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DISCREPANCIES BETWEEN THE AFFINITIES OF BINDING AND ACTION OF THE NOVEL β-ADRENERGIC AGONIST BRL 37344 IN RAT BROWN ADIPOSE TISSUE

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The novel brown adipose tissue (BAT) selective β-adrenergic agonist, BRL 37344, is 31-fold more potent than (-)-isoproterenol in stimulating the respiratory rate of interscapular BAT fragments. BRL 37344 is also more potent (9-fold) than (-)-isoproterenol in stimulating adenylate cyclase activity of IBAT purified plasma membranes whereas, in the same preparation, it is 81-fold less potent than (-)-isoproterenol in competition displacement studies with the β-adrenergic ligand, [^{125}I]cyanopindolol. We have previously demonstrated that the photoaffinity reagent [^{125}I]cyanopindolol-diazirine selectively labels a 62 kDa protein in IBAT plasma membranes that displays pharmacological properties of a β_1-adrenergic subtype. Relatively high concentrations of BRL 37344 (10 μM) are required to displace [^{125}I]cyanopindolol-diazirine binding to the 62 kDa protein. Taken together, the results suggest that two different populations of β-adrenergic receptors may co-exist in BAT plasma membranes: a small population (about 15 %) of atypical β-receptors and a large population of β_1-receptors that exhibit high and low affinities for BRL 37344, respectively.

Brown adipose tissue (BAT), which is the main effector of cold-induced and diet induced-thermogenesis in rodents (1, 2), is under the control of the sympathetic nervous system (3) which acts through β-adrenergic receptors (4). The characterization of this receptor, however, is still a matter of controversy. Various studies performed with labeled β-adrenergic ligands suggest that the BAT β-receptor consists mostly of the β_1 subtype (5 - 7). The use of a novel group of β-adrenergic agonists, however, has shown that BAT β-receptor might not conform to the β_1/β_2 classification (8). These β-agonists stimulate brown adipocyte lipolysis much more selectively than atrial rate (β_1) or tracheal relaxation (β_2). The possible atypy of BAT β-receptor is supported by another puzzling feature i.e. its unusually low affinity for β-adrenergic antagonists (8).

To date, the binding characteristics of the novel group of β-adrenergic agonists to BAT plasma membranes have to our knowledge not

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been studied. It thus seemed of interest to perform binding competition studies using the β-adrenergic ligand [^{125}I]cyanopindolol (CYP) and one of these novel β-agonists, BRL 37344, in order to determine if the high affinity of BRL 37344 for β-adrenergic action in BAT could be correlated with a high affinity for binding to the BAT β-receptor. Binding competition studies were also performed using the photoaffinity ligand [^{125}I]CYP-diazirine and labeled proteins were analyzed by SDS polyacrylamide gel electrophoresis in an attempt to characterize BRL 37344 binding proteins. The results demonstrate that BRL 37344 is much less potent in displacing the binding of β-adrenergic radioligands to IBAT receptors than in activating IBAT metabolism. These data extend previous reports on the nature of β-adrenergic responses in IBAT and suggest a new insight into the possible β-receptor subtypes of BAT plasma membranes.

MATERIALS AND METHODS

All organic and inorganic chemicals were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.), Sigma (St.Louis, MO, USA) and Fluka (Buchs, Switzerland). [^{32}P]ATP, 410 Ci/mmol, [^{125}I]CYP, 2,000 Ci/mmol and [^{125}I]CYP-diazirine, 2,000 Ci/mmol were purchased from Amersham (Amersham, U.K.). (-)-Isoproterenol was obtained from Sigma (St. Louis, MO, USA) and BRL 37344 was a gift from Beecham Pharmaceuticals (Epsom, UK). Premixed SDS-polyacrylamide gel electrophoresis standards (phosphorylase b, 92.5 kDa; albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa) were from Bio-Rad (Richmond, CA, USA). X-ray films (XAR-5) were from Eastman Kodak (Rochester, NY, USA).

Sprague-Dawley male rats weighing 200-250 g, kept at room temperature (22°C) with 12 hrs of illumination per day and fed ad libitum with Provimi Lacta chow (Cossonay, Switzerland), were used in these studies. The respiratory rate (MO_2) of IBAT fragments was measured under basal conditions and in response to increasing concentrations of β-agonists. Tissue fragments of about 12 mg were perifused with Krebs-Ringer bicarbonate solution at 30°C, and the respiratory rate measured as described in detail by Barde et al. (9). The IBAT plasma membranes were prepared as previously described (7) and atrium plasma membranes by using the same technique. Protein concentrations were determined by the method of Lowry et al. (10). The adenylate cyclase activity was measured on purified IBAT plasma membranes according to the technique described by Salomon et al. (11) as modified by Cooper et al. (12).

