Reappearance of β-Adrenergic Receptors after Isoproterenol Treatment in Intact C6-Cells

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ABSTRACT The reappearance of β-adrenergic receptors in C6-glioma cells after desensitization with isoproterenol was studied using the antagonist [3H]CGP-12177. Reappearance had the following properties: (a) it occurred in intact cells only, (b) it was temperature dependent, (c) it required an Na+/H+ gradient, low intracellular Ca2+ activity, and (d) it required ATP, and (e) intact lysosomes. The results suggest endocytosis and recycling of the β-adrenergic receptor after agonist treatment.

Cells that possess β-adrenergic receptors become refractory when they are continuously exposed to β-adrenergic hormones. Two different mechanisms have been proposed to be involved in this process: firstly, a fast, reversible "desensitization" of the agonist-induced adenylate cyclase activity and, secondly, a slow decrease in the total number of receptors, i.e., "down regulation" of the receptors (1, 2). The desensitization of adenylate cyclase is suggested to be due to a change in the conformation of the receptor enzyme complex (1). Prolonged treatment of cells with an agonist has been demonstrated to lead to internalization of the receptor (3) followed by cycling through lysosomes (4). These authors suggested that down regulation is mediated by receptor endocytosis. It is still unclear whether these two processes, i.e., desensitization of the adenylate cyclase and down regulation of the receptors, are linked and in what way this coupling occurs.

Harden et al. (5) have demonstrated that parallel to the desensitization of the adenylate cyclase, a second, physically separable receptor population appeared. This second receptor population occurred in a vesicular receptor fraction while at the same time the number of receptors bound to the plasma membrane fraction was reduced. They suggested that endocytosis of β-adrenergic receptors occurs during desensitization and not only during down regulation. The change in the properties of the β-adrenergic receptor during desensitization has been further investigated, using a new, hydrophilic β-adrenergic antagonist [3H]CGP-12177 that became recently available (6, 7). This ligand compared with the established β-adrenergic antagonists is that it cannot pass membranes and thus is not accumulated inside cells (8). Thus, using [3H]CGP-12177, the number of β-adrenergic receptors on the cell surface could be determined in intact cells. In intact cells, agonist treatment did induce a loss of agonist-stimulated adenylate cyclase (1), which also causes a decrease in the number of those β-adrenergic receptors being available for [3H]CGP-12177 (8, 9). At the same time the total number of β-adrenergic receptors, as determined with a lipophilic ligand, was still constant.

Therefore, [3H]CGP-12177 is a tool to measure in intact cells (8) the agonist-induced disappearance of cell surface receptors, which has been demonstrated before by Harden et al. (5) after lysing the cells. Using this ligand, it has been demonstrated recently that the disappearance of β-adrenergic receptors is transient and is followed by a fast reappearance of receptors after removal of the agonist (8).

In the present paper we demonstrate further characteristics of this reappearance of β-adrenergic receptors in C6 rat glioma cells following desensitization. The results suggest that the agonist-induced transient decrease in the number of β-adrenergic receptors is due to an intracellular recycling of β-adrenergic receptors.

MATERIALS AND METHODS

Cell culture media were obtained from Gibco Laboratories (Grand Island, NY). [3H]CGP-12177 (1.48 × 107 Bq/mmol) was prepared as described previously (7). [14C]Leucine (1.2 × 109 Bq/mmol) was obtained from NEN (Dreieich, FRG). Chloroquine, cycloheximide, FCCP (cyanide)-trifluoromethoxyphenylhydrazone), antimycin A, 2-deoxyglucose, l-isoproterenol bitartrate were obtained from Sigma Chemical Co. (St. Louis, MO), and valinomycin, monensin, and A 23187 from Calbiochem (Luzern, Switzerland).

Cells: C6-glioma cells (10) were grown for 4 d in 10 ml of Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum in 100-mm Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) to a density of 2 × 107 cells. The cells were then detached by treatment with 5 ml of ice-cold 0.02% buffered EDTA solution per dish (11) and suspended in ice-cold DME.

Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium.
HEPES (DME containing 36 mM sodium chloride and 10 mM HEPES titrated with sodium hydroxide to pH 7.3) at a density of ~3 x 10^6 cells/ml.

**Desensitization of β-Adrenergic Receptors: Isoproterenol**

Isoproterenol (1 μM final concentration) was added to the growth medium of confluent cells. The cells were incubated for 15–20 min at 37°C, rapidly chilled, washed twice with ice-cold DME-HEPES and detached by incubation with ice-cold buffered 0.02% EDTA solution for 4 min. After aspiration of the EDTA solution, they were resuspended in DME-HEPES to a final density of ~3 x 10^6 cells/ml.

**Ligand Binding:** To determine nonspecific and total binding, 10^6 cells were incubated with varying amounts of [3H]CGP-12177 with or without 1 μM 1-propranolol, respectively, in a total volume of 0.5 ml with shaking to prevent sedimentation of the cells. The number of binding sites, B_{max}, was determined by incubation of the cells with 0.06–1 nM [3H]CGP-12177 for 90 min at 37°C or for 24 h at 4°C.

For the determination of the rate of receptor reappearance, cells were incubated with 3 nM [3H]CGP-12177 (7 x KD) at the temperature indicated for different times. They were immediately chilled and then incubated for an additional 24 h at 4°C to allow the binding to reach equilibrium.

Before filtration, the aliquots were diluted 20-fold with ice-cold 50 mM K_2HPO_4/KH_2PO_4 buffer containing 8 mM MgSO_4, pH 7.5. They were then filtered through Whatman GF/C filters (Whatman Laboratory Products Inc., Whatman Paper Div., Clifton, NJ) and washed twice with the above buffer. The filters were then taken up in 10 ml of Aquasol-2 scintillation fluid (NEN) for the determination of radioactivity.

Cells were lysed in 5 mM HEPES containing 1 mM MgSO_4, pH 7.3. Aliquots of the lysates (0.45 ml) were incubated with 50 μl of the radioligand with or without 1 μM 1-propranolol, respectively, in 37°C for the indicated time periods.

A 23187, monensin, valinomycin, FCCP, and antimycin A were dissolved in absolute ethanol and diluted into the incubation medium to a final concentration of 1% ethanol. The same amount of ethanol was added to the controls. A 0.5 M EDTA stock solution was first buffered with KOH to pH 8.9, so that the drop in pH expected to occur by the 1.36 mM Ca^2+ present in the medium was kept to a tolerable minimum. It was diluted 100-fold into the incubation assay. Chloroquine and 2-deoxyglucose were dissolved in DME-HEPES as stated.

**Protein Synthesis:** Detached cells 2 x 10^6 cells/ml were incubated at 37°C in DME-HEPES containing 3.7 x 10^6 Bq of [3H]leucine for 2 h with and without 0.1 μg cycloheximide/ml. Incorporation of [3H]leucine into protein was determined according to Paszkowski et al. (12).

**ATP and Protein Determination:** The intracellular ATP was determined using ATP-bioluminescence (CLS) assay kit from Boehringer (Mannheim, FRG) by the method described for intact cells in their product manual. Protein was determined with the dye-binding method of Spector (13).

**Calculations:** Scatchard analysis was used to determine K_D and B_{max} (14). B_{max} was highly dependent on the number of passages through which cells have grown. Therefore, to compare independent experiments, the number of binding sites was calculated as a percentage of the control value, which was determined for each experiment. Each experiment was carried out at least three times. Results of single experiments are shown except where otherwise indicated.

**RESULTS**

**Binding of [3H]CGP-12177: Disappearance of β-Adrenergic Receptors after Isoproterenol Treatment**

For standard experiments a concentration range of 0.06 nM–1 nM [3H]CGP-12177 was used. The inset in Fig. 1 shows that the chosen concentrations cover a complete S-shaped binding curve. At least two of the concentrations used were above the inflection point of the semi-logarithmic curve. According to Klotz (15), under these conditions it is legitimate to determine B_{max} as the intercept in a Scatchard graph (14).

