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Effects of cold acclimation and palmitate on energy coupling in duckling skeletal muscle mitochondria

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Abstract Gastrocnemius subsarcolemmal and intermyofibrillar mitochondria were isolated from 5-week-old cold-acclimated and thermoneutral control ducklings. In vitro respiration (polarography) and ATP synthesis (bioluminescence) were determined at 25°C. Subsarcolemmal mitochondria showed a higher state 4 respiration and lower respiratory control and ADP/O ratio in cold-acclimated than in thermoneutral ducklings. Palmitate decreased the rate of ATP synthesis in both mitochondrial populations to about 30% of maximal but failed to abolish this process even at high concentrations. It is suggested that both expensive ATP synthesis and increased ATP hydrolysis could contribute synergistically to muscle non-shivering thermogenesis in cold-acclimated ducklings.

Key words: Cold acclimation; ATP synthesis; Fatty acid; Non-shivering thermogenesis; Muscle mitochondrion

1. Introduction

Non-shivering thermogenesis (NST), i.e. the ability to produce heat independently of muscle contractile activity, is a characteristic adaptation to cold. In mammals, especially in newborns, hibernators and small cold-acclimated rodents, brown adipose tissue (BAT) is one of the main sites of NST. In birds, despite the lack of BAT, the existence of NST has been clearly demonstrated in cold-acclimated (CA) ducklings [1], in chronic glucagon-treated ducklings reared at thermoneutrality [2] and in cold-acclimatized penguin chicks [3]. In the absence of BAT, skeletal muscle accounts for 70% of the total cold-induced NST [4].

BAT NST is mainly based on the uncoupling of oxidation and phosphorylation because of the presence in BAT mitochondria of the uncoupling protein (UCP) which allows the dissipation of the proton gradient [5]. Fatty acids (FAs) play an active role in BAT NST by activating the UCP permeability to protons. Skeletal muscle thermogenesis may be sustained by two different biochemical mechanisms pointed out so far. The first mechanism is ATP consumption by active ion transport. In CA ducklings, an ATP-consuming sarcoplasmic reticulum (SR) Ca^{2+} cycling has been involved in skeletal muscle NST [6] on account of the CA-induced increase in expression of the two proteins involved in Ca^{2+} cycling, the ryanodine receptor and the SR Ca^{2+}-ATPase [6,7]. Such Ca^{2+} cycling may be modulated by long-chain acyl esters which increase the permeability to Ca^{2+} of SR vesicles [7]. Increased ATP-consuming SR Ca^{2+} cycling may also be involved in thermogenic muscles of some fish species or in malignant hyperthermia (see [8] for review). The second mechanism of skeletal muscle NST may be the loose coupling of oxidative phosphorylation in muscle mitochondria. This phenomenon was postulated in CA mammals [9–11] and described in a case of severe human hypermetabolism of non-thyroid origin [12]. In birds, Skulachev and Maslov [13] presented the first evidence to show that cold acclimation is associated with an alteration of the coupling between oxidation and phosphorylation. This result was then confirmed by Duchamp et al. [14] who have histochemically determined in situ that cold exposure induced the development of loosely coupled mitochondria in skeletal muscle. The biochemical nature of this loose coupling is unclear because avian skeletal muscles do not express the mammalian BAT UCP [15] and there is still no experimental evidence for the presence in avian skeletal muscle of the mammalian UCP2 and UCP3, which are other members of the UCP family expressed in other tissues [16,17]. The early investigation of Levachev et al. [18] on pigeon provided the first observation that FAs are involved in thermoregulatory uncoupling. In this respect, Barré et al. [19,20] have suggested that the loose coupling of muscle mitochondria may be under the control of FAs released by secreted or injected glucagon on account of the fact that muscle mitochondria from CA and chronic glucagon-treated ducklings exhibited a higher response to the loose coupling effect of FAs.

