Biochemical Pharmacology, Vol. 44, No. 12, pp. 2333–2338, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
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PHARMACOLOGICAL CHARACTERIZATION OF A β_3-RECEPTOR AGONIST (BRL 37,344) AND A PARTIAL AGONIST (CGP 12,177A) IN NEONATAL RAT LIVER PLASMA MEMBRANES

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(*Received* 2 *February* 1992; *accepted* 15 *September* 1992)

Abstract—The pharmacological properties of BRL 37,344 (sodium-4-{2'-[2-hydroxy-2-(3-chlorophenyl)ethylamino]-propyl}phenoxyacetatesesquihydrate), a β_3-selective agonist, and CGP 12,177A) (−)-4-(3-*t*-butyl amino-2-hydroxypropoxy) benzimidazole-2-one], a non-selective β-antagonist, recently characterized as a partial β_3-agonist in rat adipose tissue, were studied in comparison with isoproterenol, a non-selective β-agonist, in plasma membranes prepared from the livers of newborn rats. Competition binding curves obtained with [^{125}I]iodocyanopindolol ([^{125}I]CYP) as ligand and isoproterenol or BRL 37,344 as competitor were characterized by the presence of a high and a low affinity binding site; the high affinity binding site was no longer detectable when guanidylimidobisphosphate (GppNHp) was present in the incubation mixture. Competition curves with CGP 12,177A were monophasic and independent of GppNHp. In the presence of 10^{-7} M of the β_2-selective antagonist ICI 118,551 [erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol], a concentration which blocks most of the β_2-receptors, ligand binding was reduced to 32% of its maximum. Under these conditions, isoproterenol further displaced the ligand, and competition curves still displayed the high and the low affinity binding sites; BRL 37,344, however, caused no further displacement of the ligand, except at the highest concentrations. This suggests that BRL 37,344 occupies only the ICI 118,551-sensitive binding sites, i.e. β_2-receptors. Isoproterenol and BRL 37,344 both stimulated adenylate cyclase (EC 4.6.1.1) activity concentration dependently, although the stimulating effect of BRL 37,344 was about half of what was found for isoproterenol. Furthermore, BRL 37,344 inhibited concentration dependently the isoproterenol-induced stimulation of adenylate cyclase, and the inhibition was dependent on the concentration of isoproterenol. The stimulating effect of isoproterenol and BRL 37,344 on adenylate cyclase was blocked by ICI 118,551, whereas the β_1-selective antagonist CGP 20,712A {(±)-(2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino)-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2-propanolmethane sulphonate} was ineffective. CGP 12,177A failed to stimulate adenylate cyclase activity. From these results we suggest that BRL 37,344 acts as a β_2-partial agonist in rat liver. The results obtained with CGP 12,177A are typical for a non-selective β-antagonist. We therefore conclude that there is no pharmacological evidence for the presence of β_3-receptors in livers from newborn rats.

Based on pharmacological and functional experiments, the existence of an atypical β-receptor or β_3-receptor was suggested, first in adipose tissue [1–6], and later in heart [7] and in rat colon [8, 9]. Using a probe against the human β_3-receptor, Emorine *et al.* [10] suggested that this receptor was also present in rat liver, ileum, soleus muscle, skin and adipose tissue [10, 11]. Recently however, the rat β_3-receptor has been cloned, and hybridization techniques suggested that this β_3-receptor was expressed in rat adipose tissues and was absent in rat liver [12, 13].

We have studied recently the effect of maturation and aging on the biochemical properties of the β-adrenergic transduction system in rat liver and we obtained evidence that a mixed β_1–β_2-receptor population is present in the livers of newborn, mature and senescent rats, and that both subtypes are involved in the regulation of glycogenolysis [14, 15]. In competition binding experiments with propranolol and CGP 20,712A {(±)-(2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino)-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2-propanolmethane sulphonate}, and after appropriate correction for non-specific binding we observed that part of the radioligand could not be displaced from its binding site. Propranolol-resistant [^{125}I]-iodocyanopindolol ([^{125}I]CYP†) binding sites were described recently in rat skeletal muscle and were suggested to be atypical β-receptor binding sites [16]. Hence, our observations could be explained by the presence of a β-receptor, different from the β_1- and β_2-receptors and possibly similar to what is found for other propranolol-resistant ligand binding.

