THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 22, Issue of November 25, pp. 13900-13908, 1983
Printed in U.S.A.

Characterization of an Altered Membrane Form of the β-Adrenergic Receptor Produced during Agonist-induced Desensitization*

(Received for publication, May 10, 1983)

Gary L. Waldo‡, John K. Northup§¶, John P. Perkins‡, and T. Kendall Harden‡‖

From the ‡Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514 and the §Department of Pharmacology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Incubation of 1321N1 human astrocytoma cells with 1 μM isoproterenol rapidly results in the conversion of a portion of the β-adrenergic receptors to a membrane form that can be separated from markers for the plasma membrane by sucrose density gradient or differential centrifugation. This "light peak" form of the receptor reaches a maximal level within 10 min of incubation of cells with catecholamine. Two types of experiments suggest that the early phase of catecholamine-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase can be separated into at least two reactions. First, the agonist-induced loss of catecholamine-stimulated adenylate cyclase activity precedes the appearance of β-adrenergic receptors in the light peak fraction by 1–2 min. Second, pretreatment of cells with concanavalin A prior to induction of desensitization blocks the formation of the light peak form of β-adrenergic receptors without blocking the "uncoupling" reaction as measured by catecholamine-stimulated adenylate cyclase activity. Specificity for the reaction that converts β-adrenergic receptors to the light peak form is indicated by the lack of a catecholamine-induced alteration in the sucrose density gradient distribution of muscarinic cholinergic receptors, adenylate cyclase or the guanine nucleotide-binding proteins, N_s and N_i. The light peak of β-adrenergic receptors migrates at a density similar to that of at least a portion of the activity of galactosyltransferase, a marker for Golgi. Enzyme marker activities for lysosomes and endoplasmic reticulum are not associated with this population of β-adrenergic receptors. Taken together, these and other data suggest that incubation of 1321N1 cells with isoproterenol results in a rapid uncoupling of β-adrenergic receptors from adenylate cyclase which is followed by a change in the membrane form of the receptor. This latter step most likely represents internalization of receptors into a vesicular form which may then serve as the precursor state from which receptors are eventually lost from the cell.

Agonist-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase of human astrocytoma (1321N1) cells has been shown to involve at least two reactions (1–3). First, cells exposed to catecholamines experience a rapid ($t_{1/2}$ ~ 2 min) functional uncoupling of receptors from adenylate cyclase which is expressed as a 50% decrease in the responsiveness of the enzyme to agonists and a loss of capacity of agonists to form a high affinity binding complex (1, 3). These alterations are rapidly ($t_{1/2}$ ~ 7 min) and completely reversible upon removal of catecholamine from the medium (3). A second reaction that results in the loss of radiolabeled β-adrenergic receptor antagonist binding occurs after a lag of 30–60 min (2–4); the formation and rate of recovery of lost receptors is much slower than the changes occurring during short-term exposure of cells to agonist (3, 4). Similar phenomena have been shown to occur in a variety of other cell types during exposure to catecholamines (5–9).

We have reported recently that short-term incubation of 1321N1 cells with isoproterenol also results in the accumulation of a subpopulation of receptors that exhibits altered sedimentation properties on sucrose density gradients (10). The time course of appearance of this population of receptors roughly coincided with the agonist-induced uncoupling reaction. Furthermore, the agonist binding properties of this receptor population were equivalent to that of "uncoupled" β-adrenergic receptors. Exposure of frog erythrocytes to catecholamines results in the appearance of a similar subpopulation of β-adrenergic receptors in supernatant fractions of lysates (11–13). We describe in the present report results from experiments that further characterize the agonist-induced modification of the membrane form of the β-adrenergic receptor in 1321N1 astrocytoma cells.

EXPERIMENTAL PROCEDURES[1,2]

Materials

The following compounds were obtained from Sigma (St. Louis, MO): ammonium molybdate, cacodylic acid, D-glucose-6-phosphate, β-mercaptoethanol, N-acetylglucosamine, 4-nitrophenylphosphate, UDP-galactose, Dowex 50-X8, and (-)-isoproterenol (+) bitartrate. Dowex AG 2-X8 anion exchange resin was from Bio-Rad Laboratories. Neutral alumina was obtained from Fisher (Pittsburg, PA). Con A was purchased from Cal-Biochem, ultra-pure sucrose from Schwarz-Mann, and 4-nitrophenol from Aldrich. Na ^{125}I was from Amersham, [^{3}H](-)-QNB (specific activity = 40.2 Ci/mmole) from New England Nuclear Corporation, and UDP-^{14}C-galactose from ICN. (-)-Pindolol and (±)-hydroxybenzylpindolol were generous gifts from Drs. Gunther Engel and Dan Hauser of Sandoz Pharmaceuticals (Basel, Switzerland). All other reagents were purchased from commercial sources.

