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Rapid and reversible disappearance of β-adrenergic cell surface receptors

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The water soluble β-adrenergic ligand [^{3}H]CGP-12177 was used to measure the cell surface receptors in intact cells. In two cell lines, C6 glioma and WEHI 7 lymphoma cells, ~50% of the cell surface receptors disappear within minutes of incubation of the cells with isoproterenol. The receptors can still be detected in homogenates and reappear on the cell surface when cells are washed and reincubated at 37°C. The data agree with a disappearance of the receptors from the cell surface by an agonist-mediated endocytosis.

Key words: receptor/β-adrenergic/cell surface/ligand binding/CGP-12177/endocytosis

Introduction

Many different cells respond to adrenalin and to noradrenalin. One of the ways through which these hormones exert their effects is the β-adrenergic receptor in the plasma membrane of these cells, which is coupled to an adenylate cyclase. A remarkable property inherent in the target cells is that they become refractory when exposed to β-adrenergic agonists. Two processes can be distinguished, a slow decrease in the total number of receptors, often referred to as "down-regulation", and a rapid desensitization of the hormonally stimulated adenylate cyclase (Su *et al.*, 1980; Green *et al.*, 1981). For down-regulation an ~2-h exposure is required for a loss of 50% of the receptors, and the reappearance of a full complement of receptors requires 10–20 h and protein synthesis. Desensitization of the adenylate cyclase response takes place within minutes and is quickly reversible.

Down-regulation has also been observed with polypeptide hormone receptors. Epidermal growth factor, after having formed a complex with its receptor, is internalized into endocytotic vesicles which later fuse with lysosomes where it is degraded (Carpenter and Cohen, 1979). The internalization leads to a concomitant decrease in cell surface receptors. This is also well documented for the insulin receptor (Baldwin *et al.*, 1980).

Agonist-induced endocytosis would explain early adenylate cyclase desensitization to β-adrenergic agonists, since the receptors would be present in endocytotic vesicles but not accessible to signals from outside. Two recent reports have described an agonist-induced shift of β-adrenergic receptors to a different membrane compartment (Harden *et al.*, 1980; Chuang and Costa, 1979). To our knowledge no direct evidence for the endocytosis of the β-adrenergic receptor, however, has been presented. We have recently described a hydrophilic β-adrenergic ligand which is not taken up by cells and therefore specifically measures the cell surface receptors (Staehelin *et al.*, submitted). We now present evidence that, within minutes of exposure to isoproterenol, ~50% of the receptors disappear from the cell surface of two cell lines, C6 glioma and WEHI 7 lymphoma cells. The receptors can still be recovered in homogenates and reappear on the cell surface after removal of the agonist. These data are compatible with a rapid and reversible agonist-induced receptor endocytosis.

Results

[^{3}H]CGP-12177 is a new, hydrophilic radioligand whose synthesis and β-adrenergic properties have been described (Staehelin *et al.*, submitted). Figure 1A shows a saturation curve obtained with intact S49 cells, demonstrating the low nonspecific binding obtained with the ligand. The Scatchard plot in Figure 1B shows that the ligand binds to a single class of binding sites with an affinity of 10^{-9} M. Figure 1C demonstrates that the ligand is stereospecifically displaced from these sites by appropriately low concentrations of (−)-and (+)-propranolol. The very low nonspecific binding observed in the presence of 10^{-6} M (−)-propranolol (Figures 1A and 1C) suggests that insignificant amounts of the ligand are taken up by cells and that therefore the ligand measures only cell surface receptors.

To determine whether the number of receptors on the cell surface changed during exposure to an agonist, cells were first incubated with 10^{-6} M isoproterenol for 15 min at 37°C. Then the cells were collected by centrifugation, washed free of isoproterenol with ice-cold medium and incubated at 0°C with the hydrophilic radioligand [^{3}H]CGP-12177. [^{3}H]CGP-12177 binding was measured at three ligand concentrations with both C6 glioma and WEHI 7 cells. Figure 2 shows the results in the form of Scatchard plots. It can be calculated that the K_D for [^{3}H]CGP-12177 is ~2 x 10^{-10} M at this temperature and that in control cells there are ~8500 receptors/C6 cell and ~850 receptors/WEHI 7 cell. The data in Figures 2A and C show that after isoproterenol treatment both cell lines display an ~50% decrease in receptor number.

