Mitochondrial uncoupling protein may participate in futile cycling of pyruvate and other monocarboxylates

PETR JEŽEK AND JIŘÍ BORECKÝ

Department of Membrane Transport Biophysics, Institute of Physiology, Academy of Sciences of the Czech Republic, CZ-14220 Prague 4, Czech Republic

Mitochondrial uncoupling protein may participate in futile cycling of pyruvate and other monocarboxylates. Am. J. Physiol. 275 (Cell Physiol. 44): C496–C504, 1998.—The physiological role of monocarboxylate transport in brown adipose tissue mitochondria has been reevaluated. We studied pyruvate, α-ketoisovalerate, α-ketoisocaproate, and phenylpyruvate uniport via the uncoupling protein (UCP1) as a GDP-sensitive swelling in K+ salts induced by valinomycin or by monensin and carbonyl cyanide-p-(trifluoromethoxy)phenylhydrazine in Na+ salts. We have demonstrated that this uniport is inhibited by fatty acids. GDP inhibition in K+ salts was not abolished by an uncoupler, indicating a negligible monocarboxylic acid penetration via the lipid bilayer. In contrast, the electroneutral pyruvate uptake (swelling in ammonium pyruvate or potassium pyruvate induced by change in pH) mediated by the pyruvate carrier was inhibited by its specific inhibitor α-cyano-4-hydroxycinnamate but not by fatty acids. Moreover, α-cyano-4-hydroxycinnamate enhanced the energization of brown adipose tissue mitochondria, which was monitored fluorometrically by 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide and safranin O. Consequently, we suggest that UCP1 might participate in futile cycling of unipolar ketocarboxylates under certain physiological conditions while expelling these anions from the matrix. The cycle is completed on their return via the pyruvate carrier in an H+ symport mode.

brown adipose tissue; uncoupling protein 1; pyruvate carrier; uniport of monocarboxylates; anion futile cycling

NONSHIVERING THERMOGENESIS in newborn mammals, including humans, or in adulthood, when induced by cold adaptation or overnutrition or under specific pathological conditions, takes place in mitochondria of the brown adipose tissue (BAT) (3, 5, 9, 23, 25). BAT mitochondria generate heat due to uncoupling protein 1 (UCP1), which is specific for BAT, unlike the ubiquitous uncoupling protein 2 (6). UCP1 dissipates a proton-motive force by mediating H+ backflow (18, 23). According to a recent hypothesis, this is carried out by a unique fatty acid cycling mechanism (7, 11, 15, 31). UCP1 is considered to conduct anionic fatty acids. Simultaneously, fatty acids are able to return in a protonated form via the lipid bilayer. The overall cycle leads to H+ translocation (7, 31) and, hence, to uncoupling. Indeed, UCP1 can be regarded as a pure anion uniporter, strictly specific for monovalent unipolar anions (14), since it translocates their wide spectrum, alkylsulfonates, oxohalogenides, and monovalent phosphate analogs (14), including anionic forms of physiologically abundant fatty acids (7). The transport of all anions is allosterically inhibited by GDP (11) and other purine nucleotides. Thus UCP1 exhibits the widest substrate specificity among the homologous anion transporters of the mitochondrial gene family (18). For UCP1, only single charged anions and their unipolarity are limiting (14).

