Determination of the desensitization of β -adrenergic receptors by [^3H]CGP-12177

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Isoprenaline treatment of C6-glioma cells induced a fast decrease in the number of β -adrenergic receptors as determined by binding of [^3H]CGP-12177, which paralleled the decrease in the hormonally stimulated adenylate cyclase activity. The total number of receptors, as determined by binding of (–)-[^3H]dihydroalprenolol, did not decrease. Separation of the β -adrenergic receptors on a sucrose density gradient showed that the decrease in the number of receptors detectable with CGP-12177 was due to a movement of the receptors from the plasma membrane to a vesicular cell compartment. By using both (–)-[^3H]dihydroalprenolol and [^3H]CGP-12177 it is thus possible to differentiate between the total number of receptors and those present at the plasma membrane in an unfractionated cell lysate.

Cells exposed to a β -adrenergic agonist react with a rise in intracellular [cyclic AMP]. After prolonged exposure, however, cells become refractory and cease responding. In membranes from such desensitized cells two phenomena have been observed (Su *et al.*, 1980; Green *et al.*, 1981): (a) a rapid decrease in hormonally sensitive adenylate cyclase (uncoupling or desensitization) and (b) a slow decrease in the number of receptors (down-regulation). It has been demonstrated that the down-regulation correlates with an increase in the number of cytosolic receptors (Chuang & Costa, 1979) or vesiculated receptors in frog erythrocytes (Stadel *et al.*, 1983).

Furthermore, Harden *et al.* (1980) have shown that even short-term exposure of human astrocytoma cells to an agonist leads to a shift of about half of the β -adrenergic receptors from the plasma membrane to a small-vesicle fraction. In addition, we have recently shown after short-term exposure to an agonist of C6-glioma cells a rapid reversible disappearance of binding sites for the β -adrenergic antagonist [^3H]CGP-12177 (Staelin & Hertel, 1983), a ligand that is not taken up into intact cells (Staelin *et al.*, 1983).

Abbreviations used: [^3H]DHA, (–)-[^3H]dihydroalprenolol; [^3H]CGP-12177, (+)-[^3H]4-(3-t-butylamino-2-hydroxypropoxy)benzimidazol-2-one hydrochloride; DMEM, Dulbecco's modified Eagle's medium; DMEM-Hepes, DMEM without NaHCO_3 but containing 10 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, pH 7.4, and 0.036 M-NaCl instead.

Using the C6-2B glioma cell line we have demonstrated that this β -adrenergic antagonist only bound to receptors in a small-vesicle fraction after they had been pre-treated with the pore-forming antibiotic alamethicin (Hertel *et al.*, 1983).

In the present paper we show that the degree of this receptor movement from plasma membrane to vesicles can be assessed in lysates under identical conditions by comparing the binding of [^3H]DHA and [^3H]CGP-12177 to the β -adrenergic receptors. Using sucrose-density-gradient centrifugation we demonstrate that [^3H]CGP-12177 binds to plasma-membrane receptors only, whereas [^3H]DHA binds to both plasma membrane and vesicle receptors. We show, furthermore, that the decrease in the number of plasma-membrane receptors parallels adenylate cyclase desensitization and occurs even in mutant cells lacking coupling of the receptor with the adenylate cyclase.

Materials and methods

Cells

C6-glioma cells were grown as described previously (Staelin & Simons, 1982). S49 wild-type and *cyc*[–] cells, obtained from the University of California Tissue Repository, San Francisco, CA, U.S.A., were grown to a density of $(0.5\text{--}1) \times 10^6$ cells/ml in RPMI medium (RPMI 1640; Gibco Europe, Basel, Switzerland) containing 10% foetal-calf serum.

