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Features of the Uncoupling Effect of Fatty Acids in Liver Mitochondria of Mammals with Different Body Weight

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Abstract—In the presence of oligomycin, EGTA, and magnesium ions, the protonophore uncoupling activity of palmitate (V_{Pal}) is determined as the ratio of the acceleration of respiration with palmitate to its concentration. Under these conditions, V_{Pal} in liver mitochondria of one-month-old rats with the body weight of 50 g is 1.46-fold higher than in liver mitochondria of adult rats with the body weight of 250 g, whereas the uncoupling activity of FCCP does not depend on the age of the animals. The difference in V_{Pal} is mainly due to its component insensitive to carboxyatractylate and glutamate (V_{Ins}). This value is 2.9-fold higher in mitochondria of one-month-old rats than in those of adult rats. The protonophore activity of palmitate is similar in liver mitochondria of four-day-old and adult rats. In liver mitochondria of adult mammals (mouse, rat, guinea pig, rabbit), V_{Pal} decreases with increase in the body weight of the animals. In double logarithmic coordinates, the dependence of the V_{Pal} value on the body weight is linear with slope angle tangent of –0.18. The V_{Pal} value is mainly contributed by its component V_{Ins}. In the presence of calcium ions, palmitate induces the nonspecific permeability of the inner membrane of liver mitochondria (pore opening). This Ca^{2+}-dependent uncoupling effect of palmitate is less pronounced in mitochondria of one-month-old rats than in those of adult rats. In mitochondria of adult animals (mice, rats, and guinea pigs), the Ca^{2+}-dependent uncoupling activity of palmitate is virtually the same. It is concluded that the protonophore uncoupling effect of palmitate in liver mitochondria of mammals, unlike its Ca^{2+}-dependent effect, is associated with thermogenesis at rest and also with production of additional heat on cooling of the animals.

Key words: liver mitochondria, fatty acids, uncoupling, ADP/ATP antiporter, aspartate/glutamate antiporter, thermogenesis

Long-chained free (un-esterified) fatty acids play an important role in oxidative metabolism not only as substrates of oxidation but also as endogenous uncouplers of oxidative phosphorylation [1-15]. Different mechanisms of the uncoupling effect of fatty acids are known. In the absence of calcium ions in the incubation medium, the uncoupling effect of fatty acids is reminiscent of the effect of classic protonophore uncouplers (DNP, FCCP), but the protonophore effect of fatty acids, in particular, in liver mitochondria, is approximately 80% caused by involvement of protein carriers of substrates: the ADP/ATP- and aspartate/glutamate-antiporters [2-7]. During the uncoupling effect of fatty acids, these carriers act as two parallel and independent pathways of $\Delta\mu_{H^+}$ dissipation [6, 7]. This hypothesis is supported by the facts that the recoupling effects of carboxyatractylate (an inhibitor of the ADP/ATP-antiporter) and glutamate (or aspartate) (a substrate of the aspartate/glutamate-antiporter) are additive and independent on the succession of the corresponding additions [6, 7]. The remaining 20% of the uncoupling effect of fatty acids is supposed to be due to involvement of carriers of dicarboxylate [4, 8], of phosphate [9], and of a special uncoupling protein UCP-2, which is an analog of the uncoupling protein of brown fatty tissue mitochondria (UCP-1) [10]. However, fatty acids seem also to function as weak uncoupling protonophores without involvement of the above-mentioned proteins [11]. This third pathway of the uncoupling effect of fatty acids is insensitive to carboxyatractylate and glutamate and exists independently of the first two pathways [12]. The appearance of calcium ions in the incubation medium significantly changes the uncoupling

Abbreviations: DNP) 2,4-dinitrophenol; FCCP) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; UCP-1) uncoupling protein of brown fat tissue mitochondria; UCP-2) uncoupling protein analog of UCP-1; $\Delta\mu_{H^+}$) difference of electrochemical potentials of protons on the inner mitochondrial membrane; V_{FCCP}) specific uncoupling activity of FCCP; V_{Pal}) specific protonophore uncoupling activity of palmitate; V_C, V_G, V_{Ins}) components of the protonophore activity of palmitate (sensitive to carboxyatractylate, to glutamate, and insensitive to both these compounds, respectively).

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effect of fatty acids. In this case, fatty acids can induce the nonspecific permeability of the inner mitochondrial membrane for low-molecular-weight hydrophilic substances (pore opening), and this, in its turn, is accompanied by collapse of $\Delta\mu_{H^+}$ and swelling of the organelles [4, 13-15]. Such an uncoupling effect of fatty acids is always suppressed with the chelating agent EGTA and in many cases, but not always, with cyclosporin A [4, 13-15]. Thus, in the presence of calcium ions, fatty acids are reminiscent of numerous other inducers of the Ca^{2+}-dependent nonspecific permeability of mitochondria, many of which are not protonophores [16].