Taking into account the results of the hybridization experiments concerning the rat β_3- and the human β_3-receptor, and the results of our own experiments we investigated whether the β-adrenergic binding site in rat liver is sensitive to BRL 37,344 (sodium-4-{2'-[2-hydroxy-2-(3-chlorophenyl)ethylamino]-propyl}phenoxyacetatesesquihydrate), an agonist of both the human and rat β_3-receptor [1, 5] and to CGP 12,177A (−)-4-(3-*t*-butyl amino-2-hydroxypropoxy)

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† Abbreviations: [^{125}I]CYP, [^{125}I]iodocyanopindolol; GppNHp, guanidylimidobisphosphate.

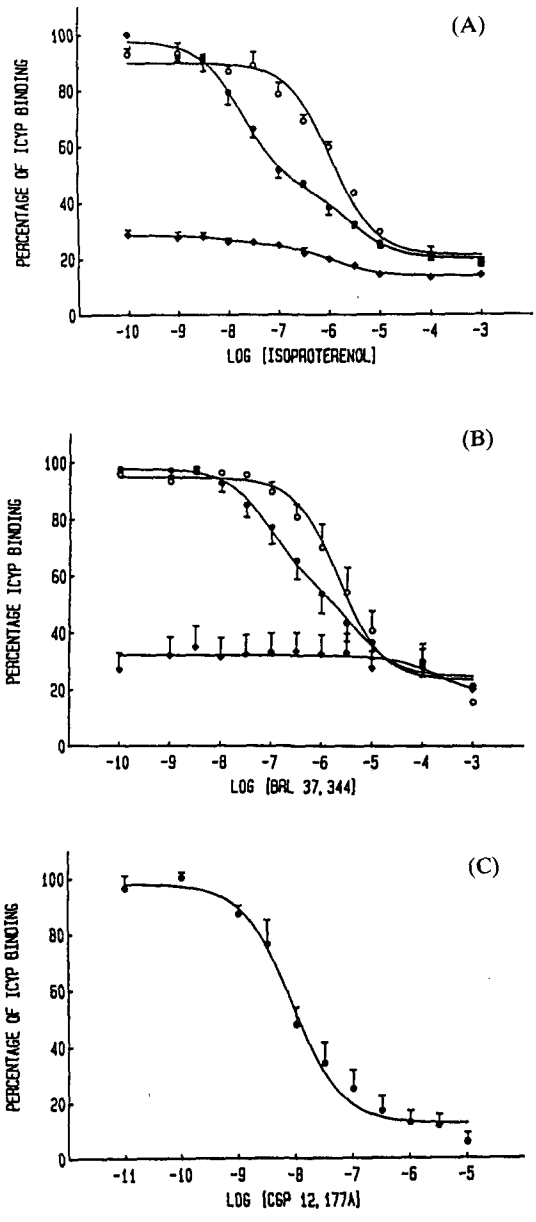

Fig. 1. (A) Mean competition binding curves with [^{125}I]-iodocyanopindolol as ligand and isoproterenol as displacer. The binding at the lowest concentration of isoproterenol is set as 100% and all other binding values are calculated relative to this value. Means ± SEM, N = 8. (●) Competition binding curve without GppNHp; (○) competition binding curve in the presence of 10^{-4} M GppNHp; (◆) competition binding curve in the presence of 10^{-7} M ICI 118,551. (B) Mean competition binding curve with [^{125}I]iodocyanopindolol as ligand and BRL 37,344 as displacer. The binding at the lowest concentration of BRL 37,344 is set as 100% and all other binding values are calculated relative to this value. Means ± SEM, N = 7. (●) Competition binding curve without GppNHp; (○) competition binding curve in the presence of 10^{-4} M GppNHp; (◆) competition binding curve in the presence of 10^{-7} M ICI 118,551. (C) Mean competition binding curve with [^{125}I]iodocyanopindolol as ligand and CGP 12,177A as displacer. The binding at the lowest concentration of CGP 12,177A is set as 100% and all other binding values are calculated relative to this value. Means ± SEM, N = 5.

benzimidazole-2-one], a non-selective β_1–β_2 antagonist, recently characterized as a β_3-partial agonist in rat adipose tissue [17]. The results were compared to those obtained with isoproterenol, a non-selective β-agonist. Our methodology included ligand binding experiments with [^{125}I]CYP and measurement of the activity of adenylate cyclase (EC 4.6.1.1). All experiments were performed on plasma membranes prepared from the livers of newborn rats, which have a high β-receptor density [14].

