Further Evidence that Desensitization of \( \beta \)-Adrenergic-sensitive Adenylate Cyclase Proceeds in Two Steps

MODIFICATION OF THE COUPLING AND LOSS OF \( \beta \)-ADRENERGIC RECEPTORS*

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In C6 glioma cells adenylate cyclase activation by a \( \beta \)-adrenergic agonist is a Michaelis-Menten function of receptor occupancy as expected in the collision-coupling model. The apparent affinity of the receptor-agonist complex for the adenylate cyclase \((K)\) is equal to 0.24 \( \times \left[ R_T \right] \), \([R_T]\) being the maximal receptor concentration.

When C6 glioma cells were incubated with \( 10^{-8} \text{ M} \) isoproterenol for 3 h, there was a 45.4 \( \pm 2.7\% \) (\( n = 10 \)) reduction in the number of total \([^3H]DHIA binding sites and the maximal adenylate cyclase activation dropped by 57 \( \pm 3.4\% \) (\( n = 6 \)). The affinity of the \( \beta \)-adrenergic receptors for both agonists and antagonists did not change during desensitization. However, the apparent affinity constant for isoproterenol stimulation of the adenylate cyclase \((K_{\text{app}})\) rose from 3.9 \( \pm 1.0 \times 10^{-8} \text{ M} \) (\( n = 5 \)) to 1.1 \( \pm 0.2 \times 10^{-7} \text{ M} \) (\( n = 5 \)). This shift is to be expected in all systems in which coupling between receptor occupancy and adenylate cyclase stimulation is not linear and in which either the number of coupled receptors decrease or the \( K \) constant is modified.

Double reciprocal plotting of equilative isoproterenol concentrations in control and desensitized adenylate cyclase systems enabled us 1) to calculate the affinity of isoproterenol for its binding sites: 1.4 \( \pm 0.2 \times 10^{-7} \text{ M} \) (\( n = 6 \)) which is very close to the \( K_D \) determined from binding experiments: 1.8 \( \pm 0.5 \times 10^{-7} \text{ M} \) (\( n = 5 \)); 2) to show that coupling changes during desensitization. This change consists in a 3.7-fold decrease in the apparent affinity of the agonist-receptor complex for the enzymes. Two hypotheses are proposed to explain this reduction.

The assumption that desensitization involves two different steps, an alteration in the coupling and a loss of \( \beta \)-adrenergic receptors, is substantiated further by the following observations: the loss of hormonal responsiveness preceded the loss of binding sites and long term treatment with cycloheximide or lowering the temperature to 4°C eliminate the loss of binding sites but not the decline in hormonal responsiveness.

Cells that respond to specific hormones possess regulation mechanisms allowing the control of their degree of responsiveness. Thus, exposure of cells to a hormone reduces their response to subsequent exposure (desensitization). On the other hand, prevention of hormone-receptor interaction, either by suppressing the hormone or adding an antagonist, enhances the response (supersensitivity). Generally, in systems in which the hormonal effect is mediated by cAMP, broken cell preparations of desensitized cells show a decrease both in adenylate cyclase stimulation by the agonists and in the density of their specific receptor sites (1). Leftkowitz and co-workers suggested that catecholamine-induced desensitization of \( \beta \)-adrenergic sensitive adenylate cyclase mainly resulted from the loss of \( \beta \)-adrenergic receptors (1).

The relationship between the reduction in the adenylate cyclase response to a \( \beta \)-adrenergic agonist and the loss of specific \( \beta \)-adrenergic receptors shows a lack of correlation between the two (2-5).

Recent results by Su et al. (2) indicated that the decline in \( \beta \)-adrenergic-sensitive adenylate cyclase of desensitized system might be a complex phenomenon. The loss of isoproterenol-stimulated adenylate cyclase preceded the loss of \( \beta \)-adrenergic receptors but, after 24 h desensitization, both activities were reduced by the same percentage. The same discrepancy was also reported by Shear et al. (4). They found that, in S49 lymphoma cells, membranes prepared after 24 h desensitization showed a 70% reduction in adenylate cyclase stimulation by isoproterenol, corresponding to a 35% drop in the number of binding sites.

Nevertheless, in order to compare these parameters, two points should be kept in mind: 1) Binding capacity and adenylate cyclase activity must be measured under the same incubation period and in the presence of the same components, especially as regards nucleotides, since these are known to influence binding of agonists (6-7). 2) It is essential to consider that the relationship between receptor occupancy and adenylate cyclase stimulation (coupling function) is not necessarily linear. For example, in ADH (8, 9) and \( \beta \)-adrenergic-sensitive adenylate cyclase systems (10-12), a 10% occupancy of receptor sites results in adenylate cyclase activities of 80% and 20% of maximally stimulated adenylate cyclase respectively.

An important question is whether the loss of \( \beta \)-adrenergic receptors is the cause or a consequence of the \( \beta \)-adrenergic-sensitive adenylate cyclase desensitization.

In the present work we attempted to answer this question by studying the relationship between the loss in \( \beta \)-adrenergic receptors and the reduction in adenylate cyclase responsiveness in C6 glioma cells, with special reference to Points 1 and 2 mentioned above. We also tried to modify this relationship by two treatments which have been shown to alter desensitization, namely changes in the temperature (13) and inhibition of protein synthesis (for review see Ref. 14).
KO, the dissociation constant of [3H]DHA for its binding sites. Iso, (-)-isoproterenol; PGE1, prostaglandin E1.

