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The β-adrenergic radioligand [^{3}H]CGP-12177, generally classified as an antagonist, is a thermogenic agonist in brown adipose tissue

Nina MOHELL and Andrea DICKER
The Wenner-Gren Institute, Biologihus F3, University of Stockholm, S-106 91 Stockholm, Sweden

The effect of CGP-12177, originally developed as a radioligand with antagonist properties for binding studies of β-adrenergic receptors, was investigated in brown adipose tissue. Contrary to expectations, CGP-12177 showed clear agonist properties in experiments with hamster brown-fat cells, with a maximal effect in stimulating oxygen consumption similar to that of the physiological stimulator noradrenaline, and also with a potency similar to that of noradrenaline [EC_{50} (50% effective concn.) approx. 70 nM]. This value could be contrasted with the very high affinity of CGP-12177 (K_D about 1 nM) for ligand-binding sites on the cells. It is therefore suggested that the high-affinity binding site may not be the one that mediates the CGP-12177-stimulated thermogenesis in isolated cells. Also, when injected into cold-adapted rats, CGP-12177 stimulated non-shivering thermogenesis similarly to noradrenaline. This observation, in conjunction with the reported low general sympathomimetic effect of CGP-12177, may indicate that CGP-12177 could be of interest for the development of anti-obesity drugs.

INTRODUCTION

Thermogenesis in brown adipose tissue is mainly mediated via β-adrenergic receptors (Nedergaard & Lindberg, 1982; Mohell *et al.*, 1983*b*, 1987) and is physiologically stimulated by noradrenaline released from the sympathetic innervation of the tissue. The binding of noradrenaline to β-adrenergic receptors activates adenylate cyclase, and the increase in intracellular cyclic AMP concentration activates the hormone-sensitive lipase. The released fatty acids serve as substrate for the mitochondria as well as an uncoupler of them, resulting in increased respiration (i.e. thermogenesis) (for review see Nedergaard & Lindberg, 1982).

The β-adrenergic receptors in brown adipose tissue have been studied by radioligand-binding techniques. Originally, the antagonist [^{3}H]dihydroalprenolol ([^{3}H]-DHA) was used as the radioligand (Bukowiecki *et al.*, 1978; Svoboda *et al.*, 1979; Kurahashi & Kuroshima, 1981; Levin *et al.*, 1982; Seydoux *et al.*, 1982; Rothwell *et al.*, 1985). However, the hydrophilic radioligand [^{3}H]CGP-12177 (Staehelin *et al.*, 1983) has proved to be superior in studies with intact cells, and particularly in studies of the agonist-induced internalization of β-adrenergic receptors (owing to its hydrophilicity; [^{3}H]CGP-12177 only labels cell-surface receptors) (Sher & Clementi, 1984; Toews *et al.*, 1984; Lacasa *et al.*, 1985, 1986).

[^{3}H]CGP-12177 has also recently been used as a radioligand to label β-adrenergic receptors in brown adipose tissue (Levin & Sullivan, 1986; Raasmaja & York, 1988; Mohell & Nedergaard, 1988*a*,*b*; Mohell *et al.*, 1989). The [^{3}H]CGP-12177-binding sites show essentially the same β-adrenergic characteristics as those previously found with [^{3}H]DHA, indicating that the two radioligands label the same β-receptor population (Mohell & Nedergaard, 1988*a*,*b*; Mohell *et al.*, 1989).

In order to correlate the characteristics of the [^{3}H]CGP-12177-binding sites with the physiological response, we attempted to investigate the inhibitory effect of CGP-12177 on noradrenaline-stimulated O_2 consumption (i.e. heat production) of isolated brown-fat cells. Unexpectedly, we found that instead of inhibiting this noradrenaline-stimulated O_2 consumption, CGP-12177 in itself was able to stimulate the respiration. The present experiments were designed to investigate this interesting phenomenon further. The results demonstrate that CGP-12177, shown to be an antagonist in other tissues (Staehelin *et al.*, 1983; Portenier *et al.*, 1984), in fact shows practically full thermogenic-agonist properties in isolated brown-fat cells and even *in vivo*. The results are discussed in relation to the question of the nature of the suggested atypical β-adrenergic receptor in brown adipose tissue (Arch *et al.*, 1984). In addition, the possible use of CGP-12177 as an anti-obesity drug is discussed.

MATERIALS AND METHODS

Materials

(−)-[^{3}H]CGP-12177 {(−)-4-(3-t-butylamino-2-hydroxypropoxy)[5,7-^{3}H]benzimidazol-2-one} (46 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.).