Binding of [3H]CGP-12177 to intact C6-glioma cells showed a K_D of 0.36 ± 0.02 nM (mean ± SE; n = 10) at 37°C (Fig. 1). Additional incubation at 4°C resulted in a decrease of K_D to 0.10 ± 0.01 nM (n = 3), similar to a K_D of 0.12 ± 0.01 nM (n = 12) determined at 4°C without preincubation at 37°C (Fig. 1). The maximal number of binding sites (B_{max}) was not altered by changing the incubation temperature. In the experiment shown in Fig. 1, B_{max} was calculated to be 14 fmol/10^6 cells. When cells were pretreated at 37°C for 20 min with isoproterenol, the K_D for [3H]CGP-12177 binding was 0.35 ± 0.04 nM (n = 9) at 37°C and 0.12 ± 0.01 nM (n = 13) at 4°C. The value for K_D was not significantly different from those obtained without isoproterenol pretreatment. After desensitization B_{max} was determined as 14 fmol/10^6 cells at 37°C and as 10 fmol/10^6 cells at 4°C (Fig. 1). Pretreatment with isoproterenol thus reduced the number of binding sites by 30%. This reduction could only be determined at 4°C, and not after incubation for 90 min at 37°C.

**Reappearance of β-Adrenergic Receptors after Desensitization**

**Rate of Appearance:** Desensitized cells were washed extensively to remove isoproterenol, then the time course of reappearance of β-adrenergic receptors was measured at 37°C, as described in Materials and Methods.

Fig. 2 shows that in intact cells within 3 min ~50% of the desensitized receptors had reappeared (○) and after 60 min the number of binding sites had reached the value determined for control cells (●).

Intactness of the cells is required for the reappearance of β-adrenergic receptors, since a hypotonic shock prevented the reappearance completely (Fig. 2, ■).

**Temperature Dependence:** The kinetics of reappearance was determined at three different temperatures (Fig. 3). At 33°C (●) all binding sites had reappeared after 60 min; at 21°C (○) after about 6 h. At 13°C (□), even after 6 h the number of β-adrenergic receptors increased only from 75% to 85% of the total number of receptors. The inset in Fig. 3 shows the temperature dependence of receptor reappearance, which is nonlinear.

**Energy Dependence:** Confluent C6-cells were incubated with 20 mM 2-deoxyglucose or 10 μM antimycin A or
bation with either one of them reduced the intracellular ATP content by 50%. As we have shown previously (16), this reduction of intracellular ATP-content did not inhibit the desensitization of the β-adrenergic receptors. To lower the ATP-concentration sufficiently during the time of receptor reappearance, we incubated the cells with 2-deoxyglucose and antimycin A for 20 min at 37°C together with isoproterenol. Afterwards, the cells were washed extensively and incubated for 90 min at 37°C with CGP-12177 (conditions where all receptors reappeared [see Fig. 1]) in the absence or presence of 20 mM 2-deoxyglucose and 10 μM antimycin A. Fig. 4A shows that β-adrenergic receptors in desensitized cells reappeared within 90 min at 37°C, giving the same number of receptors as determined in control cells (18 fmol/10⁶ cells). In contrast, in Fig. 4B the number of receptors in desensitized cells was still reduced by 30% (14 over 20 fmol/10⁶ cells), when 2-deoxyglucose and antimycin A were still present during the incubation with CGP-12177 at 37°C. This indicates that the reduction of the intracellular ATP concentration has prevented the reappearance of receptors.