Activation curve. Kaapp was calculated from the Hofstee plot of the dose-response curve given in Fig. 1B clearly shows the reductions both in maximal stimulation and apparent affinity in the desensitized system. We previously showed that [3H]DHA binding sites were identical with the β-adrenergic receptors in C6 glioma cells (10). When measured under adenylyl cyclase conditions, the affinity of [3H]DHA for its binding sites, as well as the affinity of these sites for isoproterenol, were similar in both control and desensitized systems (Fig. 1, C and D). In the isoproterenol-treated system there was no desensitization at high isoproterenol concentrations in C6 glioma cells (10).

Uncoupling of β-Adrenergic Receptors during Desensitization

RESULTS

Characteristics of β-Adrenergic-sensitive Adenylyl Cyclase and β-Adrenergic Receptors during Desensitization—C6 glioma cells were exposed for 3 h to 10−3 M isoproterenol. Characteristics of the β-adrenergic adenylyl cyclase system were observed in particulate fractions. As reported by others, maximal adenylyl cyclase stimulation diminished after desensitization (by 60%, Fig. 1A). Furthermore, we observed an increase in the apparent activation constant (KAapp) of isoproterenol from 3.5 × 10−8 M to 1.3 × 10−7 M. The Hofstee plots of the dose-response curves given in Fig. 1B clearly show the reductions both in maximal stimulation and apparent affinity in the desensitized system. We previously showed that [3H]DHA binding sites were identical with the β-adrenergic receptors in C6 glioma cells (10). When measured under adenylyl cyclase conditions, the affinity of [3H]DHA for its binding sites, as well as the affinity of these sites for isoproterenol, were similar in both control and desensitized systems (Fig. 1, C and D). In the isoproterenol-treated system there was a decline in the total number of [3H]DHA binding sites (52%) (Fig. 1C). All the results in Fig. 1 were obtained from the same experiment. Similar results were obtained from several experiments and are given in Table I. It is clear that the difference between the KAapp for adenylyl cyclase stimulation in control and desensitized systems was highly significant, whereas the respective K0.5 of [3H]DHA and isoproterenol for β-adrenergic receptors did not change significantly during desensitization. Furthermore, the loss of adenylyl cyclase stimulation was slightly greater than that of β-adrenergic receptors (Table I). Note that isoproterenol was able to displace all specific [3H]DHA binding showing that the agonist interacted with all antagonist-labeled sites (Fig. 1D).

The Hill coefficients of the isoproterenol displacement were identical (n = 0.98 and 0.96 for control and desensitized systems, respectively (Fig. 2). For several systems, the Hill coefficients of isoproterenol displacement curves are less than one in the absence of GTP and equal to one in its presence (7). The difference between these results and ours is probably due to the fact that we measured binding under adenylyl cyclase conditions, especially in the presence of nonpure ATP and of an ATP-regenerating system. Furthermore, our membrane preparation was probably contaminated by GTP and GDP. In fact, under our experimental conditions, GTP does not increase stimulation in control and desensitized systems by highly significant, whereas the respective K0.5 of [3H]DHA and isoproterenol for β-adrenergic receptors did not change significantly during desensitization. Furthermore, the loss of adenylyl cyclase stimulation was slightly greater than that of β-adrenergic receptors (Table I). Note that isoproterenol was able to displace all specific [3H]DHA binding showing that the agonist interacted with all antagonist-labeled sites (Fig. 1D).

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In all experiments, basal, NaF, and GTP-sensitive adenylate cyclase were unchanged. In addition, in each experiment, we verified that alprenolol (10−8 M) did not significantly reduce basal adenylate cyclase activity. Fig. 3 shows that the shift in the KAapp of isoproterenol for stimulating adenylate cyclase increased with the intensity of desensitization. Thus, KAapp was 3.8 × 10−8 M in the control, whereas it was equal to 7.7 × 10−8 M and 1.1 × 10−7 M after 1/8 h and 3 h of desensitization, respectively.

An important point was to check that adenylate cyclase activities were in fact measured at equilibrium and that there was no desensitization at high isoproterenol concentrations in vitro. To verify these points, we conducted an experiment comparing the dose-activation curves obtained in control and desensitized systems by measuring the adenylate cyclase activities, either between 0 to 5 min or between 8 to 15 min, in order to determine any differences as a function of time. This method is more accurate that the one that consists of measuring cyclic AMP accumulation throughout the entire period.

MATERIALS AND METHODS

Adenylyl Cyclase Assay and (-)-[3H]Dihydroalprenolol Binding in C6 Glioma Cells

Culture of C6 glioma cells, preparation of particulate fractions and measurement of [3H]DHA binding and adenylyl cyclase activity were all performed as previously described (10). [3H]DHA binding and adenylyl cyclase activities were determined under strictly identical conditions. Briefly, particulate fractions of C6 glioma cells (20 to 40 μg of protein) were incubated at 30° C in a total volume of 50 μl containing 100 mM Tris-HCl, pH 8, 5 mM MgCl2, 1 mM cyclic AMP, 0.2 mM ATP, 0.2 mg/ml of creatine kinase, 20 mM phosphocreatine, and 1 mM EDTA. For adenylyl cyclase assays, tracer amounts of [3H]AMP (2 × 10−6 μCi) and [32P]ATP (1 to 2 μCi) were added after 8 min of incubation, and the reaction was stopped 5 min later. For binding measurements, [3H]DHA was added at the beginning of incubation and the reaction was terminated 10 min later by adding 1 ml of cold (4°C) washing medium containing 50 mM Tris-HCl, pH 8, and 20 mM MgCl2. Samples were filtered through GF/C Whatman filters. Filters were then washed and the bound radioactivity determined by scintillation counting. Specific binding was defined as the difference between the amount of [3H]DHA bound in the absence (total binding) and the presence (nonspecific binding) of 10 μM unlabeled alprenolol.