The loss of receptors is only detected when the cells are cooled after incubation with isoproterenol and assayed at low temperature. The data in Figures 2B and D show experiments where the cells were treated with isoproterenol in the same way but washed at room temperature and assayed with the radioligand at 37°C. Under these conditions there is no difference in the number of available receptors between control and isoproterenol-treated cells. The decrease in available cell surface receptors can therefore only be demonstrated if the agonist-induced state of the cells is preserved by cooling the cells to 0°C in the presence of isoproterenol and maintaining them at 0°C during the receptor determination.

Using this assay, i.e., incubating the cells at 37°C with isoproterenol, cooling, washing, and assaying them at 0°C, we studied the time of exposure to 10^{-6} M isoproterenol necessary to evoke a decrease in the number of receptors. Figure 3 shows that the available cell receptors disappear very quickly. For example, for a 40% receptor decrease, 3 min of exposure to 10^{-6} M isoproterenol are required with WEHI 7 cells and ~15 min with C6 glioma cells. We have also studied

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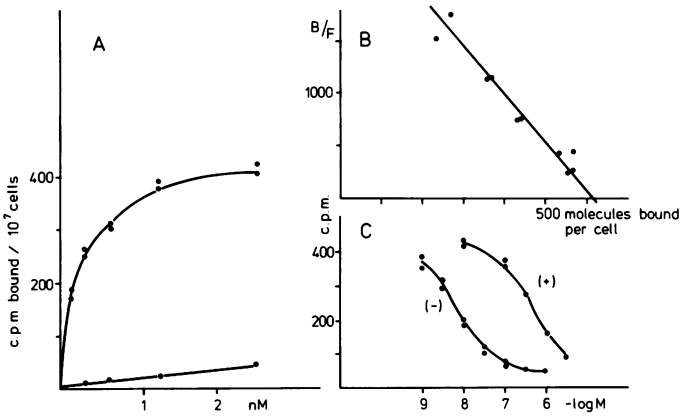


Fig. 1. Binding characteristics for [^{3}H]CGP-12177 in intact cells. Ten million S49 cells (a variant received from U.Gehring, Heidelberg) were harvested as described in Materials and methods and incubated for 30 min at 37°C with various concentrations of [^{3}H]CGP-12177. The cells were filtered as described in Materials and methods. **A**, saturation curve in presence and absence of 10^{-6} M (−)-propranolol. **B**, Scatchard plot from data in **A**. **C**, stereospecific displacement of [^{3}H]CGP-12177 (10^{-9} M) by (−)- and (+)-propranolol.

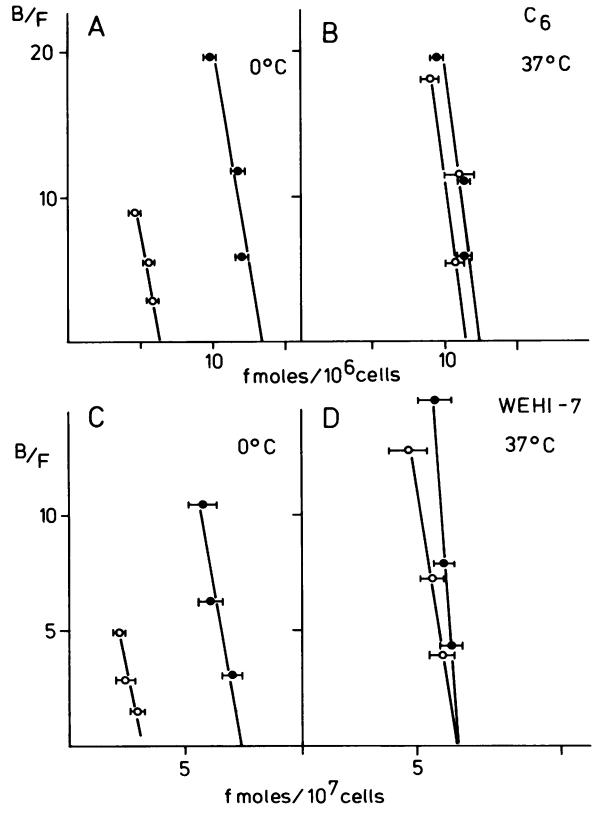


Fig. 2. Isoproterenol-induced surface receptor loss in C6 glioma and WEHI 7 cells. Top: C6 glioma cells were incubated for 15 min with or without 10^{-6} M isoproterenol, washed, and assayed for 16 h with 0.5–2.0 x 10^{-9} M [^{3}H]CGP-12177 at 0°C as described in Materials and methods (**A**). Cells treated in an identical way with isoproterenol were washed at room temperature and assayed with [^{3}H]CGP-12177 for 30 min at 37°C (**B**). (●) Control cells, (○) isoproterenol-treated cells. Bottom: WEHI 7 cells were treated with isoproterenol as above and also assayed at 0°C (**C**) or at 37°C (**D**). (●) Control cells, (○) isoproterenol-treated cells.

the concentrations of isoproterenol which cause receptor loss within 15 min. Figure 4 shows that with both cell lines there is a half-maximal receptor loss at ~3 x 10^{-8} M isoproterenol.

