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Evidence for internalization of the recognition site of β-adrenergic receptors during receptor subsensitivity induced by (−)-isoproterenol

(receptor desensitization/receptor internalization/frog erythrocytes/adenylate cyclase)

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ABSTRACT **In the supernatant (30,000 × *g*) of frog erythrocyte homogenates, by using gel filtration we detected a protein that could bind [^{3}H]dihydroalprenolol ([^{3}H]DHA) with high affinity. This binding was greatly enhanced when the erythrocytes were preincubated with (−)-isoproterenol. After various periods of incubation with (−)-isoproterenol, the extent of the increase in the density of [^{3}H]DHA binding sites in the cytosol was paralleled by a proportional decrease in the number of [^{3}H]DHA binding sites in the corresponding pellet; both events peaked after 2–3 hr of incubation with (−)-isoproterenol. The K_a of the (−)-isoproterenol-induced increase in [^{3}H]DHA binding in cytosol and the decrease in this binding in the membrane ranged between 60 and 90 nM. The changes in the cytosol and particulate [^{3}H]DHA binding sites were independent of RNA and protein synthesis. The increase in cytosol binding elicited by (−)-isoproterenol was blocked by exposure of the cells to (−)-alprenolol which *per se* failed to change the cytosol binding of [^{3}H]DHA. Scatchard analysis revealed that the enhanced [^{3}H]DHA binding to cytosol material was due to a 4-fold increase in the B_{max} with little or no change in K_d (≈9 nM). Binding displacement data show that these soluble [^{3}H]DHA binding sites resemble the surface membrane recognition sites. Moreover, the ability of various β-adrenergic agents to increase [^{3}H]DHA binding to cytosol after they were incubated with frog erythrocytes paralleled their affinity for membrane-bound β receptors. These findings support the view that the β-adrenergic receptor desensitization caused by prolonged exposure to (−)-isoproterenol is due, at least in part, to an internalization of the recognition site of β-adrenergic receptors.**

Prolonged exposure of cell surface receptors to their agonists results in a decrease in the intensity of the receptor response; this phenomenon has been termed "receptor desensitization" or "subsensitivity" (1–10). Lefkowitz and coworkers have used a cell-free system (11) or intact frog erythrocytes (12) to study the desensitization of β-adrenergic receptors caused by prolonged exposure to (−)-isoproterenol. Their results suggest that, in both systems, desensitization is mainly due to a decrease in the number of β-adrenergic receptors (7, 11, 12). Whereas in the cell-free system the decrease in the number of receptors appears to be the result of a persistent tight binding of the agonist to the receptor (13), relatively little is known about the mechanism of receptor desensitization in intact erythrocytes. In these cells, maximal desensitization requires longer incubation with (−)-isoproterenol than is required by membranes (12). Moreover, in erythrocytes a high-affinity binding of the agonist to the β receptor does not appear to be operative in causing desensitization (13, 14). These experiments suggest that, in intact erythrocytes, the mechanisms that mediate the desensitization of β-adrenergic receptors are different from those of cell-free preparations but they fail to specify the molecular nature of such mechanisms.

Certain polypeptide hormones are internalized into the cytoplasm after their binding to surface receptors (15–21); we have examined whether a similar mechanism is operative also in frog erythrocytes during desensitization of β receptors induced by prolonged exposure to (−)-isoproterenol. We present evidence that a recognition site for β-adrenergic receptor agonists is present in the soluble fraction of erythrocyte lysate and that the concentration of such recognition sites is greatly increased during receptor desensitization elicited by incubation with β-adrenergic agonists. Moreover, there is a strong correlation between the increase in the number of soluble β-adrenergic receptors and the decrease in the number of membrane-bound β receptor. This finding can be interpreted by proposing that an internalization of the recognition site of β-adrenergic receptors accounts for at least part of the decrease in the number of surface β receptors elicited by prolonged incubation of frog erythrocytes with (−)-isoproterenol.

