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Evidence for the Appearance of an Uncoupled Form of the β-Adrenergic Receptor Distinct From the Internalized Receptor

C. Hertel, M. Portenier, and M. Staehelin

Department of Research, University of Basel Medical School, CH-4031 Basel, Switzerland (M.P., M.S.), and Department of Pharmacology, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina 27514 (C.H.)

Agonist treatment of C6-glioma cells induces two altered states in β-adrenergic receptors, a low affinity for the hydrophilic antagonist CGP-12177 and a low affinity for agonists like isoproterenol. We present evidence that, in cells not treated to inhibit receptor internalization, the two properties occur with a different time course, the low affinity for isoproterenol preceding that for CGP-12177. In that the low affinity for CGP-12177 is due to the internalization of the receptor, the results indicate that uncoupling of the receptor, indicated by the low affinity for isoproterenol, occurs while the receptor is still located on the cell surface. Removal of the agonist leads to reappearance of the receptor to the plasma membrane followed by loss of the uncoupled state.

Key words: β-adrenergic receptors, CGP-12177, internalization, recycling, uncoupling

Cells containing β-adrenergic receptors (BAR) show a rapid tachyphylaxis after hormone treatment [for review, see 1]. Several groups have demonstrated in different cell lines [2–4] as well as in frog erythrocytes [5] that a rapid sequestration of the receptors from the cell surface to an intracellular compartment occurs, whereas the GTP-binding protein (N-protein) and adenylate cyclase (AC) are not sequestered to the same extent during agonist treatment [2,5]. It has been suggested by Stadel et al [5] that this physical separation of BAR from N-protein and AC per se is sufficient to account for uncoupling of BAR/AC. However, Waldo et al [6] demonstrated that loss of hormone-sensitive AC activity occurs before a sequestration of the receptor could be measured. These authors provided further evidence for a two-step mechanism by inhibiting sequestration of the receptor but not formation of an uncoupled state of the receptor with concanavalin A [6].

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In the present paper, the question of whether uncoupling between BAR and AC is due to physical separation of the receptor from N-protein and AC or to an earlier modification of the receptor while the receptor is still located on the plasma membrane is further addressed. We have previously shown that desensitized receptors have a low affinity for CGP-12177, a hydrophilic antagonist, as well as for the agonist isoproterenol [7]. We have now investigated whether these two altered properties of the receptor occur with the same time course during desensitization. In addition, we have measured the time course of their reversal during resensitization following the removal of the agonist. Our data indicate that uncoupling of the receptors precedes internalization even under conditions where the latter is not inhibited and recoupling lags behind the reappearance of the receptors on the plasma membrane.

MATERIALS AND METHODS

C6 glioma cells were grown as described previously [8]. Prior to incubation with 1 μM isoproterenol, the growth medium was aspirated and 10 ml Hepes-DMEM was added. The cells were incubated with isoproterenol for the time indicated in the experiment at 37°C and then chilled. Lysates were prepared by incubating the cells in 1 mM Tris (pH 7.4)/2 mM EDTA for 20 min on ice. The buffer was aspirated, and the cells were scraped with a rubber spatula, yielding about 2 ml of lysate. The lysates were diluted to 300–500 μg protein/ml with assay buffer (154 mM NaCl, 20 mM Tris, 5 mM $MgCl_2$, pH 7.4). Binding assays were performed with either [^{3}H]dihydroalprenolol (1 nM), [^{3}H]CGP-12177 (0.8 nM), or [^{125}I]cyanopindolol (10 pM) as the radioligand and isoproterenol or CGP-12177 as the competitor in a volume of 0.5 ml with the tritiated radioligands and a volume of 0.25 ml with the iodinated radioligand. Incubation was performed at 30°C for 2 hr and stopped by transfer onto ice. The samples were diluted with 10 ml of ice-cold 10 mM phosphate buffer (pH 7.4)/4 mM $MgSO_4$, filtered through glass fiber filters, and washed with the same buffer. Tritium was determined in a liquid scintillation counter with 10 ml of Aquasol-2 (New England Nulcear, Boston, MA), ^{125}I was determined in a gamma counter. Protein was determined by the method of Bradford [9].