**CATION DEPENDENCE:** 10 μM A 23187 (a Ca²⁺ ionophore) was added to the incubation medium in the presence of 1.36 mM CaCl₂ to increase the intracellular Ca²⁺ activity. The rate of the reappearance of the β-adrenergic receptors was determined at 32°C as described under experimental procedures (Fig. 5A). The increased intracellular Ca²⁺ activity

### Table 1

| Addition | Intracellular ATP concentration (mM) |
|----------|--------------------------------------|
| None     | 2.30 ± 0.16                          |
| 2-Deoxyglucose (20 mM) | 1.34 ± 0.04                          |
| Antimycin A* (10 μM)   | 1.62 ± 0.02                          |
| Antimycin A + 2-deoxyglucose | 0.04 ± 0.01                          |

Cells were incubated for 20 min with antimycin A and/or deoxyglucose. The ATP-content was determined after denaturation of the cells. To calculate the intracellular ATP concentration a volume of 1.8 μl/10⁶ cells was used (mean ± SE n = 6).

* n = 3.
inhibited the reappearance of the β-adrenergic receptors (■). No inhibition was measured when 5 mM EGTA was added together with 10 μM A 23187 (○), thus decreasing Ca²⁺ activity to a calculated value of 16 nM (pH 7.3). Thus the effect of A 23187 is due to the altered intracellular Ca²⁺ activity.

Diminishing monovalent cation gradients did not inhibit the desensitization of β-adrenergic receptors (16). To determine whether reappearance of β-adrenergic receptors is dependent on monovalent cation gradients, we used monovalent cation ionophores, i.e., valinomycin (an ionophore specific for K⁺), FCCP (a proton-ionophore), and monensin (an ionophore exchanging K⁺, Na⁺, H⁺). None of these ionophores affected [³H]CGP-12177 binding to β-adrenergic receptors (data not shown). Cells were incubated with 1 μM 1-isoproterenol together with 50 μM monensin for 20 min at 37°C to diminish monovalent cation gradients. After washing the cells, 50 μM monensin was added again and the reappearance of β-adrenergic receptors was determined. Fig. 5B (●) shows that monensin inhibited the reappearance of the β-adrenergic receptors completely. In order to reduce K⁺ and H⁺ gradients, cells were incubated with isoproterenol plus 5 μM valinomycin and 50 μM FCCP for 20 min at 37°C. To prevent side effects by uncoupling of mitochondrial electron transport chain by FCCP, antimycin A was added, too. After the cells were washed, FCCP, valinomycin, and antimycin A were added again to the suspension, and the reappearance of β-adrenergic receptors was determined. Fig. 5B (□) shows that under these conditions, i.e., diminishing K⁺/H⁺ gradients, the reappearance is not completely inhibited. Addition of valinomycin alone did not inhibit the reappearance of β-adrenergic receptors (data not shown).

DEPENDENCE ON INTACT LYSOSOMES: To increase the intralysosomal pH, we used the lysosomotropic agent chloroquine (17). Cells were preincubated with 0.1 mM chloroquine for 4 h at 37°C, a time period that has been described to be sufficient to accumulate chloroquine inside the lysosomes and increase their pH (18). 1 μM 1-isoproterenol was added for an additional 15 min. After the cells were washed chloroquine was added again and the number of β-adrenergic receptors was determined either at 4°C or at 37°C. Fig. 6 shows that in chloroquine-pretreated cells incubated with [³H]CGP-12177 at 4°C following desensitization the number of binding sites was reduced by 50% (15 fmol/10⁶ cells [○] vs. 30 fmol/10⁶ cells [■]). Thus, chloroquine did not inhibit desensitization. When the cells were incubated at 37°C, however, this 50% reduction is still seen (□) in contrast to the complete reappearance of receptors in cells not treated with chloroquine (see Fig. 1).

DEPENDENCE ON PROTEIN SYNTHESIS: Confluent C6 cells were incubated with cycloheximide to inhibit protein synthesis. Measuring the incorporation of [¹⁴C]leucine showed that incubating the cells for 2 h with 10 μg/ml cycloheximide at 37°C inhibited protein synthesis by ~90% (data not shown). Saturation-binding experiments with [³H]CGP-12177 showed that cycloheximide did not alter K₅₀ significantly (0.43 ± 0.4 nM, mean ± SD; n = 2) as determined by linear regression of Scatchard plot analysis (r² ≥ 0.92). Table II shows that preincubation with cycloheximide inhibited
The number of \( \alpha \)-adrenergic receptors was determined with 3 nM \( [3H] \)CGP-12177. Cells were treated with or without 1 \( \mu M \) isoproterenol for 20 min at 37°C. Preincubation with 10 \( \mu \)g/ml cycloheximide was performed at 37°C for 2 h. The number of \( \alpha \)-adrenergic receptors was determined with 3 nM \( [3H] \)CGP-12177 incubating for 24 h at 0°C with or without 90-min preincubation at 30°C (mean ± SD; \( n = 2 \)).

