P)H level but decreased it. The similar results were obtained in EAC and when DNP was used instead of CCCP (not shown). Moreover, both glucose and glutamine partially reduced NAD(P)H (Table III) and this correlated with the recovery of the respiration (Fig 1, Table II). This means that the uncouplers had no direct effect on the respiratory chain. Hereof it is obvious that the observed effect of uncouplers on the respiration may be explained by their effects on respiratory chain substrate (NADH) availability. The first-order kinetics of the respiratory inhibition and lag-phase before recovery of respiration after exogenous substrates addition (Fig. 1) also supports the above suggestion.

Finally, the effect of Ca^{2+} on respiration was studied in permeabilized EAC cells, where EGTA and NADH-independent substrate, succinate, can be applied. EGTA significantly decreased both endogenous (not shown) and glutamine-supported respiration while Ca^{2+} reversed the effect of EGTA; succinate-supported respiration was not inhibited by EGTA (Table IV). Thus, these results directly demonstrate involvement of Ca^{2+} in regulation of NADH-dependent respiration in tumor cells.

4. DISCUSSION

Stimulation by uncouplers of Ca^{2+}_m efflux is well documented both in isolated mitochondria and intact cells, including tumor ones; this efflux occurs via Ca^{2+} uniporter which is specifically inhibited by RR [2,7,14]. Addition of A23187 to uncoupler-treated cells is widely used as a standard method for measuring of total cell Ca^{2+} by arsenaso-III [13,14]. However, to our knowledge, there was only one report where respiratory inhibition was correlated with mitochondrial Ca^{2+} efflux, namely in anoxia-treated hepatocytes [15]. Nevertheless, in the latter study, the respiratory inhibition was not strong (40–70%) since mitochondria of hepatocytes could substantially maintain their membrane potential under anoxic conditions.

Considering possible ways of mitochondrial Ca^{2+} efflux, we observed that intracellular acidification significantly accelerated the effect of uncouplers on respiration; moreover, at pH 6.0, A23187 alone inhibited it to the same extent as uncouplers (Fig. 1, Table I). Apparently, the activity of endogenous $Ca^{2+}/2G^{+}$-antiporter is low (or absent) in the mitochondria of EL-4 and EAC cells, but exogenous antiporter, A23187, stimulated Ca^{2+} efflux, especially at low pH, when mitochondrial pH gradient is high. Another antiporter, namely Ca^{2+}/Na^{+}-antiporter, may operate in the tumor cells [2].

Third way of Ca^{2+} efflux, cyclosporin A-sensitive pore [2,16], probably did not play any role under our conditions since: (i) low pH inhibited this pore [17]; (ii) CCCP + RR activated this pore [16] while we observed prevention of respiratory inhibition under these conditions; (iii) cyclosporin A (2 μg/ml) did not prevent the effect of uncouplers at pH 7.3 (Gabai, unpublished).

The effect of Ca^{2+} on the activity of key mitochondrial NADH-generating enzymes of Krebs cycle in normal cells is well documented [3], but, to our knowledge, there was only one report about their Ca^{2+} dependence in tumor cells, AS-30D hepatoma [9]. The latter study was performed, however, on the mitochondrial extracts. The present work on intact EL-4 and EAC cells supports the above study and suggest that Ca^{2+}_m indeed can regulate the activity of these enzymes and respiration in vivo.

Table III

Changes in cellular NAD(P)H fluorescence in uncoupler- and rotenone-treated EL-4 cells with different substrates

Substrates	Changes in F NAD(P)H (rel. units)		
	Control	CCCP	Rotenone
Endogenous	0	−29 ± 2	+15 ± 3
Glucose	−7 ± 2	−18 ± 2	+1 ± 2
Glutamine	+1 ± 1	−19 ± 2	+13 ± 2

CCCP = 4 μM; rotenone = 2 μM; glucose = 1 mM; glutamine = 1 mM. NAD(P)H fluorescence was determined after 10 min of incubation with the indicated compounds.

Table IV

Ca^{2+} dependence of glutamine and succinate-supported respiration in permeabilized EAC cells

Substrates	Respiration (% of control)		
	EGTA	EGTA + Ca^{2+}	EGTA + Ca^{2+} + CCCP
Glutamine	53 ± 7*	100 ± 6	19 ± 7*
Succinate	86 ± 9	60 ± 6*	89 ± 10

*$P < 0.05$ compared to controls. EGTA = 0.8 mM; Ca^{2+} = 1 mM; CCCP = 2 μM; glutamine = 1 mM; succinate = 1 mM. The cells were permeabilized with digitonin as described in section 2.

Summarizing, the following scheme may be proposed from our results:

$$\text{uncouplers} \rightarrow \bar{\mu}H^+ \text{ decrease} \xrightarrow[RR]{A23187,\ EGTA} Ca_m^{2+} \text{ efflux} \rightarrow \text{NADH-dehydrogenase(s) inhibition} \rightarrow \text{NADH decrease} \rightarrow \text{respiratory inhibition}$$

When glutamine or glucose were added to uncoupled cells, respiration can be partially recovered (Fig. 1a, Table II) since NADH generation increased (Table III). RR suppressed, and A23187 or EGTA (in permeabilized cells) stimulate the respiratory inhibition (Tables I, IV). No effect of uncouplers or EGTA was observed with succinate as respiratory substrate (Table IV). Thus, Ca_m^{2+} is involved in regulation of respiration in tumor cells, apparently being necessary for NADH generation.

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