MATERIALS AND METHODS

Materials. (−)-[propyl-^{3}H]Dihydroalprenolol hydrochloride [(−)-[^{3}H]DHA; 51.1 Ci/mmol; 1 Ci = 3.7 × 10^{10} berquerels] was obtained from New England Nuclear. (−)-Isoproterenol bitartrate, (+)-isoproterenol bitartrate, (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, and (−)-alprenolol tartrate were from Sigma Chemical Company. (±)-Hydroxybenzylisoproterenol was a generous gift of New England Nuclear. Sephadex G-50 (superfine) was purchased from Pharmacia. Pronase (nonspecific) was from Boehringer Mannheim.

Preparation of Frog Erythrocytes. Blood was obtained by decapitation of bullfrogs (*Rana catesbeiana*) about 15.0–20.0 cm long, which were purchased from Mogul-Ed (Oshkosh, WI). Erythrocytes were collected and washed by centrifugation at 700 × *g* twice in 20 mM Tris·HCl, pH 7.4/100 mM NaCl/5 mM KCl/0.8 mM $MgCl_2$/0.01% heparin. These erythrocytes then were washed twice in medium A (20 mM potassium phosphate, pH 7.4/85 mM NaCl/1 mM $CaCl_2$/2 mM KCl/8 mM theophylline) and were resuspended in medium A at a hematocrit value of 20–25%. The erythrocytes were freshly prepared before each experiment.

Preparation of Soluble and Particulate Fractions from Erythrocytes Incubated with (−)-Isoproterenol. Erythrocytes at 20% hematocrit in 2 ml of medium A were incubated at 30°C in the presence of (−)-isoproterenol in a water bath with constant shaking. At the times indicated, cells were washed in 5 ml of ice-cold medium A by centrifugation at 700 × *g* for 2 min. The supernatant was carefully aspirated and the cells were

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Abbreviation: [^{3}H]DHA, [^{3}H]dihydroalprenolol.

washed an additional eight times. In control experiments in which (−)-isoproterenol was omitted, erythrocytes were incubated and washed in parallel. The final cell pellet was lysed by adding 2 ml of 25 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$ followed by gentle and brief vortexing; 200 μl of 25 mM Tris·HCl, pH 7.4/100 mM $MgCl_2$ was added and the resulting lysate was centrifuged at 30,000 × *g* for 20 min. The supernatant was further clarified by centrifugation at 30,000 × *g* and then assayed for β-adrenergic receptor binding activity with (−)-[^{3}H]DHA as the ligand. This soluble receptor is stable for up to 2 weeks when kept frozen at −20°C. The 30,000 × *g* pellet of erythrocyte lysate was washed by homogenization in 5 ml of medium B (25 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$) followed by centrifugation at 30,000 × *g*. The pellet was resuspended by grinding in 1 ml of medium B and was used immediately for (−)-[^{3}H]DHA binding studies.

Binding of (−)-[^{3}H]DHA to the Soluble Fraction of Erythrocyte Lysate. The binding mixture contained, in a total volume of 450 μl of medium B, 20 nM (−)-[^{3}H]DHA and 15–20 mg of 30,000 × *g* supernatant protein prepared as described above. After incubation at 30°C for 10 min, the binding mixture was cooled in ice water and loaded onto a freshly prepared Sephadex G-50 (superfine) column (0.6 × 13 cm) equilibrated with 25 mM Tris·HCl (pH 7.4). The column was then washed with 0.3 ml of this Tris buffer and the eluate was discarded. An additional 1.2 ml of the same buffer was added as soon as the column was drained and the eluate, which contained most of the void volume of the column, was collected into a test tube. Trichloroacetic acid (100%), 60 μl, was added to each eluate which then was heated at 95°C for 5 min to precipitate hemoglobin (hemoglobin was found to quench ^{3}H radioactivity). After centrifugation at 3000 × *g* for 10 min, 1 ml of the supernatant was removed and assayed for radioactivity in 10 ml of Aquasol (New England Nuclear). The binding in the presence of 5 μM unlabeled (−)-DHA was taken as the blank and subtracted from the values measured in its absence. The specific activity of [^{3}H]DHA bound was about 25 cpm/fmol.