The two NST mechanisms described so far obviously appear mutually exclusive, since SR Ca^{2+} cycling heat production requires ATP supply while loose coupled mitochondria would have a depressed ATP production. The present study attempted to investigate whether this is the case or whether these two thermogenic mechanisms could contribute together to muscle NST in CA ducklings. This could be achieved only if loose-coupled muscle mitochondria can still produce ATP. The biochemical properties of isolated muscle intermyofibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM) have therefore been evaluated in vitro by investigating (i) mitochondrial respiratory parameters with a classical polarographic method, (ii) mitochondrial membrane potential with a spectrophotometric method using the dye safranine, and (iii) mitochondrial ATP production with a bioluminescence method under various degrees of loose coupling induced by increasing concentrations of palmitate.

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Abbreviations: $\Delta\psi$, transmembrane electric potential; UCP, uncoupling protein; BAT, brown adipose tissue; CA, cold-acclimated; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAs, fatty acids; FAF-BSA, fatty-acid-free bovine serum albumin; NST, non-shivering thermogenesis; IFM, intermyofibrillar mitochondria; SR, sarcoplasmic reticulum; SSM, subsarcolemmal mitochondria; TN, thermoneutral control

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2. Materials and methods

2.1. Animals

Twenty male muscovy ducklings (*Cairina moschata* L., pedigree R31, Institut National de la Recherche Agronomique, France) from a commercial stockbreeder (Ets Grimaud, France) were used. These were fed ad libitum with commercial mash (Genthon 5A) and had free access to water. The cold acclimation schedule described by Barré et al. [1] was used: from the age of 1 week the ducklings were caged for a period of 5 weeks at either 4°C (cold-acclimated) or 25°C (thermoneutral control) in a constant photoperiod (light/dark: 8/16).

2.2. Isolation of skeletal muscle mitochondria

Six-week-old ducklings were killed by decapitation. Gastrocnemius muscle samples were rapidly taken out and mixed in cold isolation medium with Teflon glass. Muscle IFM and SSM populations were isolated as previously described [20] from the red part of the gastrocnemius muscle by a modification of the differential centrifugation procedure of Palmer et al. [21]. The isolation medium contained 100 mM sucrose, 50 mM KCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.4. Final SSM and IFM pellets were resuspended in a minimal volume of storage medium and kept on ice. After determination of protein by the biuret method with bovine serum albumin as standard, mitochondria were diluted to 20 mg/ml. The final storage medium contained 250 mM sucrose, 1 mM EGTA, and 20 mM Tris base, pH 7.4.

2.3. Mitochondrial respiration

The respiration of isolated mitochondria (0.5 mg mitochondrial protein/ml) was determined polarographically with a Clark oxygen electrode (oxygraph Gilson 5/6 H), in a glass cell of 1.5 ml volume, thermostatted at 25°C. The respiratory reaction medium contained 200 mM sucrose, 5 mM KH_2PO_4, and 20 mM Tris-HCl, pH 7.4 with a final fatty-acid-free bovine serum albumin (FAF-BSA) concentration of 2 mg/ml (0.2% w/v). The controlled state of respiration (state 4) was initiated by the addition of 5 mM succinate (sodium salt) in the presence of rotenone (5 μM) and the active state of respiration (state 3) was initiated by the addition of 100 μM ADP. The method of Estabrook [22] was used for the calculation of state 4 and state 3 respiration and the respiratory control ratio (RCR). The latter respiratory parameter is a measure of the degree of control imposed on oxidation by phosphorylation, and we assumed that values lower than 2 indicated mitochondria to be loose coupling. ADP/O ratio was calculated by using the total oxygen (O) consumed during phosphorylation of a pulse of ADP. ADP/O was obtained by dividing the nmol of ADP added to initiate state 3 respiration and the nmol of oxygen consumed during state 3 respiration [22]. In parallel with the latter determination, we have the method of Beavis and Lehninger [23] to measure an upper limit of the mechanistic ADP/O ratio which is referred to as 'P/O ratio' throughout the present study.