Preparation of intact and lysed cells

Confluent 10 cm dishes of C6 glioma cells were treated with or without $1\mu\text{M}$ (–)-isoprenaline and incubated as indicated at 37°C in an incubator with air/ CO_2 (19:1). The dishes were then placed on ice and washed twice with DMEM-Hepes. Cells were treated with 0.02% EDTA in phosphate-buffered 0.9% NaCl for 15 min at 0°C to detach intact cells. This buffer was then removed and the cells were suspended in ice-cold DMEM-Hepes to a density of about 2×10^6 cells/ml.

Cell lysates were prepared by incubating cells in 1 mM-Tris (pH 7.4)/2 mM-EDTA for 30 min at 0°C . The lysed cells were taken up in 10 mM-Tris (pH 7.5)/154 mM-NaCl/5 mM-MgCl₂.

Density-gradient centrifugation

Sucrose (60%; 2 ml) was overlaid with 30 ml of a linear sucrose gradient (55–30% sucrose). All sucrose solutions contained 10 mM-Tris, pH 7.4. Lysates were prepared as described above after treatment of the cells with or without concanavalin A (Harden *et al.*, 1980). After lysis the cells were scraped off with a rubber policeman, dispersed in a final volume of 1 ml per dish and layered on top of the gradient. Centrifugation was carried out in a Beckman SW27 rotor for 60 min at 25 000 rev./min at 4°C . Fractions (2 ml) were collected by piercing the tubes at the bottom and pumping water through the top at a rate of 2 ml/min.

Binding assay

[³H]DHA (sp. radioactivity 71 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The preparation of [³H]CGP-12177 (sp. radioactivity 40 Ci/mmol) has been described previously (Staehelin *et al.*, 1983). To cells or membranes (0.45 ml containing 10^6 cells or 0.2 ml of sucrose fractions) 50 μl of radioligand was added.

After incubation the samples were cooled on ice, diluted with 10 ml of ice-cold 10 mM-phosphate buffer (pH 7.4)/4 mM-MgSO₄, filtered through glass-fibre filters (GF/C; Whatman, Maidstone, Kent, U.K.) and washed twice with 10 ml of the same buffer. The filters were added to 10 ml of Aquasol-2 (New England Nuclear, Boston, MA, U.S.A.) while still wet, shaken for 30 min and counted in a Packard scintillation counter at 44% counting efficiency.

Adenylate cyclase assay

Homogenates were prepared by lysing cells in 10 mM-Hepes (pH 8.0)/1 mM-MgSO₄ and homogenization in a tight-fitting Dounce homogenizer. Crude membranes were prepared as described by Maguire *et al.* (1976) and incubated in the assay medium described by Lucas & Bockaert (1977) at a concentration of 10–20 μg of protein/50 μl assay mix. At the end of the incubation period samples

were cooled and the nucleotides separated by the method of Salomon *et al.* (1974).

5'-Nucleotidase assay

Lysates or sucrose fractions (20 μl in each case) were incubated for 60 min under the conditions described by Aronson & Touster (1974). Phosphate determinations were carried out by Pollard & Korn's (1973) modification of the method of Martin & Doty (1949).

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Analysis of experimental data

Best estimates of the binding parameters were calculated using non-linear least-squares regression by an extended version of the numerical procedure described by Duggleby (1981). The data were fitted by the exact mathematical model of ligand-binding systems (Munson & Rodbard, 1980). The deviations of the experimental points from the predicted values were weighted according to the reciprocal of either their standard deviations or their predicted variance (Munson & Rodbard, 1980). Goodness-of-fit and significance of additional parameters were analysed using the *F*-test based on the 'extra sum of squares' (Munson & Rodbard, 1980). Since DeLean *et al.* (1982) have shown that using the negative logarithm ($\text{p}K_D$) rather than K_D itself improved the statistical analysis, the dissociation constants are given as $\text{p}K_D \pm \text{s.e.m.}$ *R*(1) refers to the percentage of receptors having $\text{p}K_D$ (1).