(−) Cyanopindolol was iodinated with chloramine T according to Barovsky and Brooker [10]; the specific radioactivity of the product was 8.1×10^{10} Bq/μmol. [^{3}H](±)CGP-12177 (1.5×10^{12} Bq/mmol) was prepared as described previously [11]. [^{3}H](−)dihydroalprenolol (2.6×10^{12} Bq/mmol) was obtained from The Radiochemical Centre (Amersham, UK). (−)Isoproterenol bitartrate and (−)propranolol were obtained from Sigma (St. Louis, MO).

ANALYSIS OF EXPERIMENTAL DATA

The number of receptors per cell was highly dependent on the number of passages through which the cells had 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 in triplicate.

The standard deviation was always $< 10\%$ unless otherwise indicated. Analysis of competition binding experiments was done as described previously [7]. Since DeLean et al [12] have shown that using the negative logarithm (pKd) rather than Kd

itself improved the statistical analysis, the dissociation constants are given as pKd ± SEM.

RESULTS

Low Affinity of BAR From Desensitized Cells for Agonists and Membrane-Impermeable Antagonists

Desensitization of C6 glioma cells induces the formation of a subpopulation of BAR possessing low affinity for CGP-12177 [7,13]. Figure 1a shows the displacement of [^{3}H]dihydroalprenolol by various concentrations of CGP-12177 in lysates from

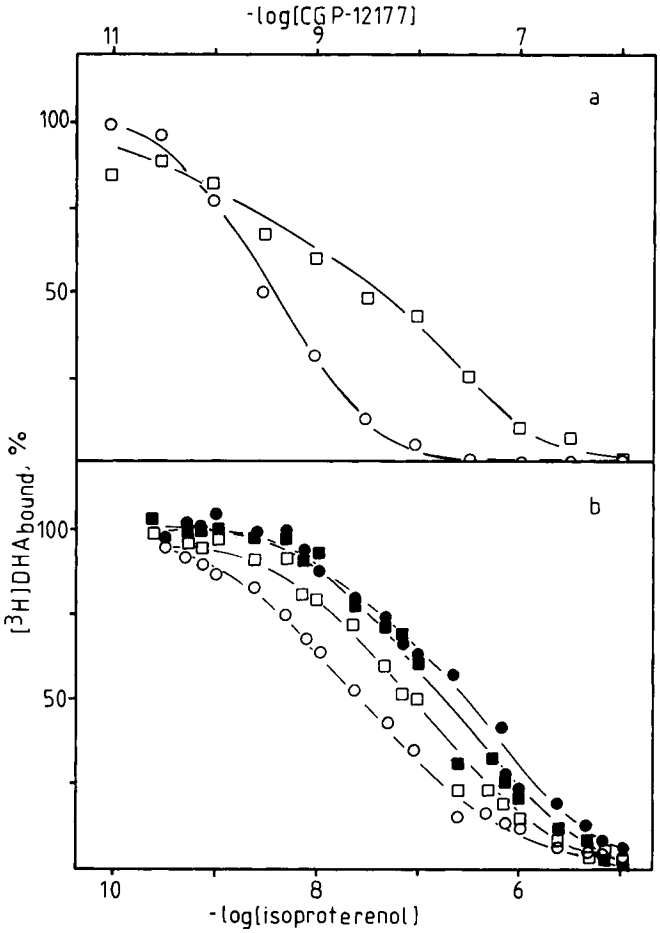

Fig. 1. Effect of desensitization of C6 cells on binding of CGP-12177 and isoproterenol. C6 cells were incubated either with (□,■) or without (○,●) 1 μM isoproterenol for 20 min at 37°C, chilled, washed, and lysed by hypotonic shock. Competition binding experiments were performed with 1 nM [^{3}H]dihydroalprenolol and CGP-12177 (a) or isoproterenol (b); 100 μM GTP was added to the binding assay with isoproterenol (b) for control (●) and desensitized (■) lysates. [^{3}H]dihydroalprenolol specifically bound was 31 and 33 fmol/mg protein for control and desensitized cells, respectively. Results from one experiment performed in triplicate out of a series of six are shown.