Among mitochondrial metabolite anions (19), pyruvate has been demonstrated to be a UCP1 substrate (14). It is not trivial to ask, What is the physiological role of such monocarboxylate transport via UCP1, since a futile cycling of pyruvate might proceed in a manner similar to that of the cycling of fatty acids? The anionic monocarboxylates would be expelled from the matrix because of the negative membrane potential (Δψ) generated by the respiratory chain, whereas their return in an H+ symport (electroneutral) mode could proceed via specific metabolite carriers, the pyruvate or the α-ketoisovalerate carriers. Such a mechanism is plausible under the following conditions: 1) UCP1 should provide monocarboxylate uniport, 2) BAT mitochondria should contain carriers allowing for electroneutral uptake (H+ symport) of monocarboxylates, the pyruvate carrier (4, 19), or the α-ketoisovalerate carrier (10), and 3) these carriers should be active at an intermediate thermogenic state, when the anion uniport pathway of UCP1 is not completely inhibited by ATP and is not saturated by fatty acids. In this state a minimum change in pH (ΔpH) should be established to drive the pyruvate-H+ symport. Condition 1 is fulfilled, because a GDP-sensitive pyruvate uniport in BAT mitochondria was described previously (14). However, other unipolar ketocarboxylates were not studied, and it is not known whether fatty acids can compete with ketocarboxylates. Moreover, regulation of UCP1 activity is complex and includes variations in the levels of free fatty acids and cytosolic purine nucleotides (20). The nucleotide inhibitory ability is negatively modulated by increasing pH and Mg2+ (16). It has long been known that full coupling of BAT mitochondria in vitro is possible only when the fatty acids are removed (by BSA or combustion via the carnitine cycle) and, at the same time, purine nucleotides such as GDP are present (17, 26, 28). Why simple removal of the cycling agent, a fatty acid, is not sufficient to cause full coupling of BAT mitochondria has not been explained.

Condition 2 is also valid: because BAT mitochondria can respire with pyruvate (21), they exhibit an uptake of [14C]pyruvate and swell in ammonium pyruvate (4). However, condition 3 needs to be investigated, and this is the main issue of this article. We have found that, in addition to pyruvate, UCP1 also translocates other unipolar ketocarboxylates and that their transport is

1 Unipolarity refers to a condition stating that, if there is a polar group besides the carboxyl (or other charged group), it should be located close to the carboxyl group.
inhibited by fatty acids. The presence of the pyruvate carrier in BAT mitochondria was confirmed, and inhibition of this carrier by α-cyano-4-hydroxycinnamate (α-CHC) was shown to enhance mitochondrial energization. Hence, it is suggested that futile cycling of ketocarboxylates might partially contribute to uncoupling of mitochondria in BAT.

MATERIALS AND METHODS

Most of the chemicals were purchased from Sigma Chemical or Fluka: 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASMPI) was a kind gift of Prof. J. Rafael (University of Heidelberg, Heidelberg, Germany). Syrian hamsters were cold adapted for at least 3 wk at 5°C. BAT mitochondria were isolated in a medium containing 250 mM sucrose, 10 mM Tris-MOPS, 1 mM Tris-EGTA, pH 7.4, and BSA (5 mg/ml); the final washing was performed in a BSA-free medium. BSA was omitted during the isolation of mitochondria for some Δψ measurements.

Anion uniport indicated by osmotic swelling was measured by following the decrease in apparent absorbance given by light scattering in the 530- to 550-nm range with use of a diode-array spectrophotometer (Spectronics 3000, Milton Roy). Light-scattering intensity reflects the inverse volume (2). All media contained 0.1 mM Tris-EGTA, 2–8 µM rotenone, and 1 µM oligomycin. Passive swelling in the absence of respiration was assayed routinely in the presence of 0.25 µg/ml antimycin. Usually, 0.2 mg protein/ml of BAT mitochondria were used per assay in a medium of 40% isotonic osmolarity (270 mosmol taken as 100%), i.e., in 54 mM salts of monovalent anions buffered to pH 7.2 with 5 mM Tris-MOPS. The transport rates (in min⁻¹) were calculated as the time changes in a normalized light-scattering parameter β (2), which was calculated as follows

\[ \beta = \frac{P}{P_{s}} + (A^{-1} - \alpha) \]

where \( P \) is the mitochondrial concentration (mg/ml), \( P_{s} \) is the standard mitochondrial concentration of 1 mg/ml, \( A^{-1} \) is the inverse absorbance of the suspension, and \( \alpha \) is a machine constant (2) equal to 0.1163 for the Spectronics 3000 diode-array spectrophotometer.