2.4. Membrane potential

The transmembrane electric potential ($\Delta\psi$) was estimated with the dye safranine at a concentration of 20 μM in a cuvette containing 2 ml of the incubation medium and 0.5 mg mitochondrial protein as described by Nedergaard [24]. The incubation medium consisted of 200 mM sucrose, 5 mM Tris-PO_4, 20 mM Tris-HCl, 5 μM rotenone and 0.2% FAF-BSA. The quenching of the color was measured at 511–533 nm in an Aminco DW-2000 dual-wavelength spectrophotometer, at 25°C. To calibrate the response, diffusion membrane potentials were artificially imposed on the mitochondria by the use of the K^+ concentrations: valinomycin was first added to 9 μM, and KCl was then added to the concentrations ranging from 0.1 to 30 mM. To estimate the absorbance at zero membrane potential, at the end of all experiments 5×10 mM NaOH was added to obtain full disintegration of the mitochondrion and ensure that no membrane potential could be maintained.

2.5. Mitochondrial ATP synthesis

The ATP synthesis of mitochondria was determined by the bioluminescence procedure of Wibom et al. [25] at 25°C with some modifications. The lyophilized monitoring reagent mixture (Bioorbit, Finland) containing firefly luciferase, D luciferin, inorganic pyrophosphate (0.1 μmol), magnesium acetate (500 μmol) and FAF-BSA (50 mg) was dissolved in 10 ml bidistilled water. The final medium was extemporaneously prepared and contained 40 μl of monitoring reagent mixture plus 160 μl respiratory reaction medium. Measurement of ATP synthesis was performed with a Kontron luminometer (model 1250) connected to a Linseis recorder (model L6512). The apparatus was calibrated by standard ATP solution (5×10^{-7} M) in the presence of substrate (sodium succinate). Then, 10 μl succinate (750 mM), 10 μl ADP (2 mM) and 10 μl of the mitochondrial suspension (20 μg/ml of IFM or 40 μg/ml of SSM) were added to the final medium (200 μl) and total ATP synthesis rate was recorded during 6 min. 10 μl of oligomycin (100 μg/ml) was added to obtain residual ATP synthesis rate. The basal rate of mitochondrial ATP synthesis was the difference between the total and residual ATP synthesis rates. To titrate ATP synthesis rate in the presence of fatty acids, sodium palmitate was added in the final reaction medium after the initial ATP production was registered in the presence of succinate and ADP. Because sodium palmitate was dissolved in 50% ethanol solution, a blank assay with a 50% ethanol solution without palmitate was also measured to correct ATP synthesis values from any ethanol effect.

2.6. Chemicals and statistics

FAF-BSA (V fraction) was from Boehringer; the bioluminescence lyophilized monitoring reagent mixture was from Bioorbit (Finland); the others were purchased from Sigma.

Analysis of variance (ANOVA) and Student's *t*-test were used for statistical calculations, and regression lines were calculated by the least-squares method.

3. Results

3.1. Comparison of biochemical parameters between intermyofibrillar and subsarcolemmal mitochondria in TN ducklings

Respiration rates of the IFM and SSM were measured with succinate as substrate. To allow only oxidation of this flavoprotein-coupled substrate, the respiratory medium contained rotenone, which inhibits NADH-linked respiration (i.e. FA oxidation). Control SSM showed significantly lower respiratory activity than did IFM (Table 1). In addition, SSM exhibited a 60% lower RCR than did IFM, suggesting that control SSM might be in a loose coupling state (RCR < 2) when compared with corresponding IFM (Table 1). From this result, the question arose whether the isolation procedure could produce the observed loose coupling state of TN SSM. Indeed, SSM are isolated from the supernatant of the first centrifugation, where high levels of FAs released during Potter homogenization might be expected. To test this point, we resuspended the pellet of the first centrifugation, which contains IFM with the 'subsarcolemmal supernatant', instead of isolation medium. Respiration and RCR were then determined but were no different from those obtained with the normal procedure (data not shown). These results suggest that loose coupling may be a biochemical and physiologically related property of the subsarcolemmal fraction rather than an artifactual uncoupling action of the FAs released during the isolation procedure. Table 1 also shows that the phosphorylating efficiency of TN SSM was 10% lower than in IFM. It is known that the ADP/O ratio underestimates mechanistic stoichiometry both because mitochondria are never fully coupled and because ATP synthesis always proceeds against a large 'back pressure' in the form of a positive phosphorylation potential (ΔG_p). The method of Beavis and Lehninger [23] was therefore used to estimate an upper limit of the mechanistic stoichiometry of mitochondrial oxidative phosphorylation. This was achieved by plotting ADP/O ratio as a function of 1/RCR. Linear plots were obtained (Fig. 1).