Unlabelled CGP-12177 was generously given by Ciba–Geigy (Vastra Frölunda, Sweden). Noradrenaline was obtained as (−)-Arterenol bitartrate (Sigma). Both agents were dissolved in 0.04% ascorbate for the studies *in vitro* and in physiological saline for the studies *in vivo*. Prazosin hydrochloride was a gift from Pfizer (Brussels, Belgium). (±)-Propranolol hydrochloride and (−)-alprenolol d-tartrate were from Sigma.

Methods

Measurement of cell respiration. Brown-fat cells from adult hamsters (*Mesocricetus auratus*) living at 21±2 °C were isolated by a collagenase digestion method, and the O_2 consumption by the isolated cells was measured polarographically with a Clark-type oxygen electrode, as described previously (Mohell *et al.*, 1983*b*).

Abbreviations used: [^{3}H]DHA, [^{3}H]dihydroalprenolol; EC_{50}, 50% effective concn.

Measurement of thermogenesis. The O_2 consumption *in vivo* was measured in an open-circuit metabolic chamber, principally as described by Heldmaier & Steinlechner (1981). The animals used were adult warm-adapted (pre-acclimatized to 28 °C) or cold-adapted (more than 8 weeks at 4 °C) female Sprague–Dawley rats.

Radioligand-binding studies. The radioligand-binding assay was performed with crude membrane preparations or with intact isolated cells (similar to those used for the cell-respiration studies) made from the brown adipose tissue of adult hamsters as previously described (Mohell *et al.*, 1983*a*; Raasmaja & York, 1988). For total binding, (−)-[^{3}H]CGP-12177 was added in 11 concentrations from about 0.05 to 4.0 nM; 1 μM-(−)alprenolol was present in parallel incubations for determination of non-specific binding. The IC_{50} value for CGP-12177 (i.e. the molar concentration causing 50% inhibition of specific [^{3}H]CGP-12177 binding) was determined by competition experiments, and the K_i value (i.e. the inhibitor constant) was estimated from the equation (Cheng & Prusoff, 1973):

$$K_i = IC_{50}/1 + (L/K_D)$$

where K_D is the affinity of [^{3}H]CGP-12177 (0.7 nM) and L the concentration of the radioligand (3 nM).

RESULTS

Stimulation of O_2 consumption *in vitro*

One of the most characteristic features of brown-fat cells is their extremely high capacity for catecholamine-stimulated O_2 consumption.

Fig. 1 shows the dose–response curves for CGP-12177 and noradrenaline stimulation of O_2 consumption by brown-fat cells. The EC_{50} values were 20±4 nM for noradrenaline and 73±17 nM for CGP-12177, and the maximal increases in the rates of oxygen consumption were 480±10 and 450±30 nmol of O/min per 10^6 cells

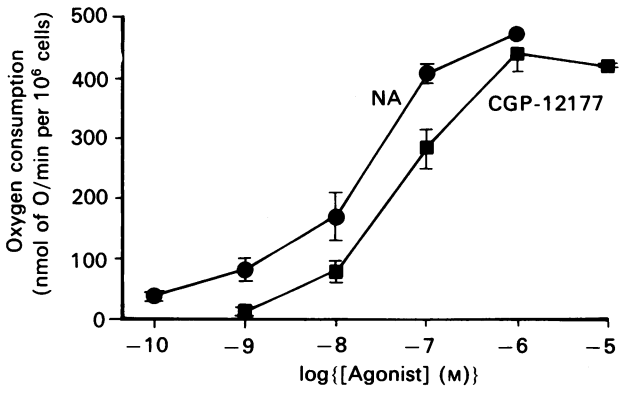

Fig. 1. Dose–response curve for CGP-12177 and noradrenaline stimulation of O_2 consumption in hamster brown-fat cells

To obtain the indicated concentration, CGP-12177 (■) or noradrenaline (NA; ●) was added successively in increasing concentrations to the cell suspension, and the rate of O_2 consumption was measured for 2–3 min for each concentration. Basal respiration was subtracted (40 nmol of O). Results are means±S.E.M. for eight determinations on three different cell preparations.