To ascertain that the reduction in the number of binding

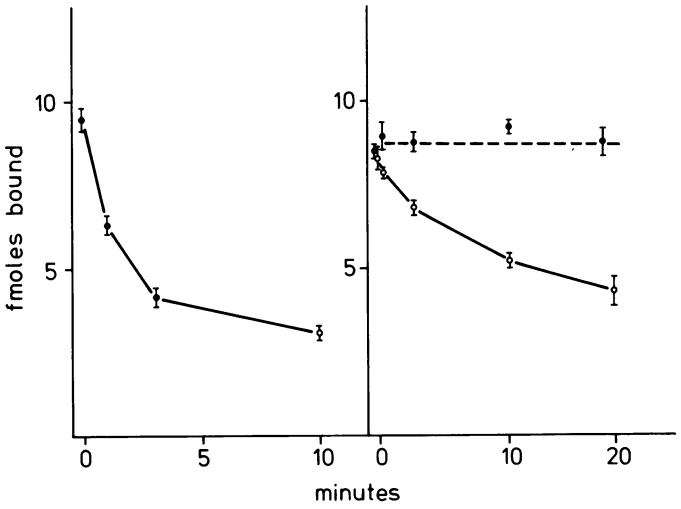


Fig. 3. Time course of the surface receptor loss in C6 and WEHI 7 cells. Cells were assayed in the standard assay described in Materials and methods. The isoproterenol concentration was 10^{-6} M, the [^{3}H]CGP-12177 concentration used for the assay was 10^{-9} M and the time of incubation was varied. **Left panel**: WEHI 7 cells; **right panel**: C6 glioma cells. Dashed line: control cells (treated with ascorbic acid alone).

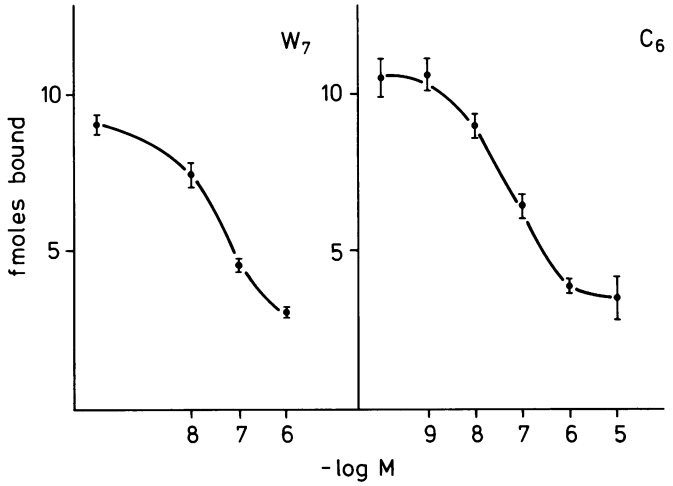


Fig. 4. Dependence of the surface receptor loss on isoproterenol concentration. Assays were done as in Figure 7. The incubation time with the various isoproterenol concentrations was 15 min. **Left panel**: WEHI 7 cells; **right panel**: C6 glioma cells.

sites for [^{3}H]CGP-12177 was not due to persistent occupancy of the receptors by isoproterenol at 0°C, C6 glioma cells were first incubated with 10^{-5} M isoproterenol for 2 h at 0°C, washed, and then assayed with [^{3}H]CGP-12177 for 16 h at 0°C. Under these conditions the receptor numbers were similar for control and isoproterenol-treated cells, i.e., 10.8 fmol/10^6 cells and 11.5 fmol/10^6 cells, respectively. That the receptors had actually interacted with isoproterenol during the 2-h preincubation at 0°C could be demonstrated. With 10^{-9} M [^{3}H]CGP-12177 present during the 2-h preincubation at 0°C, 10 fmol/10^6 cells of the radioligand were bound in the absence of 10^{-5} M isoproterenol and <0.02 fmol/10^6 cells in its presence. Two conclusions can be drawn from these experiments; first, isoproterenol binds to the receptor at 0°C, but the half-life of the complex is so short that it does not interfere with radioligand binding after cells are washed; and second, the interaction of intact cells with isoproterenol at