control and desensitized cells. Furthermore, desensitized receptors possess a low affinity for agonists (Fig. 1b) similar to astrocytoma cells [14]. Figure 1b shows that higher concentrations of isoproterenol are required to displace [^{3}H]dihydroalprenolol from lysates of desensitized cells compared to those of control cells. In lysates from control cells, a low affinity for isoproterenol can also be induced by addition of GTP to the binding assay (Fig. 1b), whereas GTP has no effect on the binding of CGP-12177 or other agonists [7].

To determine whether these two phenomena occuring in lysates of desensitized cells, ie, low affinity for a membrane-impermeable antagonist and low affinity for agonists, are due to a common alteration of BAR, the time courses of appearance of these two alterations of the BAR were determined. Intact cells were preincubated for various times with 1 μM isoproterenol, chilled, washed, and lysed. The appearance of low affinity for either CGP-12177 or isoproterenol was determined in lysates in competition binding experiments with [^{3}H]dihydroalprenolol. Concentrations of CGP-12177 (10 nM) and isoproterenol (100 nM) were chosen at which the difference in the efficacy to compete with [^{3}H]dihydroalprenolol in control and desensitized lysates was maximal (see Fig. 1a,b).

Figure 2 shows the time course of appearance of low affinity of BAR for isoproterenol and CGP-12177 plotted as percent of specific binding of the radioligand. Specific binding in the absence of the nonlabeled ligand was defined as 100%. The formation of the low affinity for isoproterenol is completed within 5 min, with a half-time of approximately 1 min. The formation of low affinity for CGP-12177 is almost linear for 10 min and is complete after 20–30 min [13].

Agonist Binding of the Plasma Membrane Receptors After Desensitization for 20 min

In lysates from control cells, a low affinity of BAR for agonists can be induced by addition of GTP to the lysates, when the radioligand is either [^{3}H]dihydroalprenolol (Fig. 1b) or [^{3}H]CGP-12177 (Fig. 3). In lysates from desensitized cells, [^{3}H]dihydroalprenolol measures both vesicular and plasma membrane BAR, and under these conditions only a small shift to low affinity can be determined by addition of GTP (Fig. 1b). However, when [^{3}H]CGP-12177 is used, and thus only the remaining plasma membrane receptors are measured in lysates from desensitized cells, addition of GTP to the lysates induces a shift to lower affinity for isoproterenol similar to the one observed in control cells (Fig. 3).

Reversibility of Low Affinity of BAR for Agonists and Membrane-Impermeable Antagonists

After removing agonists from the medium, BAR reappear to the cell surface [15]. In Figure 4, a comparison of the reappearance of high affinity for isoproterenol and for CGP-12177 is shown. The experiment is performed under conditions similar to those described for Figure 2, but instead of [^{3}H]dihydroalprenol [^{125}I]cyanopindolol was used. It is demonstrated that the reappearance of high affinity for isoproterenol is slightly slower than for CGP-12177, with a lag phase of about 10 min.