Results

Reduction of binding sites for CGP-12177 by incubation with agonists

C6-cells were incubated with $1\mu\text{M}$ -isoprenaline at 37°C for 15–20 min and then lysed afterwards by hypo-osmotic shock. The number of β -adrenergic receptors was determined in an iso-osmotic medium at 30°C .

Fig. 1(b) shows that this agonist treatment results in a 50% decrease in the number of binding sites of [³H]CGP-12177 compared with controls, whereas K_D is not altered. This decrease in the number of binding sites observed at 30°C is similar to that observed at 0°C (results not shown) or in an intact-cell binding assay at 0°C (Staehelin & Simons, 1982). Using [³H]DHA, only a 10% decrease in the number of binding sites in lysates of agonist-treated cells compared with controls is observed at 30°C (Fig. 1a).

In order to determine whether those receptors that bind [³H]DHA only but not [³H]CGP-12177 bind CGP-12177 at elevated concentrations, a com-

Fig. 1. Saturation binding of [³H]DHA and [³H]CGP-12177 to lysates from control and agonist-pretreated cells

Cells were incubated with (○) or without (●) 1 μ M (—) isoprenaline for 20 min at 37°C, lysed and incubated at 35°C for 45 min with either [³H]DHA (0.15–3 nM) (a) or [³H]CGP-12177 (0.15–1.5 nM) (b). Non-specific binding was determined in the presence of 1 μ M-propranolol. A Scatchard plot of the data is shown (Scatchard, 1949).

Fig. 2. Competition binding of [³H]DHA and unlabelled CGP-12177

Cells were incubated with (▲) or without (●) 1 μ M (—) isoprenaline for 20 min at 37°C, lysed and incubated at 30°C for 90 min with 0.9 nM-[³H]-DHA plus CGP-12177 (10 pM–1 μ M). Non-specific binding was determined in the presence of 1 μ M-propranolol. In the absence of competing ligand, control cells bound 22 ± 1 fM-[³H]-DHA and agonist-pretreated cells bound 19.7 ± 0.6 fM-[³H]-DHA.

petition experiment with [³H]DHA and unlabelled CGP-12177 was performed. Fig. 2 shows that in lysates of control cells the competition curve is monophasic ($pK_D = 9.79 \pm 0.10$; mean $\pm$ S.E.M., $n = 6$), whereas it is biphasic in lysates of agonist-treated cells [$pK_D(1) = 9.76 \pm 0.15$; $pK_D(2) = 7.9 \pm 0.12$; $R(1) = 42 \pm 4\%$; means $\pm$ S.E.M., $n = 5$]. The K_D of

the low-affinity binding site is 100-fold higher than the K_D of the high-affinity binding site. Thus the second binding site cannot be determined in a saturation binding experiment (Fig. 1), because the concentration range in which the radioligand can be used without interference from non-specific binding is not wide enough.

By using this method of lysing cells after agonist treatment, we compared the reduction in the number of binding sites for [³H]CGP-12177 and [³H]DHA with the decrease of hormone-sensitive adenylate cyclase activity. Fig. 3(a) shows that the decrease in the number of binding sites for [³H]CGP-12177 depends on the concentration of isoprenaline used. The decrease in the number of binding sites of [³H]CGP-12177 parallels the decrease in the hormone-sensitive adenylate cyclase activity. The decrease in the [³H]DHA binding, however, is much less pronounced.

Fig. 3(b) shows a time course of incubation with 1 μ M-isoprenaline. Again the decrease in binding sites for [³H]CGP-12177 parallels the decrease in hormone-sensitive adenylate cyclase activity, both showing a 50% reduction after 20 min of incubation with 1 μ M-isoprenaline. The decrease in binding sites for [³H]DHA is much smaller.

