Acute effects of a β -adrenoceptor agonist (BRL 26830A) on rat brown-adipose-tissue mitochondria

Increased GDP binding and GDP-sensitive proton conductance without changes in the concentration of uncoupling protein

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GDP binding, proton conductance and the specific concentration of uncoupling protein were measured in brown-adipose-tissue mitochondria of rats treated acutely with the novel β -agonist, BRL 26830A. At 1 h after dosing with BRL 26830A, mitochondrial GDP binding was increased more than 2-fold. The increase in binding resulted from an increase in the number of binding sites. An iterative analysis of Scatchard binding data suggested that there is only one high-affinity GDP-binding site (K_d 0.3 μ M) in brown-adipose-tissue mitochondria. The acute increase in GDP binding produced by treatment with BRL 26830A occurred without any alteration in the specific mitochondrial concentration of uncoupling protein, as determined by radioimmunoassay. Treatment with the β -agonist did, however, lead to a small increase in the GDP-sensitive component of mitochondrial proton conductance. These results indicate that GDP-binding sites on uncoupling protein can be rapidly unmasked after treatment with a brown-fat-specific β -agonist, and that the increase in binding reflects an increase in the activity of the mitochondrial proton-conductance pathway.

INTRODUCTION

Brown adipose tissue (BAT) is now viewed as a major site of both the non-shivering and diet-induced forms of thermogenesis in small mammals (for reviews, see Himms-Hagen, 1986; Rothwell & Stock, 1986). The thermogenic activity of the tissue results from the uncoupling of respiration from ATP synthesis, by dissipation of the proton electrochemical gradient (for reviews, see Nicholls & Locke, 1984; Nicholls *et al.*, 1986). BAT mitochondria contain a tissue-specific protein (M_r 32000) that acts as a 'proton short-circuit', controlling the permeability of the inner membrane to protons. This protein, generally termed 'uncoupling protein', is thereby the principal factor regulating the uncoupling of respiration within the brown adipocyte (for review, see Nicholls & Locke, 1984).

Uncoupling protein has a high affinity for purine nucleotides (Nicholls, 1976; Heaton *et al.*, 1978), and the binding of GDP in particular has been used as a measure of the amount of the protein within BAT mitochondria. Whether GDP binding is in fact a direct measure of the uncoupling-protein content of the mitochondria has been the subject of several conflicting reports. Some studies suggest that the ability of uncoupling protein to bind GDP can change rapidly, and that the binding sites can therefore be 'masked/unmasked' (Desautels *et al.*, 1978; Desautels & Himms-Hagen, 1979; Gribskov *et al.*, 1986; Trayhurn *et al.*, 1987). Other reports, however, suggest that there is a fixed stoichiometry between the

amount of the protein and the extent of GDP binding (Rial & Nicholls, 1984), even when a measure of uncoupling protein independent of the binding assay has been employed (Nedergaard *et al.*, 1984; Ricquier *et al.*, 1984; Nedergaard & Cannon, 1985).

In the present experiments a pharmacological agent, the β -adrenoceptor agonist BRL 26830A, was used to provide an acute and highly specific stimulus to BAT in order to ensure a direct and substantial activation of thermogenesis. With this agonist we show that GDP binding to BAT mitochondria increases acutely (within 1 h), without any alteration in the concentration of uncoupling protein (measured independently by radioimmunoassay). BRL 26830A also leads to an increase in the GDP-sensitive component of the mitochondrial proton conductance, indicating that the acute changes in GDP binding reflect an alteration in the 'activity' of uncoupling protein. BRL 26830A was used in this study, since the β -adrenoceptor in BAT has been reported to be neither β_1 nor β_2 , but a novel β -subtype (Arch *et al.*, 1984a), and BRL 26830A is a potent activator of thermogenesis in BAT through its selective interaction with this receptor (Arch *et al.*, 1984b).

EXPERIMENTAL

Chemicals

[8- 3 H]GDP and [U- 14 C]sucrose were from Amersham International (Amersham, Bucks., U.K.). GDP, bovine

Abbreviation used: BAT, brown adipose tissue.