Binding of (−)-[^{3}H]DHA to the Particulate Fraction of Erythrocyte Lysate. The binding condition was essentially the same as described (11). The reaction mixture, in a volume of 150 μl of medium B, contained 20 nM (−)-[^{3}H]DHA and 0.5–1.0 mg of 30,000 × *g* particulate protein prepared as described in the previous section. After incubation at 30°C for 10 min, the reaction was terminated by adding 4 ml of ice-cold medium B and the mixtures were filtered immediately under reduced pressure through a Whatman GF/B filter. The filter was washed with two 4-ml portions of ice-cold medium B and then placed in 10 ml Aquasol for assay of ^{3}H radioactivity. Nonspecific binding was determined in the presence of 5μM unlabeled (−)-DHA and subtracted from the total binding obtained in its absence. The specific activity of (−)-[^{3}H]DHA bound was 30–35 cpm/fmol.

Protein Determination. Proteins in the soluble and particulate fractions were measured by the method of Lowry *et al.* (22) with bovine serum albumin as the standard.

RESULTS

Isolated frog erythrocytes were incubated without or with 5 μM (−)-isoproterenol for various periods of time; this incubation was followed by extensive washing of the cells to remove the agonist. The final cell pellet was lysed and the 30,000 × *g* supernatant (termed "cytosol") of the lysate was assayed for [^{3}H]DHA binding by filtration with a Sephadex G-50 column. The extent of the [^{3}H]DHA binding to cytosol was enhanced by more than 4-fold when the cells were pretreated with (−)-isoproterenol (Fig. 1*A*). This increase was evident after 15 min

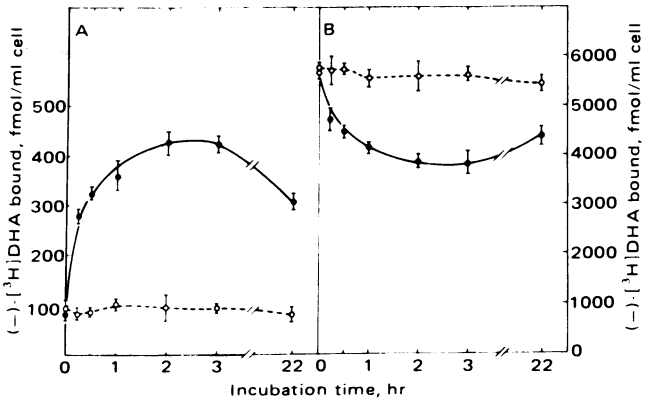

FIG. 1. Time course of isoproterenol effect on [^{3}H]DHA binding in the supernatant (*Left*) and particulate (*Right*) fractions of frog erythrocytes. Erythrocytes were incubated without (○) or with (●) 5 μM (−)-isoproterenol for the times indicated and the binding activities for (−)-[^{3}H]DHA in the 30,000 × *g* supernatant and pellet were determined. When the erythrocytes were incubated for long periods (22 hr), penicillin (100 units/ml) and streptomycin (100 μg/ml) were included in the incubation medium to retard the growth of bacteria. Values are mean ± SEM for four experiments.

of incubation; it approached the maximum after 1–2 hr of incubation and remained at a plateau for at least an additional hour. After 22 hr of exposure to (−)-isoproterenol the binding activity appeared to be lower than the maximum but still was higher than in control cells. A detectable amount of [^{3}H]DHA binding to erythrocyte cytosol was present in the control, cells incubated in medium A without isoproterenol, but this activity failed to increase with incubation time (Fig. 1*A*). Thus, despite the incubation in medium A, when the number of surface receptors remained unchanged, the numbers of cytosol binding sites failed to change.