In ligand binding studies, IBAT and atrium plasma membranes were incubated 30 min at 37°C in 20 mM PO_4 buffer at pH 7.4 containing [^{125}I]CYP alone or in the presence of increasing concentrations of β-agonists (total volume: 0.5 ml). The binding of the radioligand to the membranes was determined by the polyethylene glycol precipitation technique (13). Specific binding was defined as the difference between total binding obtained in the absence of competing ligand and the non specific binding value obtained in the presence of an excess of (-)-isoproterenol. The mean specific binding represented 88 % of the total binding. Estimation of the K_i as well as that of the maximal number of binding sites at saturation were calculated from a Scatchard plot (14) using the LIGAND program (15). Plasma membranes were photoaffinity labeled as described previously (7). SDS-polyacrylamide

gel electrophoresis was performed according to the method of Laemmli (16) using 9-15 % acrylamide gradient slab gels as described previously (7).

RESULTS

Oxygen consumption of IBAT fragments

As shown in Fig. 1, (-)-isoproterenol and BRL 37344 produce dose-dependent increases in the MO_2 values of rat IBAT fragments. Maximal stimulation of MO_2 by both agonists is similar (7.8-fold over the basal value) however BRL 37344 is 31-fold more potent ($p<0.001$) than (-) -isoproterenol in stimulating MO_2 in IBAT fragments (Fig. 1 and Table). When BRL was added in the presence of (-)-propanolol (1 μM) a 9-fold shift to the right in the dose-response curve for MO_2 was observed (Fig. 2).

Adenylate cyclase activity of IBAT plasma membranes

Fig. 3 shows the adenylate cyclase activity of rat IBAT plasma membranes in the presence of increasing concentrations of (-)-isoproterenol or of BRL 37344. Maximal stimulations of adenylate cyclase activity are 5.8- and 4.1-fold over basal levels for (-)-isoproterenol and BRL 37344, respectively. The maximally stimulated values are 340 ± 31 and 232 ± 16 pmol of $_cAMP$ formed per mg protein per min for (-)-isoproterenol and BRL 37344, respectively. The difference between these two values is significant ($p<0.05$). As found for MO_2, BRL 37344 is more potent than (-)-isoproterenol in stimulating adenylate cyclase activity in IBAT membranes ($p<0.005$). Comparison of the K_{act} values (see Table) demonstrates that the potencies differ by a factor of 9.

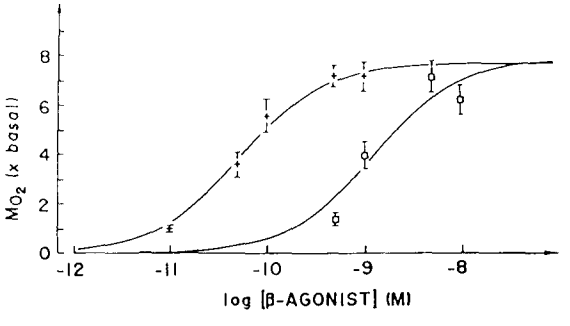


Fig. 1: Steady state increase of MO_2 in IBAT fragments in the presence of increasing concentrations of (-)-isoproterenol (□) or BRL 37344 (+). The values are the means ± SEM of 5 experiments. Mean basal value is 40 ± 2 nmol O_2 per mg wet weight per hour.

TABLE

EC_{50}, K_{act} and K_i values of BRL 37344 and of (-)-isoproterenol on MO_2, adenylate cyclase activity and [^{125}I]CYP binding

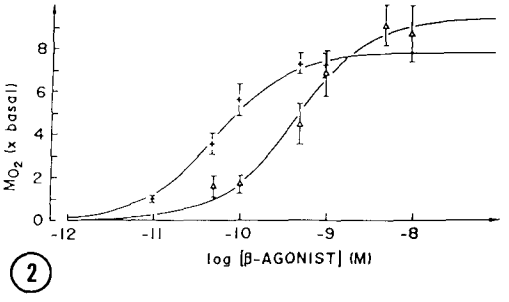
	MO_2	Adenylate cyclase	[^{125}I]CYP binding
(-)-isoproterenol	1.4 ± 0.1	2,130 ± 230	140 ± 50
BRL 37344	0.045 ± 0.010	230 ± 7	11,300 ± 1,800

The results are expressed in nmol/L and represent the mean of the number of experiments in the corresponding figures.

[^{125}I]CYP and [^{125}I]CYP-diazirine binding in IBAT plasma membranes

As illustrated in Fig. 4, the binding of 50 pM [^{125}I]CYP to IBAT plasma membranes can be displaced by increasing concentrations of BRL 37344 or (-)-isoproterenol. (-)-Isoproterenol is a more potent inhibitor of [^{125}I]CYP binding to IBAT plasma membranes than BRL 37344. Comparison of the K_i values (see Table) shows that the binding affinities of these ligands for the IBAT β-receptor differ by a factor of 81.