One of physiological functions of the uncoupling effect of fatty acids in mammals is production of heat to maintain the necessary temperature of the body [1-3]. The metabolically active liver of mammals significantly contributes to thermoproduction of the body both at rest under conditions of optimal temperature [17, 18] and on cooling [19]. The specific rate of thermoproduction (i.e., related to unit of body weight) at rest at optimal temperature (E) in mammals decreases with increase in the body weight (m) according to equation: $E = am^{-b}$, where a is a coefficient not depending on the animal species and b is an exponent equal, on average, to –0.25 [17, 18]. The rate of passive leakage of protons (J) in mitochondria isolated from the liver of various mammals, from a 20-g-weight mouse to a 200-kg-weight horse, decreases with increase in the body weight according to the equation: $J = am^{-0.14}$ [18]. The difference in values of the exponents in these two equations is explained by the existence, in addition to more intensive passive leakage of protons, of other mechanisms providing higher thermoproduction in animals with lower body weight, in particular, the increased number of mitochondria in hepatocytes [17]. The rate of thermoproduction in homoiothermic animals increases with decrease in the environmental temperature [1, 19]. This additional thermoproduction may be evaluated by the so-called thermoregulatory efficiency of metabolism, which is determined as a ratio of the increase in the thermoproduction rate by the increase in the difference between the animals' body and environmental temperatures [19]. Similarly to the specific rate of thermoproduction at rest, this parameter referred to the body weight significantly increases with decrease in the animals' body weight according to the above-presented equation, and the exponent (b) is –0.19 [19]. In work [18], the passive leakage of protons in mitochondria was determined in the absence of fatty acids. However, these endogenous uncouplers are always present in liver mitochondria, and their number can increase under some extreme conditions, in particular, on cooling of animals [1, 2, 20, 21]. Thus, if biological significance of the protonophore uncoupling effect of fatty acids in mitochondria is determined by production of additional heat in the liver cells, the intensity of this process will be higher in mitochondria isolated from the liver of animals with the lower body weight and follow the same pattern as the dependence of the rate of thermogenesis on the body weight.

In some diseases, the uncoupling effect of fatty acids is thought to be one of the causes of cell damage [20, 21]. Fatty acids were recently found to induce in animal cell cultures cell death of apoptotic and necrotic types [22-24]. These processes are suggested to be associated with induction by fatty acids and/or their metabolites of the calcium-dependent nonspecific permeability of mitochondria [22-24]. It is suggested that the biological significance of the calcium-dependent uncoupling effect of fatty acids should be induction of cell death and not production of additional heat on cooling; therefore, the intensity of this process should depend not on the animals' body weight but on other factors.

To elucidate the biological significance of uncoupling of oxidative phosphorylation by fatty acids, the purpose of this work was to comparatively study the protonophore and calcium-dependent mechanisms of the uncoupling effect of palmitate in liver mitochondria of animals with different body weight: rats of different age and other animals.

MATERIALS AND METHODS

The following animals were used: white randomly bred 6-8-month-old male rats with body weight of 240-260 g (adult rats), 30-35-day-old male rats with body weight of 45-55 g (one-month-old rats), four-day-old rats of both sexes, white randomly bred 2-3-month-old male mice with body weight of 25-29 g, randomly bred male guinea pigs with body weight of 550-650 g, and male rabbits with body weight of 3010-3300 g. All adult animals and also one-month-old rats were kept in a vivarium with food *ad libitum* at the temperature of 14-15°C. Four-day-old rats were taken from mothers immediately before sacrifice. Mitochondria were isolated from the liver of the animals by differential centrifugation [25]. To remove endogenous fatty acids, the mitochondria were preincubated with BSA made free of fatty acids as described in [25]. The isolation medium contained 250 mM sucrose, 1 mM EGTA, and 5 mM Mops-KOH (pH 7.4). The suspension of mitochondria (60-70 mg protein per ml) was stored on ice. The protein was determined by the biuret method with BSA as the standard. Mitochondria were isolated from livers of ten four-day-old rats, two one-month-old rats, three adult mice, one guinea pig, and one rabbit, and every time from the liver of one adult rat.

Respiration of mitochondria was recorded with a Clark type oxygen electrode in a thermostatted cell at 25°C. The incubation medium contained 200 mM sucrose, 5 mM potassium succinate, 20 mM KCl, 0.5 mM EGTA, 2 mM $MgCl_2$, and 5 mM Mops-Tris (pH 7.4). Immediately on addition of mitochondria (1.1-1.2 mg/ml), oligomycin (2 μg/ml) and rotenone (2 μM)

were added into the cell. To determine the maximal respiration rate, 50 μM DNP was added to the mitochondria. The dependence of the respiration rate of mitochondria on the concentration of DNP in the range from 10 to 100 μM was studied in special experiments, and 50 μM DNP was found to induce the maximal stimulation of respiration of liver mitochondria of all animals studied.

To quantitatively evaluate the protonophore uncoupling activity of palmitate or FCCP, new values were introduced: the specific uncoupling activity of palmitate (V_{Pal}) and the specific uncoupling activity of FCCP (V_{FCCP}). These two values are determined as the uncoupler-caused increase in the respiration rate referred to the uncoupler concentration according to the formula: $(J_u - J_o)/[U]$, where J_o and J_u are respiration rates of mitochondria (μM O_2 per min) without considering their concentration before and after the addition of the uncoupler, respectively, [U] is concentration of the uncoupler (μM for palmitate or nM for FCCP). It was preliminarily found that the respiration rate of mitochondria of all animals under study depended linearly on the concentration of palmitate or FCCP, as shown in Fig. 1 for liver mitochondria of adult rats. Fatty acids and also FCCP are known to have a very high lipid/water distribution coefficient [26, 27]; therefore, nearly all molecules of these uncouplers added to the mitochondria will bind to membranes of the organelles and will be involved in the uncoupling. Obviously, the number of fatty acid or FCCP molecules involved in the uncoupling will not depend on concentration of the mitochondria. Figure 1 shows that both palmitate and FCCP stimulate respiration of the mitochondria similarly, independently of concentration of the mitochondria. Values of V_{Pal} and V_{FCCP} were calculated in the presence of 25 μM palmitate and 20 nM FCCP, respectively. In the rat liver mitochondria the V_{Pal} value at the protein concentration of 0.8 mg/ml was 0.565 ± 0.023 μM O_2/min per 1 μM palmitate, and at the protein concentration of 1.2 mg/ml it was 0.581 ± 0.037 μM O_2/min per 1 μM palmitate, i.e., did not depend on concentration of the mitochondria. V_{FCCP} also did not depend on concentration of the mitochondria.