Fig. 3. Adenylate cyclase activity and [³H]CGP-12177 and [³H]DHA binding after agonist treatment

C6-glioma cells were washed and crude membranes prepared by the method of Maguire *et al.* (1976). Portions were assayed for [³H]CGP-12177 binding (○) and [³H]DHA binding (●) at 0.3 and 1 nM concentrations respectively (i.e. $1 \times 10 K_D$), and for hormonally sensitive adenylate cyclase (×) as described in the Materials and methods section. (a) Cells were treated for 20 min with various concentrations of (—) isoprenaline. (b) Cells were treated with 1 μ M (—) isoprenaline for various lengths of time. Control values were: [³H]CGP-12177 binding, 171 ± 10 fmol/mg of protein; [³H]-DHA binding, 210 ± 12 fmol/mg; basal adenylate cyclase activity, 38 ± 2 pmol/min per mg; 1 μ M-isoprenaline-stimulated adenylate cyclase, 191 ± 6 pmol/min per mg.

Table 1. [^3H]CGP-12177 binding to β -adrenergic receptors in control and agonist pretreated ($1\mu\text{M}$ -isoprenaline for 20 min) S49 wild-type and *cyc*⁻ mutant cells

Saturation binding experiments (0.15 – 2.5 nM [^3H]CGP-12177) were analysed by linear regression of a Scatchard plot analysis.

	Wild-type		<i>cyc</i> ⁻ mutant	
	Control	Agonist-treated	Control	Agonist-treated
K_D (pM)	244	258	276	180
B_{max} (pM)	14	7	26	17
r^2	0.93	0.92	0.92	0.84

Table 2. Agonist-induced reduction in the number of binding sites for [^3H]CGP-12177 and [^3H]DHA in S49 wild-type and *cyc*⁻ mutant cells

[^3H]CGP-12177 (1 nM) and 1 nM [^3H]DHA were used. The results are expressed as percentages of the control value and are means $\pm$ S.E.M. ($n = 3$).

	Binding (%)			
	Wild-type		<i>cyc</i> ⁻	
Preincubation period with $1\mu\text{M}$ -isoprenaline (min)	CGP-12177	DHA	CGP-12177	DHA
15	41 ± 12	92 ± 7	43 ± 15	92 ± 7
30	43 ± 9	92 ± 7	39 ± 4	92 ± 7

In order to determine whether this reduction in the number of binding sites for [^3H]CGP-12177 is coupled to the adenylate cyclase activity, we compared S49 wild-type cells and their *cyc*⁻ mutants. The latter lack a functional coupling protein and thus no longer respond to β -adrenergic agonists with a rise in [cyclic AMP] (Ross *et al.*, 1978). Table 1 shows that the affinity of β -adrenergic receptors in S49 wild-type and *cyc*⁻ mutant cells for CGP-12177 is almost the same ($K_D = 250 \pm 16\text{ pM}$). Agonist treatment did not significantly alter K_D in both cell lines. However, as determined from the linear regression of saturation binding experiments according to Scatchard (1949), B_{max} (maximum binding) was reduced by agonist pretreatment in both cell lines. Since the affinity of the receptors for CGP-12177 was not altered, the effect of agonist pretreatment on the number of receptors was verified at a concentration of 1 nM [^3H]CGP-12177. Table 2 shows that the number of receptors was decreased by the same amount after 15 min pretreatment with $1\mu\text{M}$ (–)-isoprenaline as well as after 30 min pretreatment in S49 *cyc*⁻ and wild-type cells. In both cell lines the number of receptors as determined by [^3H]DHA binding remained constant.

Fig. 4. Ligand binding and 5'-nucleotidase activity in sucrose-gradient fractions of desensitized cells

Eight 10 cm dishes of C6-glioma cells were treated for 15 min with $1\mu\text{M}$ (–)-isoprenaline at 37°C . Cells were washed twice with ice-cold DMEM-Hepes and incubated for 30 min at 0°C with concanavalin A (0.25 mg/ml). After two additional wash-steps cells were lysed in 1 mM -Tris ($\text{pH } 7.4$)/ 2 mM -EDTA and applied (9 ml) to a linear sucrose gradient (60 – 30% , w/v; Δ). Fractions (2.4 ml) were collected. Ligand binding was performed in $200\mu\text{l}$ portions with either 0.7 nM [^3H]CGP-12177 ($\bullet$) or 1.3 nM [^3H]DHA ($\circ$). 5'-Nucleotidase activity ($\times$) was determined in $30\mu\text{l}$ portions.