Analysis of the competition displacement curves by the LIGAND program suggests the existence of two populations of binding sites for BRL 37344 with K_i of 5.9 and 14,000 nM, respectively. The high affinity BRL 37344 binding sites represent only 14 % of the total BRL 37344 binding sites of the IBAT plasma membranes. In contrast, although two populations of binding sites for (-)-isoproterenol are present in IBAT, the majority of these sites (86 %) are of high affinity with a K_i of 92 nM. It is of note that the small population



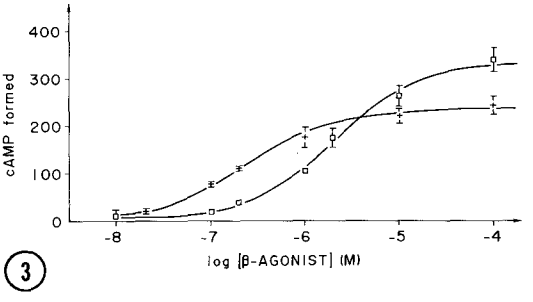
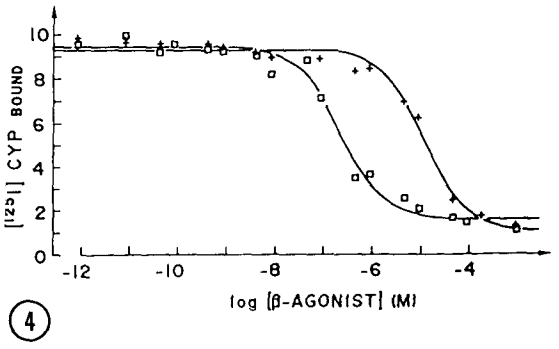


Fig. 2: Steady state increase of MO_2 in IBAT fragments in the presence of increasing concentrations of BRL 37344 alone (+) or with 1 μM (-)-propranolol (Δ). The values are the means ± SEM of 3 experiments.

Fig. 3: Stimulation of adenylate cyclase activity in IBAT plasma membranes by increasing concentrations of (-)-isoproterenol (□) or BRL 37344 (+). The values which are the mean of 3 experiments ± SEM are expressed in pmol of cAMP formed per mg protein per min. Mean basal value is 58 pmol per mg protein per min.



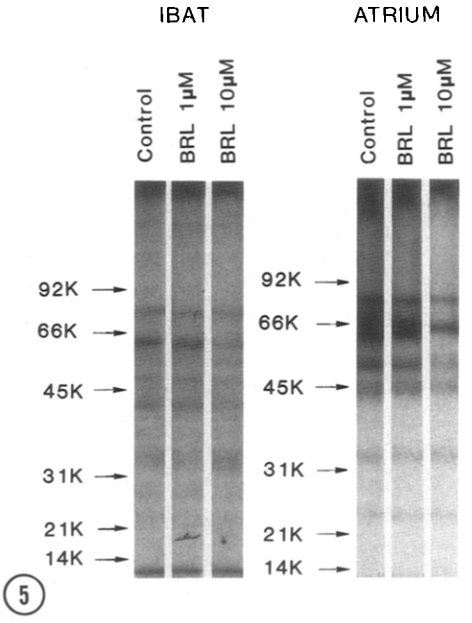


Fig. 4: Displacement of [^{125}I] CYP to IBAT plasma membranes by increasing concentrations of (-)-isoproterenol (□) or BRL 37344 (+). Membranes were incubated with 50 pM [^{125}I]CYP and the indicated concentrations of agonist for 30 min at 37°C. Assays were stopped and binding determined as described. The values, which are the means of duplicates, were obtained in 3 separate experiments and are expressed in fmol of ligand bound per mg of protein.

Fig. 5: Displacement of the specific binding of 0.5 nM [^{125}I]CYP diazirine to IBAT or atrium plasma membranes by BRL 37344. The figure shows autoradiograms obtained after incubation of the plasma membranes with [^{125}I]CYP-diazirine followed by photolysis and polyacrylamide gradient SDS gel electrophoresis (see Methods). The mobility of the protein standards is shown to the left of the figures.

of high affinity BRL 37344 binding sites has an affinity for BRL 37344 16-fold higher than the high affinity (-)-isoproterenol binding sites.