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serum albumin (fraction V, essentially fatty acid-free), rotenone and oligomycin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Tetraphenylphosphonium bromide was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Other chemicals were from BDH Chemicals (Poole, Dorset, U.K.). BRL 26830A, or (R*,R)-($\pm$)-methyl 4-{2-[(2-hydroxy-2-phenylethyl)amino]propyl}benzoate, (E)-but-2-enedioate (2:1) (salt), was synthesized by Beecham Pharmaceuticals.

Preparation of mitochondria

Male Sprague-Dawley rats were housed in pairs in plastic cages at 23 °C, with free access to a standard rodent diet. One animal of each pair was dosed with BRL 26830A by oral gavage (10 mg/kg body wt.). Rats were killed by cervical dislocation, and BAT was rapidly removed from the interscapular and subscapular sites. Connective tissue and white adipose tissue were removed, and BAT was homogenized in a medium containing 250 mM-sucrose, 1 mM-Hepes and 0.2 mM-potassium EDTA (pH 7.2). Mitochondria were prepared as described by Slinde *et al.* (1975), except that the buffer used in the first 'wash' contained 0.5% (w/v) bovine serum albumin (essentially fatty acid-free). The final mitochondrial pellet was resuspended in sucrose buffer, except where stated otherwise. Mitochondrial protein was determined by a biuret method, with bovine serum albumin as a standard.

GDP binding

GDP binding was measured as described previously (Goodbody & Trayhurn, 1981). Mitochondria were incubated for 6 min at room temperature in a buffer at pH 7.1, but with a series of concentrations of [3 H]GDP ranging from 28 nM to 0.2 mM. The incubation buffer contained 100 mM-sucrose, 10 mM-choline chloride, 20 mM-Tes, 1 mM-EDTA (disodium salt) and 5 μ M-rotenone. For each study where Scatchard analysis of the data was performed, mitochondria were prepared from pooled homogenates of BAT from ten animals. At the end of the incubation period, mitochondria were collected by centrifugation and solubilized in 0.75 M-NaOH. [14 C]Sucrose was included, before centrifugation, as a marker for the extra-mitochondrial space.

Mitochondrial proton conductance

For the measurement of mitochondrial inner-membrane conductance, the mitochondrial pellet was resuspended in 100 mM-KCl/10 mM-Tes (pH 7.0). Mitochondrial conductance was measured in an oxygen-electrode chamber modified to take two electrodes. Mitochondrial respiration was monitored with a Clark oxygen electrode, and membrane potential was measured simultaneously by monitoring tetraphenylphosphonium uptake into the mitochondria by using an electrode sensitive to tetraphenylphosphonium (Kamo *et al.*, 1979; Locke *et al.*, 1982). The membrane potential was calculated from the measured concentration of tetraphenylphosphonium, taking protein concentration and mitochondrial volume into account.

Mitochondria (0.3 mg/ml) were incubated in 1.5 ml of a medium (pH 7.0) containing 50 mM-KCl, 10 mM-Tes, 2 mM-MgCl₂, 5 mM-K₂HPO₄ and oligomycin (1 μ g/ml). Tetraphenylphosphonium was added to give a concentration of 5 μ M. Pyruvate (5 mM), malate (2 mM) and α -

Fig. 1. Scatchard plots of GDP binding to BAT mitochondria of control rats (●) and rats treated acutely with BRL 26830A (○).

Rats were dosed with BRL 26830A, and BAT was removed 60 min later. For full experimental details see the text. (a) shows the total binding curve for a representative experiment. (b) shows the data for the high-affinity binding site after iterative analysis.

glycerophosphate (5 mM) were added as substrates for respiration. Steady-state membrane potentials and corresponding respiration rates were then measured after the addition of bovine serum albumin (16 μ M; essentially fatty acid-free) and subsequently GDP (3 mM).

The effective proton conductance of the mitochondrial inner membrane was estimated from the measured membrane potential and the respiration rate, assuming an average H⁺/O ratio of 7 for the oxidation of the substrates provided (Rial & Nicholls, 1984). The matrix volume of the mitochondria was measured by incubating with 3.6 μ Ci of 3 H₂O for 10 min. [14 C]Sucrose was added before centrifugation, as a marker for the extra-mitochondrial space.