The V_{Pal} value is considered to consist of three parts: sensitive to carboxyatractylate (V_C), sensitive to glutamate (V_G), and insensitive to carboxyatractylate and glutamate (V_{Ins}). The first two values quantitatively characterize the involvement in the uncoupling of the ADP/ATP-antiporter and aspartate/glutamate-antiporter, respectively, and the third value characterizes in total all other structures involved in the uncoupling. Values of V_C or V_G were determined as the ratio of the carboxyatractylate- or glutamate-induced decrease in the respiration rate of mitochondria in the presence of palmitate (ΔJ_u) to con-

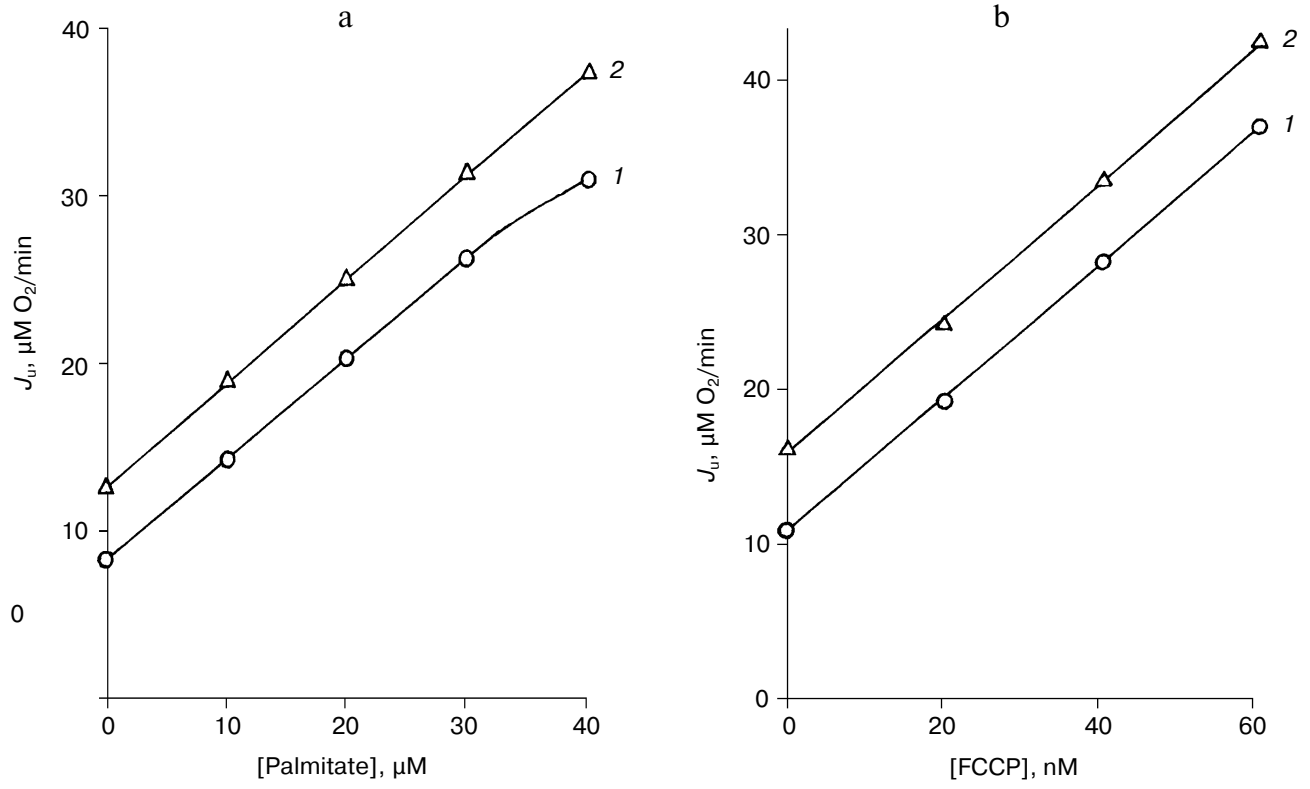

Fig. 1. Dependence of the respiration rate of mitochondria on concentration of palmitate (a) and FCCP (b) at the concentration of mitochondrial protein of 0.8 (*1*) and 1.2 mg/ml (*2*).

centration of the corresponding uncoupler by the formula: $\Delta J_u/[U]$. In the absence of palmitate, carboxyatractylate and glutamate failed to affect the respiration of mitochondria. The V_{Ins} value was determined as the difference between the respiration rates of mitochondria in the presence (J_{ur}) and in the absence of palmitate, carboxyatractylate, and glutamate to the concentration of palmitate by the formula: $(J_{ur} - J_o)/[U]$. On calculating all these values, the respiration rates of mitochondria were determined without considering their concentration at the palmitate concentration of 25 μM. Preliminary studies showed the independence of V_C, V_G, and V_{Ins} values of the concentration of mitochondria.