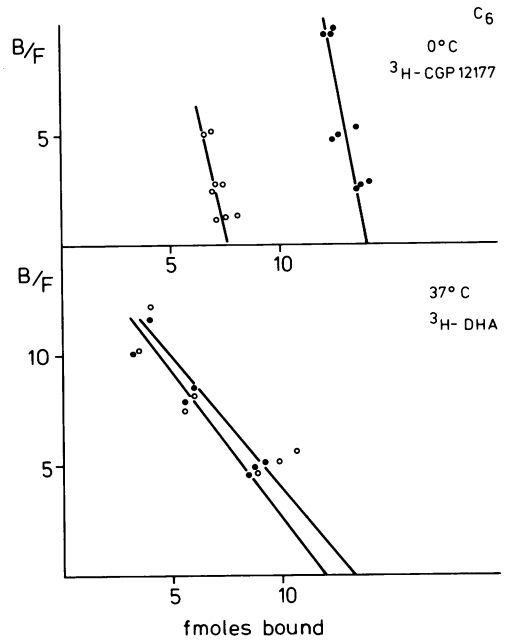


Fig. 5. Effect of isoproterenol treatment on cell surface receptors and receptors in homogenates. **Top**: C6 glioma cells were incubated for 15 min with 10^{-6} M isoproterenol, washed, and assayed for 16 h with 0.5–2.0 x 10^{-9} M [^{3}H]CGP-12177 at 0°C as described in Materials and methods. **Bottom**: cells incubated and washed identically were resuspended in 10 mM Hepes - 1 mM $MgSO_4$ instead of Dulbecco-Hepes, allowed to swell for 20 min at room temperature, and homogenized in a dounce homogenizer. The homogenates were incubated with 0.5–2.0 x 10^{-9} M [^{3}H]-DHA at 37°C, cooled and filtered 30 min after the addition of 10 mM phosphate pH 7.4 - 4 mM $MgSO_4$. (●) Control cells, (○) isoproterenol-treated cells.

0°C does not lead to a loss of available receptors, in contrast to an interaction at 37°C.

Down-regulation of receptors in membrane preparations is a slow process requiring at least 2 h for a 50% receptor decrease (Su *et al.*, 1980). This process is different from the phenomenon described in this paper, namely the fast disappearance of cell surface receptors in intact cells. To demonstrate the difference between the two processes the experiment described in Figure 5 was performed. C6 glioma cells were treated with or without isoproterenol for 15 min at 37°C, cooled, and washed. Half the cells were assayed as described in Figure 2A and again showed a 50% decrease in receptor number (Figure 5, top). The other half of the cells was homogenized and the homogenates assayed with [^{3}H]-dihydroalprenolol (DHA). Under these conditions the full complement can still be detected (Figure 5, bottom). It was important to use [^{3}H]DHA for the assay of the homogenates since only 70–80% of the receptors can be detected with [^{3}H]CGP-12177 (data not shown). This is to be expected. If internalization of the receptors is the reason for their disappearance from the cell surface, the receptors inside unbroken endocytotic vesicles will still not be accessible to the hydrophilic ligand. Similarly, it should be mentioned that in intact cells many more receptors are detected with [^{3}H]DHA than with [^{3}H]CGP-12177 after agonist treatment. This is also to be expected since [^{3}H]DHA diffuses into intact cells even at 0°C.