MATERIALS AND METHODS

Materials. [^{125}I]CYP and [^{3}H]cAMP were obtained from Amersham (U.K.). (*S*)(−)-propranolol and (*S*)(−)-isoproterenol were obtained from the Sigma Chemical Co. (Poole, U.K.). Guanidylimidobisphosphate (GppNHp) and GTP were purchased from Boehringer-Mannheim (F.R.G.). CGP 12,177A and CGP 20,712A were gifts from Ciba-Geigy (Groot-Bijgaarden, Belgium). ICI 118,551 [erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol], was a gift from I.C.I. (Destelbergen, Belgium). BRL 37,344 was a gift from Smith Kline Beecham Pharma (Genval, Belgium).

Animals. Wistar rats of 2–4 days old (average weight of 12 ± 1 g, range 7–20 g; N = 75; further called newborn rats) were used. For each experiment sufficient animals were killed so as to obtain approximately 1 g of liver tissue wet weight (four to nine animals).

Tissue preparation. A crude plasma membrane preparation was obtained as described before [14, 18]. Briefly, rats were decapitated and the livers were homogenized three times for 15 sec with 1 min intervals in homogenization buffer (sucrose–Tris–EGTA, 250–50–2 mM, pH 7.4) at 1/20 dilution (g/mL) with an Ultra Turrax; the homogenate was centrifuged at low speed (3000 *g* for 10 min) and the supernatant at high speed (27,000 *g* for 30 min) (Sorvall, SS-34 rotor). The pellet was washed three times by gentle homogenization (Potter–Elvehjem, four strokes) and centrifugation (27,000 *g* for 30 min) in homogenization buffer. The final membrane pellet was resuspended in assay buffer (Tris–Mg^{2+}–EGTA, 50–20–2 mM, pH 7.4) at 2–4 mg protein/mL. The membrane preparation was used the same day in competition binding experiments with agonists and in experiments involving stimulation of adenylate cyclase.

The protein content was determined with the dye-binding method of Bradford as modified by Macart and Gerbaut [19] using bovine serum albumin as standard.

Ligand binding experiments. Ligand binding experiments were carried out using the manual filtration method as described before [14, 15].

In the saturation binding experiments, nine concentrations of the β-receptor ligand [^{125}I]CYP, ranging from 5 to 300 pM, were incubated for 90 min at 37° with 20–30 μg proteins in a total volume of 250 μL. After separation of bound and free ligand through GF/C filters (Whatman), radioactivity retained on the filters was counted in a γ-teller (Packard instruments). Receptor density is expressed in fmol/mg protein. Preliminary experiments showed

Table 1. Characteristics of the competition binding curves with [^{125}I]iodocyanopindolol as ligand and isoproterenol and BRL 37,344 as displacer in plasma membranes from the livers of newborn rats

	Isoproterenol (N = 8)	BRL 37,344 (N = 7)
Without GppNHp		
K_i, HA (10^{-9} M)	10.5 ± 3.7	34.5 ± 1.5
K_i, LA (10^{-7} M)	13.0 ± 7.4	12.0 ± 0.6
% HA	49.2 ± 4.1	42.5 ± 5.7
With GppNHp		
% LA	100	100
K_i, GppNHp (10^{-7} M)	9.0 ± 1.6	9.7 ± 0.2

K_i, HA and K_i, LA: equilibrium inhibition constants of the high and low affinity binding sites respectively.

% HA: percentage of high affinity binding sites in the absence of GppNHp.

% LA: percentage of low affinity binding sites in the presence of 10^{-4} M GppNHp.

K_i, GppNHp: equilibrium inhibition constants of the agonist binding site in the presence of GppNHp (10^{-4} M).

Values are means ± SEM.

N, number of experiments.

that non-specific binding determined with 10 μM (−)propranolol was linear over the concentration range of the ligand. Similar results were obtained with 100 μM (−)isoproterenol. In all further experiments, non-specific binding was routinely measured at the highest ligand concentration using 10 μM (−)propranolol.

In the competition binding experiments, one concentration of [^{125}I]CYP (between 100 and 150 pM) was incubated with 20–30 μg membrane proteins for 90 min at 30° with different concentrations of either isoproterenol (10^{-10}–10^{-3} M), BRL 37,344 (10^{-10}–10^{-3} M) or CGP 12,177A (10^{-11}–10^{-5} M). Experiments were performed in the absence and presence of GppNHp (10^{-4} M). In some experiments ICI 118,551 (10^{-7} M) together with the different concentrations of BRL 37,344 or isoproterenol was included in the incubation mixture.