\( O_{2} \) consumption of mitochondria was measured at 37°C in a 5-ml thermostatically controlled chamber equipped with a Clark polarographic electrode in a medium containing 250 mM sucrose, 10 mM Tris-MOPS, 1 mM Tris-EGTA, pH 7.4, and 40 µM rotenone. Energization (uncalibrated Δψ) of a mitochondrial suspension was continuously monitored (17, 22, 28) in the same medium by a fluorescence probe, DASMPI (3 µM), with excitation at 467 nm and emission at 561 nm with use of 2-nm slit widths on an Ortec fluorescence spectrophotometer (model 9200, EG & G) or a Shimadzu fluorometer (model RF5301 PC), with excitation vertically polarized (5-nm slit width) and the emission collected through a 10-nm slit and a polarizer in cross orientation to eliminate scattering. Δψ was estimated according to Mewes and Rafael (22). For calibration, we assumed that under maximum energization (GDP + BSA) Δψ of 170 mV is established and with BSA only Δψ of 37.5 mV is established (26), while ΔpH is 50 mV at pH 7.2 (26) and overall protonmotive force is 220 and 87.5 mV, respectively. A net fluorescence intensity was obtained when light scattering and a fluorescence increase due to BSA interaction with the probe were taken into account. Alternatively, Δψ was monitored by 12.5 µM safranin, as described elsewhere (13), in the same medium in which DASMPI was used.

RESULTS

Uncoupling protein mediates uniport of monovalent ketocarboxylates. In accordance with our previous observations and conclusions (14), we reevaluated the ability of UCP1 to conduct physiologically relevant "metabolic" anions. We studied valinomycin-induced uptake of K⁺ salts of pyruvate (Fig. 1A) and phenylpyruvate (Fig. 1B) in nonrespiring BAT mitochondria pretreated with BSA. An inhibitor of the pyruvate carrier (27), α-CHC, was routinely present to eliminate the pyruvate

![Fig. 1. Uniport of pyruvate and other unipolar ketocarboxylates via uncoupling protein in brown adipose tissue (BAT) mitochondria (A–D) and its absence in liver mitochondria (E). Uniport of pyruvate (A), phenylpyruvate (B), α-ketoisovalerate (C), and α-ketoisocaproate (D) was measured as a passive swelling monitored by light scattering. Swelling was induced by 1 µM valinomycin (Val) in 55 mM K⁺ salts buffered to pH 7.2 with 5 mM Tris-MOPS (A and B) or by a mixture of 1 µM monensin (Mon) and 1 µM carbonyl cyanide- (trifluoromethoxy)phenylhydrazide (FCCP) in 55 mM Na⁺ salts (C and D) and 5 mM Tris-MOPS, pH 7.2, all in presence of 1 mM α-cyano-4-hydroxycinnamate (α-CHC). +GDP and +GDP + FCCP, traces measured with 1 mM GDP and with 1 mM GDP and 1 µM FCCP, respectively; oxamate, trace measured in K⁺ salt of oxamate (β-amino-α-ketocacette). All assays contained 0.1 mM Tris-EGTA, 2 µM rotenone, 0.25 µg/ml antimycin, and 0.2 mg protein/ml of BAT or liver mitochondria.](http://ajpcell.physiology.org/DownloadedFrom10.220.32.247)
carried out by valinomycin-induced passive swelling of rat liver mitochondria in potassium pyruvate. Measurements were performed under conditions described in Fig. 1 legend. Inhibitory ability in percent is expressed as 100% \( (v_0) \) minus remaining activity in percent at given GDP dose \( (v) \). Curves were drawn on assumption that infinite GDP would yield 100% inhibition. Dose responses were fitted on basis of linearization of Hill plots (inset), and fits were drawn according to Hill’s equation (solid lines). This procedure yielded Hill’s coefficient \( (n_H) \) of 0.95 for pyruvate and phenylpyruvate and inhibition constants \( (K_i) \) of 155 and 2,076 \( \mu M \), respectively.