| Addition                          | Bound, fmol/10⁶ cells |
|----------------------------------|----------------------|
| None                             | 19.6 ± 0.1           |
| Isoproterenol                    | 15.7 ± 0.2           |
| Isoproterenol + cycloheximide    | 14.5 ± 0.1           |

**TABLE II**

Effect of Cycloheximide on Reversible Disappearance of \( \beta \)-Adrenergic Receptors

Cells were treated with or without 1 \( \mu M \) isoproterenol for 20 min at 37°C. Preincubation with 10 \( \mu \)g/ml cycloheximide was performed at 37°C for 2 h. The number of \( \beta \)-adrenergic receptors was determined with 3 nM \( [3H] \)CGP-12177 incubating for 24 h at 0°C with or without 90-min preincubation at 30°C (mean ± SD; \( n = 2 \)).

| Addition                          | Bound, fmol/10⁶ cells |
|----------------------------------|----------------------|
| None                             | 19.6 ± 0.1           |
| Isoproterenol                    | 15.7 ± 0.2           |
| Isoproterenol + cycloheximide    | 14.5 ± 0.1           |

neither the disappearance of binding sites for CGP-12177 nor the reappearance of these binding sites.

**DISCUSSION**

The desensitization of adenylate cyclase in cells containing \( \beta \)-adrenergic receptors is induced by exposure to adrenergic agonists such as isoproterenol (1, 19). This desensitization of the adenylate cyclase is not accomplished by a loss in the total number of receptors (20). It has been proposed that this is due to a change of the conformation of the receptor enzyme complex (21). However, it has been shown that when astrocytoma (5) or C6-glioma cells were desensitized, a second vesicular receptor population occurred, which was not coupled to adenylate cyclase. Perkins (22) has shown that the occurrence of this vesicular receptor population is transient. He and Harden et al. (5) proposed that the desensitization of the \( \beta \)-adrenergic receptor is due to a reversible endocytosis of the receptors. This has been supported by Staehelin and Simons (9), who demonstrated a reversible disappearance of \( \beta \)-adrenergic receptors in C6-glioma and WEH17 lymphoma cells, using the new hydrophilic antagonist \( [3H] \)CGP-12177. In contrast to more lipophilic antagonists, such as dihydroalprenolol (DHA), it has been demonstrated that CGP-12177 possesses very little nonspecific binding in intact cells (7), which is due to the fact that CGP-12177 is not accumulated inside cells, while DHA is accumulated (8). A comparison of the binding of CGP-12177 and DHA to intact cells pretreated with 1 \( \mu M \) isoproterenol for up to 20 min showed that DHA still bound to as many receptors as in control cells, while CGP-12177 bound to only 60–70% of the receptors present in control cells (9). It was therefore concluded that because of the special properties of CGP-12177, i.e., membrane impermeability, those receptors that still bind DHA but no longer CGP-12177 are located inside the cells. In addition, in desensitized cells it has been possible to separate, by sucrose density gradient centrifugation, those receptors that bind only DHA, from plasma membrane receptors that bind DHA as well as CGP-12177 (34). The former are localized in vesicles and become accessible for \( [3H] \)CGP-12177 in the presence of the pore-forming agent alamethicin.

We used \( [3H] \)CGP-12177 to investigate further the properties of the reappearance of \( \beta \)-adrenergic receptors after the desensitization.

De novo synthesis of the receptors was not involved in their reappearance, because treatment with cycloheximide (10 \( \mu \)g/ml; 2 h), which inhibited \( [14C] \)leucine incorporation into proteins by 90%, did not prevent the reappearance of the receptors.