Recoupling effects of carboxyatractylate or glutamate were determined as the ratio between the inhibition of respiration with one of these recoupling agents and the stimulation of respiration with palmitate by the formula: $\Delta J_u/(J_u - J_o)$.

The calcium-dependent uncoupling effect of palmitate was assessed turbidimetrically at 620 nm by swelling of mitochondria using an SF-46 spectrophotometer at 20°C. In these experiments, the EGTA concentration in the isolation medium of mitochondria was decreased to 0.5 mM. The incubation medium contained 250 mM sucrose, 5 mM potassium succinate, and 5 mM Mops-Tris (pH 7.4). Concentration of the mitochondrial protein in the cuvette of the spectrophotometer was 0.6 mg/ml. In the experimental sample, oligomycin (2 μg/ml) and rotenone (2 μM) were added into the cuvette immediately on introduction of mitochondria, 1 min later 30 μM $CaCl_2$, and still 1 min later 20 μM palmitate were added, and the recording of changes in the optical density was started. Instead of $CaCl_2$, into one of the control samples 3 μl of H_2O was added and 3 μl of ethanol into another. In both cases, the optical density of the suspension of mitochondria was unchanged for 30 min. The rate of change in the optical density of the suspension of mitochondria (ΔA/min per mg protein) was calculated as the change in A within the first 30 sec of the recording.

The results are presented as mean values ± mean square error of the mean value.

Mops, Tris, palmitic acid, oligomycin, potassium succinate, potassium glutamate, carboxyatractylate, and BSA made free of fatty acids were from Sigma (USA); rotenone and EGTA were from Serva (Germany); sucrose, KCl, and $MgCl_2$ were from Merck (Germany). Solution of palmitic acid (20 mM) in ethanol was used.

RESULTS

In the absence of fatty acids, i.e., in state 4, and in the presence in the incubation medium of EGTA, magnesium ions, and oligomycin, the respiration of liver mitochondria is virtually completely determined by the passive leakage of protons [28, 29]. Therefore, the rate of passive leakage of protons can be assessed by the respiration rate of mitochondria under the above-mentioned conditions. In the presence of EGTA, magnesium ions, and oligomycin, fatty acids stimulate the respiration of mitochondria only due to their protonophore effect [7, 30]. This allows us, as reasoned earlier [30] and described in "Materials and Methods", to characterize the protonophore uncoupling effect of palmitate by a value determined as the difference between the respiration rate of mitochondria before and after the addition of fatty acid, with respect to concentration of the latter.

To study dependence of the uncoupling effect of fatty acids on the body weight of animals, in the present work two approaches were used: first, animals of the same species were used but of different age and, consequently, with different body weight; second, adult animals of various species different in body weight. In the first case, we used rats of three age groups: four-day-old rats, one-month-old rats taken from their mothers and existing independently for a week, and adult rats.

Compared to liver mitochondria of adult rats, liver mitochondria of newborn rats have significantly lower respiration rates in state 3 and at the complete uncoupling of oxidative phosphorylation and also higher rate of leakage of protons through the inner membrane [31-33]. During the first hours after the birth, these differences decrease but failed to fully disappear even after some days [32, 33]. Our studies have shown that in four-day-old rats the respiration rate of mitochondria at the complete uncoupling of oxidative phosphorylation with DNP is slightly different from the respiration rate of mitochondria of adult animals (Table 1). However, the respiration rate of mitochondria of four-day-old rats in state 4 was significantly higher than in adult animals. Addition of BSA free of fatty acids into the incubation medium fails to affect the respiration in state 4 of mitochondria of adult rats, but nearly twofold decreases the respiration of mitochondria of four-day-old rats (Table 1). Note that in the course of isolation these mitochondria were already deprived of endogenous fatty acids (see "Materials and Methods"). The inhibitory effect of BSA on the respiration of liver mitochondria of four-day-old rats seemed to indicate that these mitochondria should contain endogenous fatty acids.

Table 1 shows that in the absence of BSA, 25 μM palmitate stimulated the respiration of mitochondria of four-day-old and adult rats by 12.1 and 11.9 nmol O_2/min per mg protein, i.e., similarly. The V_{Pal} values were 0.574 ± 0.017 and 0.547 ± 0.023 μM O_2/min per 1 μM palmitate in liver mitochondria of four-day-old and adult rats, respectively. It is interesting to compare the protonophore uncoupling effect of palmitate with the effect of an artificial uncoupler, the protonophore FCCP, which has another mechanism of action [2]. In our experiments, V_{FCCP} values were 0.533 ± 0.012 and 0.528 ±

Table 1. Comparison of respiration rates of liver mitochondria (nmol O_2/min per mg protein) of adult and four-day-old rats in state 4 and on subsequent addition of palmitate, carboxyatractylate, glutamate, and DNP in the absence of BSA (A) and in the presence of BSA (0.5 mg/ml) (B). Additions in section A: 25 μM palmitate (Palm), 1 μM carboxyatractylate (Catr), 2 mM glutamate (Glu), and 50 μM DNP (DNP). Additions in section B: 40 and 30 μM palmitate (Palm) to mitochondria of adult and four-day-old rats, respectively; other additions as in section A