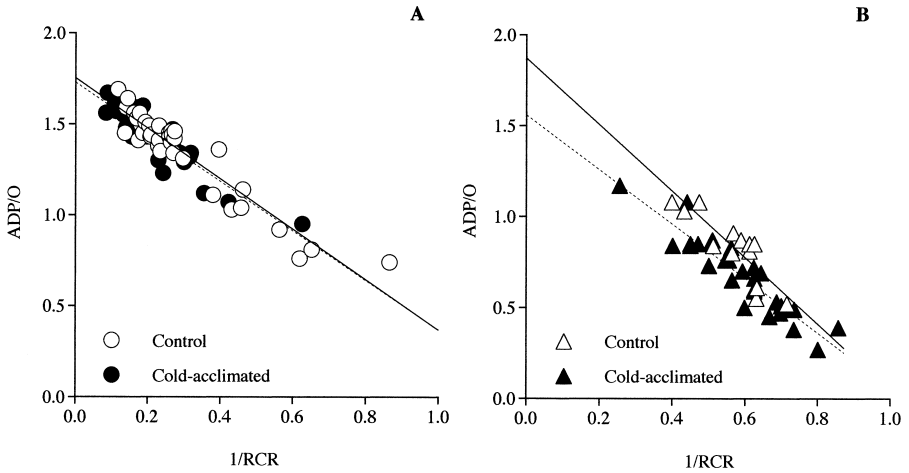

Fig. 1. Determination of the upper limit of the mechanistic 'P/O ratio' using carboxyatractyloside ranging from 20 to 500 nM. The ADP/O is plotted versus 1/RCR. A: Regression lines for IFM in CA ducklings: $y=-1.36x+1.73$ ($r=0.91$) and in thermoneutral control: $y=-1.38x+1.75$ ($r=0.95$), lines intersect y-axis at 'P/O' = 1.73 and 'P/O' = 1.75, respectively. B: Regression lines for SSM in CA ducklings: $y=-1.50x+1.56$ ($r=0.93$) and in thermoneutral control: $y=-1.83x+1.87$ ($r=0.87$), lines intersect y-axis at 'P/O' = 1.56 and 'P/O' = 1.87, respectively. All slopes of regression lines were not significantly different either in CA and TN control ducklings or in IFM and SSM. The y-axis intercept value of the regression line for SSM in CA ducklings was significantly lower than those obtained in IFM and in TN control SSM.

The mechanistic stoichiometry was estimated from the extrapolation of the curve to the y-axis. The obtained 'P/O ratios' were not different between IFM and SSM (1.75 and 1.87 respectively). This implies that control SSM have a similar capacity to produce ATP as corresponding IFM.

The membrane potential was estimated with the lipophilic cationic dye safranine in IFM and SSM respiring on succinate. Table 1 shows that control SSM inner membrane potential was significantly lower than in IFM. From the $\Delta\psi$ values and the state 4 respiration values reported in Table 1, it is possible to estimate an effective proton conductance of the mitochondrial inner membrane by using Ohm's law. Assuming constant stoichiometry of 6 H^+/O for the respiratory complex from succinate to oxygen, we calculated a similar proton conductance of inner membrane in energized IFM and SSM from TN ducklings (Table 1).