Fatty acids and monocarboxylates share the same pathway in UCP1. The mutation competition of fatty acids and other anionic substrates on UCP1 has repeatedly been reported (7, 11, 15, 29). Because some artificial derivatives such as azido fatty acid, 12-(4-azido-2-nitrophenylamino)dodecanoic acid (AzDA), were previously shown to inhibit \( \text{Cl}^- \) uniport via UCP1 more potently than natural fatty acids (15), we investigated whether the UCP1-mediated pyruvate uniport is also inhibited by AzDA. Figure 3 shows strong inhibition by 40 \( \mu M \) AzDA in the dark (i.e., not photoactivated).  

\[ \text{H}^+ \text{ transport mediated by UCP1 is most probably a result of fatty acid cycling (6). Residual endogenous fatty acids that were not removed by BSA treatment could therefore provide such an H}^+\text{-conducting pathway.} \]
independently of the presence of \( \alpha \)-CHC. A similar result was also obtained with lauric acid, a natural fatty acid, but at a much higher concentration of 500 \( \mu \)M (Fig. 3B, -laurate). As a gauge for nonspecific changes that could be caused by AzDA and lauric acid, we tested swelling in potassium acetate with nigericin, which acts independently of a protein carrier (not shown). This swelling was not affected by concentrations of up to 50 \( \mu \)M with AzDA or up to 500 \( \mu \)M with lauric acid. Figure 4 illustrates the dose responses for inhibition of pyruvate uniport by AzDA and lauric acid. Apparent \( K_i \) was \(-10 \mu \)M, irrespective of whether \( \alpha \)-CHC was present. The inhibitory effect of lauric acid was lower (\( K_i = 306 \mu \)M). Similar data were found for other monocarboxylates (not shown).

Pyruvate carrier in BAT mitochondria is inhibited by \( \alpha \)-CHC but not by fatty acids. A symport of \( H^+ \) and monocarboxylates can be provided in mitochondria by the pyruvate and the \( \alpha \)-ketoisovalerate carrier (10, 19). The former has been assumed to allow for respiration of BAT mitochondria with pyruvate (21). Therefore, our further goal was to reevaluate a non-UCP1-mediated electroneutral pyruvate uptake in BAT mitochondria that can be ascribed to the pyruvate carrier. For this purpose, we first studied the swelling of BAT mitochondria in ammonium pyruvate (Fig. 5). The pyruvate uptake representing such swelling (4) must be electroneutral, proceeding as a symport with \( H^+ \), since only the neutral \( NH_3 \) is able to pass through the membrane.

The transport was inhibited by \( \alpha \)-CHC with a \( K_i \) of 5.5 mM (Fig. 5C). On the contrary, it was not inhibited by AzDA (Fig. 4) or by lauric acid. The electroneutral nigericin-induced uptake of pyruvate in the presence of GDP and the absence of \( \alpha \)-CHC was quite slow in nonrespiring BAT mitochondria (0.05 min\(^{-1}\)) as well as its \( \alpha \)-CHC-sensitive part (0.03 min\(^{-1}\), Fig. 5A). This shows that the activity of the pyruvate carrier is low under these conditions. However, another electroneutral transport induced by nigericin, a phosphate-\( H^+ \) symport via the phosphate carrier, was not obstructed, as shown in a parallel experiment (Fig. 5A).

We have also developed a new assay for electroneutral pyruvate transport in which we employ the valinomycin-induced swelling in potassium pyruvate under conditions when fully coupled BAT mitochondria (by GDP and BSA) are respiring with \( \alpha \)-glycerophosphate (Fig. 6). Propranolol was also present to exclude most of the inner membrane anion channel-mediated flux (1). Under these conditions, respiratory \( H^+ \) pumping compensates the pyruvate-\( H^+ \) symport and the simultaneous \( K^+ \) uniport (influx). Moreover, addition of valinomycin to coupled mitochondria is known to create an initial \( \Delta pH \) jump (1). Thus \( \Delta pH \) drives the pyruvate-\( H^+ \) symport. As demonstrated in Fig. 6, such electroneutral pyruvate-\( H^+ \) symport was sensitive to \( \alpha \)-CHC with a \( K_i \) of 9 mM (Fig. 5C), indicating the participation of the pyruvate carrier. Similar results were obtained with the other known substrates of the pyruvate carrier such as phenylpyruvate (Fig. 6B), lactate (Fig. 6C), and chloroacetate (Fig. 6D). The residual inhibitor-insensitive portion could be attributed to the nonionic diffusion of pyruvic and other ketocarboxylic acids across the lipid bilayer or to the uninhibited part of the inner membrane anion channel-mediated flux. A contribution of the former process should be minor, as documented by the very slow passive swelling of BAT mitochondria in potassium pyruvate in the presence of nigericin and \( \alpha \)-CHC (Fig. 5A).