Additions	Adult rats ($n = 5$)	Four-day-old rats ($n = 5$)
A		
Without additions	10.3 ± 0.5	18.6 ± 0.6
Palm	22.2 ± 0.7	30.7 ± 0.9
Palm + Catr	16.9 ± 0.7	21.8 ± 0.8
Palm + Catr + Glu	12.4 ± 0.6	15.3 ± 0.9
Palm + Catr + Glu + DNP	80.9 ± 3.6	69.8 ± 2.0
B		
Without additions	10.2 ± 0.5	10.5 ± 0.4
Palm	23.8 ± 1.5	23.0 ± 0.6
Palm + Catr	17.9 ± 1.1	17.4 ± 0.4
Palm + Catr + Glu	12.7 ± 0.7	12.6 ± 0.4
Palm + Catr + Glu + DNP	81.3 ± 5.0	71.3 ± 6.8

Table 2. Comparison of the respiration rates of liver mitochondria (nmol O_2/min per mg protein) of adult and one-month-old rats in state 4 and on subsequent addition of palmitate (Palm), carboxyatractylate (Catr), glutamate (Glu), and DNP. Additions as in Table 1A

Additions	Adult rats ($n = 7$)	One-month-old rats ($n = 7$)
Without additions	9.9 ± 0.4	13.9 ± 0.5
Palm	21.4 ± 0.7	31.0 ± 0.9
Palm + Catr	16.2 ± 0.5	23.9 ± 0.8
Palm + Catr + Glu	11.9 ± 0.5	19.6 ± 0.8
Palm + Catr + Glu + DNP	80.4 ± 3.7	82.0 ± 4.0

0.011 μM O_2/min per 1 nM FCCP in liver mitochondria of four-day-old and adult rats, respectively. These findings suggest a similar manifestation of the protonophore uncoupling activity of palmitate and activity of FCCP in liver mitochondria of four-day-old and adult rats. The respiration of mitochondria of both groups of animals in the presence of palmitate was effectively suppressed with carboxyatractylate and glutamate (Table 1). In the absence of BSA, the combined effect of these recoupling agents inhibited the respiration of mitochondria of four-day-old rats to a value lower than the respiration rate in state 4 (Table 1). Consequently, in mitochondria of these animals carboxyatractylate and glutamate suppressed the effect not only of exogenous palmitate but also of endogenous fatty acids.

In the presence of BSA, when endogenous fatty acids able to uncouple oxidative phosphorylation were bound to this protein, carboxyatractylate and glutamate similarly suppressed the protonophore uncoupling effect of palmitate in liver mitochondria of both adult and four-day-old rats (Table 1). In this case, the recoupling effects of carboxyatractylate and glutamate in mitochondria of adult rats were, respectively, 44 ± 3 and 38 ± 1% and in mitochondria of four-day-old rats were, respectively, 45 ± 2 and 38 ± 2%. As stated in the introduction, in the presence of magnesium ions in the medium, the ATP/ADP- and aspartate/glutamate-antiporters are responsible for two parallel and independent pathways of $\Delta\mu_{H^+}$ dissipation [6, 7]. Therefore, it was reasonable to consider values of recoupling effects of carboxyatractylate and glutamate as a measure of involvement in the uncoupling of the ADP/ATP- and aspartate/glutamate-antiporter, respectively [6]. Thus, the findings suggested that these carriers should be similarly involved in the uncoupling in liver mitochondria of adult and four-day-old rats.

The respiration rate of mitochondria of one-month-old rats in state 4 was 40% higher than the similar value for mitochondria of adult rats, whereas the respiration rate at the maximal uncoupling with DNP was the same (Table 2). This difference was not associated with the presence of endogenous fatty acids because the addition of BSA failed to affect the respiration of one-month-old rats in state 4 (data not presented). Palmitate stimulated the respiration of mitochondria of one-month-old rats more strongly than the respiration of adult rats (Table 2). FCCP (20 nM) stimulated the respiration of mitochondria of adult rats similarly to the stimulation with 25-μM palmitate. But unlike palmitate, FCCP similarly stimulated the respiration of one-month-old and adult rats (data not presented). In the presence of palmitate, the respiration of mitochondria of both groups of animals was effectively suppressed with carboxyatractylate and glutamate (Table 2). In these experiments the recoupling effects of carboxyatractylate and glutamate were, respectively, 45 ± 2 and 38 ± 2% in mitochondria of adult rats and 41 ± 2 and 25 ± 2% in mitochondria of one-month-old rats. Consequently, the contribution of the aspartate/glutamate antiporter to the uncoupling effect of palmitate in mitochondria of one-month-old rats was lower than in mitochondria of adult rats.

Table 3. Comparison of the uncoupling activities of FCCP (V_{FCCP}, μM O_2/min per 1 nM FCCP) and palmitate (V_{Pal}, μM O_2/min per 1 μM palmitate) and also of the components of the uncoupling activity of palmitate: sensitive to carboxyatractylate (V_C), sensitive to glutamate (V_G), and insensitive to carboxyatractylate and glutamate (V_{Ins}) in liver mitochondria of adult and one-month-old rats. Additions as in Table 1A

Symbols	Adult rats ($n = 7$)	One-month-old rats ($n = 7$)
V_{FCCP}	0.543 ± 0.014	0.541 ± 0.011
V_{Pal}	0.540 ± 0.016	0.783 ± 0.013
V_C	0.245 ± 0.009	0.326 ± 0.011
V_G	0.200 ± 0.003	0.200 ± 0.008
V_{Ins}	0.095 ± 0.008	0.257 ± 0.009

In mitochondria of one-month-old rats, V_{Pal} was 1.46-fold higher than in mitochondria of adult rats, whereas V_{FCCP} did not depend on age of the animals (Table 3). Unlike the V_G value, the value of V_C was 1.34-fold higher and that of V_{Ins} 2.9-fold higher in mitochondria of one-month-old rats compared to corresponding values in mitochondria of adult animals (Table 3).