Fig. 5 shows the result of a typical experiment performed with the photoaffinity ligand [^{125}I]CYP-diazirine. A protein of 62 kDa as well as others at 50 and 42 kDa are specifically labeled with [^{125}I] CYP-diazirine. A recent study from our laboratory suggested that these proteins most likely represent the intact β-adrenergic receptor and two major proteolytic fragments (7). In the atrium the pattern of photoaffinity labeling is comparable; specific labeling of peptides at 65, 50 and 45 kDa are observed. It can be seen that BRL 37344 is no more potent in displacing the specific binding of 0.5 nM [^{125}I]CYP-diazirine to the β-receptor in the IBAT than in the atrium In fact, at a concentration of 1 μM BRL 37344, no displacement of [^{125}I]CYP-diazirine binding is seen in plasma membrane proteins of either tissue. It is only when the concentration of BRL 37344 is increased to 10 μM that displacement of the stereoselectively labeled bands is seen in the plasma membranes of both tissues.

DISCUSSION

The MO_2 value measured in BAT fragments is a good index of thermogenesis in this tissue. The β-agonist BRL 37344 was found to be more potent in stimulating MO_2 in brown adipose tissue fragments than (-)-isoproterenol. This result extends the finding by Arch et al. (8) that BRL 37344 was more potent in stimulating lipolysis in brown adipocytes than (-)-isoproterenol. The EC_{50} of BRL 37344 for the stimulation of thermogenesis was even lower than the EC_{50} described by Arch et al. (8) for stimulation of lipolysis in brown adipocytes by a factor of 38-fold, confirming that BRL 37344 is a very powerful thermogenic agent in BAT. The inhibition of BRL 37344-stimulated MO_2 by (-)-propanolol confirms that BRL 37344 acts through the β-receptor.

Adenylate cyclase activity and β-adrenergic binding were both measured on the same purified IBAT plasma membrane system to allow for a more valid comparison of the results. The K_{act} of (-)-isoproterenol and BRL 37344 were 3 orders of magnitude higher than the EC_{50} for MO_2 observed in tissue fragments. Such a change in potency has already been described by Svartengren et al. (17). The K_{act} and the activity at maximal stimulation obtained in the present study are very close to those reported in the literature (17, 18). The main finding of these experiments is that the K_{act} of BRL 37344 for adenylate cyclase activity in IBAT plasma membranes is lower than the K_{act} of (-)-isoproterenol. It is interesting to note that BRL 37344 stimulates less adenylate cyclase activity than (-)-isoproterenol. The reason for this difference is currently under study.

The situation is quite different when the K_i of (-)-isoproterenol and of BRL 37344 for [^{125}I]CYP displacement are studied. It was found that BRL 37344 is much less potent that (-)-isoproterenol in competing with [^{125}I]CYP for binding to IBAT β-receptors. Analysis of the curves using the LIGAND program (15) suggests that a small population of binding sites (14 %) has a high affinity for BRL 37344. This population would represent the atypical β-receptor proposed by Arch et al. (8) which mediates the selective thermogenic effect of BRL 37344 in BAT. The major part of the binding sites i.e. the (-)-isoproterenol binding sites have a low affinity for BRL 37344. It cannot be determined from these data wether the thermogenic effect of (-)-isoproterenol is mediated by the atypical β-receptor displaying high affinity for BRL 37344 and/or by the other β-receptors present in IBAT.

The autoradiograms reveal that the concentration of BRL 37344 required for displacement of [^{125}I]CYP-diazirine binding to the IBAT β-receptor is significantly higher than that which elicits maximal adenylate cyclase activation. Furthermore, the tissue selectivity described for BRL 37344 action is not observed, since BRL 37344 is no more potent in inhibiting [^{125}I] CYP-diazirine binding to the β-receptor in IBAT plasma membranes than in atrium. It should be noted

that the labeled 62 kDa protein has the characteristics of a β_1 adrenergic receptor subtype since the β_1-antagonist, betaxolol, was found to be 100 times more potent in displacing the labeling of this protein than the β_2-antagonist ICI 118,551 (7). One interpretation of these data is that both the atypical β-receptor and the β_1-receptor have the same molecular weight of 62 kDa. The small population of atypical β-receptors with a high affinity for BRL 37344 would be displaced by low concentrations of BRL 37344 but, since they represent only a small percentage of the labeled receptor this displacement would not be detectable. The binding of [^{125}I]CYP-diazirine to the large population of β_1-receptors, having a low affinity for BRL 37344, is only displaced by high concentrations of BRL 37344 and to the same extent in IBAT and in atrium. Another interpretation of these data is that, due to the low affinity of the atypical receptor for β-antagonists (8), [^{125}I]CYP-diazirine does not bind to the atypical receptor and thus cannot be detected on the autoradiograms.

Taken together the results of this study suggest that two different populations of β-adrenergic receptors may co-exist in IBAT plasma membranes: a small population of atypical β-receptor that exhibit high affinity for BRL 37344 but that is difficult to detect by competition displacement studies with radiolabeled β-adrenergic antagonists and a large population of β_1-receptor that exhibit low affinity for BRL 37344. The cloning and sequence analysis of β-adrenergic receptors in brown adipose tissue will provide the most definitive evidence for the existence of more than one receptor subtype.

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