Adenylate cyclase activity. The activity was assayed as described before [18], using approximately 90–100 μg proteins in 100 μL membrane suspension; cAMP was determined according to Tovey *et al.* [20]. In the first series of experiments the samples were incubated in the presence of 5×10^{-5} M GTP with either isoproterenol, BRL 37,344 or CGP 12,177A (10^{-10}–5×10^{-4} M) for 20 min at 30°. The increase in adenylate cyclase activity was either expressed as pmol cAMP/mg protein × min or as per cent stimulation above the activity in the presence of GTP alone. In a second series of experiments, isoproterenol (5×10^{-7}, 5×10^{-6} or 5×10^{-5} M) in the presence of 5×10^{-5} M GTP was incubated together with BRL 37,344 (5×10^{-11}–5×10^{-4} M). The net increase in adenylate cyclase activity (increase above the activity in the presence of GTP alone) obtained with isoproterenol in the absence of BRL 37,344 was set as 100% and other values were calculated relative to this value. In a third series of experiments, ICI 118,551 or CGP 20,712A (5×10^{-10}–5×10^{-4} M) was incubated together with 5×10^{-5} M isoproterenol or 5×10^{-5} M BRL 37,344. In these experiments, the net increase in adenylate cyclase activity obtained with 5×10^{-5} M isoproterenol or with 5×10^{-5} M BRL 37,344 in the presence of 5×10^{-5} M GTP was set as 100% and the inhibition was calculated relative to this value.

Calculations. Analysis of the saturation binding curves was done using Scatchard plot analysis. Curve fitting of the data from competition binding experiments was done using the program "GraphPad" [21]. Binding at the lowest concentration of competitor was set as 100% and all other binding data were calculated relative to this value. Curves were routinely fit according to a one- and a two-site model, and the best model was chosen on the basis of the F-test [22]. K_i values were calculated from IC_{50} values according to Cheng and Prusoff [23]. Mean values are given ± SEM.

Differences between two independent groups of observations were evaluated using the Student's *t*-test. Differences between curves were evaluated using one-way or two-way analysis of variance (ANOVA). Statistical difference was accepted at the $P < 0.05$ level.

RESULTS

Ligand binding experiments

The binding of [^{125}I]CYP to the rat liver plasma membranes was saturable and of high affinity. Scatchard plot analysis of the saturation hyperboles (N = 7) yielded linear curves with an average correlation coefficient of 0.97 ± 0.03; receptor density was 124.4 ± 7.7 fmol/mg protein and the equilibrium dissociation constant was 61.3 ± 5.9 pM. Non-specific binding was 18.9 ± 2.7% of total binding at the highest ligand concentration.

The results of the competition binding experiments with isoproterenol, BRL 37,344 and CGP 12,177A are shown in Fig. 1A, B and C, respectively; quantitative data are summarized in Table 1.

In the absence of GppNHp, competition binding curves of isoproterenol and BRL 37,344 were biphasic. The affinity of the high affinity binding site for BRL 37,344 was approximately three times lower than for isoproterenol. In the presence of GppNHp, only one binding site with low affinity for both agonists was present. When in competition experiments, 10^{-7} M ICI 118,551 was added to the incubation mixtures, the ligand was displaced from its binding site to 32.0 ± 5.1% of its maximal binding (N = 6). Competition binding with isoproterenol under these experimental conditions resulted in a further reduction of the binding of ligand. All individual competition binding curves were biphasic, with an average K_i value of $11.0 \pm 7.6 \times 10^{-9}$ M for the high affinity binding site and an average K_i value of $12.0 \pm 9.9 \times 10^{-7}$ M for the low affinity binding site; 43.0 ± 10.2% of the receptors were of high affinity. In similar experiments with BRL 37,344, no further reduction in binding of the ligand was found except at the highest concentrations (10^{-4} and 10^{-3} M).