In other experiments, the protonophore uncoupling effect of palmitate was comparatively studied in liver mitochondria of various species of adult mammals different in body weight: mice, rats, guinea pigs, and rabbits. The respiration rate of liver mitochondria in state 4 was found to decrease with increase in the body weight of the animals, whereas at the maximal uncoupling effect of DNP the respiration rate of mitochondria of all the animals was virtually the same (Table 4). This difference was not due to the uncoupling effect of endogenous fatty acids because the addition into the medium of BSA free of fatty acids did not affect the respiration of mitochondria of these animals (data not presented). It has been noted in the introductory part that in the absence of fatty acids the activity of passive leakage of protons in liver mitochondria decreases with increase in body weight of mammals; this dependence expressed in double logarithmic coordinates is linear with slope angle tangent of –0.14 [18]. In our experiments, logarithm of the respiration rate of mitochondria in state 4 also depended linearly on logarithm of the body weight of the animals, with the slope angle tangent of –0.16 (data not presented). Since the respiration of liver mitochondria in state 4 is virtually completely determined by the passive leakage of protons [28, 29], our findings support the data published earlier [18].

Stimulation of the respiration of mitochondria with palmitate decreased with increase in the body weight of the animals (Table 4). In all cases, the respiration rate of mitochondria was effectively suppressed with carboxyatractylate; however, glutamate suppressed the respiration of mouse mitochondria significantly less than the respiration of mitochondria of other animals (Table 4). In these experiments the recoupling effects of carboxyatractylate and glutamate in mitochondria of mice were, respectively, 46 ± 5 and 12 ± 1%, of rats, respectively, 42 ± 2 and 37 ± 2%, of guinea pigs, respectively, 29 ± 3 and 54 ± 3%, and of rabbits, respectively, 32 ± 3 and 54 ± 3%. The V_{Pal} value in liver mitochondria decreased with increase in the body weight of the animals (Table 5). The V_C and especially the V_{Ins} values displayed similar dependence (Table 5). Surprisingly, the V_G value had no such dependence on the body weight of the animals (Table 5). Note that according to the data of Table 5 the sum of V_C and V_G values significantly less depended on the body weight of the animals. Unlike V_{Pal}, the V_{FCCP} value was virtually the same in liver mitochondria of mice and rats but nearly twofold lower in mitochondria of guinea pigs and rabbits (Table 5). The dependence of the V_{Pal} value on the body weight of animals in double logarithmic coordinates was linear, with the slope angle tangent of –0.18 (Fig. 2). Thus, the V_{Pal} value decreased with increase in the body weight of the animals according to the equation $V_{Pal} = am^{-0.18}$.

Table 4. Respiration rate of liver mitochondria (nmol O_2/min per mg protein) of various adult mammals in state 4 and on subsequent addition of palmitate (Palm), carboxyatractylate (Catr), glutamate (Glu), and DNP. Additions as in Table 1A

Additions	Mice ($n = 6$)	Rats ($n = 12$)	Guinea pigs ($n = 5$)	Rabbits ($n = 5$)
Without additions	15.3 ± 0.6	10.5 ± 0.2	9.1 ± 0.2	7.5 ± 0.8
Palm	32.1 ± 0.8	23.5 ± 0.5	21.2 ± 0.6	15.6 ± 1.3
Palm + Catr	24.5 ± 0.7	17.9 ± 0.4	17.7 ± 0.3	13.0 ± 1.3
Palm + Catr + Glu	22.4 ± 0.5	13.4 ± 0.3	11.2 ± 0.3	8.6 ± 1.0
Palm + Catr + Glu + DNP	60.0 ± 3.4	69.5 ± 4.5	70.1 ± 6.0	58.8 ± 6.9

Table 5. Comparison of the uncoupling activities of FCCP (V_{FCCP}, μM O_2/min per 1 nM FCCP) and palmitate (V_{Pal}, μM O_2/min per 1 μM palmitate) and also of the components of the uncoupling activity of palmitate: sensitive to carboxyatractylate (V_C), sensitive to glutamate (V_G), and insensitive to carboxyatractylate and glutamate (V_{Ins}) in liver mitochondria of various mammals. Additions as in Table 1A

Symbols	Mice (n = 6)	Rats (n = 12)	Guinea pigs (n = 5)	Rabbits (n = 5)
V_{FCCP}	0.465 ± 0.038	0.501 ± 0.015	0.283 ± 0.018	0.286 ± 0.025
V_{Pal}	0.898 ± 0.043	0.608 ± 0.021	0.513 ± 0.030	0.382 ± 0.030
V_C	0.409 ± 0.016	0.262 ± 0.010	0.148 ± 0.015	0.123 ± 0.007
V_G	0.110 ± 0.016	0.219 ± 0.011	0.276 ± 0.024	0.208 ± 0.019
V_{Ins}	0.379 ± 0.037	0.127 ± 0.007	0.089 ± 0.005	0.052 ± 0.006