DISCUSSION

By now it is a well described phenomenon that BAR are internalized during agonist treatment in astrocytoma cells [2,7,16], frog erythrocytes [5], lymphoma cells

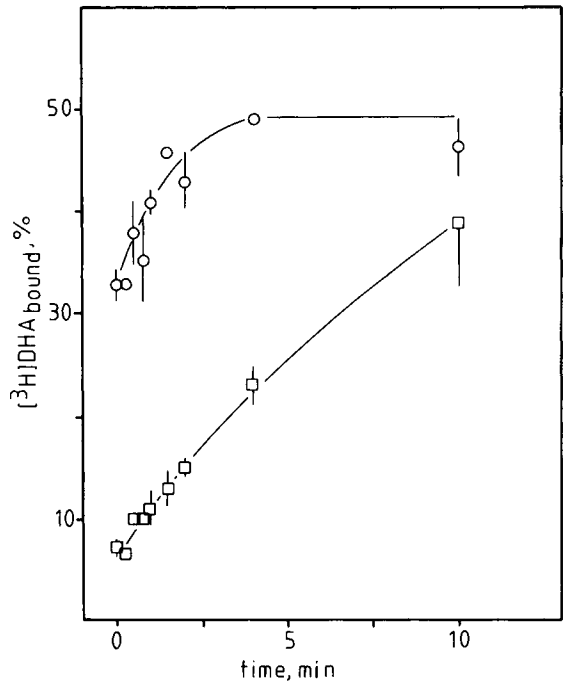

Fig. 2. Time course for the appearance of low affinity for CGP-12177 and isoproterenol. C6 cells were incubated at 37°C with 1 μM isoproterenol for the time indicated, chilled, washed, and lysed. Each lysate was incubated with 1 nM [^{3}H]dihydroalprenolol to determine total binding, radioligand and 1 μM propranolol to determine nonspecific binding, radioligand and 100 nM isoproterenol (○), or radioligand and 10 nM CGP-12177 (□). The percentage of binding of the radioligand in the presence of the competing ligand was calculated using the specific binding. [^{3}H]dihydroalprenolol bound specifically was 30–35 fmol/mg protein. The results from a single experiment performed in triplicate out of a series of four are shown. SD are indicated in the graph.

[8], and C6 glioma cells [3,13]. Evidence has been published [6] that agonist-induced uncoupling of adenylate cyclase can occur without internalization of the receptor. Recently, uncoupled receptors have been demonstrated on the plasma membrane after inhibiting receptor loss [17]. In the present paper, we have further investigated the early alterations of the binding properties of BAR during agonist treatment in cells that are still able to internalize their receptors.

Comparison of the appearance of low affinity for CGP-12177, which is due to internalization of BAR [7,13,18], and low affinity for isoproterenol demonstrates that first a low affinity for the agonist occurs (t1/2 = 2 min) followed by internalization of the receptor. This low affinity for agonists seems to be due primarily to uncoupling from the N-protein. The affinities for isoproterenol [Kd(1) = 10 nM, Kd(2) = 400 nM] determined in this assay, ie, lysates of control or desensitized cells, probably reflect the affinity of BAR resulting from coupling or uncoupling from the N-protein have high affinity binding sites for agonists, whereas mutant cells lacking a functional N-protein show only low affinity binding sites for agonists. The internalized receptor as determined in intact cell binding assays has an even lower apparent affinity for isoproterenol, probably because of the additional factor of a plasma membrane barrier [16].