Effect of \( \alpha \)-CHC on coupling of BAT mitochondria. It is well known (17, 26, 28) that addition of GDP to fatty acid-depleted BAT mitochondria leads to their maximum coupling (\( \Delta \psi \) of 170 mV) (26). Here we demonstrate that addition of \( \alpha \)-CHC to fatty acid-depleted BAT mitochondria leads to some coupling (Fig. 7). Monitoring \( \Delta \psi \) fluorometrically by DASMPI (22), we found that a nearly uncoupled state of fatty acid-depleted BAT mitochondria in the absence of GDP can be further coupled by \( \alpha \)-CHC in the presence of endogenous (not shown) or externally added pyruvate (Fig. 7). This intermediate coupling with \( \alpha \)-CHC and BSA
was estimated to reach 112–135 mV (the maximum $\Delta \psi$ and the sole BSA-induced energization served as the 2 calibration points). We have explained this as a result of the elimination of pyruvate cycling. $\alpha$-CHC enhanced the energization, independently of whether it was added before (not shown) or after BSA (Fig. 7A) or before (Fig. 7B) or after (Fig. 7A) the respiratory substrate $\alpha$-glycerophosphate. Pyruvate served only as a cofactor of the putative pyruvate cycling, since rotenone was always present. Figure 7B shows that the energization in the presence of pyruvate and rotenone was higher with than without $\alpha$-CHC. A sole pyruvate addition rather led to a slight uncoupling (Fig. 7B, only pyr). We have also confirmed that $\alpha$-CHC added after FCCP did not cause any effect, nor did it interfere with the fluorescence of DASMP1 (not shown). We have also confirmed the well-known fact (17, 26, 28) that the sole addition of GDP (not shown) or sole fatty acid removal (Fig. 7) did not lead to complete coupling, but only to small $\Delta \psi$. Also, GDP added after $\alpha$-CHC was still able to induce maximum energization (Fig. 7A, dashed trace). Note that the scale in millivolts is nonlinear with regard to the fluorescence; consequently, this energization appears to be exaggerated.

Parallel measurements of respiration by a Clark O$_2$ probe confirmed that the $\alpha$-CHC-induced coupling decreases O$_2$ consumption of BAT mitochondria, whereas the addition of an uncoupler accelerates their respiration (Fig. 7C). Rat liver mitochondria respiring with endogenous substrates (i.e., without rotenone, Fig. 8A) or nonenergized (with rotenone), in the presence (Fig. 8A, bottom trace) or absence of pyruvate (Fig. 8B), exhibited no increase in $\Delta \psi$ after addition of $\alpha$-CHC. Instead, a slight decrease of $\Delta \psi$ was noted. Succinate, when added after $\alpha$-CHC, was still able to energize rat liver mitochondria to a maximum coupling (Fig. 8B).

The alternative monitoring of $\Delta \psi$ by safranin also confirmed the coupling effect of $\alpha$-CHC in BAT mitochondria (Fig. 9). In contrast to DASMP1, safranin fluorescence is quenched with increasing $\Delta \psi$, so one could expect almost "mirror" changes in the fluorescence records, and this was indeed the case. $\alpha$-CHC enhanced the energization independently of whether added after BSA (Fig. 9A) or before BSA (Fig. 9B and C). The latter effect of $\alpha$-CHC was higher in the presence of externally added pyruvate (Fig. 9, B vs. C). As expected, ATP added after BSA induced the maximum energization (Fig. 9, B and C). These results again suggest that the inhibition of the pyruvate carrier may eliminate pyruvate cycling, in which this carrier participates with UCP1.

