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Fatty Acid Activation of the Reconstituted Brown Adipose Tissue Mitochondria Uncoupling Protein*

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The effect of fatty acids, palmitoyl-CoA, and N',N'-dicyclohexylcarbodimide on the ion conductance of the reconstituted brown adipose tissue mitochondria uncoupling protein was investigated. 1, 5, and 10 μM palmitoyl-CoA induced a marked increase in proton conductance in proteoliposomes containing the uncoupling protein but not in proteoliposomes prepared with purified protein extracts of liver mitochondria. 10 μM oleic acid, like palmitic acid, increased proton conductance in proteoliposomes prepared with the uncoupling protein. Palmitoyl-CoA and caprylic acid had no effect on increasing proton conductance. Similar to the observation in mitochondria, there was no effect of palmitic acid on Cl\textsuperscript- conductance, but unlike mitochondria its activation by palmitoyl-CoA or inhibition by N',N'-dicyclohexylcarbodimide was lost. The results, obtained in an isolated system, provide support for the contention that long chain fatty acids act as an acute physiological activator of the uncoupling protein.

Brown adipose tissue mitochondria (BATM) possess the unique ability to uncouple respiration from oxidative phosphorylation, permitting a thermogenic response to be elicited by the tissue (for review, see Ref. 1). A unique ability to uncouple respiration from oxidative phosphorylation via a non-specific, GTP inhibitable, increase in proton conductance in proteoliposomes containing the reconstituted brown adipose tissue mitochondria uncoupling protein was demonstrated (2, 3). This uncoupling protein is a peripheral membrane protein that is activated and inhibitable by GDP, GTP, ATP, and ADP. In vivo the UP would be inactive unless some mechanism was available to overcome the inhibitory effects of the cytosolic purine nucleotides. Evidence that fatty acids or their acyl-CoA derivatives mediate this acute response has been indicated by a number of reports (2-4). A high acyl-CoA synthetase activity in BATM (5), among other factors, makes the effects that added fatty acids have on UP activation difficult to clearly differentiate from those of their acyl-CoA derivatives. We have recently reported the reconstitution of the UP into liposomes (6). This isolated system offers a unique opportunity to investigate the effects of fatty acids and their acyl-CoA derivatives on the activation of the UP without the interference of other proteins. This communication presents results which indicate that proton conductance by the reconstituted UP is specifically activated by long chain fatty acids. Furthermore, the effects of palmitoyl-CoA on UP activation in BATM (2, 3) were lost with reconstitution.

MATERIALS AND METHODS

BATM were prepared from Syrian hamsters, cold-adapted for 3-6 weeks, by the method of Cannon and Lindberg (7). Hamster liver mitochondria were prepared according to the method of Johnson and Lardy (8). Washed mitochondrial pellets were resuspended in 20 mM MOPS-NaOH, 20 mM NaSO\textsubscript{4}, 0.16 mM EDTA, pH 6.7, rapidly frozen on a dry ice/methanol bath and stored overnight at -70 °C. The BATM UP was partially purified using octyl-d-glucopyranoside as previously described (6). Initial treatment to remove proteins peripheral to the membranes was done by a modification of the method of Lin and Klingenberg (9). The frozen BATM pellet was thawed and mixed with 6.4% Lubrol WX in 20 mM MOPS-NaOH, 20 mM NaSO\textsubscript{4}, 0.16 mM EDTA to a final concentration of 3.2% and 1.15 mg of Lubrol WX/mg of mitochondrial protein. The detergent-mitochondrial suspension was stirred at 4 °C for 30 min and the insoluble membrane fraction was removed by centrifugation at 100,000 g for 90 min. The sedimented membranes were then washed for 30 min at 4 °C with 250 mM sucrose, 5 mM TES-KOH, 1 mM EDTA, pH 7.2, and centrifuged again at 100,000 g for 30 min. The UP was extracted from the washed residual membrane fraction by solubilization in 40 mM octyl-d-glucopyranoside, 20 mM MOPS-NaOH, 20 mM NaSO\textsubscript{4}, 0.16 mM EDTA, pH 6.7, at 4 °C for 60 min. Insoluble membrane material was removed by centrifugation at 100,000 g for 30 min. The octyl-d-glucopyranoside to BATM protein ratio was 1:2.1 (w/w). Optimal UP solubilization, as determined by the specific binding capacity for GDP, is achieved using an octyl-d-glucopyranoside concentration of 40 mM.