In addition to studies on the protonophore uncoupling effect of palmitate, we compared its calcium-dependent uncoupling effect in liver mitochondria of various animals. As in the previous case, animals of the same species but of different age were used (adult and one-month-old rats) and adult animals of various species different in body weight. It was mentioned in the introduction that the induction with fatty acids of the calcium-dependent nonspecific permeability of the inner membrane of mitochondria (pore opening) is accompanied by their swelling [13, 15]. Therefore, intensity of the calcium-dependent uncoupling effect of palmitate may be assessed by changes in the optical density (ΔA) of a suspension of mitochondria. Changes in the optical density were more pronounced in liver mitochondria of adult rats than in those of one-month-old rats (Fig. 3). The rate of this process was 0.310 ± 0.030 and 0.132 ± 0.036 ΔA/min per mg protein for mitochondria of adult and one-month-old rats, respectively. Consequently, the calcium-dependent uncoupling effect of palmitate, unlike its protonophore activity, was higher in mitochondria of adult rats than in mitochondria of one-month-old rats. In the presence of calcium ions and palmitate, the rate of changes in the optical density of suspension of liver mitochondria from adult animals (mice, rats, and guinea pigs) was, respectively, 0.260 ± 0.025, 0.247 ± 0.029, and 0.236 ± 0.015 ΔA/min per mg protein. Thus, unlike the protonophore uncoupling effect of palmitate, its calcium-dependent effect did not depend on the animal species.

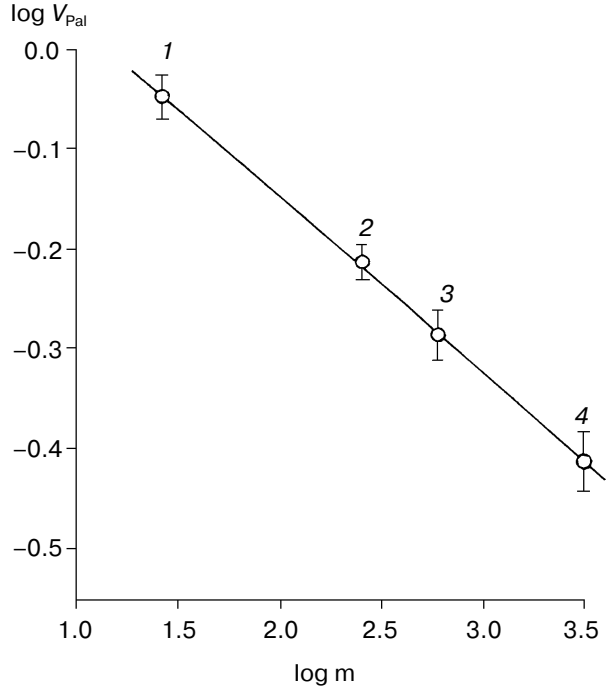

Fig. 2. Dependence of logarithm of the protonophore uncoupling activity of palmitate (log V_{Pal}) on logarithm of the mean body weight of animals (log m): mice, 27 g (*1*); rats, 252 g (*2*); guinea pigs, 601 g (*3*); and rabbits, 3170 g (*4*). Values of V_{Pal} are taken from Table 5. Parameters of the empirical equation of linear regression (log V_{Pal} = 0.21 – 0.18 log m) are determined by the least square method. The correlation coefficient (r) is –0.98, $p < 0.01$.

DISCUSSION

It has been established earlier [18] and confirmed in the present work that in the absence of fatty acids the rate of passive leakage of protons in mitochondria isolated from the liver of adult mammals decreases with increase in body weight in accordance with the equation: $J = am^{-b}$. The exponent (b) is –0.14 [18], or –0.16 in the present work. The body weight of one-month-old rats is 20% of the body weight of adult rats. Our calculations on the basis of this equation have shown that in state 4, the respiration rate of liver mitochondria of one-month-old rats

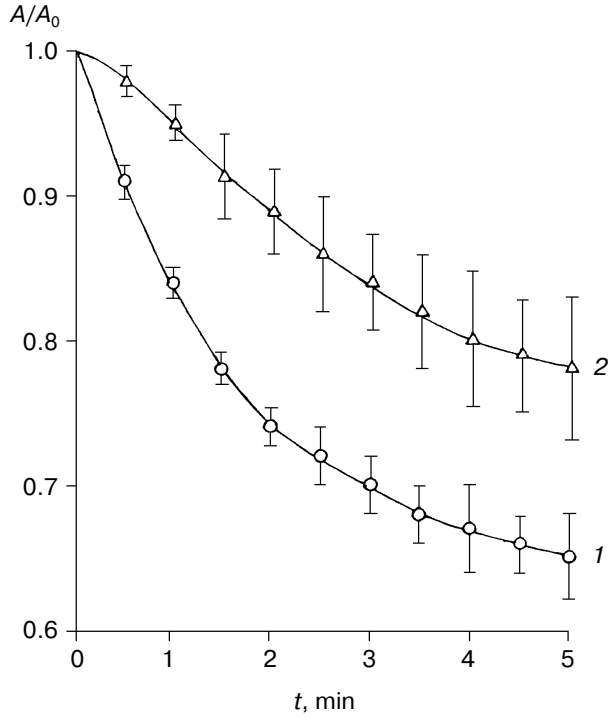

Fig. 3. Changes in the optical density (ΔA) of suspensions of liver mitochondria from adult rats (*1*) ($n = 6$) and one-month-old rats (*2*) ($n = 6$).