**DISCUSSION**

Ježek and Garlid (14) reported for the first time that UCP1 can translocate pyruvate and acetate. We have now extended these findings to the entire class of
unipolar ketocarboxylates. Our data show that UCP1 allows for the uniport of phenylpyruvate, α-ketoisovalerate, and α-ketoisocaproate, which have not been identified as the UCP1 substrates. We can also suggest that fatty acids and unipolar monocarboxylates compete within a single pathway of the UCP1. A model fatty acid, AzDA (15, 29, 30), and the natural lactic acid inhibit the uniport of pyruvate via UCP1, but not electroneutral pyruvate transport, mediated by the pyruvate carrier. The latter was clearly identified in BAT mitochondria, and its inhibition by α-CHC resulted in an increased coupling of BAT mitochondria (which was incomplete). Our data suggest that, even in the absence of fatty acids, futile cycling of pyruvate and other monocarboxylate anions might mimic the fatty acid cycling uncoupling mechanism (7, 31). All monocarboxylate anions, which may enter into the matrix by a symport with H⁺, could be involved. This could be ensured by the pyruvate carrier or by the α-ketoisovalerate carrier. Hence, the existence of the futile cycling of monocarboxylates could contribute, at least partially, to the uncoupling and enable a fine regulation of coupling in BAT mitochondria (Fig. 10).

Features of the uncoupling protein. The UCP1 is now a well-characterized uniporter of monovalent anions. Striking analogy between the character and size of fatty acids and UCP1 amphiphilic substrates such as alkylsulfonates [translocation of which was directly proven (7, 14)] and the mutual competition of fatty acids and alkylsulfonates (7, 11, 15, 29) supports the fatty acid cycling mechanism (7). We now suggest that not only can long-chain fatty acids undergo such cycling but also the compounds with a short chain, namely, the monocarboxylic α-ketoacids (Fig. 10). We have unambiguously characterized pyruvate and other unipolar ketocarboxylates as the transport substrates of UCP1. We may assume that maximal reaction velocity (Vmax) for pyruvate uniport via UCP1 will be close to the value found for Cl⁻ uniport (7) and that the Michaelis-Menten constant is on the order of 10 mM. Even if Vmax is lower and the assumed affinity (the inverse Michaelis-Menten constant) is much lower than the corresponding parameters for the fatty acid uniport, pyruvate uniport in the absence of fatty acids would still be able to contribute to the uncoupling at a pyruvate physiological concentration of 0.1 mM (10). Under these conditions, 1% of Vmax amounts to 100 nmol H⁺·min⁻¹·mg UCP1⁻¹, which is equal to the turnover of 7 s⁻¹. Because it is known that free Mg²⁺ also prevents nucleotide inhibition of anion uniport via UCP1, we can expect that at least 35% of in vitro measured pyruvate uniport activity will remain (16) at the physiological concentrations of Mg²⁺ and ATP, 0.5 mM in a thermogenic state (20).

The ketocarboxylate uniport via UCP1 is presumably competitively inhibited by fatty acids (Figs. 3 and 4) and allosterically (11) by GDP (Figs. 1 and 2). With unipolar ketocarboxylates, we confirmed both trends, which were previously revealed for the UCP1-substrate pattern, namely, that the transport rates are enhanced with increasing hydrophobicity of the anion (14), e.g., phenylpyruvate vs. pyruvate. A second feature, that GDP inhibition of anion uniport is decreasing with the hydrophobicity of the anion (with increasing Kᵢ), was also confirmed for ketocarboxylates. Moreover, residual GDP-insensitive transport in Na⁺ salts of α-ketoisovalerate and α-ketoisocaproate might indicate that BAT mitochondria contain a branched-chain α-ketoacid carrier (10).