Further purification of the UP was achieved by chromatography of the solubilized BATM proteins on ATP-agarose. The BATM octyl-d-glucopyranoside extract (1.0-1.2 mg/ml ATP-agarose) was applied to a 3-ml ATP-agarose affinity column equilibrated with 40 mM octyl-d-glucopyranoside, 20 mM MOPS-NaOH, 20 mM NaSO\textsubscript{4}, 0.16 mM EDTA, pH 6.7, at 4 °C for 60 min. Flow-through fractions which contained the UP (6) were collected and pooled for use in subsequent reconstitution studies.

A partially purified protein extract from liver mitochondria used in the reconstitution experiments was prepared in exactly the same manner. Liposomes composed of phosphatidylethanolamine/phosphatidylcholine/cardiolipin in a ratio of 49.5:49.5:1 were prepared with purified UP or liver mitochondrial protein extract essentially as previously described (6). A chloroform solution of the phospholipids was dried under nitrogen at room temperature and then vortex-suspended in a medium containing 40 mM octyl-d-glucopyranoside, 20 mM NaSO\textsubscript{4}, 20 mM MOPS-NaOH, pH 6.8, and 100 μM GDP. Purified...
UP, liver mitochondrial protein extract, or an equal volume of ATP-agarose octyl-D-glucopyranoside equilibration buffer (control) was added to the phospholipid-detergent suspension at a concentration of 5–10 μg/ml phospholipid. The final solution had an octyl-D-glucopyranoside-to-phospholipid molar ratio of 10:1 and a phospholipid concentration of 3.13 mg/ml. Two 6-ml aliquots were placed in separate Spectrapor 2 dialysis bags and dialyzed 40–44 h at 4 °C against five 2-liter changes of 20 mM NaSO₄, 20 mM MOPS-NaOH, pH 6.8, and 100 μM GDP. Dialysis buffer was changed at 8–10-h intervals and GDP was omitted from the final buffer change.

Proton conductance was measured by a slight modification of our previously described method (6). Medium external to the liposomes was replaced with 20 mM NaSO₄, 0.5 mM TES-NaOH, pH 6.8, by gel chromatography on a 1 x 30-cm Sephadex G-50 column at 4 °C. The void volume fractions containing the liposomes were collected and used for proton conductance assays. A 1-ml aliquot of the liposomes was diluted with 2 ml of 20 mM NaSO₄, 0.5 mM TES-NaOH, pH 6.8, containing 150 μg of carbonic anhydrase. The sample was stirred for 35–40 min at 25 °C under a gentle stream of water-saturated Nz in a water-jacketed sample vial fitted with a combination pH electrode connected to a pH meter and chart recorder. System half-time response was less than 1 s and full scale deflection was 0.2 pH units. Potassium was added to the sample as 0.0625 ml of 240 mM KSO₄, 20 mM NaSO₄, 0.5 mM TES-NaOH, pH 6.8, and the sample was stirred until a stable pH was reached (approximately 1 min). Valinomycin in ethanol was added (final concentration, 0.5 μg/ml) to generate a membrane potential, and the resulting change in external pH was monitored. Proton conductance was analyzed by measuring the initial rate (V0) of pH change induced by H⁺ efflux from the liposome interior (see Fig. 1). The external buffering capacity of the system was determined by monitoring the decrease in pH upon addition of 50 mM of HSO₄. Addition of GDP to the system was made 5 min prior to initiation of conductance by valinomycin. Other additions were made 1 min prior to starting the reaction.