Separation of β -adrenergic receptors from C6-cells on a sucrose density gradient

To determine which receptors are able to bind [^3H]CGP-12177 as well as [^3H]DHA and which can only bind [^3H]DHA we separated the receptors on a sucrose density gradient as described by Harden *et al.* (1980) for astrocytoma cells and Hertel *et al.* (1983) for C6-2B cells.

As determined by [^3H]DHA binding, β -adrenergic receptors from control cells are mainly located at a density of 40% sucrose (results not shown), whereas desensitized cells show two populations of receptors, one at 40% and a second at about 30% sucrose (Fig. 4). Fig. 4 shows the separation of these two different β -adrenergic receptor populations from C6-cells on a sucrose density gradient. Whereas the receptors located in a peak at 40% sucrose (heavy peak) bind both [^3H]CGP-12177 and [^3H]DHA those located at 30% sucrose (light peak) can only bind [^3H]DHA. Determinations of the activity of the plasma-membrane marker enzyme 5'-nucleotidase showed that only the heavy peak contained high amounts of plasma membrane, whereas the light peak contained almost no plasma membrane, indicating that the receptors in the light peak are no longer located on the plasma membrane.

Fig. 5 shows the distribution of [³H]CGP-12177-binding sites, which were labelled in intact cells. For this experiment C6-cells were first treated with 1 μ M-isoprenaline for 15 min at 37°C, chilled, washed extensively to remove isoprenaline and then incubated for 20 h at 0°C with 1 nM-[³H]CGP-12177 ($5 \times K_D$). Cells were rewashed, lysed and fractionated on a sucrose density gradient. Portions of each fraction were filtered afterwards. Almost all binding sites for [³H]CGP-12177 were then found in the heavy peak and none in the light peak. In order to determine in this same gradient the total amount of β -adrenergic receptors, portions from each fraction were incubated with 1 nM-[³H]DHA for 45 min at 37°C and the total amount of radioactivity bound was determined by a filtration assay. The amount of [³H]CGP-12177 still bound after this time (two half-lives) was then determined for each fraction in the presence of 1 μ M-propranolol and subtracted from the total amount of radioactivity bound. The result shown in Fig. 5 indicates that in the light peak almost an equal number of binding sites for

[³H]DHA was revealed as in the heavy peak. These receptors in the light peak therefore have not interacted with [³H]CGP-12177 in the intact cells.

Discussion

Uncoupling of hormonally sensitive adenylate cyclase occurs rapidly and is also rapidly reversible, whereas down-regulation, i.e. disappearance of receptors, is a slow process and is only reversed when new receptors are synthesized (Su *et al.*, 1980). It has been demonstrated by Harden *et al.* (1980) in astrocytoma cells that even a brief agonist incubation induces the formation of a vesicular β -adrenergic receptor population in addition to plasma-membrane receptors. We have recently demonstrated in intact cells a fast reversible decrease in the number of β -adrenergic binding sites after agonist treatment using the new β -adrenergic antagonist CGP-12177. By using CGP-12177 in C6-2B cells we showed that this ligand did not bind to vesicular receptors, but that the receptors could be unmasked by using a pore-forming antibiotic, alamethicin (Hertel *et al.*, 1983), suggesting that a membrane barrier caused the inability of CGP-12177 to bind the vesicular receptors.

We have also shown that the re-appearance of the receptors on the cell surface requires intact cells and does not occur any more in lysates. It requires intracellular ATP and is inhibited by lysosomotropic agents such as chloroquine (Hertel & Staehelin, 1983).