For desensitization experiments, isoproterenol (10−8 M, except otherwise mentioned) was added to the culture medium (HAM F 10 supplement with 10% fetal calf serum). Isoproterenol (10−8 M) was added every 90 min throughout incubation. Cells were then washed four times with 20 mM NaCl and particulate fractions prepared as previously described (10). We verified that 90 min after addition of isoproterenol (10−8 M), the concentration of this agonist was still sufficient for maximal stimulation of cAMP production. Thus, another addition of 10−8 M isoproterenol did not raise further the level of intracellular cAMP (5 pmol/mg of protein). The basal intracellular concentration of cAMP was 5 pmol/mg of protein. Measurements were made by radioimmunoassay.

Adenylyl Cyclase Assay in Neuroblastoma Cells

Neuroblastoma cells were grown on Falcon Petri dishes in MEM (Eagle’s minimal essential medium) with Earle’s salts, supplemented with 10% fetal calf serum. Cells were incubated for 4 days at 37°C in a humidified atmosphere (90% air, 10% CO2).

Membrane Preparation—After: removal of the culture medium, cells were suspended in 0.9% NaCl at 4°C, centrifuged at 300 × g for 5 min, and washed three times in the same medium. After the third centrifugation, cells were lysed by resuspension for 15 min in hypotonic buffer composed of 5 mM Tris-HCl (pH 8) and 1 mM MgCl2. Lysates were disrupted in a Dounce homogenizer (20 strokes). After partial purification according to the method of Brunton et al. (15), membranes were suspended in the hypotonic buffer and used immediately.

Adenylyl Cyclase Assay—Adenylyl cyclase was assayed at 30°C in a 50-μl incubation medium containing 25 mM Tris-HCl, pH 8, 0.1 mM ATP, 1 to 2 μg of [32P]ATP, 0.65 mM MgCl2, 0.1 mM papaverine, 1 mM cyclic GMP, 20 mM creatine phosphate, 50 μg of creatine kinase, 2 × 10−4 μCi of [3H]AMP, and 5 μg of bovine serum albumin. Membranes were incubated for 10 min and the reaction was stopped and cyclic AMP isolated as previously described (10).

Calculations

The isoproterenol dissociation constant for β-adrenergic receptors (K0.5) was determined experimentally: it was calculated from the isoproterenol concentration that inhibited [3H]DHA binding by 50%: IC50 = K0.5 (1 + [S]/KD) where [S] is the [3H]DHA concentration and KD the dissociation constant of [3H]DHA for its binding sites. KAapp is the agonist activation constant for the adenylyl cyclase system and is equal to the agonist concentration yielding 50% maximal activation. Kapp was calculated from the Hofstee plot of the dose-activation curve.

Chemicals

(-)-(3H)Dihydroalprenolol, cyclic (3H)AMP, and [32P]ATP were purchased from the New England Nuclear Corp. (-)-Isoproterenol was obtained from the Sigma Chemical Co. (-)-Alprenolol was kindly supplied by the Ciba-Geigy Laboratories and prostaglandin E1.

The abbreviations used are: [3H]DHA, (−)-(3H)dihydroalprenolol; Iso, (−)-isoproterenol; PG6, prostaglandin E1.
Uncoupling of β-Adrenergic Receptors during Desensitization

**FIG. 1.** Effects of β-adrenergic desensitization on isoproterenol-sensitive adenylate cyclase and β-adrenergic receptor characteristics. C6 glioma cells were exposed for 3 h to 10^{-3} M isoproterenol. A and B, maximal stimulation and apparent affinity of isoproterenol-sensitive adenylate cyclase from control (●) and isoproterenol-treated (○) cells. A, Dose-activation curves. Theoretical dose-activation curve of the desensitized system calculated from the equation

$$E^* = E_T \frac{[RH']}{K'} + [RH']$$

where \(E_T\) is the maximal theoretical adenylate cyclase activity of control membranes (see Fig. 8A), \([RH']\) varies from 0 to 0.48 × \([R_T]\) (\([R_T]\) being the concentration of receptors in membranes before desensitization, \(K_D\) of isoproterenol for binding sites = 1.7 × 10^{-7} M), and \(K'\) the apparent affinity of the receptor agonist complexes for adenylate cyclase in the desensitized system. \(K' = 3.7 K = 0.89 [R_T].\) B, Hofstee plots of the dose-activation curves. \(b\) = basal activity of adenylate cyclase. C, Scatchard plots of \([3H]DHA\) binding to particulate fractions from control (●) and isoproterenol-treated (○) cells. \([3H]DHA\) concentration was 1.9 × 10^{-5} M.

**TABLE I**

Comparison of binding and adenylate cyclase characteristics of control and desensitized β-adrenergic systems

| Experimental determinations | Control | Desensitized | \(p^*\) |
|-----------------------------|---------|--------------|---------|
| \(K_D [3H]DHA\) (nM)        | 3.2 ± 0.6 × 10^{-9} M | 3.7 ± 0.4 × 10^{-9} M | NS |
| (n = 7)                     | 100     | 54.6 ± 2.7   | (n = 10) |
| [3H]DHA binding sites (% of control) | 1.9 ± 0.5 × 10^{-7} M | 2.0 ± 0.5 × 10^{-7} M | NS |
| (n = 5)                     | 100     | 1.1 ± 0.2 × 10^{-7} M | (n = 5) |
| \(K_{app} Iso\) (nM)        | 3.9 ± 1.0 × 10^{-8} M | 1.1 ± 0.2 × 10^{-7} M | \(< 0.01\) |
| (n = 5)                     | 100     | 43.0 ± 3.4   | (n = 6) |

The results of this experiment showed that basal activity was strictly linear. Maximal activities were slightly higher when measured between 8 to 13 min than between 0 to 5 min (less than 10% difference from linearity during the entire period). However, the important point is that the \(K_{app}\) values in control and desensitized system were identical when measured either between 0 to 5 min or 8 to 13 min (data not shown).