[1] "Experimental Procedures" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1278, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations are: ConA, concanavalin A; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ^{125}I-PIN, (-)-^{125}I-pindolol; [^{3}H]QNB, (-)-[^{3}H]quinuclidinyl benzilate; IAP, islet-activating protein; N_s, the stimulatory guanine nucleotide regulatory component of adenylate cyclase; N_i, the inhibitory guanine nucleotide regulatory component of adenylate cyclase; C, the catalytic component of adenylate cyclase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTPγS, guanosine 5′-3(thio)triphosphate.

* This work was supported by Grant NS 18153 to Dr. A. G. Gilman and Grants GM 27820 and GM 29522 from the United States Public Health Service. A preliminary report of portions of this work has been presented (47). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address, University Calgary Faculty Medicine, 3330 Hospital Drive, Calgary, Alberta, Canada T2N 4N1.

‖ Established Investigator of the American Heart Association. To whom correspondence should be addressed.

This is an Open Access article under the CC BY license.

Cell Culture

Human astrocytoma cells (1321N1) were grown in the absence of antibiotics on 150 mm plastic culture dishes (Falcon) in 20 ml of Dulbecco's modification of Eagle's medium supplemented with 5% fetal calf serum. Cells were subcultured at a density of 10,000–15,000 cells/cm^2 and maintained in a 37° humidified incubator in an atmosphere of 92% air and 8% CO_2. All experiments were carried out with confluent cultures 4–5 days after subculture. Growth medium was replenished 24 hr before each experiment, and replaced with 10 ml of Eagle's minimal essential medium buffered with 20 mM Hepes (pH 7.5) just prior to incubation with drugs. All subsequent 37° incubations were in a humidified incubator in air. S49 cell culture and membrane preparation were performed as described (14,15).

Drug Treatment

Cells were desensitized by incubation at 37° with 1 μM (–)-isoproterenol in the presence of 1 mM Na ascorbate for the times indicated. Control cells were incubated with Na ascorbate alone. Unless specified otherwise, incubations with isoproterenol were for 20 min.

Membrane Preparation

Four to seven confluent 150 mm dishes were used per gradient in a typical experiment. Marker enzyme experiments required up to eighteen dishes per gradient. Following drug treatment, 5 ml of ice cold Hepes-buffered (20 mM, pH 7.5) Eagle's medium containing 0.25 mg/ml Con A was added directly to the desensitization medium (10 ml). As has been previously described (10,16,17), treatment of cells with Con A results in the formation upon cell lysis of plasma membrane fragments that migrate as more uniform particles to heavier densities during sucrose density gradient centrifugation. After incubation of the dishes with Con A on ice for 20 min, the medium was aspirated, and the dishes were washed once with 10 ml of cold hypotonic buffer (2 mM EDTA, 1 mM Tris, pH = 7.4). The cells were then allowed to swell in an additional 10 ml of hypotonic buffer for 20 min on ice. The swollen cells were lysed in a small volume (~1 ml/dish) by aspirating the buffer and scraping the dishes with a rubber policeman. Lysates of the dishes were combined and diluted to a final volume of 10.5 ml with hypotonic buffer. Nine and one half ml of this preparation was layered on each gradient.

Density Gradients

Sucrose density gradients were prepared with an ISCO model 570 gradient former in a 4° cold room. The most frequently used system was a 30 ml linear gradient running from 30 to 60% (w/v) sucrose. For some experiments it was necessary to construct shallower gradients (e.g. Fig. 8). In these experiments a 27 ml linear gradient running from 15 to 30% sucrose with a 3.6 ml step of 5% (w/v) sucrose on top was used. All sucrose solutions were buffered to pH 7.4 at 4° with 10 mM Tris.

Gradients were centrifuged for 1 hour at 114,000 x g in a Beckman SW-27 rotor and a model L5-65 or L8-70 ultra-centrifuge. Fractions (1.8 ml) were collected from the top of each gradient with an ISCO model 568 fractionator at a flow rate of 3.0 ml/min. The gradient fractions were diluted with 145 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tris, pH 7.5 at 25° for receptor assays.

Receptor Binding Assays

(–)-Pindolol was iodinated and ^{125}I-PIN purified as previously described (18,19). Binding assays consisted of 180 μl of sample in 145 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris, pH 7.5 at 25°, 50 μl of ^{125}I-PIN (usually 80-90 pM, final concentration) in 1 mM Na ascorbate, and 20 μl of 1 mM HCl or 1 mM HCl containing (–)-isoproterenol (100 μM, final concentration) to define nonspecific binding. Incubations were for 60 min at 25° and the binding reaction was terminated by filtration over glass fiber filters as described elsewhere (19). Nonspecific binding was usually ≤ 5% of total counts retained by the filter. In several experiments ^{125}I-hydroxybenzylpindolol (^{125}I-HYP) was used as the radioligand to label β-adrenergic receptors. The protocol for ^{125}I-HYP assays was the same as described for ^{125}I-PIN except that incubations were for 60 min at 37°.