3.2. *Effect of cold acclimation in muscle mitochondrial biochemical parameters*

Polarographic measurements indicated that IFM and SSM were differently affected by cold exposure (Table 1). In both mitochondrial populations, state 4 respiration and RCR were significantly increased (+24%) and decreased (−14%) with cold acclimation, respectively. In IFM, neither the ADP/O ratio nor the 'P/O ratio' obtained from the y-axis intercept of the line in Fig. 1A was changed after 5-week cold exposure. Contrary to IFM, the ADP/O ratio was significantly lower in CA SSM. This result was confirmed in Fig. 1B with the intercept of the curve with the y-axis which indicated a significant 17% decrease in 'P/O ratio' of CA SSM (Table 1).

Table 1 also shows that cold acclimation lowered $\Delta\psi$ by 13% and 8% in IFM and SSM, respectively, while inner membrane proton conductance was in turn increased in both CA mitochondrial populations.

3.3. *Effect of palmitate on mitochondrial ATP synthesis of muscle mitochondria from TN and CA ducklings*

The determination of ATP synthesis by bioluminescence was performed in fully coupled mitochondria. In TN duck-

Table 1
Respiratory activities, membrane potential and proton membrane conductance of duckling gastrocnemius muscle mitochondria

	Intermyofibrillar mitochondria		Subsarcolemmal mitochondria	
	Control	Cold-acclimated	Control	Cold-acclimated
Respiratory activity (nmol O/min/mg protein)				
State 4	23.1 ± 0.7	29.3 ± 1.7‡	20.2 ± 1.2†	24.9 ± 0.8†‡
State 3	124.9 ± 5.5	132.7 ± 8.4	39.0 ± 1.5†	42.3 ± 1.1†
Respiratory parameters				
RCR	5.39 ± 0.15	4.57 ± 0.18‡	1.98 ± 0.09†	1.72 ± 0.08†*
ADP/O	1.41 ± 0.03	1.40 ± 0.03	1.24 ± 0.08†	1.09 ± 0.03†*
'P/O'	1.75	1.73	1.87	1.56‡
Membrane potential (mV)				
$\Delta\psi$	145.7 ± 3.1	126.5 ± 3.3‡	125.6 ± 6.7†	115.4 ± 3.5
Membrane conductance (nmol H^+/min/mg/mV)				
C_H	0.96 ± 0.02	1.40 ± 0.04‡	0.98 ± 0.05	1.30 ± 0.04‡

Mitochondria were treated as described in Section 2. Values are means ± S.E.M. ($n=12$). †$P<0.05$, significantly different from intermyofibrillar mitochondria; *$P<0.05$ and ‡$P<0.01$, significant effect of cold exposure. RCR, respiratory control ratio; 'P/O', the upper limit of the mechanistic ADP/O ratio was determined as described in Fig. 1; C_H, the proton conductance of the mitochondrial inner membrane was calculated from $\Delta\psi$ values and corresponding state 4 respiration values assuming a constant stoichiometry of 6 H^+/O for the respiratory complex from succinate to oxygen.