Table 1. Acute effect of BRL 26830A on GDP binding and the concentration of uncoupling protein in rat BAT mitochondria

Rats were dosed with BRL 26830A, and BAT was removed 60 min later. The results are given as means $\pm$ S.E.M., with the numbers of observations shown in parentheses. * $P < 0.05$ compared with the control group.

	Control	Dosed
GDP bound (pmol/mg of mitochondrial protein)	65.9 $\pm$ 7.3 (4)	139.1 $\pm$ 19.8* (3)
K_d (μ M)	0.35 $\pm$ 0.05 (4)	0.29 $\pm$ 0.06 (3)
Uncoupling protein (μ g/mg of mitochondrial protein)	13.3 $\pm$ 1.4 (12)	11.9 $\pm$ 0.9 (12)
Binding ratio (mol of GDP/mol of uncoupling protein dimer)	0.32	0.75

Table 2. Acute effects of BRL 26830A on the GDP-sensitive component of the mitochondrial proton conductance

Rats were dosed with BRL 26830A, and BAT was removed 60 min later. For full experimental details see the text. The results are given as means $\pm$ S.E.M. for four separate measurements in each group. * $P < 0.05$ compared with the control group (Student's paired t test).

	Membrane potential (mV)	Respiration rate (nmol of O/min per mg)	Conductance (nmol of H ⁺ /min per mg per mV)
Control			
+ Albumin	155.6 $\pm$ 4.7	193.5 $\pm$ 16.7	8.7 $\pm$ 0.7
+ GDP	173.7 $\pm$ 3.2	136.9 $\pm$ 13.0	5.5 $\pm$ 0.6
GDP-sensitive component	18.1 $\pm$ 3.0	56.4 $\pm$ 12.2	3.2 $\pm$ 0.6
Dosed with BRL 26830A			
+ Albumin	139.8 $\pm$ 7.2*	207.2 $\pm$ 33.1	10.3 $\pm$ 1.2
+ GDP	170.1 $\pm$ 5.4	143.2 $\pm$ 16.1	5.9 $\pm$ 0.6
GDP-sensitive component	29.7 $\pm$ 2.9*	63.9 $\pm$ 19.0	4.4 $\pm$ 0.8*

Radioimmunoassay of uncoupling protein

The mitochondrial uncoupling-protein concentration was measured by solid-phase radioimmunoassay, with Tween 20 included in all stages of the assay to increase sensitivity and specificity (Lean *et al.*, 1986). A rabbit anti-(rat uncoupling protein) serum was used, with a rat uncoupling-protein standard prepared from cold-acclimated animals (Lin & Klingenberg, 1980).

Statistical analysis

The statistical significance of differences between groups was assessed by Student's t test, paired or unpaired. A paired t test was used in the experiments on the measurement of mitochondrial proton conductance in order to eliminate day-to-day variation in the measured conductance values. The rats were divided into dosed and control groups at the start of each individual experiment, and were then treated identically in every respect.

RESULTS

Acute effects of BRL 26830A on GDP binding: Scatchard analysis of binding data

In the first experiments the acute effects of BRL 26830A on GDP binding to BAT mitochondria were

determined, binding being measured over an extended range of ligand concentration. Scatchard analysis of the binding data produced curves of the type shown in Fig. 1(a). The curve for the rats dosed with BRL 26830A was shifted markedly to the right. The binding curves were subjected to an iterative analysis, in order to resolve them into their component lines, each of which represents a different binding site (Mendel & Mendel, 1985). The curves for mitochondria from both control and dosed animals resolved as in Fig. 1(b), showing a single high-affinity site (K_d 0.3 μ M), with a very-low-affinity, presumably non-specific, component (K_d 80–100 μ M).

Repeat studies showed that dosing animals with BRL 26830A consistently induced this rapid increase in GDP binding, which was due to an increase in the maximum number of binding sites with no change in the K_d of the high-affinity site (Table 1). The number of binding sites increased more than 2-fold after treatment with the β -agonist. The ' K_d ' of the very-low-affinity component of the binding curve was also unaffected by BRL 26830A.