**Influence of the Degree of Desensitization on the \(K_{app}\) of PGE<sub>1</sub> for Adenylate Cyclase System—**To ascertain whether the shift in the \(K_{app}\) which occurs during desensitization concomitantly with the decrease in the maximal adenylate cyclase stimulation by an agonist is found in other systems, we studied the desensitization of the prostaglandin-sensitive adenylate cyclase system in neuroblastoma cells. Fig. 4 shows that when neuroblastoma cells were incubated for 6 h with increasing concentrations of PGE<sub>1</sub>, the maximal stimulation of adenylate cyclase by PGE<sub>1</sub> diminished and the \(K_{app}\) gradually shifted to the right.
Uncoupling of $\beta$-Adrenergic Receptors during Desensitization

Kaapp sensitization was maximal after reduction of maximally stimulated $\beta$-adrenergic-sensitive adenylate and $\beta$-adrenergic receptor losses. Time course for the $\beta$-adrenergic receptors still continued after 6 h. Further evidence that the rate of adenylate cyclase desensitization was greater than the velocity of $\beta$-adrenergic receptor reduction is given in Fig. 5B which is the mean of three experiments.

Intensity of Desensitization at 4°C—$\text{C}_6$ glioma cells were incubated with isoproterenol ($10^{-5}$ M) for 3 h at 4°C. As shown in Fig. 6, the degree of desensitization of the adenylate cyclase system was less than that obtained at 37°C. There was only a 32% reduction of the maximal adenylate cyclase stimulation, compared to 57% at 37°C (Table I). Note that in this case too, there was an increase in the Kaapp for isoproterenol adenylate cyclase stimulation in the desensitized system (Fig. 6A). The intensity of adenylate cyclase desensitization did not rise when cells were incubated for 5 instead of 3 h at 4°C. At this temperature, there was no significant reduction in the number of $\beta$-adrenergic receptors (<10%) following treatment of cells with $10^{-8}$ M isoproterenol for 3 h (Fig. 6B) or 5 h (data not shown).

Effects of Cycloheximide on the Degree of Desensitization—When $\text{C}_6$ glioma cells were incubated with cycloheximide (50 $\mu$g/ml) for 1 h before desensitization, the latter was not reduced. There was a 69% drop in the $\beta$-adrenergic stimulation of the adenylate cyclase and 51% loss of $\beta$-adrenergic receptors (Fig. 7, A and B). This loss in adenylate cyclase stimulation was the same whether or not cycloheximide was present throughout washing (data not shown).

However, when $\text{C}_6$ glioma cells were incubated for 15 h with cycloheximide before desensitization started, there was no significant decrease in the number of $\beta$-adrenergic receptors (Fig. 7C), whereas desensitization of adenylate cyclase, although reduced, still persisted (38%, Fig. 7D). Mean results of four experiments were as follows: 1) loss of $\beta$-adrenergic

**Fig. 3.** Influence of the degree of desensitization on the Kaapp of isoproterenol for the $\beta$-adrenergic receptor. $\text{C}_6$ glioma cells were incubated without (A) or with $10^{-8}$ M isoproterenol for 3 h (B) and 3 h (C). Hofstee plots of the adenylate cyclase dose-activation curves. Lines are computer-derived from least square regression analysis ($r = 0.99$). $V_{max}$, $b$ were 1.173 (A), 0.712 (C), and 0.326 (B) nmol of cAMP/5 min/mg of protein. $b$ = basal activity of adenylate cyclase.

**Fig. 4.** Evolution of the Kaapp and maximal activity of PGE$_1$-stimulated adenylate cyclase for different degrees of desensitization. Neuroblastoma cells were exposed for 6 h without (A) or with different PGE$_1$, concentrations $5 \times 10^{-9}$ M (+), $10^{-7}$ M (○), and $3 \times 10^{-6}$ M (△). Kaapp were, respectively, 1.4 $\times 10^{-7}$ M (○), 3.8 $\times 10^{-7}$ M (+), and 4.5 $\times 10^{-7}$ M (△). Respective basal adenylate cyclase activities were 0.1 (○), 0.1 (+), 0.08 (△), and 0.08 (△), nmol of cAMP/10 min/mg of protein and were subtracted from stimulated activities.

**Time Course for $\beta$-Adrenergic-sensitive Adenylate Cyclase and for $\beta$-Adrenergic Receptor Losses—**Time course for the reduction of maximally stimulated $\beta$-adrenergic-sensitive adenylate cyclase and of $\beta$-adrenergic receptor density were not identical (Fig. 5A). In this experiment, adenylate cyclase desensitization was maximal after 1 h, whereas the loss of $\beta$-adrenergic receptors still continued after 6 h. Further evidence that the rate of adenylate cyclase desensitization was greater than the velocity of $\beta$-adrenergic receptor reduction is given in Fig. 5B which is the mean of three experiments. A, adenylate cyclase dose-activation curves in the control (○) and isoproterenol-treated cells (△). B, Scatchard plots of $[^3H]$DHA binding to particulate fractions in control (A) and isoproterenol-treated cells (B).
Uncoupling of β-Adrenergic Receptors during Desensitization

![Graphs and equations]

Fig. 7. Influence of cycloheximide on desensitization. Cycloheximide (50 μg/ml) was added 1 h (A, B) and 15 h (C, D) before 10−3 M isoproterenol. Desensitization lasted for 3 h. A and C, binding of [3H]-DHA to particulate fractions prepared from control (●) and isoproterenol-treated cells (○). D, cAMP produced in the presence of 10−3 M isoproterenol in particulate fractions prepared from control (●) and isoproterenol-treated cells (○). Basal adenylate cyclase activities were: B, 0.05 nmol of cAMP/5 min/mg of protein in both control and isoproterenol-treated cells. D, 0.09 nmol of cAMP/5 min/mg of protein in both control and isoproterenol-treated cells.