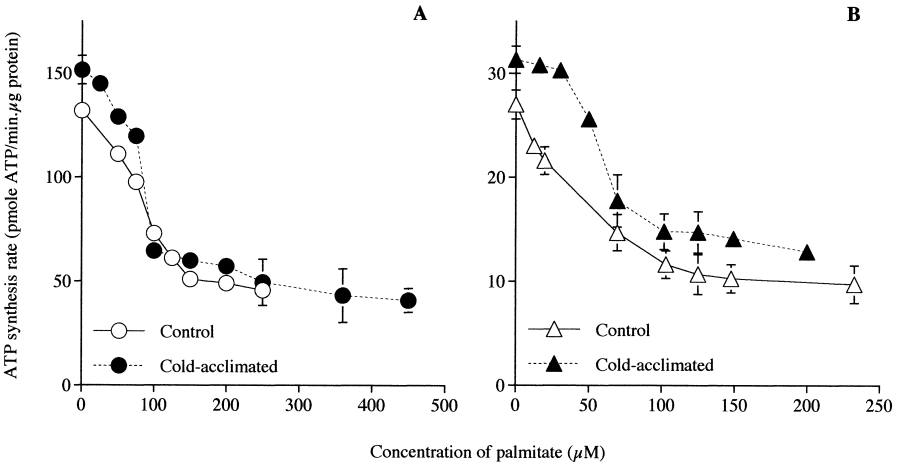

Fig. 2. Effects of palmitate on the ATP synthesis in IMF (A) and SSM (B) isolated from Tn control and CA ducklings. Values are means ± S.E.M. Conditions for the measurement were described in Section 2.

lings, the ATP synthesis rate of SSM was 4.9 times as low as in IFM (Table 2). As a result of cold acclimation, the rates of basal ATP synthesis (J_{ATP0}) in both mitochondrial populations were significantly increased by 15% of their control values (Table 2). We measured the rate of ATP synthesis in IFM and SSM from TN and CA ducklings at increasing doses of palmitate ranging from 10 to 450 μM. Palmitate inhibited the rate of ATP synthesis in both IFM and SSM (Fig. 2). However, palmitate failed to completely abolish mitochondrial ATP synthesis, even at the highest concentration. Accordingly, the palmitate-inhibited ATP synthesis reached a maximal threshold and further addition of palmitate did not affect the rate of ATP synthesis. These results indicate that even when maximally loose-coupled by palmitate, both IFM and SSM populations are still able to produce ATP. The relative decrease in ATP synthesis under maximal inhibition by palmitate was 60% in both IFM and SSM of TN ducklings. Cold acclimation did not significantly change the response of both mitochondrial populations to the uncoupling effect of palmitate. Nevertheless, there was a trend that the relative decrease in ATP synthesis induced by palmitate addition was greater in CA than in TN IFM (70.5 ± 3.3% vs. 63.7 ± 1.3% in CA and TN ducklings, respectively). The sensitivity of these mitochondria to palmitate was, however, not changed by cold acclimation, as measured with the IC_{50} values (Table 2). By contrast, cold acclimation specifically decreased the sensitivity SSM to the uncoupling effect of palmitate as indicated by their higher IC_{50} value (Table 2).

4. Discussion

The present results showed that (i) IFM and SSM exhibited different functional properties, (ii) cold acclimation resulted in a loose coupling of SSM, and (iii) maximal loose coupling by increasing doses of palmitate failed to abolish ATP production in both muscle mitochondrial populations.

The present study showed that IFM exhibited a higher oxidative capacity and a greater ATP synthesis rate than SSM. These results are consistent with previously reported measurements which imply distinct biochemical properties in these two mitochondrial populations either in heart [21] and in skeletal muscle [20,26–28] of mammals and birds. All these data indicate that the IFM display a higher oxidative phosphorylation capacity than the related SSM.

Cold acclimation induced an increase of state 4 respiration while RCR values were in turn significantly decreased in both IFM and SSM populations (see also [19]). These results together with the increase in inner membrane proton conductance suggest that cold-increased state 4 respiration would be ascribed to an increase of proton leak across the mitochondrial inner membrane [29]. However, in the absence of oligomycin, we cannot exclude that one part of the cold-increased state 4 respiration can be accounted for by an increased turnover of intramitochondrial ATP. It also arises from the oxidative phosphorylation study achieved in this paper that the phosphorylating efficiency of SSM as measured polarographically by either the ADP/O ratio or the 'P/O ratio' was specifically decreased by cold acclimation. These data are consistent

Table 2
Palmitate uncoupling effect on ATP synthesis rate of gastrocnemius muscle mitochondria

	Intermyofibrillar mitochondria		Subsarcolemmal mitochondria	
	Control	Cold-acclimated	Control	Cold-acclimated
ATP synthesis rate (pmol ATP/min/μg protein)				
J_{ATP0}	132.0 ± 3.7	151.6 ± 6.9*	27.0 ± 1.4†	31.3 ± 1.3†*
J_{ATPmin}	47.9 ± 1.7	44.7 ± 4.9	10.7 ± 0.7†	13.9 ± 0.8†*
Palmitate sensitivity (μM)				
IC_{50}	81.3 ± 1.4	85.9 ± 0.7	41.4 ± 4.4†	57.2 ± 1.1†‡