Regulation of thermogenesis in BAT. Regulation of thermogenesis in BAT has not yet been satisfactorily explained. A central mechanism should involve a single third messenger (or several such messengers) between norepinephrine-stimulated cAMP levels and UCP1 (23). The most plausible candidate according to LAnoue et al. (20) is ATP, which drops to 0.5 mM in thermogenic BAT cells. At physiological free Mg²⁺ concentrations and pH, this ATP level is not inhibitory and allows the UCP1 transport pathway to open (16). At the same
time, fatty acid levels are elevated, and this leads to the thermogenic state.

We may hypothesize that the sole removal (combustion) of fatty acids can arrest the uncoupling cycle of fatty acids but not of monocarboxylates. The latter would be blocked only when high levels of ATP or other purine nucleotides are also present. Thus, in the absence of fatty acids as cycling agents, pyruvate cycling

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**Fig. 7.** Coupling effect of α-CHC in BAT mitochondria. A and B: energization (Δψ) of mitochondria was monitored by increasing fluorescence of 6 µM 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASMPI), added at beginning of each trace. BAT mitochondria were resuspended to 1 mg protein/ml in an O₂-saturated medium containing 250 mM sucrose, 10 mM Tris-MOPS, 1 mM Tris-EGTA, pH 7.4, and 40 µM rotenone. A: BAT mitochondria containing natural endogenous fatty acids. Solid trace, effect of α-CHC added after α-glycerophosphate (α-GP) and pyruvate. First, BSA (5 mg/ml) was added to eliminate fatty acids; corresponding fluorescence increase is mostly due to BSA interaction with DASMPI. Subsequently, a substrate, 10 mM α-glycerophosphate, Na⁺ salt, and a cofactor, 5 mM Tris-pyruvate, were added (arrow, α-GP + pyr), causing a slight signal decrease. Addition of 1 mM α-CHC caused a biphasic fluorescence (Δψ) increase, which was terminated by anoxia (no O₂). Dashed trace, coupling effect of 1 mM GDP added after 1 mM α-CHC. Order of additions before GDP was as described above. A complete uncoupling induced by 2 µM FCCP is also demonstrated; fluorescence (Δψ) dropped to original low value. B: BAT mitochondria were isolated with BSA, which was also present in assay medium (5 mg BSA/ml). Top trace, α-CHC effect when 1 mM α-CHC was added before α-glycerophosphate and pyruvate (α-GP + pyr). Because 40 µM rotenone was present, only former represents a respiratory substrate. This is compared with same measurement without α-CHC (dashed trace) and without both α-CHC and α-glycerophosphate (bottom trace, only pyr). C: α-CHC-induced coupling of BAT mitochondria monitored by O₂ consumption. BAT mitochondria (0.4 mg protein/ml) were injected into a 5-ml chamber with a Clark polarographic O₂ probe containing an O₂-saturated medium of same composition used for Δψ monitoring, supplemented by 7.5 mg BSA/ml. As indicated by arrows, 10 mM Tris-pyruvate (pyr), 4 mM α-glycerophosphate, Na⁺ salt (α-GP), 8 mM α-CHC, and 4 µM FCCP were added. Numbers under trace are respiratory rates in nmol O₂·min⁻¹·mg protein⁻¹.

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**Fig. 8.** Lack of α-CHC effect in rat liver mitochondria. Energization (Δψ) of rat liver mitochondria (1 mg protein/ml) was monitored by increasing fluorescence of DASMPI as described in Fig. 7 legend. In A, 1 mM α-CHC was added to mitochondria energized almost completely by endogenous substrates [no rotenone, top trace; note that 10 mM Tris-succinate (succ) did not further increase Δψ] or to nonenergized mitochondria (bottom trace), where effect of 5 mM Tris-pyruvate was also evaluated (pyr). In B, 10 mM succinate (succ) added after α-CHC was still able to induce maximum coupling; uncoupling induced by 2 µM FCCP is illustrated as well.
may play an important regulatory role for thermogenesis, when fatty acids are rapidly depleted from the triglyceride droplets. Our current data support this point of view. Despite the fact that \(\alpha\)-CHC in fatty acid-depleted BAT mitochondria does not induce the highest coupled state, the observed partial \(\Delta c\) increase is sufficient for delicate regulation of coupling. This partial \(\Delta c\) increase can be interpreted as a result of blockage of pyruvate cycling by inhibition of pyruvate uptake via the pyruvate carrier.