Receptors: 44.0 ± 3.6% for the control and 6 ± 3% after cycloheximide pretreatment and 2) loss of adenylate cyclase activity: 67 ± 2% in the control and 29 ± 8% after cycloheximide pretreatment. We checked that the cAMP production system was still working after this long term treatment with cycloheximide by measuring intracellular cAMP by radioimmunoassay. In control cells, cAMP rose from 4.9 to 868 pmol/mg of protein when 10−3 M isoproterenol was present for 10 min, and in cycloheximide-treated cells, cAMP rose from 3.2 to 539 pmol/mg of protein. Stimulations by NaF and Gpp(NH)p were not modified by pretreatment with cycloheximide.

In another experiment, we used 20 μg/ml of puromycin instead of cycloheximide for 15 h of pretreatment. We did not observe any change in the number of β-adrenergic receptors during desensitization, whereas in the corresponding control experiment, there was a 41% reduction. Although there was also a reduction in adenylate cyclase desensitization after puromycin treatment, the results were difficult to interpret, since there was also a 72% decrease in basal adenylate cyclase activity in puromycin-treated cells.

Coupling Function between Receptor Occupancy and Adenylate Cyclase Stimulation—We previously found that in C6 glioma cells, the coupling function between receptor occupancy (RH) and adenylate cyclase stimulation (E) was not linear (50% occupancy). Denyadenylate cyclase stimulation was obtained with less than 50% of receptor occupancy). In the present report, this function was analyzed in more detail. An Eadie plot of this function is shown in Fig. 8A. The receptor occupancy [RH] was normalized to maximal occupancy (RHmax). A straight line was obtained indicating that adenylate cyclase activation can be described as a Michaelis-Menten function of receptor occupancy. Thus,

\[ E^* = E_T \frac{[RH]}{K + [RH]} \]  

with \( E^* \) = adenylate cyclase minus basal activity, \( E_T \) is the maximal theoretical adenylate cyclase activity (\( RH \to \infty \)), whose value is shown on this line intercept with abscissa (1.22 nmol of cAMP/5 min/mg). Fig. 8A shows that this value is 120% of the maximal experimental adenylate cyclase activity. The slope of this line allows calculation of \( K \) (apparent affinity of \( RH \) for the enzyme) expressed relatively to \( [RH] \) concentration (\( R_T = \) maximal concentration of hormone receptor complex measured in binding experiments). In this case, \( K = 0.24 \times [R_T] \). Note that these reactions occur within the membrane, whose volume is unknown. Thus, it is impossible to ascertain the absolute values of \( [R_T] \) and \( K \).

The interaction of H with the receptor may be described as a Michaelis-Menten function of [H] since the Hill coefficient for agonist binding is very close to 1 (see Fig. 2).

\[ [RH] = \frac{[R_T]}{K_D + [H]} \]  

From Equations 1 and 2, it follows that

\[ E^* = \frac{E_T}{K} \frac{[R_T]}{[R_T] + 1} \frac{K_D + [H]}{K} \]

Hence, adenylate cyclase activation is a Michaelis-Menten function of the agonist concentration. Data presented here (Fig. 1B) and in the literature (9, 10, 12, 16) indicate that this is observed generally. This Michaelis-Menten function should be obtained whatever the value of \( [R_T] \).

The apparent activation constant of the agonist for adenylate cyclase stimulation is:

\[ K_{app} = \frac{K_D}{1 + [R_T]} \]

Fig. 8. Coupling function relating receptor occupancy and adenylate cyclase activation. A, Eadie plot of the function relating adenylate cyclase activation to receptor occupancy. Data are taken from Fig. 1A. • control, ○ desensitized; E = [RH]/[H]; E* = adenylate cyclase activity minus basal; [RH]/[H] = fractional receptor occupancy by isoproterenol calculated taking \( K_D = 1.7 \times 10^2 \) (Fig. 1D) and assuming that the binding follows a Michaelis-Menten function, as suggested by the Hill coefficient of the displacement curves (Fig. 2); \( K_D \) is apparent affinity of the receptor-agonist complex for the enzyme. The line is computer-derived from least square regression analysis (r = 0.99). O, desensitized system; \( E^* \) = adenylate cyclase activity minus basal; \( [RH]/[H] \) = fractional receptor occupancy by isoproterenol taking \( K_D = 1.7 \times 10^2 \) and \( [RH]/[H] \) varying from 0 to 0.48 \( [RH]/[H] \) = fractional receptor occupancy by isoproterenol taking \( K_D = 1.7 \times 10^2 \) and \( [RH]/[H] \) varying from 0 to 0.48 \( [RH]/[H] \) = fractional receptor occupancy by isoproterenol taking \( K_D = 1.7 \times 10^2 \) and \( [RH]/[H] \) varying from 0 to 0.48. B, theoretical coupling function relating receptor occupancy and adenylate cyclase activation. This function is given by:

\[ E^* = \frac{E_T ([RH] \times [R_T])}{K + [RH]} \]  

\([RH] \) is normalized to \( [R_T] \) and \( E^* \) to \( E_T \) with \( E_T = \) maximal theoretical adenylate cyclase activity and \( K = 0.24 \times [R_T] \).
and the maximal experimental stimulation is:

\[ V_{\text{max}} = \frac{E_T}{K + [RH]} \]

(Equation 1)

\[ E_T = \frac{[RH]}{K + [RH]} \]

Thus:

\[ \frac{[RH]}{K + [RH]} = \frac{[RH']}{K' + [RH']} \]

The observation that basal, NaF, and Gpp(NH)p-stimulated adenylate cyclase activities do not change suggests that \( E_T \) is the same in control and desensitized systems. Furthermore, the Eadie plot of the function relating adenylate cyclase stimulation to receptor occupancy for the desensitized system (Fig. 8A) shows that \( E_T \) is equal to 1.20 nmol of cAMP/5 min/mg which is identical with the \( E_T \) for the control (1.22 nmol of cAMP/5 min/mg).