In confirmation of previous reports (12), the 30,000 × *g* pellet of lysate derived from erythrocytes incubated with (−)-isoproterenol contained a smaller number of [^{3}H]DHA binding sites than did the corresponding fraction derived from untreated cells (Fig. 1*B*). The time course of the decline in [^{3}H]DHA binding paralleled that of the increase in the number of binding sites in the cytosol fraction. At 2 hr the increased binding of [^{3}H]DHA to the cytosol (about 340 fmol/ml of packed cells) could account for 20% of the β-adrenergic receptor lost from the plasma membrane (about 1700 fmol/ml of packed cells). Under these experimental conditions the number of membrane-bound β receptors was decreased by 31% and the isoproterenol-stimulated adenylate cyclase activity in the membrane preparation was decreased by 48% (data not shown). The dose-response curve of (−)-isoproterenol-induction of a decrease in membrane [^{3}H]DHA binding and an increase in the [^{3}H]DHA binding to the cytosol is shown in Fig. 2. The potency of (−)-isoproterenol to increase the [^{3}H]DHA binding to the cytosol fraction and to decrease it to the particulate fraction was about the same. When erythrocytes were incubated with actinomycin D (30 μg/ml) or cycloheximide (100 μg/ml) prior to the addition of isoproterenol, the number of cytosol and particulate [^{3}H]DHA binding sites remained unchanged in the control and treated cells. Under these conditions, the incorporations of [^{3}H]uridine and [^{3}H]leucine into erythrocyte RNA and protein, respectively, were blocked by more than 90% (data not shown).

Scatchard analysis of the [^{3}H]DHA binding revealed a single class of binding sites in the cytosol of cells incubated without or with isoproterenol for 2 hr. The maximal number of binding sites present in the cytosol of cells incubated with (−)-isoproterenol (2.3 fmol/mg of protein) was 4 times greater than in

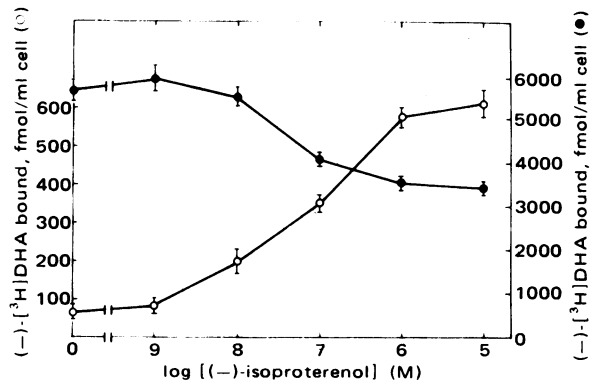

FIG. 2. Dose-response for (−)-isoproterenol incubated with frog erythrocytes. Erythrocytes were incubated with various concentrations of (−)-isoproterenol for 2 hr at 30°C, and the binding of [^{3}H]DHA was determined in the cytosol (○) and particulate (●) fractions. Values are mean ± SEM for three determinations.

cytosol of cells incubated with medium A (Fig. 3). However, little or no change in the K_d was found in these two preparations (9.0 vs 8.5 nM; Fig. 3). Various β-receptor-acting drugs were tested for their potency to compete with the binding of [^{3}H]DHA to cytosol obtained from erythrocytes incubated with (−)-isoproterenol. We found the order of effectiveness to be (−)-alprenolol > (±)-hydroxybenzylisoproterenol > (−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine > (+)-isoproterenol (Fig. 4). Such a rank order is characteristic for β_2-adrenergic receptors which are present in frog erythrocyte