The nonlinear temperature dependence of the receptor reappearance suggests that this process is membrane mediated. An estimation of the \( Q_{10} \) gave two values of ~4.0 for \( T \) below 20°C and ~2.6 for those above 20°C (by definition: \( Q_{10} = V_{37°}/V_{0°} \), where \( V \) is rate of reaction, \( T \) is temperature in °C). Such a biphasic profile is characteristic of a membrane-mediated process, because membranes have a melting point which is roughly at 20°C (23).

Moreover, the reappearance of the receptors is dependent on an intact cell structure, because it was completely inhibited by lysing the cells (Fig. 2). Ion gradients will be dissipated by the cell lysis. Therefore we have investigated the possible influence of the different ion gradients on reappearance. Increased intracellular Ca\(^{2+} \) activity did inhibit the reappearance of the receptors, but had no effect on their disappearance (Fig. 5). A possible explanation may be the disassembly of microtubules induced by high Ca\(^{2+} \) activity (24). Thus the reappearance of the \( \beta \)-adrenergic receptors may require intact microtubules.

The other cation gradients were altered by means of the following ionophores: monensin; exchanging Na\(^+ \), K\(^+ \), H\(^+ \) against each other; valinomycin; transporting K\(^+ \) and FCCP; transporting H\(^+ \). Decreasing K\(^+ \)/H\(^+ \) gradients by valinomycin plus FCCP slightly inhibited reappearance of receptors, while diminishing H\(^+ \)/Na\(^+ \)/K\(^+ \) gradients by monensin inhibited the latter completely (Fig. 5). Gradients of H\(^+ \)/Na\(^+ \)/K\(^+ \) occur at the plasma membrane (25) and intracellular membranes, e.g., membranes of endocytotic vesicles (26), Golgi membranes (27, 28), and lysosomal membranes (17). H\(^+ \)/Na\(^+ \)/K\(^+ \) gradients are considered necessary for fusion of endocytotic vesicles with Golgi-derived particles and lysosomes (26). Moreover, it has been shown that monensin inhibits recycling only, but not internalization of the low density lipoprotein–receptor (29). Our results show that monensin inhibited the reappearance but not the disappearance (16) of the \( \beta \)-adrenergic receptors. Therefore, we suggest that the transient disappearance of the \( \beta \)-adrenergic receptors is caused by a mechanism analogous to that described for the low density lipoprotein–receptor, namely an endocytosis cycle (29).

Furthermore, in contrast to the disappearance, the reappearance of the \( \beta \)-adrenergic receptors was ATP dependent (Fig. 4). Preventing the ATP production by inhibiting glycolysis and the mitochondrial respiratory chain did impede the reappearance of the \( \beta \)-adrenergic receptor. It has been suggested that the intracellular recycling of receptors is ATP dependent (30, 31).

The fact that the lysosomotropic agent chloroquine, which is accumulated by intact cells into the lysosomes (17), inhibited the reappearance of the \( \beta \)-adrenergic receptors (Fig. 6), provides additional supporting evidence for an intracellular cycling of the \( \beta \)-adrenergic receptor. The inhibitory effect of chloroquine on the recycling of membrane antigens (32) and mannose glycoconjugate receptors (33) has been described recently. This suggests that intact lysosomes, with a normal low pH, may be a prerequisite for reappearance of any receptors, including the \( \beta \)-adrenergic receptor.

The results obtained so far show that recycling of \( \beta \)-adrenergic receptors occurs and that this requires a low intracellular Ca\(^{2+} \) activity, as well as H\(^+ \)/Na\(^+ \) gradients, ATP, and intact lysosomes. However, it is not entirely elucidated whether this process is taking place in the plasma membrane, or intracellularly. Although the present results support the latter possibility, we cannot exclude the occurrence of a
primary step in which receptors become inaccessible within the plasma membrane, followed by endocytosis and intracellular recycling as a second step.

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