Chloride influx was assayed as previously described (6) by incubation of liposomes with 36Cl− followed by gel filtration on Sephadex G-50 to remove external 36Cl−. To prevent in the assay, external GDP was removed from the prepared liposomes by passage over Sephadex G-50 as described for the proton conductance assay. The elution buffer was 20 mM NaSO₄, 20 mM MOPS-NaOH, pH 6.8. Addition of palmitic acid or palmitoyl-CoA was made prior to initiating the influx. DCCD-treated liposomes were prepared by incubation with varying concentrations of ethanolic DCCD on ice for 2–3 h prior to use in influx experiments. Controls treated with an equal volume of ethanol were unaffected.

Mitochondrial protein was determined by the Lowry method (10). Detergent extract protein and liposome protein were determined in 1% sodium dodecyl sulfate by a modified Lowry procedure (11). Bovine serum albumin was used as a standard.

Radioisotopes were obtained from ICN. ATP-agarose and palmitoyl-CoA were purchased from P-L Pharmacia Biochemicals. Palmitic acid, caprylic acid, oleic acid, GDP, valinomycin, and DCCD were from Sigma. Phosphatidylcholine (egg, >99% pure), phosphatidylethanolamine (egg, >99% pure), and cardiolipin (beef heart) were purchased from Avanti Polar Lipids, Inc.

RESULTS AND DISCUSSION

We have previously reported (6) the successful reconstitution of the UP using a partially purified octyl-D-glucopyranoside extract of BATM. The observed H⁺ and Cl− conducting activities of the proteoliposomes was attributed to the UP based on the inhibition produced by GDP. A lower concentration of GDP (100 μM) was similarly effective in this study (Fig. 1 and Table I). Additional evidence that the UP mediates the increased H⁺ and Cl− conductance in proteoliposomes was obtained by use of ATP-agarose purified octyl-D-glucopyranoside extracts of liver mitochondria. No significant increased H⁺ or Cl− conductance was observed in these proteoliposomes (see Tables I and II). Liver mitochondria do not contain the UP (1) and no protein of 32,000 M, was apparent on sodium dodecyl sulfate electrophoretic gels of octyl-D-glucopyranoside/ATP-agarose extracts from liver mitochondria.

Whether long chain fatty acids or their acyl-CoA derivatives act as acute physiological activators of the BATM UP is controversial. Bukowiecki et al. (12) observed that an increased respiration in brown adipocytes could be mediated by inclusion of fatty acids in the incubation medium and found the response to be independent of the norepinephrine-CAMP pathway. The data suggested that either fatty acids or their acyl-CoA derivative were acting as the acute activator of the UP. Evidence for each as an activator has been reported previously (1). Locke et al. (4) demonstrated that palmitic acid could induce a rapid reversible membrane depolarization and concomitant increase in oxygen consumption in respiring BATM. However, the depolarization was not sensitive to GDP inhibition. In a subsequent report, Rial et al. (13) observed

TABLE I

Effect of GDP, fatty acids, palmitoyl-CoA, and FCCP on the initial rate of proton efflux from liposomes and proteoliposomes

The proton efflux assays were carried out as described under "Materials and Methods." The amount of protein used in the reconstituted system was 5 μg/mg phospholipid. A, control liposome; B, control liposome plus 5 μM FCCP; C, proteoliposome prepared with uncoupling protein; D, proteoliposome prepared with uncoupling protein plus 100 μM GDP; E, proteoliposome prepared with uncoupling protein plus 10 μM palmitic acid; F, proteoliposome prepared with uncoupling protein plus 100 μM GDP and 10 μM palmitic acid.