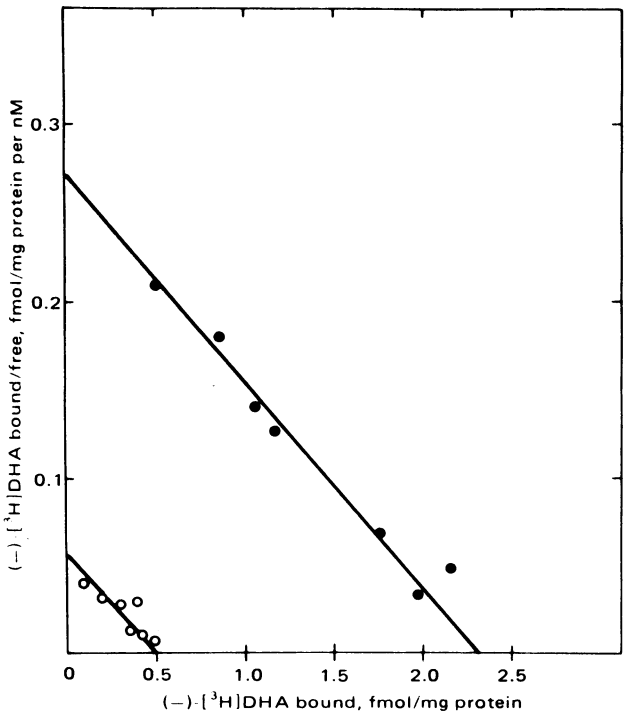

FIG. 3. Scatchard plot of [^{3}H]DHA binding in the cytosol derived from frog erythrocytes incubated with or without isoproterenol. The 30,000 × g supernatant was derived from erythrocytes incubated without (○) or with (●) (−)-isoproterenol (5 μM) for 2 hr at 30°C and the binding of [^{3}H]DHA was measured as described in *Materials and Methods*, except that the ligand concentrations were varied (4–100 nM). Specific binding at each ligand concentration was determined by subtracting the nonspecific binding measured in the presence of 5 μM unlabeled (−)-alprenolol. K_d: 9.0 nM without isoproterenol; 8.5 nM with isoproterenol.

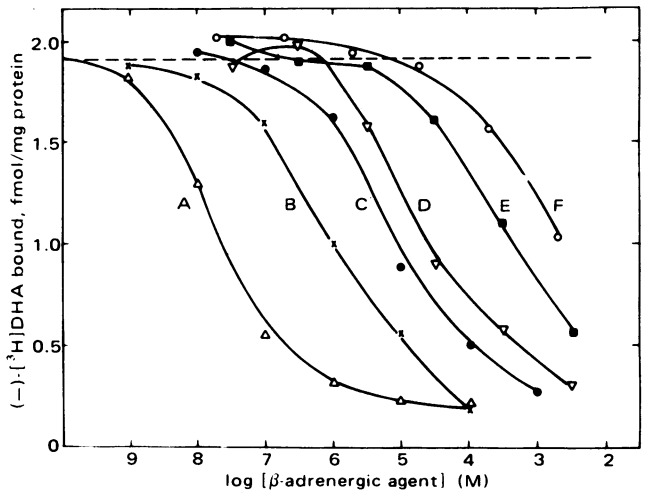

FIG. 4. Effects of various β-adrenergic-acting drugs on the binding of [^{3}H]DHA in the cytosol derived from frog erythrocytes incubated with isoproterenol. Erythrocytes were incubated with (−)-isoproterenol (5 μM) for 2 hr at 30°C. Cytosol was prepared and binding was performed as described in *Materials and Methods* except that the indicated concentrations of various β-adrenergic agents were present in the binding mixtures: curve A, (−)-alprenolol; B, (±)-hydroxybenzylisoproterenol; C, (−)-isoproterenol; D, (−)-epinephrine; E, (−)-norepinephrine; F, (+)-isoproterenol. Broken line indicates the binding value obtained in the absence of any added agent. Results in this figure are typical for three such experiments.

membrane (7, 11, 12). From the data reported in Fig. 4 the following values of concentration of agents causing 50% displacement of specific binding were calculated: (−)-alprenolol, 3×10^{-8} M; (±)-hydroxybenzylisoproterenol, 7×10^{-7} M; (−)-isoproterenol, 8×10^{-6} M; (−)-epinephrine, 3×10^{-5} M; (−)-norepinephrine, 5×10^{-4} M; (+)-isoproterenol, 3×10^{-3} M. In addition, the data reported in Fig. 4 show that the specific binding of [^{3}H]DHA to the cytosolic β-adrenergic receptor represented about 90% of the total radioactive ligand bound to cytosol. The specific [^{3}H]DHA binding of cytosol was almost obliterated by proteolysis with Pronase (10 μg/ml) for 10 min at 30°C. The protein that bound with high affinity [^{3}H]DHA was not pelleted by centrifuging at 100,000 × g for 1 hr.