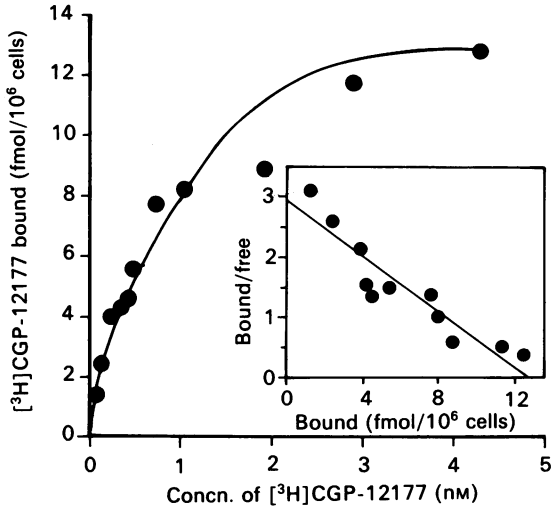

Fig. 2. Specific [^{3}H]CGP-12177 binding to isolated hamster brown-fat cells as a function of radioligand concentration

Binding experiments were performed as described under 'Methods'. Each value represents the mean of duplicate determinations. The experiment shown is representative of three such experiments performed with different cell preparations. Inset: Scatchard (1949) analysis of specific [^{3}H]CGP-12177 binding. The slope of the plot, $-1/K_D$, was determined by regression analysis. The maximal binding capacity, $B_{max.}$, was obtained as the intercept with the abscissa. Results: $B_{max.}$ = 13 fmol/10^6 cells; K_D = 0.59 nM; r = 0.92.

respectively (n = 8). Thus the surmised antagonist CGP-12177 showed pronounced agonist properties in its ability to stimulate brown-fat-cell respiration.

It has been demonstrated that the major part of the noradrenaline-stimulated respiration in hamster brown-fat cells is due to stimulation of β-adrenergic processes (Mohell *et al.*, 1983*b*). That the effect of CGP-12177 was also mediated via β-adrenergic receptors was indicated by experiments in which the ability of the β-antagonist propranolol to shift the dose–response curve was tested. The presence of 10 μM-propranolol led to a 10-fold shift to the right in the dose–response curve for both noradrenaline and CGP-12177 (results not shown).

In control experiments, we demonstrated that the radioactively labelled compound (−)-[^{3}H]CGP-12177 also showed agonist properties, similar to those of the parent compound (results not shown).

The EC_{50} value of CGP-12177 for stimulating O_2 consumption (73 nM) was about 100-fold higher than the K_D of [^{3}H]CGP-12177 binding generally reported (about 0.5 nM) and also shown in brown fat (Raasmaja & York, 1988; Mohell *et al.*, 1989; and see below). It could be envisaged that CGP-12177 at lower concentrations demonstrated the expected antagonist properties. Therefore we tested the effect of 1 nM-CGP-12177 on noradrenaline-stimulated respiration. Neither stimulatory nor inhibitory effects on the noradrenaline dose–response curve were found.

Binding of [^{3}H]CGP-12177

As stated in the Introduction, [^{3}H]CGP-12177 is normally used in radioligand-binding studies of β-adrenergic receptors, generally on the assumption that it is a β-

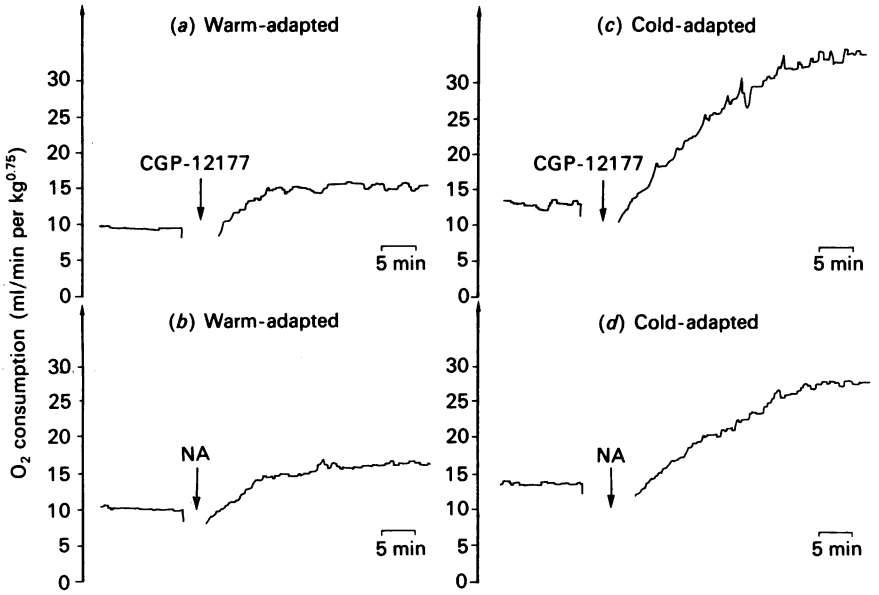

Fig. 3. CGP-12177 (*a*, *c*) and noradrenaline (*b*, *d*) stimulation of thermogenesis