In contrast with the large increase in GDP binding, there was no acute effect of BRL 26830A on the concentration of uncoupling protein (Table 1). This protein is considered to exist as a dimer, each dimer having one binding site for GDP (Lin & Klingenberg, 1980). The binding ratio (mol of GDP/mol of dimer) for control and treated animals was calculated (Table 1).

The ratio more than doubled in the treated group, to a value suggesting that three-quarters of the theoretical number of binding sites were occupied.

Effect of BRL 26830A on proton conductance

Table 2 shows the measurements of respiration rate and membrane potential, which were made after bovine serum albumin (to bind fatty acids) and then GDP had been added to recouple the mitochondria. Conductances were calculated from the steady-state measurements after each addition. The results show that, after the addition of albumin, the mitochondria from dosed animals established a significantly lower membrane potential than did those from controls, with no significant difference in respiration rate. On addition of GDP, the mitochondria from both groups of animals established similar membrane potentials, the change in potential being significantly greater for the dosed animals. Again, the respiration rates were not significantly different.

The decrease in conductance of BAT mitochondria on addition of GDP is termed the GDP-sensitive component of conductance (Rial & Nicholls, 1984). When conductances were calculated, the results indicate that there was a small, but statistically significant, increase in the GDP-sensitive component in mitochondria from dosed animals compared with controls, with no significant difference in the final conductance values between the groups.

DISCUSSION

GDP-binding studies have been widely used as a method of assessing the thermogenic state of BAT mitochondria *in vitro*, but whether the binding studies are a measure of the concentration of uncoupling protein or some index of the 'activity' of the protein is not yet clear. There is evidence to suggest that the extent of GDP binding can be dissociated from the uncoupling-protein content of BAT mitochondria (Desautels *et al.*, 1978; Desautels & Himms-Hagen, 1979; Gribskov *et al.*, 1986; Trayhurn *et al.*, 1987); that is, rapid changes in GDP binding have been measured without apparent changes in the amount of uncoupling protein. Other studies, however, have failed to detect any rapid change in the ability of uncoupling protein to bind GDP, and a fixed stoichiometry for the binding of the ligand to uncoupling protein has been postulated (Holloway *et al.*, 1984; Rial & Nicholls, 1984; Ricquier *et al.*, 1984; Nedergaard & Cannon, 1985). In this case the extent of GDP binding would represent a direct measure of uncoupling-protein concentration.

There are several possible factors underlying these conflicting results, which may largely relate to the diversity of experimental approaches used. Investigation of 'unmasking' requires an assessment of mitochondrial uncoupling-protein content that is independent of GDP binding, yet this has not always been done. In some cases uncoupling protein has been determined by using densitometric scanning of mitochondrial proteins separated by SDS/polyacrylamide-gel electrophoresis, but this method is neither sensitive nor necessarily specific for the protein. In order to detect rapid 'unmasking' or 'activation' of uncoupling protein it may also be necessary for the tissue to receive a very specific and

substantial thermogenic stimulus. Cold exposure has generally been used to activate thermogenesis, but it provides a variable (and possible weak) stimulus to BAT in short-term studies, the acute physiological response to cold involving a complex series of changes, including the initiation of shivering.

In the present study we have attempted to circumvent some of these problems. A β -agonist with a selective action on β -adrenoceptors in BAT (Arch *et al.*, 1984a,b) was used to provide a direct stimulus, sufficient to activate thermogenesis rapidly and substantially. Parallel measurements of mitochondrial uncoupling protein were made by radioimmunoassay, a method that is highly sensitive as well as being specific to the protein. Since the primary parameter diagnostic of functional uncoupling protein is the nucleotide-sensitive proton conductance of the mitochondrial membrane (Nicholls, 1974; Nicholls & Locke, 1984), direct measurements of proton conductance were also included.