| Condition                  | H⁺/min | μmol H⁺/min/mg protein |
|----------------------------|--------|------------------------|
| Control liposomes          | 10 ± 1 |                        |
| 5 μM FCCP                  | 842 ± 31 |
| BATM proteoliposome        |        |                        |
| No additions               | 27 ± 1 | 1.69 ± 0.77            |
| 100 μM GDP                 | 11 ± 1 | 0.69 ± 0.06            |
| 100 μM GDP plus 10 μM palmitoyl-CoA | 11 ± 1 | 0.69 ± 0.06 |
| 100 μM GDP plus 20 μM palmitoyl-CoA | 12 ± 1 | 0.75 ± 0.06 |
| 100 μM GDP plus 5 μM palmitic acid | 20 ± 3 | 1.78 ± 0.13 |
| 100 μM GDP plus 10 μM palmitic acid | 28 ± 3 | 1.78 ± 0.19 |
| 1 μM palmitic acid         | 31 ± 2 | 1.99 ± 0.08            |
| 5 μM palmitic acid         | 44 ± 2 | 2.84 ± 0.12            |
| 10 μM palmitic acid        | 80 ± 12| 5.11 ± 0.77            |
| 10 μM oleic acid           | 75 ± 4 | 4.78 ± 0.28            |
| 10 μM caprylic acid        | 29 ± 2 | 1.83 ± 0.12            |
| 10 μM palmitoyl-CoA        | 23 ± 1 | 1.40 ± 0.06            |
| Liver proteoliposome       |        |                        |
| No additions               | 7 ± 1  | 0.42 ± 0.29            |
| 10 μM palmitic acid        | 8 ± 1  | 0.50 ± 0.29            |

Fig. 1. Recordings of the decrease in pH induced by proton efflux from liposomes and proteoliposomes after imposition of a membrane potential. The initial rate (V0, nmol of H⁺/min/mg protein) was calculated from the pH change resulting from valinomycin addition as shown. The assay for proton efflux is further described under "Materials and Methods." The amount of protein used in the reconstituted system was 5 μg/mg phospholipid. A, control liposome; B, control liposome plus 5 μM FCCP; C, proteoliposome prepared with uncoupling protein; D, proteoliposome prepared with uncoupling protein plus 100 μM GDP; E, proteoliposome prepared with uncoupling protein plus 10 μM palmitic acid; F, proteoliposome prepared with uncoupling protein plus 100 μM GDP and 10 μM palmitic acid.

![Fig. 1](attachment:image.png)
that GDP could affect the fatty acid-induced increase in proton-limited swelling of BATM in K-acetate, and they postulated that GDP was effective only at the low membrane potentials generated in nonrespiring BATM. Fatty acids would act only to lower the membrane potential “break point” (13) at which proton conductance through the UP increased. However, a high membrane potential would be expected in vivo, and the reported concentration of fatty acids in BAT (1) is greater than that needed to elicit the in vivo membrane depolarization (4). Additionally, fatty acids have long been known to act as nonspecific uncouplers in liver mitochondria (14), and recently nanomolar concentrations have been shown to have an uncoupling effect in isolated perfused liver (15). The fatty acyl-CoA could also potentially act as the acute activator as it can displace bound nucleotides from, and stimulate ion conductance through, the UP (2, 3). A large potential for acyl-CoA production certainly exists in BATM (5). Moreover, Locke et al. (4) observed that palmitoyl-CoA induced a membrane depolarization in respiring BATM. However, they found that the effect was not reversible and considered it to be due to nonsel- ective detergent effects of palmitoyl-CoA. Results demonstrating a specific interaction of palmitoyl-CoA with the BATM UP (3) rule out detergent effects as its mechanism of action.

Using the reconstituted UP, we have examined the effects of added palmitic acid or palmitoyl-CoA on the proton conducting activity. This isolated system eliminates the influence of other proteins such as the acyl-CoA synthetase. An increase in the initial rate of H⁺ conductance in the presence of 100 µM GDP was observed only with added palmitic acid (Fig. 1 and Table I). Palmitic acid, without added GDP, was effective at concentrations as low as 1 µM, whereas 10 µM concentrations were sufficient to completely overcome GDP inhibition. Palmitoyl-CoA at concentrations up to 20 µM had no effect on overcoming the inhibition by GDP. 10 µM palmitoyl-CoA did slightly diminish the observed initial rate indicating its ability to interact with the purified UP is not completely lost. A similar inhibitory effect by palmitoyl-CoA alone on Cl⁻ conductance has been observed in nonrespiring BATM (3). Both palmitoyl-CoA and palmitic acid can induce an increase in proton permeability in nucleotide-inhibited BATM (3, 4, 13). It is clear from this investigation that palmitic acid is considerably more potent.