The values of \( E_T \) in control and desensitized systems were computer-derived from least square regression analysis. The accuracy of \( E_T \) determination was however less in desensitized than in control systems, since \( E_{\text{max}} \) was far from \( E_T \). In two other experiments in which all the parameters for binding and adenylate cyclase were measured on the same enzyme, the \( E_T \) values in the desensitized system were 93 and 87% of the control.

For concentrations of agonist \([H]\) and \([H']\) producing the same adenylate cyclase activation in control and desensitized systems (\( E^* = E^{*+} \)), we obtain from Equation 1

\[ \frac{[RH]}{K + [RH]} = \frac{[RH']}{K' + [RH']} \]

Thus:

\[ \frac{[RH]}{K + [RH]} = \frac{[RH']}{K' + [RH']} \]

Since the isoproterenol constant \( K_D \) for \( \beta \)-adrenergic receptors did not change during desensitization,

\[ \frac{[RH]}{K + [RH]} = \frac{1}{Q} \]

If

\[ \frac{K^1}{K} \times \frac{[RH]}{[RH']} = \frac{1}{Q} \]

then

\[ \frac{1}{Q} = \frac{1}{[H']} \times \frac{1}{[H]} + \frac{1 - Q}{QK_D} \]

This formulation is similar to that proposed by Furchgott and Bursztyn (17) who compared the physiological response of acetylcholine before and after irreversible blockade of the receptor.

The plotting of 1/[\(H]\) against 1/[\(H]\) (double reciprocal plot for equiactive agonist concentrations in control and desensitized systems) should give a straight line with a slope equal to 1/Q and an intercept with the ordinate equal to (1 - Q)/QK_D.

In all desensitization experiments with C6 glioma cells, straight lines were in fact obtained (Fig. 9, A, B, C). Similar plotting of the dose-activation curves for prostaglandin-sen-

\[ f(i) = \frac{E_T}{K + [RH]} \]

sitive adenylate cyclase in neuroblastoma cell membranes also gave straight lines (Fig. 9D).

One advantage of this method is that it allows the agonist dissociation constant \( K_D \) for the receptor implicated in adenylate cyclase stimulation to be determined from adenylate cyclase experiments. As shown in Fig. 9, A, B, C, this \( K_D \) is very close to that determined by binding experiments. The mean \( K_D \) value determined from cyclase experiments is 1.4 ± 0.2 × 10^{-7} M (n = 6) compared to 1.9 ± 0.5 × 10^{-7} M (n = 5) from binding measurements. Within a single experiment, the above method of plotting adenylate cyclase activity resulted in very close \( K_D \) values whether membranes were prepared after cells had been incubated for different periods at the same agonist concentration (Fig. 9B) or for the same period at different agonist concentrations (Fig. 9D).

The experiment shown in Fig. 1, in which all binding and adenylate cyclase parameters were measured on the same membranes, indicated that, in membranes from desensitized cells the number of \( \beta \)-adrenergic receptors determined by binding was 48% of the control (Fig. 1C). If desensitization only involved a loss of \( \beta \)-adrenergic receptors without any change in the coupling, the maximal adenylate cyclase stimulation would, according to the coupling function shown in

\[ \frac{[RH]}{K + [RH]} = \frac{[RH']}{K' + [RH']} \]

**FIG. 9.** Double reciprocal plots of equiactive agonist concentrations in the control and desensitized adenylate cyclase systems. A, double reciprocal plots for the experiments reported in Fig. 1. [\(H]\] and [\(H]\) are equiactive isoproterenol concentrations in the control and desensitized adenylate cyclase systems, respectively. [\(H]\) concentrations are taken arbitrarily from a concentration range varying from 10^{-8} M to 3.1 × 10^{-7} M every two intervals, and the corresponding [\(H]\) concentrations are measured from the control dose-activation curve. A straight line was obtained (r = 0.99) permitting determination, according to Equation 6, of the dissociation constant of isoproterenol, \( K_D = 1.9 × 10^{-7} M \). B, double reciprocal plots for experiments reported in Fig. 3. [\(H]\) concentrations are taken arbitrarily from a concentration range varying from 10^{-6} M to 3.1 × 10^{-6} M every two intervals (1) and from 5 × 10^{-7} M to 6 × 10^{-7} M every 2% intervals (2). The corresponding [\(H]\) concentrations are measured on the experimental control curve. Lines were computer-derived from a least square regression analysis (r = 0.98). C, double reciprocal plots for experiments reported in Fig. 6A performed at 4°C. [\(H]\) concentrations are taken arbitrarily from a concentration range varying from 6.4 × 10^{-7} M to 2 × 10^{-6} M every two intervals. The corresponding [\(H]\) concentrations were measured on the experimental control curve. Lines were computer-derived from a least square regression analysis (r = 0.98). [\(H]\) concentrations are taken arbitrarily from a concentration range varying from 2.3 × 10^{-6} M to 1.3 × 10^{-6} M every 10% intervals (1) and from 2.3 × 10^{-5} M to 4.8 × 10^{-7} M every 1/4 intervals (3). The corresponding [\(H]\) concentrations are measured on the experimental control curve.
Fig. 2B, amount to 68% \( E_T \), i.e. 82% of the maximal stimulation of the control. This value is obviously far higher than the one observed experimentally after desensitization (41% of the control).