Warm (*a*, *b*)- and cold (*c*, *d*)-adapted rats (body wt. about 300 g) were transferred to the thermostatically maintained (28 °C) metabolic chamber, and the basal metabolic rate was estimated for a period of 2 h. After this, the rats were injected intraperitoneally with 1 mg (per kg body wt.) of a saline solution of either CGP-12177 or noradrenaline (NA), and the non-shivering thermogenesis was measured as described under 'Methods'. The unit $kg^{0.75}$ refers to the 'metabolic body weight' (Hemmingsen, 1960).

adrenergic antagonist. Also in brown-adipose-tissue membrane preparations, [^{3}H]CGP-12177-binding sites have been identified and shown by competition experiments to have β-adrenergic specificity (Levin & Sullivan, 1986; Raasmaja & York, 1988; Mohell & Nedergaard, 1988*a*,*b*; Mohell *et al.*, 1989). In order to be able to compare directly [^{3}H]CGP-12177-binding affinity with its potency to elicit the biological response, we performed radioligand-binding experiments with the isolated intact-cell preparations also used in O_2-consumption studies. As shown in Fig. 2, [^{3}H]CGP-12177 bound to receptors on isolated cells with as high affinity (K_D 0.59 nM) as in crude membrane preparations (see below). There was about 100-fold difference between the EC_{50} and K_D values.

Agonist- and antagonist-binding affinities to adrenergic receptors are expected to show differential responses to guanine nucleotides (Rodbell, 1980). Thus in membrane preparations the affinity for agonists is decreased in the presence of GTP, as has been shown also in brown adipose tissue for the classical β-receptor studied with [^{3}H]DHA and noradrenaline (Svartengren *et al.*, 1984). Assuming that the agonist effect of CGP-12177 reported above was mediated via the classical β-adrenergic receptor also labelled with the radioligand, it would be expected that the affinity of [^{3}H]CGP-12177 for this binding site should be influenced by GTP. However, we were unable to find any significant effect of the presence of 100 μM-GTP on the binding affinity of [^{3}H]CGP-12177; it was in both cases about 0.7 nM. In a series of four such experiments, performed on two different membrane preparations, the K_D values were 0.8 ± 0.1 and 0.9 ± 0.1 nM in the absence and presence of GTP respectively, as analysed according to Scatchard (1949). The maximal number of binding sites was not affected. The binding data constituted a straight line in the Scatchard analysis, indicating the existence of only one type of binding site in this concentration range (see under 'Methods').

In control experiments, we checked by competition studies the affinity of unlabelled CGP-12177 for the radioligand-binding site labelled with [^{3}H]CGP-12177. The K_i of CGP-12177 was 3.7 ± 1.6 nM (two determinations on two different membrane preparations) (results not shown).

Stimulation of thermogenesis *in vivo*

In order to investigate whether the agonist effect of CGP-12177 could also be observed *in vivo*, the ability of CGP-12177 to stimulate non-shivering thermogenesis in conscious control warm-adapted and cold-adapted rats was investigated.

A low dose of either CGP-12177 or noradrenaline (0.1 mg/kg body wt.) had no appreciable effect on the rate of O_2 consumption by warm-adapted or cold-adapted rats (results not shown). When a larger dose of CGP-12177 or noradrenaline (1 mg/kg body wt.) was injected in warm-adapted rats, the O_2 consumption was increased about 70% (Figs. 3*a* and 3*b*). In cold-adapted rats a typical pronounced thermogenic response to noradrenaine as well as to CGP-12177 was seen: more than a doubling of the metabolic rate (Figs. 3*c* and 3*d*). Thus the effect of CGP-12177 was similar to the effect of noradrenaline in both warm- and cold-adapted rats. The stimulation of non-shivering thermogenesis in cold-adapted rats was blocked by propranolol (20 mg/kg body wt.), but not by prazosin (0.5 mg/kg body wt.), suggesting that it was a β-receptor-mediated process (results not shown).

It was concluded that CGP-12177 could induce non-shivering thermogenesis also *in vivo*, with a potency and

maximal effect similar to that of the physiological agonist noradrenaline.

DISCUSSION

In this investigation, we demonstrate that CGP-12177, which is normally considered to be a β-adrenergic antagonist (Staehelin *et al.*, 1983; Portenier *et al.*, 1984; Sher & Clementi, 1984; Toews *et al.*, 1984; Lacasa *et al.*, 1985, 1986), was able to stimulate both isolated brown-fat-cell respiration *in vitro* as well as non-shivering thermogenesis *in vivo* with an efficiency and potency similar to that of the natural agonist noradrenaline.