In the present publication we have compared the binding of CGP-12177 and DHA to β -adrenergic receptors in agonist-treated cells. Reduction in the number of binding sites for [³H]CGP-12177 is not only detectable in intact cells at 0°C (Staehelin & Simons, 1982), but also in cell lysates at 30°C. Thus we could compare receptor binding of [³H]DHA and [³H]CGP-12177 in lysates of agonist-treated cells under identical conditions.

A comparison of DHA and CGP-12177 binding to β -adrenergic receptors showed that agonist treatment induced the formation of a second receptor population with a low affinity for CGP-12177, whereas the affinity for DHA was not altered. This low-affinity binding site for CGP-12177 was not evident in a saturation binding experiment, because the concentration could not be increased to more than one-tenth of the K_D of the low-affinity binding site without increasing the non-specific binding to a too high percentage of the total binding of the radioligand. Therefore saturation binding experiments, shown as Scatchard plots, permitted determination of the decrease in the number of those receptors binding [³H]CGP-12177 after agonist treatment.

This decrease in the number of binding sites

Fig. 5. Prelabelling with [³H]CGP-12177 of β -adrenergic receptors separated on a sucrose density gradient

Confluent dishes of C6-glioma cells were incubated in DMEM containing 10% foetal-calf serum at 37°C with 1 μ M-(—)-isoprenaline for 15 min. Cells were washed twice with ice-cold DMEM-Hepes and incubated for 24 h at 0°C with 1 nM-[³H]CGP-12177. Cells were washed again and lysed in Tris/EDTA. Fractionation on a sucrose gradient was carried out as described in the Materials and methods section using a 56–25% (w/v) sucrose gradient. Of each 12 ml fraction, 0.5 ml was analysed by filtration to measure [³H]CGP-12177 binding (●). A further 0.45 ml was incubated with 50 μ l of [³H]DHA (final concentration 1 nM) in the presence or in the absence of 1 μ M-(—)-propranolol for 45 min at 37°C (○).

paralleled the decrease in hormone-sensitive adenylate cyclase activity. However, as we have demonstrated by using the *cyc⁻* mutant of S49 cells, this decrease in the number of binding sites is not coupled to an activation of adenylate cyclase, i.e. formation of cyclic AMP.

Separation of β -adrenergic receptors on a sucrose gradient showed that those receptors that are located in a low-density peak were unable to bind [³H]-CGP-12177, but still able to bind [³H]DHA, which has been similarly demonstrated in C6-2B cells (Hertel *et al.*, 1983). The high-density peak, however, contained β -adrenergic receptors that bound [³H]CGP-12177 as well as [³H]DHA. This high-density peak contained high amounts of 5'-nucleotidase activity, indicating that it is plasma-membrane-enriched. As has been shown for astrocytoma cells (Harden *et al.*, 1980), we found that this plasma-membrane fraction contained adenylate cyclase activity, whereas the low-density peak contained almost no activity (results not shown).

We therefore conclude that those receptors that are able to bind [³H]CGP-12177 as well as [³H]DHA are located on the plasma membrane. Since the agonist-induced reduction in the number of plasma-membrane receptors parallels the decrease in the hormonally sensitive adenylate cyclase activity, we propose naming this phenomenon 'receptor-desensitization' in comparison with adenylate cyclase desensitization. Furthermore these results indicate that those receptors that have low affinity for CGP-12177 ('desensitized receptors') are located in the low-density peak, which represents another cellular compartment, probably of some intracellular vesicles. As we have demonstrated elsewhere (Hertel *et al.*, 1983) the receptors in these vesicles become available for CGP-12177 in the presence of the pore-forming agent alamethicin. These results suggest that the apparent low affinity of these receptors for CGP-12177 may be due to the impermeability of membranes to CGP-12177.

Further investigation of these vesicle receptors is required to determine the properties of the 'desensitized receptors' and to compare them with plasma-membrane receptors.

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