Competition binding curves with CGP 12,177A

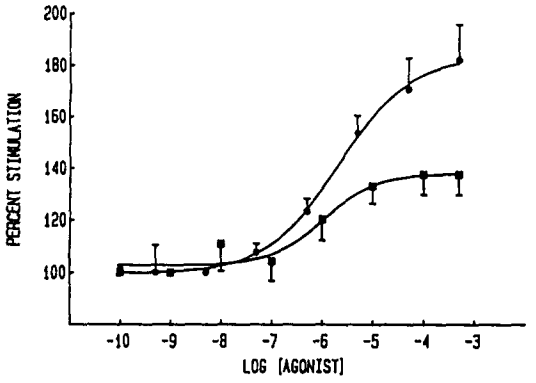

Fig. 2. Adenylate cyclase activity as a function of the concentration of isoproterenol (●) and BRL 37,344 (■) in the presence of 5×10^{-5} M GTP. Means ± SEM, N = 5. The results are presented as per cent increase above the stimulation in the presence of 5×10^{-5} M GTP alone.

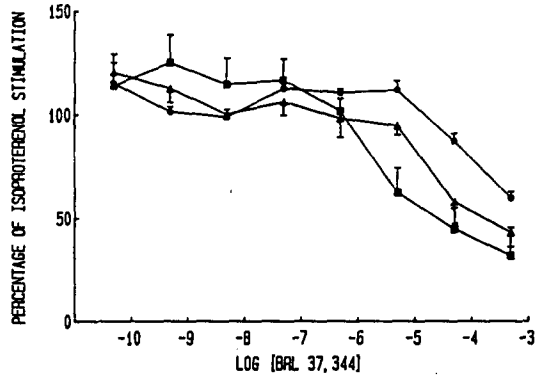

Fig. 3. Adenylate cyclase activity as a function of the concentration of BRL 37,344 and in the presence of one concentration of isoproterenol. The results are presented as per cent of the net isoproterenol-stimulated adenylate cyclase activity, which is set as 100%. Means ± SEM, N = 5. (■) 5×10^{-7}, (▲) 5×10^{-6} and (●) 5×10^{-5} M isoproterenol.

were monophasic, with a K_i value of $2.7 \pm 1.1 \times 10^{-9}$ M (N = 5) and were independent of GppNHp (N = 5, results not shown).

Adenylate cyclase activity

The results of the stimulation of adenylate cyclase are shown in Fig. 2. In the presence of GTP alone the activity of adenylate cyclase was 75.1 ± 10.7 pmol cAMP/mg protein × min (N = 5). Stimulation with isoproterenol (N = 5) and BRL 37,344 (N = 5), both in the presence of GTP, resulted in a concentration-dependent further increase in the activity of adenylate cyclase, with EC_{50} values of $2.0 \pm 0.1 \times 10^{-6}$ and $0.7 \pm 0.3 \times 10^{-6}$ M, respectively. Maximal effect was obtained between 5×10^{-5} and 5×10^{-4} M isoproterenol; a net increase of 54.4 ± 6.3 (N = 5) pmol cAMP/mg protein × min above the activity in the presence of GTP was noticed (74.4 ± 13.0% above GTP). Within the same concentration range, BRL 37,344 caused a smaller but significant ($P < 0.05$) net increase of 28.0 ± 4.1 (N = 5) pmol cAMP/mg protein × min above the activity in the presence of GTP alone (37.6 ± 7.9% above GTP).

The effect of BRL 37,344 on adenylate cyclase activity stimulated with 5×10^{-5}, 5×10^{-6} or 5×10^{-7} M isoproterenol is shown in Fig. 3. BRL 37,344 significantly inhibited concentration dependently adenylate cyclase activity stimulated with either 5×10^{-5}, 5×10^{-6} or 5×10^{-7} M isoproterenol (one-way ANOVA, $P < 0.05$). The three inhibition curves differed significantly (two-way ANOVA, P = 0.0034). IC_{25} values of the curves were significantly different: $58.3 \pm 27.8 \times 10^{-6}$, $8.2 \pm 4.0 \times 10^{-6}$ and $0.49 \pm 0.2 \times 10^{-6}$, respectively (two-way ANOVA, P = 0.0036).

The stimulation of adenylate cyclase with 5×10^{-5} M isoproterenol and with 5×10^{-5} M BRL 37,344 was inhibited by ICI 118,551, with an IC_{50} value of $2.7 \pm 1.0 \times 10^{-6}$ M and $1.4 \pm 0.8 \times 10^{-6}$ M, respectively (N = 5, Fig. 4A and B). CGP 20,712A did not inhibit the increase in activity upon β-receptor stimulation with BRL 37,344 (N = 5, results not shown).