This agonist effect of CGP-12177 was unexpected. However, in one cell type (C6 rat glioma cells; Staehelin *et al.*, 1983), CGP-12177 has been shown to be a weak partial agonist for stimulation of cyclic AMP production [but not in, e.g., S49 cells (Portenier *et al.*, 1984)]. As it has been shown that the EC_{50} of noradrenaline for stimulation of adenylate cyclase (increase in cyclic AMP) in isolated brown-fat cells is rather high (800 nM) as compared with the EC_{50} for stimulation of respiration (38 nM) (Svartengren *et al.*, 1982), it is possible that also in brown-fat cells CGP-12177 is a partial agonist for cyclic AMP production. In brown-fat cells, with a high coupling between cyclic AMP and the respiratory response, a small increase in cyclic AMP may be sufficient to stimulate thermogenesis fully. This may be the basis for the pronounced agonist properties of CGP-12177.

However, not even a high coupling efficiency can explain the discrepancy between the high binding affinity (K_D) of [^{3}H]CGP-12177 and its low potency (EC_{50}) to stimulate respiration. In fact, a high coupling efficiency between receptor binding and biological response results in high potency (i.e. low EC_{50}) to elicit biological response when compared with binding affinity. The unique properties of CGP-12177 as a thermogenic agonist are consequently further stressed by a comparison with the classical β-agonist isoprenaline. We have previously (in competition studies) shown that the K_i of isoprenaline for the [^{3}H]CGP-12177-binding site in membrane preparations (no GTP added) is rather high (55 nM) (Mohell *et al.*, 1989), but that its EC_{50} value to stimulate O_2 consumption is low (8 nM) (Mohell *et al.*, 1983*b*), i.e. for isoprenaline the relation between binding affinity and potency is opposite to that of CGP-12177.

In the light of the discussion of atypical β-receptors in brown adipose tissue (Arch *et al.*, 1984), a possible interpretation of these unique properties of CGP-12177 could be as follows. In brown adipose tissue, two β_1-receptor subtypes (Mohell & Nedergaard, 1988*b*; Mohell *et al.*, 1989) or alternatively two different binding sites, or two different affinity states of the receptor (Lang & Lemmer, 1985; Takayanagi *et al.*, 1987), may exist. One β_1-receptor (or binding site) would correspond to the traditional β_1-receptor generally labelled with antagonist radioligand. To this, [^{3}H]CGP-12177 binds with high affinity (less than 1 nM), and so does the traditional β-antagonist propranolol (about 11 nM) (Mohell & Nedergaard, 1988*a*,*b*; Mohell *et al.*, 1989). The novel β-adrenergic drugs (BRL series) show only poor affinity for this binding site (about 1 μM) (Mohell & Nedergaard, 1988*b*). In this interpretation, the binding of adrenergic drugs to this site would not be coupled to a stimulation of O_2 consumption (thermogenesis).

The other receptor (or site) must so far remain a postulate, as it has not been demonstrated with radioligand-binding studies. This site would be the one coupled to stimulation of O_2 consumption, and to it CGP-12177 would bind with lower affinity, as would propranolol (which is a comparatively poor antagonist of thermogenesis). Here also the BRL compounds would exert their thermogenic effect.

We emphasize that, regardless of which molecular explanation is valid, there are several important implications of the present study.

First, since CGP-12177 is apparently without significant stimulatory sympathomimetic effects (German Patent 2700193 cited in Staehelin *et al.*, 1983; Portenier *et al.*, 1984) on systems other than brown adipose tissue, the ability of CGP-12177 to stimulate thermogenesis competently in intact animals must be considered as a demonstration that the increased heat production under these circumstances is a consequence of stimulation of brown adipose tissue, a point still under debate (Foster, 1984).

Secondly, the directed ability of CGP-12177 to stimulate thermogenesis in brown adipose tissue, apparently without showing significant sympathomimetic effects, makes this compound a very interesting candidate for an anti-obesity drug, with a selective action in stimulating metabolic degradation of stored and consumed substrate.

Thirdly, we wish to point out that caution is needed when [^{3}H]CGP-12177 is used to characterize β-adrenergic receptors in various tissues. Its possible agonist properties also in other tissues may have important implications for interpretation of the binding data, particularly when agonist-induced internalization of the receptor is investigated.

Note added in proof (received 11 May 1989)

We have recently been informed that CGP-12177 has been observed to have some toxicological effects, and is therefore not in itself feasible as an anti-obesity drug.

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