Features of the pyruvate carrier in BAT mitochondria. The presence of the pyruvate carrier in BAT mitochondria has previously been indicated by the high capacity of BAT mitochondria (21) and BAT cells (25) to oxidize pyruvate as a respiratory substrate by the existence of swelling of BAT mitochondria in ammonium pyruvate and by the respiratory-driven uptake of \([14C]\)pyruvate into BAT mitochondria (4). We have confirmed the existence of swelling in ammonium pyruvate, and we have demonstrated the respiratory-driven \(\Delta p\)H-induced electroneutral transport of pyruvate and other monocarboxylates in BAT mitochondria. Both processes are sensitive to \(\alpha\)-CHC, a specific inhibitor of the pyruvate carrier (27), but are insensitive to fatty acids (Fig. 10). The former is driven by \(NH_3\) permeation into the membrane, and subsequent matrix alkalinization results from the formation of \(NH_4^+\), whereas the latter process is driven by \(\Delta p\)H of the same orientation, i.e., by the increased \(\Delta p\)H under conditions when \(K^+\) uptake by valinomycin discharges the \(\Delta p\) component of the proton electrochemical gradient (\(\Delta \mu_{\text{H}^+}\)). Swelling in ammonium pyruvate is fast (4), because a much greater \(NH_4^+\) gradient is established and concomitant matrix alkalinization creates \(\Delta p\)H comparable in magnitude to that during respiration.

Slow electroneutral pyruvate transport was detected only when we attempted to induce passive swelling of BAT mitochondria in potassium pyruvate by nigericin in the presence of GDP. First, it shows that pyruvate, unlike acetate, is poorly permeant through the mitochondrial membrane itself. Otherwise, swelling in potassium pyruvate and nigericin should be as rapid as any swelling independent of a protein carrier. Because during a passive swelling nigericin collapses \(\Delta p\)H, we might conclude that electroneutral pyruvate transport is slow at low \(\Delta p\)H. This has been suggested also for the dicarboxylate carrier (12). Such a \(\Delta p\)H regulation would represent a rate-limiting step in pyruvate cycling. Consequently, pyruvate cycling is expected to be a fine regulatory mechanism rather than a major thermogenic mechanism.

In conclusion, pyruvate (monocarboxylate) cycling might contribute only partially to the overall thermogenesis but should play an important role in the fine control of coupling. This could be exerted even by sequential fluxes via the pyruvate carrier and UCP1. Because of the limited penetration of pyruvate acid via the lipid bilayer, pyruvate cycling cannot proceed as
freely as fatty acid cycling but is regulated on both proteins involved. With a partially inhibited pyruvate carrier (by low ΔpH), such cycling will play only a minor role. However, on combustion of fatty acids and concomitantly enhanced Δψ and ΔpH, the activated pyruvate carrier will enable the pyruvate cycling in concert with the open UCP1 pathway (unsaturated with nucleotides and fatty acids).

The use of a fluorometer provided by Drs. Martin Nikl and Karel Palka (Institute of Physics, Prague, Czech Republic) is gratefully acknowledged.

This work was partly supported by Academy of Sciences of the Czech Republic Grant 51151 and later by Grant Agency of the Czech Republic Grants 301/95/0620 and 301/98/0568 and by US-Czechoslovak Science and Technology Program Grant 94043, which aided the purchase of a new fluorometer.

Address for reprint requests: P. Jezek, Dept. of Membrane Transport Biophysics (No. 375), Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Prague 4, Czech Republic.

Received 26 February 1996; accepted in final form 15 April 1998.

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