CGP 12,177A did not cause an increase in adenylate cyclase activity (N = 4, results not shown).

DISCUSSION

Our studies on the effect of maturation and aging on the β-adrenergic transduction system in the liver of the rat [14, 15] and the results of hybridization techniques using the human β_3-receptor probe [10] suggested the presence of an atypical receptor in rat liver. When, however, a rat β_3-receptor probe was used, the presence of mRNA coding for β_3-receptors in rat liver was not confirmed, while its presence was established in adipose tissue [12, 13]. In view of these contradictory results, we investigated whether pharmacological evidence could be found for the presence of β_3-receptors in plasma membranes prepared from the livers of newborn rats. We therefore compared the effects of BRL 37,344 and CGP 12,177A with those of isoproterenol, using ligand binding experiments and measurements of the activity of adenylate cyclase.

Our results indicate that isoproterenol as well as BRL 37,344 has agonist properties in the rat liver. Indeed, competition binding of [^{125}I]CYP with either isoproterenol or BRL 37,344 was characterized by the presence of a high and a low affinity binding site in the absence of GppNHp, although the affinity of the high affinity binding site for BRL 37,344 was approximately 3.4 times lower than that for isoproterenol. In the presence of GppNHp, all β-receptors were converted into the low affinity state for the agonists, most probably due to uncoupling from the G-protein. These results suggest that the expression of high affinity binding sites for both isoproterenol and BRL 37,344 requires interaction between the β-receptor and G-protein [24]. The use of [^{125}I]CYP as ligand to investigate atypical β-receptors was discussed before [2, 25]. The

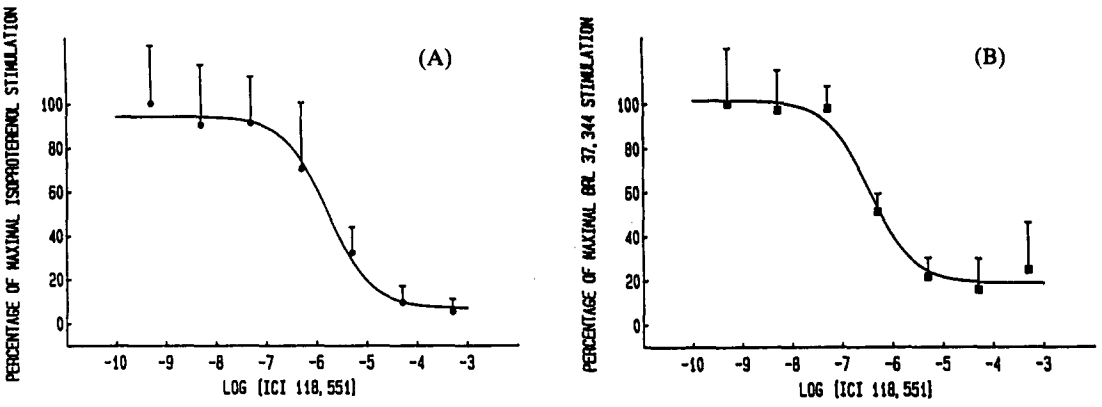

Fig. 4. (A) Inhibition of adenylate cyclase activity, stimulated with 5×10^{-5} M isoproterenol in the presence of 5×10^{-5} M GTP, as a function of the concentration of ICI 118,551. Means ± SEM, N = 5. The results are expressed as per cent decrease of the activity obtained in the presence of agonist and GTP which is set as 100%. (B) Inhibition of adenylate cyclase activity, stimulated with 5×10^{-5} M BRL 37,344 in the presence of 5×10^{-5} M GTP, as a function of the concentration of ICI 118,551. Means ± SEM, N = 5. The results are expressed as per cent decrease of the activity obtained in the presence of agonist and GTP.

concentration of [^{125}I]CYP in our competition binding experiments was below the K_d value of [^{125}I]CYP for the atypical β-receptor in Chinese hamster ovary cells transfected with the cloned β_3-gene [4], but was, in view of the respective K_d value, 3–4-fold higher than the concentration needed to label β_1- and β_2-receptors and 3-fold higher than used by others [4, 25]. We can therefore assume that if β_3-receptors were present in our preparation, they would be labeled with the ligand, albeit not maximally.