Palmitic acid alone, as reported for nonrespiring BATM (13), further enhanced the initial rate of proton conductance but not to the extent of the artificial proton translocator FCCP (Table I). To investigate whether the palmitic acid effect was a property of the incorporated UP and not just added protein, we tested the response obtained with proteoliposomes prepared from purified liver mitochondria protein extracts. No increase in proton conductance was apparent in these proteoliposomes (Table I), indicating that palmitic acid is not acting as a nonspecific protein protonophore. This observation together with the one showing that the palmitic acid effect is inhibited by GDP indicate that the fatty acid is specifically activating the UP.

Activation of the UP in BATM has been shown to be specific for long chain fatty acids (13) as caprylic acid (8:0) was ineffective in eliciting a membrane depolarization in respiring BATM. Similarly, with the reconstituted UP, caprylic acid did not stimulate proton conductance while oleic acid (18:1) like palmitic acid (16:0) increased proton conductance in the reconstituted UP (Table I). The preference for long chain fatty acids agrees with that seen in intact BATM (13). The data provide additional support that long chain fatty acids act specifically to activate the UP as implied for BATM (4, 13).

In BATM, palmitoyl-CoA can act as an activator of the UP as shown by its ability specifically to overcome GDP inhibited Cl⁻ permeability (2, 3). The UP is considered to mediate both H⁺ and Cl⁻ conductance through the same pathway (13, 18) although this is disputed (16). Proteoliposomes prepared with purified UP retain a GDP-sensitive Cl⁻ conductance (6) and the activity is certainly a function of reconstituted UP as proteoliposomes prepared with liver extracts do not have a similar response (Table II). As previously reported in BATM (3), neither the activation of GDP-inhibited Cl⁻ conductance by 10 or 20 µM palmitoyl-CoA nor the suppression of Cl⁻ conductance by 20 µM palmitoyl-CoA alone was observed in the reconstituted system. As it is not yet clear whether Cl⁻ and H⁺ conductance are mediated by the same pathway (1, 13, 16) a possibility exists that the Cl⁻ conducting pathway is modified during purification and/or reconstitution. The mitochondrial membrane proteins carnitine palmitoyltransferase and adenine nucleotide translocase are altered by solubilization and reconstitution. The Ki for palmitoyl-CoA inhibition of carnitine binding to the carnitine palmitoyltransferase is increased, and sensitivity to malonyl-CoA inhibition is lost after treatment with detergents (17, 18). The concentration of palmitoyl-CoA required for inhibition of the adenine nucleotide translocase is increased after reconstitution (19).

The effectiveness of DCCD in inhibiting Cl⁻ conductance in BATM (3, 20) is also lost in this reconstituted system (Table II). The lack of sensitivity to DCCD may be related to an alteration of the reactive region of the protein due to the solubilization-reconstitution process. Loss of sensitivity to DCCD is seen in the chromaffin granule ATPase after solubilization in cholate or Nonidet P-40 (21, 22). DCCD sensitivity can be restored by reconstitution of the cholate but not the Nonidet P-40 extracts. It is postulated that the junction between the membranous and extrinsic portions of the protein is altered by extraction and is not restored upon reconstitution.

The present results using an isolated reconstituted system clearly support a specific interaction and the role of long chain fatty acids as the acute activator of the UP. Additionally, it is clear that GDP can inhibit the activation by palmitic acid alone which suggests, in vivo, purine nucleotides may act to suppress the fatty acid effect. The mechanism by which fatty acids affect the UP requires further study. In particular, it is important to understand why fatty acids affect proton but not chloride conductance if, in fact, both conductances...
are mediated by the same mechanism. It seems apparent that fatty acids do not interact directly at the GDP binding site since nucleotide binding to BATM (13) or purified UP2 is unaffected by palmitic acid. Additionally, palmitic acid binding to purified UP2 is unaffected by GDP. The nature of the fatty acid interaction with the UP remains unknown and future efforts will be directed towards its elucidation.

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