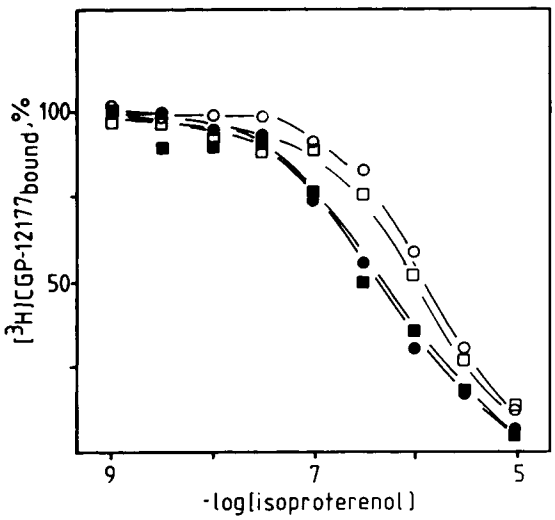

Fig. 3. Isoproterenol binding to plasma membrane receptors. C6 cells were either preincubated for 20 min at 37°C without (●,○) or with (■,□) 1 μM isoproterenol, chilled, washed, and lysed. Competition binding experiments were performed using 0.8 nM [^{3}H]CGP-12177 and isoproterenol in the presence (○,□) or absence (●,■) of 100 μM GTP. Nonspecific binding was determined in the presence of 1 μM propranolol. [^{3}H]CGP-12177 specifically bound was 20 and 12 fmol/mg protein for control and desensitized cells, respectively. Results from a single experiment performed in triplicates out of a series of three are shown.

Recovery of cell surface receptors is rapid after removing agonist from the cells. This process takes about 60 min to be completed if the incubation is performed in the absence of antagonist; however, addition of antagonist during the recovery period will shorten it to 30 min [15]. The appearance of high affinity for isoproterenol is slightly slower, suggesting that the receptor that reappears to the plasma membrane is not immediately coupled to N-protein, at least not in the absence of hormone. There is evidence, however, that the reappearing receptor can immediately couple to adenylate cyclase, and thus to the N-protein, when stimulated by isoproterenol [14,19].

The results obtained demonstrate that agonist treatment of C6-cells induces two distinguishable alterations in the β-adrenergic receptor: first, a decrease in the affinity for agonists, probably because of uncoupling from the N-protein while the receptor is still located on the plasma membrane [17]. Second, this "uncoupled receptor" is internalized, and the receptors remaining on the cell surface measured with [^{3}H]CGP-12177 are as tightly coupled as those of control cells indicated by a similar GTP-shift. Third, after the agonist is removed, the receptors reappear on the cell surface again, but the formation of a "coupled" high-affinity state appears more slowly than the reappearance of the receptor on the plasma membrane.

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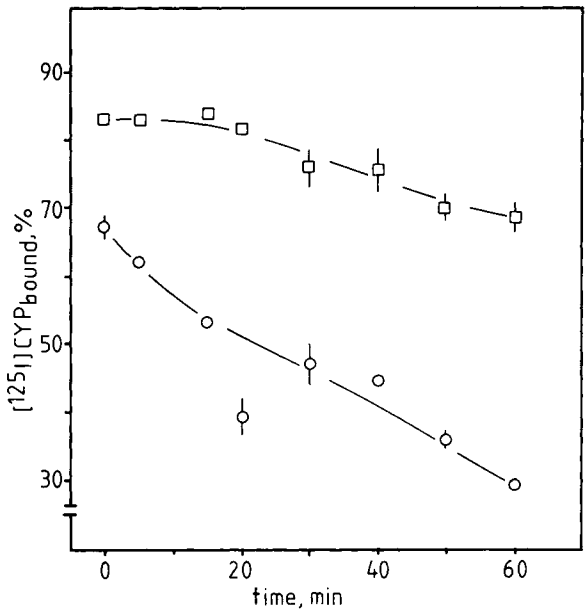

Fig. 4. Reappearance of high affinity for CGP-12177 and isoproterenol. C6 cells were treated for 20 min at 37°C with 1 μM isoproterenol, chilled, washed, supplemented with Hepes-EMEM (37°C), and incubate for the times indicated at 37°C. They were then chilled, washed, and lysed. Binding assays were performed with 10 pM [^{125}I]cyanopindolol, radioligand and 1 μM propranolol, radioligand and 100 nM isoproterenol (□), radioligand and 10 nM CGP-12177 (○). The percentage of binding of the radioligand in the presence of the competing ligand was calculated using the specific binding. [^{125}I]cyanopindolol bound specifically was 20–25 fmol/mg protein. Results from a single experiment performed in triplicate out of a series of four are shown. SD are indicated.

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