Both isoproterenol and BRL 37,344 stimulated adenylate cyclase concentration dependently although the stimulation with BRL 37,344 was only about half of that obained with isoproterenol. In rat brown adipose tissue, a tissue which contains β_3-receptors, Muzzin *et al.* [26] showed that BRL 37,344 was nine times more potent than isoproterenol in stimulating adenylate cyclase in spite of the fact that BRL 37,344 was 80 times less potent than isoproterenol in competition binding studies with the β-adrenergic ligand [^{125}I]CYP. When comparing these observations with our data, we can conclude that there are few arguments in favor of the hypothesis that BRL 37,344 binds in rat liver to an atypical or β_3-adrenergic binding site.

In order to investigate further the pharmacological properties of BRL 37,344 in rat liver, we studied the interaction between isoproterenol- and BRL 37,344-stimulated adenylate cyclase activity. We found that BRL 37,344 inhibits concentration dependently isoproterenol-induced stimulation of adenylate cyclase and that the IC_{25} values are dependent on the concentration of isoproterenol. From these results, we conclude that BRL 37,344 is a partial β-agonist in neonatal rat liver plasma membranes.

The next question is as to which β-receptor subtype BRL 37,344 binds to in rat liver. This was investigated using competition binding experiments and stimulation of adenylate cyclase in the presence of β_1- and β_2-subtype selective antagonists. In the presence of 10^{-7} M ICI 118,551, which, in view of its K_i value for β_2-receptors [27], blocks 90–95% of the β_2-receptors, the binding of [^{125}I]CYP to rat liver plasma membranes was reduced to 32% of its maximal binding. Isoproterenol caused a small but significant further displacement of the ligand and the curves were statistically better fit by a two-site model. In similar competition experiments with BRL 37,344, no further displacement was found. These results are interpreted as follows. We found recently that in livers from newborn rats 10–20% of the receptors are of the β_1-subtype [15], as also suggested by Snell and Evans [27]. Hence, the high and the low affinity binding sites obtained with isoproterenol, a non-selective β-agonist, under conditions where most β_2-receptors are blocked, represent most probably the high and the low affinity binding of isoproterenol to β_1-receptors. Since BRL 37,344 showed no further displacement of ICI 118,551- insensitive binding sites and since BRL 37,344 has a low affinity for β_1-receptors [26], it can be concluded that, apart from β_1- and β_2-receptors, no additional β-receptor binding site was labeled, and that BRL 37,344 and ICI 118,551 occupied the same binding sites (i.e. β_2-receptors). The small displacement of the ligand at the highest concentrations of BRL 37,344 in the presence of ICI 118,551 is probably irrelevant. Furthermore, the stimulatory effect of BRL 37,344 on the adenylate cyclase activity was blocked concentration dependently by the β_2-selective antagonist ICI 118,551, whereas the β_1-selective antagonist CGP 20,712A did not inhibit the stimulatory effect of BRL 37,344. This is another argument in favor of the hypothesis that BRL 37,344 is a β_2-agonist. It has to be mentioned that, although BRL 37,344 is accepted as a β_3-selective agonist, Granneman [5] suggested that in rat brown adipose tissue BRL 37,344 activates adenylate cyclase via neither typical (β_1 and β_2) nor atypical (e.g. β_3) receptors.

Finally, we observed that CGP 12,177A, recently described to be a β_3-partial agonist in adipose tissue [17], acted as a non-selective β-antagonist in competition binding experiments, and competition curves were independent of GppNHp. CGP 12,177A did not cause an increase in adenylate cyclase activity.

As mentioned in the introduction, these experiments were initiated by the observation that part of the ligand could not be displaced by β_1- and by non-selective antagonists [15]. From our results, it is clear that these observations can not be explained by the presence of non-β_1-/non-β_2-adrenergic binding sites.

In conclusion, our results suggest that BRL 37,344, a selective β_3-agonist in adipose tissue, is a partial agonist at the β_2-receptor in rat liver plasma membranes, while the pharmacological properties of CGP 12,177A are typical for a non-selective β-antagonist. No pharmacological evidence for the presence of β_3-receptors in plasma membranes prepared from the livers of newborn rats was found.

Acknowledgements—The authors thank Dr M. G. Bogaert and Dr R. A. Lefebvre for their critical review of the text. The financial support of the F.G.W.O. grant 3.9006.87 is acknowledged. Ann Van Ermen is a bursary of the Belgian I.W.O.N.L.

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