Thus, it is reasonable to suppose that some change in coupling occurs. Since, in the experiment presented in Figs. 1A and 4A, \( Q = K/K' \times [R_T^i]/[R_T] = 0.13 \) (for six experiments, the mean value of \( Q \) after 3 h desensitization with \( 10^{-5} \) M isoproterenol was 0.16 ± 0.02) and \([R_T^i]/[R_T] = 0.48\), it follows that \( K' = 3.7 \) K. On the basis of the \([R_T^i]\) and \( K' \) values a theoretical isoproterenol dose activation curve can be computed from Equation 1 for the desensitized system. This curve fits the experimental one reasonably well (Fig. 1A). The significance of \( K \) in terms of molecular events and its possible modification during desensitization will be discussed later.

**DISCUSSION**

A careful comparison between the respective losses in hormonal responsiveness and hormonal receptors during desensitization should give interesting indications on the relations between the two. Several authors have already compared the loss of maximal hormonal responses with that of binding sites (2-5). However, direct comparison between the percentage of receptors accessible to a ligand and the percentage of maximal hormonal stimulation after desensitization can lead to erroneous conclusions as regard receptor uncoupling during desensitization, unless the relationship between receptor occupancy and adenylate cyclase stimulation is linear, which is generally not the case (8-12). This is why we carefully analyzed this relationship in the system under study, i.e. C6 glioma cells.

We showed that a Michaelis-Menten type of function relates \( \beta \)-adrenergic receptor occupancy to adenylate cyclase stimulation (see Fig. 8). This was expected since both the binding and the adenylate cyclase stimulation are Michaelis-Menten functions of isoproterenol concentration. An apparent affinity constant (\( K \)) of the receptor ligand complex \( (RH) \) for the adenylate cyclase was calculated \( (K = 0.24 \times [R_T]) \). This is a relative value with respect to the total receptor concentration.

An interesting feature of this finding is that, in our system, when \([RH] = [R_T] \) the adenylate cyclase stimulation is only 81% of the theoretical maximal stimulation (see Fig. 8). This is because \([RH] \) is limited to \([R_T] \) and \( K \) is not negligible with respect to \([R_T] \) (see Equation 4). We replotted the relationship between receptor occupancy and adenylate cyclase stimulation from various data reported in the literature: the isoproterenol response of \( S_m \) lymphoma cells (4), frog and turkey erythrocytes (12), the glucagon response of liver membranes (16) and the [Arg.sup.8]-vasopressin response of beef kidney membranes (9). In all these systems, the relation is of Michaelis-Menten type and \( K \) and \( Y \) were equal to 0.95 × [\( R_T \)], 0.3 × [\( R_T \)], 3 × [\( R_T \)], 0.16 × [\( R_T \)], and 0.28 × [\( R_T \)], respectively. Note that maximal theoretical adenylate cyclase stimulation was never reached in any of these systems. Furthermore, if several specific receptors are coupled with the same adenylate cyclase molecule, the responses will be more or less additive and limited to \( E_T \), depending on the respective apparent affinities of the different hormone-receptor complexes for the enzyme and the concentration of these receptors within the membrane.

It is difficult to demonstrate that \( E_T \) represents the total enzyme pool activity in our system. The fact that \( E_T \), like basal, Gpp(NH)p, and NaF-stimulated activities, does not change significantly during desensitization is a good argument for this assumption. It has been suggested that the activity measured in the presence of Gpp(NH)p plus isoproterenol reflects total enzyme activity (18). However, in our system, Gpp(NH)p reduced the maximal stimulation produced by isoproterenol (10). We calculated that, in turkey erythrocytes (12) \( K = \frac{[R_T]}{[R_T]} \) (see above). Thus, according to Equation 4, \( E_T/E_{max} \) should be equal to 4. In fact, in this system, the activity measured in the presence of Gpp(NH)p plus isoproterenol was reported to be 5 times higher than in the presence of the natural effector GTP (18). Considering that these data are taken from two different laboratories, this concordance is rather good.

In pig kidney plasma membranes, on the other hand, the coupling function is not a Michaelis-Menten function, because the adenylate cyclase stimulation is not a Michaelis-Menten function of the concentration of the agonist (vasopressin). This is probably due to a partial irreversibility of the adenylate cyclase stimulation (8, 19).

A coupling model for the C6 glioma cell system would have to take into account the following observations: 1) the binding for the agonist is noncooperative (Fig. 2); 2) activation of E is a Michaelis-Menten function of \( RH \); and 3) \( E_T \) does not change when the number of receptors is reduced. The literature gives at least one model which fits these facts, i.e. the collision coupling proposed by Levitzki and colleagues (20) which is set out as follows

\[
R + H \rightleftharpoons RH \rightleftharpoons RHE \rightarrow RH + E^+ \]

where \( (RHE) \) is a very transient complex and \( K = \frac{k_{out}}{k_{in}} \), \( k_i \) = the bimolecular rate constant that describes the collision between the enzyme and the agonist-bound receptor. \( k_{out} \) = rate constant of enzyme deactivation.

However, we do not know if all the \( (RH) \) complexes are able to activate the adenylate cyclase. It has been proposed (21, 22) that, even with a full agonist, there are two interconvertible populations of complexes: \( (RH^*) = (RH^*) \), \( (RH) = (RH^+) \) \( + (RH^-) \) in which only one \( = (RH^*) \) is able to activate the adenylate cyclase. With an antagonist \( (RH^+) = (RH) \), whereas with a partial agonist, the ratio \( (RH^+)/[RH] \) is probably lower than with a full agonist (22).