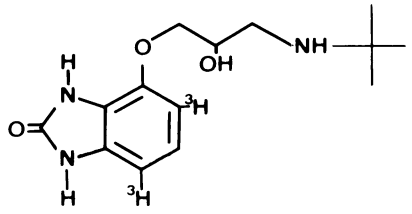
Discussion

Several recent reports have summarized the evidence for receptor-mediated endocytosis of various polypeptide hormones and other receptor-bound proteins, as well as for the recycling of the respective receptors (Doyle and Baumann, 1979; Goldstein *et al.*, 1979; Pastan and Willingham, 1981; Pearse and Bretscher, 1981; Schneider *et al.*, 1979). As far as the β-adrenergic receptor is concerned, a major difficulty has been that the receptors can only be assayed using labeled antagonists and not agonists. The lipophilic antagonists described in the literature (Teraski and Brooker, 1978; Insel and Stoolman, 1978) show extremely high unspecific binding to intact cells. These radioligands probably dissolve in the plasma membrane and enter the cells, where they may interact with internalized receptors. Several reports in the literature, however, indicate that β-adrenergic receptors might also undergo receptor-mediated endocytosis. Harden *et al.* (1980) have analyzed the membranes of lysed human astrocytoma cells after centrifugation in a sucrose gradient. After cells had been exposed to isoproterenol for 15 min the receptor distribution in the gradient showed a shift of about half the receptors to a slower moving fraction which the authors suggested might be endocytotic vesicles. Internalization of β-adrenergic receptors from frog erythrocytes was also suggested by Chuang and Costa (1979) who found that after a 1-h incubation with isoproterenol some receptors shifted from the pellet to the 30 000 *g* supernatant. Pittman and Molinoff (1980) have shown that in intact cells isoproterenol initially competes with the binding of hydroxybenzylpindolol, but after a few minutes there is no more competition. This result is to be expected when receptors are internalized and only accessible to the lipophilic ligand and not to the hydrophilic isoproterenol.

Our results show that within minutes of stimulation by isoproterenol the β-adrenergic receptors available to the hydrophilic ligand disappear from the cell surface. This is reminiscent of the time course of adenylate cyclase desensitization. The concentrations of isoproterenol that induce a reduction in the number of cell surface receptors are also very similar to those required for adenylate cyclase desensitization (Su *et al.*, 1980). We suggest, therefore, that adenylate cyclase desensitization might be the consequence of an agonist-mediated receptor endocytosis. The rapid reversibility of adenylate cyclase desensitization and our finding that this loss of cell surface receptors can only be demonstrated when measured in the cold and not when cells are reincubated at 37°C, are also compatible with this concept, since rapid recycling of the receptors has been described for cell surface antigens (Schneider *et al.*, 1979).

Materials and methods

[^{3}H]CGP-12177 differs from the well known propranol mainly by the nature of the aromatic ring system which is a phthalimide residue instead of a naphthyl residue; its radiosynthesis as well as the β-adrenergic blocking properties have been described (Staehelin *et al.*, submitted).



CGP-12177

[^{3}H]DHA (72 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, UK, and (−)-isoproterenol-bitartrate from Sigma (St. Louis, MO). Cell culture media were obtained from GIBCO (Grand Island, NY).

C6 glioma cells (Benda *et al.*, 1968) were grown for 4 days in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum to a density of 2 x 10^7 cells/10 cm Falcon dish and suspended by treatment with EDTA (Dibner *et al.*, 1981). They were then dispersed in DMEM containing 10 mM Hepes pH 7.4, and 0.0136 M sodium chloride instead of bicarbonate (DMEM-Hepes) at a density of 2.2 x 10^6 cells/ml. WEHI 7 (Harris *et al.*, 1973) cells were grown in 75 ml Falcon flasks to a density of $1-2$ x 10^6 cells/ml in RPMI medium containing 10% fetal calf serum and collected by centrifugation. Cells were resuspended in RPMI medium at a density of 2.2 x 10^7 cells.

Isoproterenol pretreatments

Cells were preincubated for 5 min at 37°C. Isoproterenol from stock solutions prepared in 0.02% ascorbic acid was added, usually at 10^{-6} M final concentration for 15 min at 37°C. Unless indicated otherwise, the tubes were cooled in ice water, spun for 4 min at 4000 r.p.m. in a refrigerated Sorvall centrifuge, resuspended in ice-cold DMEM-Hepes, and spun again.

Ligand binding

The washed cells were resuspended in the original volume of cold DMEM-Hepes. To 0.45 ml, 50 μl of radioligand was added and the cells were kept on ice overnight. Prior to filtration they were diluted with 10 ml of 10 mM phosphate pH 7.4, 4 mM $MgSO_4$. The cells were collected on GF/C glass fiber filters (Whatman, Maidstone, Kent, UK), the filters were washed twice with 10 ml of the same buffer, and counted in 10 ml of Instagel (Packard) at 44% counting efficiency. The nonspecific binding of 2 x 10^{-9} M [^{3}H]CGP-12177 was ~10% of the total binding.

When homogenates were assayed the washed cells were taken up in 0.01 M Hepes pH 8, 1 mM $MgSO_4$, kept at 0°C for 30 min to allow the cells to swell, and homogenized in a tight fitting dounce homogenizer. Aliquots of the homogenate (0.45 ml) were then incubated with 50 μl of various concentrations of [^{3}H]DHA for 16 h at 0°C and filtered as described above.

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