should be 1.29-fold higher than that of adult rats, and this is close to the experimentally found difference. The respiration rate of liver mitochondria of four-day-old rats in state 4 was nearly twofold higher than that of liver mitochondria of adult rats. But, unlike the case of mitochondria of one-month-old rats, this difference was not observed in the presence of BSA, i.e., it was entirely caused by the uncoupling effect of endogenous fatty acids. In the absence of fatty acids, the passive leakage of protons in mitochondria occurred by simple diffusion, possibly through channels of the inner mitochondrial membrane [30]. Thus, with decrease in the body weight of the animals, the rate of passive leakage of protons by this pathway increases.

The V_{Pal} value in mitochondria isolated from the liver of adult mammals decreases with increase in the body weight of the animals also in accordance with the above-presented equation with the exponent of –0.18 (Fig. 2). Based on this observation, the V_{Pal} value in liver mitochondria of one-month-old rats should be 34% higher than in mitochondria of adult rats. And this value is close to the value found by us experimentally (Table 3).

Unlike activity of the natural uncoupler palmitate, the activity of the artificial protonophore FCCP is similar in liver mitochondria of mice and of adult and one-month-old rats, but nearly twofold lower in liver mitochondria of guinea pigs and rabbits. This uncoupler, in distinction to fatty acids, can easily cross the phospholipid bilayer in both neutral and anionic form [27], although involvement of some proteins in this process cannot be excluded [2]. Obviously, different changes in the values of V_{FCCP} and V_{Pal} are caused by different mechanisms of the uncoupling effect of FCCP and palmitate.

Thus, our findings suggest that changes in the protonophore uncoupling activity of palmitate in various mammals follow the same pattern as changes in the passive leakage of protons without involvement of fatty acids. As noted in the introduction, thermoproduction at rest is caused, in particular, by free oxidation in liver mitochondria due to the passive leakage of protons without involvement of fatty acids. However, the liver cells always contain a quantity of free fatty acids, which is sufficient for a noticeable acceleration of the respiration of isolated mitochondria in state 4 [20]. It is suggested that the protonophore uncoupling effect of endogenous fatty acids should be another cause of thermoproduction in liver cells at rest.

On decrease in the environmental temperature, the rate of thermoproduction in mammals significantly increases, and this correlates with increase in free fatty acids in the cells [1, 2]. In the present work, changes in the V_{Pal} value in liver mitochondria of various mammals follow the same pattern as dependence of the thermoregulatory efficiency of metabolism on the body weight of homoiothermic animals [19]. In particular, the thermoregulatory efficiency of metabolism calculated by data of this work was 2.4-fold higher in mice than in rabbits, and the protonophore uncoupling activity of palmitate in liver mitochondria of mice was also 2.4-fold higher than in liver mitochondria of rabbits. This is in good agreement with the hypothesis that the biological significance of the protonophore uncoupling effect of fatty acids is the production of additional heat on cooling of the animals [1, 2]. It is known that thermoregulatory mechanisms in newborn rats, which are constantly in contact with the mother, are not yet developed [34], and the protonophore uncoupling activity of palmitate in their liver mitochondria is significantly lower than expected based on their body weight. It seems that the higher content of free fatty acids in the liver cells of newborn rats could compensate the low activity of other mechanisms of thermogenesis.

Our studies have shown that the increase in the V_{Pal} value in liver mitochondria with decrease in the body weight of mammals is mainly due to the component V_{Ins} of the uncoupling. This third pathway of the proton returning into the matrix with fatty acids is at present the least studied. No doubt, liver mitochondria of mice and one-month-old rats will be the most suitable object for such studies. Note that in liver mitochondria of various animals the degree of involvement of the ADP/ATP- and aspartate/glutamate-antiporters varies strongly. We suggest that this should be due to varied contents of cationic and anionic groups of phospholipids and/or proteins on

the surface of the inner membrane. In fact, we have shown [6, 7, 35] that appearance of additional cationic groups induced by decrease in pH of the incubation medium or addition of a cationic detergent cetyltrimethylammonium bromide (CTAB) increases the contribution of the aspartate/glutamate-antiporter and decreases the contribution of the ADP/ATP-antiporter to the uncoupling effect of fatty acids.

Unlike the protonophore uncoupling effect of palmitate, its calcium-dependent uncoupling effect was similar in liver mitochondria of various adult mammals. Moreover, liver mitochondria of one-month-old rats were more resistant to the palmitate-induced calcium-dependent nonspecific permeability than liver mitochondria of adult rats, whereas in the case of the protonophore uncoupling effect of this fatty acid the picture was opposite. Note that in principle any uncoupling of oxidative phosphorylation results in the dissipation of energy as heat released during the oxidation not coupled with synthesis of ATP [1, 2]. However, the calcium-dependent effect of fatty acids is rather a "strict" form of uncoupling. In this case, the initial activation of free oxidation is replaced by suppression of oxidative processes at the cost of loss of cytochrome *c*, which in its turn triggers cell death by apoptosis or necrosis [36]. Obviously, this type of uncoupling of oxidative phosphorylation in mitochondria of homoiothermic animals is not associated either with thermoproduction at rest under conditions of normal temperature or with production of additional heat on cooling of the animals.

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