Under these conditions, if \( [RH^*] = \alpha [RH] \) then \( [RH] = (RH^+) (1 + 1/\alpha) \); and, taking the collision coupling as a model, the activation of adenylate cyclase is given by:

\[
E^+ = E_T \frac{[RH]}{k_i \alpha + [RH]} + [RH]
\]

The constant \( K \) defined here is thus equal to:

\[
k_{out}
\]

The rate constant for enzyme activation is

\[
k_{in} = k_1 \frac{\alpha}{1 + \alpha} [RH]
\]

Having thus defined the relationship between adenylate cyclase activation and receptor occupancy in the membranes from control cells, we were able to compare the loss of adenylate cyclase response to the loss of \( \beta \)-adrenergic receptors during desensitization.

When C6 glioma cells were incubated with \( 10^{-5} \) M isoproterenol for 3 h, the adenylate cyclase system became desensitized and the following observations were made: 1) the number of \( \beta \)-adrenergic receptors accessible to [H]DHA decreased (45.4 ± 2.7%, \( n = 10 \)). The affinity of these receptors for both the antagonist [H]DHA and the agonist isoproterenol did not change (Table 1). 2) The maximal adenylate cyclase response
Uncoupling of β-Adrenergic Receptors during Desensitization

Although, like cycloheximide, puromycin eliminates the loss of binding sites, it is not impossible that this drug exerts some kind of action not mediated by protein synthesis inhibition. In any case, cycloheximide treatment was able to dissociate desensitization of the β-adrenergic adenylyl cyclase from the loss of β-adrenergic receptors. Such a dissociation was also shown when desensitization was conducted at 4°C (Fig. 6). It is tempting to propose that the loss of binding sites is the result of internalization as suggested recently by Chuang et al. (27) for the β-adrenergic receptor of frog erythrocytes. Note that internalization of epidural growth factor is suppressed at 4°C (28, 29).

In another C6 glioma cell line (C62B), Terasaki et al. reported different characteristics for desensitization than those described here (30). The main difference is that these authors did not see any change in the number of receptors after desensitization. This is probably due to a difference in the cell lines used in these two studies. Furthermore, a decrease in the number of β-adrenergic receptors after desensitization was found recently in C6 glioma cells by Mallorga et al. (31) with cells which originated from the same source as those used in our experiments (Benda, 32, 33).

The third type of finding in support of two-step desensitization was obtained from the analysis of the different parameters for β-adrenergic-sensitive adenylyl cyclase system during desensitization, as described under “Results.” The double reciprocal plots of agonist concentrations in control and desensitized systems always produced straight lines both in the β-adrenergic- and prostaglandin-sensitive adenylyl cyclase systems (Fig. 9). The result of this analysis is interesting for three reasons: 1) it allows determination of the affinity of the agonist for the receptor (Ko) involved in adenylyl cyclase stimulation without binding experiments. Note that, in C6 glioma cells, the Ko determined by this method (1.4 ± 0.2 x 10⁻⁵ m, n = 6) is very close to that determined by binding experiments (1.9 ± 0.5 x 10⁻⁵ m, n = 5). This confirms that [³H]DHA really labels the β-adrenergic receptors coupled to adenylyl cyclase. Furthermore, the low affinity of PGE₂; for its receptors calculated by this method (Ko = 2.6 ± 0.6 x 10⁻⁵ m, n = 3) explains why it is impossible to measure PGE₂ binding under adenylyl cyclase assay conditions. 2) Double reciprocal plot analysis indicates that the Michaelis-Menten nature of the function relating binding occupancy to adenylyl cyclase stimulation does not change during desensitization; otherwise no straight lines would be obtained for these plots.

3) Such analysis enables calculation of the value Q which is equal to K/(K¹ x [Rf⁻¹]/Kf). When C6 glioma cells are desensitized for 3 h with 10⁻² m isoproterenol, the value of Q is 0.13 (Fig. 9A), which is different from the ratio of total receptor concentration in the desensitized system over the receptor concentration in the control system, as determined in binding experiments (0.48, Fig. 1). A change in the coupling function therefore occurs during desensitization. A 3.7-fold increase in the constant K is occurring (K² = 3.7 K). Since K = k₁/k₂(α + a), the increase observed during desensitization might result from changes in three parameters.

1) k₉ might be modified. Although this is unlikely, such a change would explain heterologous desensitization as described in astrocytoma cells (5).

2) k₉ might diminish either because of a reduction in k₉, or in a (ratio between active and inactive agonist receptor complexes).

Note that these changes in either k₉ or a have completely different meanings in terms of molecular events. When k₉ changes, all the efficient receptor-agonist complexes (RH) are capable of activating the adenylyl cyclase but with a lower apparent affinity. In the case of a change in α, the ratio...
between the active and inactive form of the receptor agonist complexes is reduced during desensitization. It is of interest to note that the Gpp(NH)p-induced decrease in agonist affinity for β-receptors seems to be reduced by desensitization (24, 35). A report by Kent et al. (22) shows that when agonist binding was measured in Tris buffer on control membranes of turkey and frog erythrocytes, β-adrenergic receptors were found in both low and high affinity states. The ability of an agonist to activate adenylate cyclase correlated with the amount of high affinity state receptors formed in the presence of the agonist. During desensitization there was impairment of high affinity state formation (22).

Although further experiments are needed to discriminate between the proposed hypotheses explaining the decrease of $K$ during desensitization, our results clearly show a modification in the coupling between β-adrenergic receptors and adenylate cyclase during desensitization and also that experimental conditions can be found for dissociating this change in coupling from the loss of β-adrenergic receptors.

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