The increase in protein with high-affinity binding for [^{3}H]DHA elicited by (−)-isoproterenol was abolished when the erythrocytes were incubated with (−)-isoproterenol in the presence of (−)-alprenolol (10 μM) (Fig. 5). Moreover, when the erythrocytes were incubated with (−)-alprenolol alone, the number of [^{3}H]DHA binding sites in the cytosol failed to change. When the capability of various β-receptor agonists to increase [^{3}H]DHA binding of cytosol was studied, there was good correlation between the extent of increase in [^{3}H]DHA binding in cytosol and the decrease in the membrane binding of this ligand elicited by the various agonists (Table 1). Among the agonists tested, (−)-isoproterenol was the most effective in causing both events. Second in potency was (−)-epinephrine followed by (−)-norepinephrine; (+)-isoproterenol was practically inactive.

DISCUSSION

Our results show that the cytosol of frog erythrocytes contains a material that binds [^{3}H]DHA with an affinity similar to that of β-adrenergic receptors bound to erythrocyte membranes. This material is destroyed by Pronase and remains in the supernatant after 1 hr of centrifugation at 100,000 × g. This binding activity is probably due to a protein. Because it is greatly enhanced when frog erythrocytes are incubated for 15 min or longer with 5 μM (−)-isoproterenol, this increase

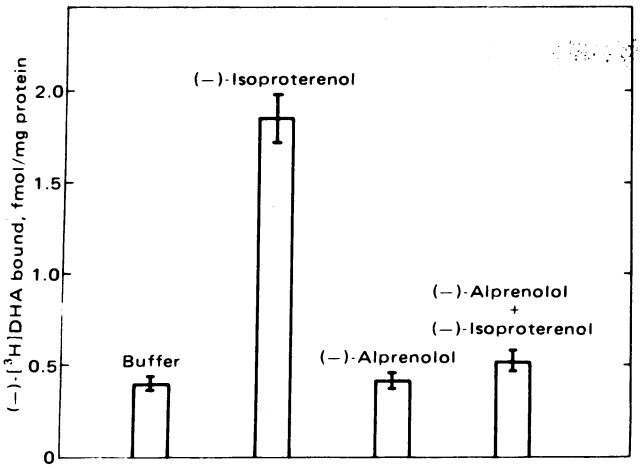

FIG. 5. Effect of (−)-alprenolol on the [3H]DHA binding activity in cytosol derived from frog erythrocytes incubated without or with isoproterenol. Erythrocytes (20%) were incubated in 2 ml of medium A at 30°C for 10 min in the absence or presence of (−)-alprenolol (10 μM). Medium A or (−)-isoproterenol, in 50 μl, was added to give a final concentration of 5 μM, and the mixtures were further incubated for 2 hr. Binding of [3H]DHA in the cytosol was then measured. Values are mean ± SEM for three determinations.

probably relates to the desensitization of β-adrenergic receptors. In fact, when erythrocytes are incubated with (−)-isoproterenol, the number of recognition sites of receptors located in the plasma membrane decreases (7, 11, 12). Binding competition studies with various drugs acting on β receptors shows that the activity profile of the cytosolic binding is of β_2 type and it appears to be identical to that of the β receptors bound to the membrane of frog erythrocytes.

By changing the incubation time or the concentrations of (−)-isoproterenol and by incubating frog erythrocytes with other β-receptor agonists, it can be shown that the extent of the increase in [3H]DHA binding to cytosol protein and of the decrease in the binding to particulate β receptors are inversely correlated. That is, when the membrane receptor shows desensitization, the amount of binding protein found in the cytosol increases. These changes in the cytosol and membrane receptors

Table 1. Effects of incubation of erythrocytes with various β-adrenergic agonists on [3H]DHA binding activity in the cytosol of erythrocyte lysate

Agent added	[3H]DHA bound, fmol/ml cell: Soluble fraction	Particulate fraction
None	54	4094
(−)-Isoproterenol		
0.1 μM	342	2762
1.0 μM	392	2640
(−)-Epinephrine		
0.1 μM	99	3871
1.0 μM	234	3207
(−)-Norepinephrine		
0.1 μM	72	4009
1.0 μM	154	3604
(+)-Isoproterenol		
0.1 μM	57	4105
1.0 μM	80	3904