Mitochondria were treated as described in Section 2. Values are means ± S.E.M. ($n = 8$). $^{\dagger}P < 0.05$, significantly different from intermyofibrillar mitochondria; $^{*}P < 0.05$ and $^{\ddagger}P < 0.01$, significant effect of cold exposure. J_{ATP0}, ATP synthesis rate in the absence of palmitate addition; J_{ATPmin}, minimal ATP synthesis rate at maximal effective dose of palmitate; IC_{50}, palmitate concentration giving half-maximal inhibition.

with the previous report of Skulachev and co-workers who found that the ADP/O ratio was decreased in SSM isolated from skeletal muscle of cold-exposed mice [9] and pigeons ([13], review in [30]). From the fact that SSM showed both a low RCR (<2) and a specific decrease of ADP/O in CA ducklings while no changes were found in IFM, it could be assumed that their energetic efficiency would be lower than that in CA IFM. This lower efficiency of SSM to use the energy of oxidation for ATP synthesis suggests that more energy is released as heat in respiring SSM and therefore that SSM would display a greater part in muscle thermogenesis. Similar conclusions have been previously reported in CA mammals [10,31] and birds [27,32]. All these data allow us to hypothesize that the ATP production of CA skeletal muscle cells would be less effective, and so, that the energetic cost of any biochemical process involving ATP hydrolysis would increase in CA ducklings. This assumption requires further investigation.

The present study also showed in vitro that cold acclimation induced an increase of ATP synthesis rate in recoupled IFM and SSM populations. The increase of ATP synthesis allows us to suggest a potentially greater energy production of muscle in response to cold stress, which would furnish ATP to cellular energetic processes (i.e. muscular contraction) or would be dissipated as heat (i.e. Ca^{2+} cycling across SR membranes). This assumption may be true in vivo, if no processes will further alter the mitochondrial ATP synthase activity and/or the adenine transport across the mitochondrial inner membrane, as FAs and acyl-CoA esters are able to do [33]. To date in birds, it is known that cold acclimation produces an increase of long-chain acyl ester levels in skeletal muscle [7] as a result of fat deposit mobilization [1] and an increase of FA supply to skeletal muscles [4,27]. These FAs or their related metabolites may contribute to muscle NST in birds either by enhancing the ATP-consuming SR Ca^{2+} cycling and its associated heat production [7], or by uncoupling mitochondrial oxidative phosphorylation processes ([14,19], see also [33]). For that purpose, the present study shows that palmitate inhibited the rate of ATP synthesis in both mitochondrial populations but failed to abolish this process even at high concentrations. These results therefore show that even when maximally loose-coupled by FAs, mitochondria are still able to furnish ATP to muscular energetic processes. These results demonstrate that SR Ca^{2+} cycling and loose-coupled mitochondria could both contribute to muscular NST of CA ducklings.

In conclusion, from the result previously reported for sarcoplasmic reticulum [6,7] and for loosely coupled mitochondria [14] together with those of the present study, it is suggested that expensive ATP synthesis (e.g. loose-coupled mitochondria) and increased ATP hydrolysis could contribute synergistically to muscle NST in CA ducklings. These muscle NST mechanisms would be regulated by FAs mobilized during cold exposure in response to the lipolytic action of glucagon on adipose tissue. These metabolites would assume two non-exclusive actions in muscle mitochondria: (i) as substrates for respiration which would increase flux through the respiratory chain, especially in CA loosely coupled SSM that in turn would increase heat lost during the oxidative phosphorylation process; (ii) as uncouplers which would directly uncouple oxygen consumption from phosphorylation but never entirely impair the mitochondrial ATP production of cell muscle.

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