The results obtained clearly show that the acute stimulation of thermogenesis by a single dose of the β -agonist BRL 26830A induces a large increase in GDP binding, without any change in the concentration of uncoupling protein. The increase in binding was accompanied by an increase in the GDP-sensitive component of proton conductance. Changes in the 'activity' of uncoupling protein occurring in synchrony with the changes in GDP binding would be predicted, since the extent of GDP binding has previously been shown to parallel the proton conductance of the mitochondria (Rial & Nicholls, 1984). In addition, acute increases in GDP binding have been shown to be associated with an increase in the GDP-sensitive component of the passive swelling of the mitochondria in potassium acetate (Trayhurn *et al.*, 1987). However, another study reported acute increases in GDP binding without changes in mitochondrial swelling rates in rats exposed to the cold (Swick & Swick, 1986). Only after 10 days of cold exposure was an increase in swelling observed, suggesting that acute changes in GDP binding may not necessarily reflect alterations in proton conductance.

The explanation for the disparity between these results is not clear. However, in the report by Swick & Swick (1986) mitochondrial swelling was measured in potassium acetate without the addition of fatty acid-free albumin, so that the GDP-sensitive component of the swelling would not be separated from the effects of endogenous fatty acids. This may have obscured any differences in the GDP-sensitive component of the swelling in mitochondria from control and cold-exposed animals.

The changes in mitochondrial GDP binding documented here were measured by using Scatchard (1949) analysis. Scatchard analysis of radioligand binding data from early studies identified a single class of binding site for GDP on BAT mitochondria with a K_d of approx. 1–2 μM (Nicholls, 1976; Sundin & Cannon, 1980; Goodbody & Trayhurn, 1981). More recently, two distinct high-affinity binding sites have been reported, a 'low-capacity/very-high-affinity' site being found in addition to the previously recognized 'high-affinity' site (Bryant *et al.*, 1983, 1984; French *et al.*, 1985). The existence of a third, 'very-low-affinity', site has also been reported, but this site is non-functional with respect to the regulation of mitochondrial proton permeability (Rial & Nicholls, 1983) and may well represent non-specific binding.

In the studies in which two high-affinity sites were reported, non-specific binding was assessed by measuring binding in the presence of excess unlabelled ligand (Bryant *et al.*, 1983, 1984; French *et al.*, 1985). In the present study we have used an approach to the analysis of mitochondrial GDP binding in which a 'total binding' curve is produced, which is then resolved into its component lines by iterative analysis procedures (Mendel & Mendel, 1985). This approach is attractive because it does not rely on assumptions about non-specific binding, but instead directly measures the 'total binding' by using an extended range of ligand concentrations. With this approach only one high-affinity GDP-binding site (K_d 0.3 μ M) was found, the affinity of which was unaffected by the acute thermogenic stimulus used. No evidence was found for a second, 'very-high-affinity', binding site.

In conclusion, the present study is consistent with the view that binding sites for GDP on uncoupling protein can be rapidly 'masked/unmasked' (Desautels *et al.*, 1978; Desautels & Himms-Hagen, 1979). The results also demonstrate a functional significance of acute changes in GDP binding in that 'masking/unmasking' appears to relate to an 'inactive/active' state of uncoupling protein. GDP binding would seem to be primarily a measure of thermogenic activity, rather than of the amount of uncoupling protein as such, although over the long term the two parameters show parallel changes (see Trayhurn *et al.*, 1987).

There are several mechanisms that could be involved in the change in activity of uncoupling protein. The protein could undergo a conformational change, although it has been suggested that this is unlikely, since unmasking persists despite freeze-thawing and sonication of the mitochondria (Swick & Swick, 1986). However, GDP binding to uncoupling protein alters the products of proteolysis of the protein, and it is suggested that this may be due to a change in the conformation of the protein (French & York, 1986). Another possibility is that uncoupling protein is phosphorylated/dephosphorylated. In a series of experiments, using whole-tissue homogenates or isolated mitochondria from BAT of rats in thermogenically active and inactive states, we have been unable to find any evidence that covalent modification of uncoupling protein by phosphorylation occurs (R. E. Milner, unpublished work). A final possibility is that alterations in oligomeric structure may explain changes in the number of binding sites on uncoupling protein.

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