Erythrocytes were incubated for 2 hr at 30°C in the presence of indicated concentration of each catecholamine. Soluble and particulate fractions were prepared and the binding of [3H]DHA was determined as described in *Materials and Methods*. These values were the mean of two independent experimental results.

can take place in the absence of RNA and protein synthesis. We interpret these results to suggest that, during desensitization, the recognition site of β-adrenergic receptors translocates from plasma membrane to the cytoplasm. This receptor internalization is therefore a mechanism that is operative in the decrease of the receptor density in the membrane elicited by prolonged exposure of cells to agonists. Several lines of evidence contradict the possibility that the increment in the number of receptor recognition sites in cytosol results from a release of some loosely bound receptors from the membrane during the rupture of cells *in vitro*. These cells were gently lyzed in a hypotonic buffer without homogenization. Magnesium (1 mM) was included to stabilize the structure of plasma membranes. Moreover, a strong homogenization of the 30,000 × *g* pellet prepared from cells treated with isoproterenol failed to increase the number of recognition sites present in cytosol. Finally, we confirmed a previous report (23) that the β receptor bound to frog erythrocyte membranes can be solubilized effectively only by treatment with 1% digitonin.

The number of recognition sites of β-adrenergic receptors present in the cytosol of erythrocytes incubated with (−)-isoproterenol can account for approximately 20% of the specific membrane-bound protein lost during desensitization. This percentage may be lower than the actual value for several reasons: (*i*) the soluble protein that functions as the recognition site of β receptors was determined by gel filtration column chromatography whereas the membrane receptor was estimated by glass fiber filtration under vacuum. The gel filtration assay is relatively lengthy and therefore the ligand–receptor complex present in cytosol has a greater chance to dissociate than does the complex present in membranes, which are rapidly filtered. (*ii*) A portion of the soluble receptor may have been degraded or otherwise inactivated after translocation to the cytoplasm. This view is strengthened by the findings that receptors for epidermal growth factor and human chorionic gonadotropin are degraded by the lysosomal system after their internalization (19, 20). (*iii*) β-Adrenergic receptors may be internalized as a complex with the (−)-isoproterenol or other agonists, which have an affinity greater than that of [3H]DHA, and therefore they may not be readily dissociated by [3H]DHA used as the ligand to detect the protein.

Indeed, our preliminary results show that, in frog erythrocytes incubated with (±)-[3H]hydroxybenzylisoproterenol, significant amounts of 3H were present in the cytosol fraction. Moreover, a fraction of the cytosolic 3H could be recovered in the void volume of a Sephadex G-50 column, suggesting that this ligand was complexed to a receptor fraction. Little or no radioactivity was found in the cytosol when cells were incubated with (−)-[3H]DHA. Nevertheless, our results do not rule out the participation of other mechanisms such as the release of β receptor into the incubation medium during desensitization.

Because internalization and desensitization are agonist specific, it seems logical to assume that production of cyclic AMP and activation of cyclic AMP-dependent protein kinase are involved in both events. We have found (unpublished data) that, when desensitization is evident, phosphorylation of two membrane proteins with molecular weights of 60,000 and 38,000 is selectively enhanced. Thus, one might speculate that membrane bound β receptor is internalized through the phosphorylation of these membrane proteins. In this regard, it is of interest to note that the activator protein for cyclic AMP phosphodiesterase and adenylate cyclase can be released from the synaptic membrane by phosphorylation of membrane proteins through a cyclic AMP-dependent protein kinase (24, 25). Our results show that the internalization is triggered when the recognition site of the receptor is occupied by the agonist;

if the antagonist is present alone or in combination with (−)-isoproterenol, internalization fails to occur. This finding supports the possibility that the activation of cyclic AMP-dependent protein kinase participates in controlling the number of receptors present in the membrane and ultimately that the turnover rate of membrane-bound receptor recognition sites changes when the extent of receptor occupancy increases for protracted time periods. In any event, our results do not exclude that receptor internalization involves aggregation of β receptors induced by isoproterenol in frog erythrocytes (26).

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