Muscarinic cholinergic receptors were quantitated as previously described (20). Briefly, ^{3}H-QNB (100-200 pM) was incubated with gradient samples in 145 mM NaCl, 5 mM $MgSO_4$ and 20 mM Tris (pH = 7.5) in a final volume of 1.0 ml. The incubation time was 45 min at 37°. Assays were terminated by the addition to the reaction tubes of 10 ml of a wash buffer consisting of 145 mM NaCl, 10 mM Tris (pH=7.4) and filtration over glass fiber filters (Schleicher and Schuell, #30). The filters were washed with an additional 10 ml of wash buffer, dried and placed in scintillation vials. Radioactivity was determined at an efficiency of 33%. Specific binding was defined as the amount of radioactivity bound in the absence of competing drug minus the amount bound in the presence of 1 μM atropine.

Adenylate Cyclase

Adenylate cyclase activity was measured by a modification (20) of the method described by Salomon et al (21). Assay tubes with a final volume of 150 μl/assay contained the following reagents (final concentration): 5.0 mM $MgCl_2$, 1 mM EGTA, 8 mM creatine phosphate, creatine phosphokinase (6 units/assay), 1 mM [^{3}H]-cAMP (~30,000 cpm/assay), 0.4 mM [α-^{32}P]ATP (0.5 μCi/assay), 10 μM GTP, 1.4 mM dithiothreitol, and 50 mM Tris, pH 7.4 at 30°. The reaction was started by adding 100 μl of the reagents listed above to tubes containing 50 μl of sample. After 30 min at 30°, the reaction was terminated by the addition of 850 μl of 5% (w/v) trichloroacetic acid. [^{32}P]-cAMP was separated from [^{32}P]-ATP by sequential column chromatography over Dowex 50-X8 and neutral alumina. The columns were prepared and eluted as previously described (20). The recovery of ^{3}H-cAMP from the columns was 60–75% and assay blank values were < 60 cpm.

Assays for adenylate cyclase components.

The assay of N_s activity in extracts from 1321N1 membranes was accomplished by reconstitution of adenylate cyclase in membranes from the cyc$^-$ variant of the mouse S49 lymphoma line. The procedures used for reconstitution of hormonally regulated adenylate cyclase were those of Sternweis and Gilman (22). Assays of guanine nucleotide-activated G/F were performed as described (Assay II, 23). Adenylate cyclase activity restored in the cyc$^-$ membranes was measured by the method of Salomon et al. (21) also as described (23).

The activity of catalytic adenylate cyclase was assayed by activation with GTPγS-activated N_s or by forskolin. For the former, a saturating amount (1 μg) of N_s was added to 40 μg of membranes and adenylate cyclase was assayed in the presence of 5 mM $MgCl_2$. Forskolin-stimulated adenylate cyclase was assayed in the presence of 100 μM forskolin and 2 mM $MnCl_2$. Either procedure yields the maximal activity attainable for adenylate cyclase.

ADP-ribosylation of adenylate cyclase components.

The GTP-binding regulatory proteins (N_s and N_i) of adenylate cyclase were ADP-ribosylated by incubation of 1321N1 cell membrane fractions with [α-^{32}P]NAD and cholera toxin or the islet activating protein (IAP) of Bordetella pertussis as described (24,25). Briefly, membrane fractions (120 μg protein) were incubated in a final reaction volume of 500 μl containing 100 mM Tris, pH 8 (IAP labeling) or 100 mM sodium phosphate, pH 8 (cholera toxin labeling), 10 μM NAD with about 10^7 cpm [α-^{32}P]NAD, 1 mM thymidine, 6 mM potassium phosphoenol pyruvate, 10 μg/ml pyruvate kinase, 0.5 mM ATP, 1 mM dithiothreitol, and 15 μg/ml IAP or 60 μg/ml cholera toxin. The reactions were terminated after 90 min at 30°C by the addition of (final concentrations) 2% sodium dodecylsulfate and 12.5% trichloroacetic acid. Samples were allowed to precipitate overnight at 0° C and sedimented at 9,000 rpm in a Beckman JA20 rotor for 30 min. The pellets were rinsed twice with 1 ml ether and then dissolved with 100 μl of Laemmli sample buffer for application on 11% discontinuous polyacrylamide gels (26). Gels were fixed, stained with Coomassie Brilliant Blue, dried, and autoradiograms were developed using Kodak XAR-5 film and Chronex lighting plus intensifying screens.

Detergent extraction of membrane fractions.

Membrane fractions were washed by dilution with a solution containing 50 mM sodium Hepes (pH 8), 1 mM sodium EDTA, 1 mM dithiothreitol, and 50 mM NaCl and sedimented at 50,000 rpm for 30 min in a Beckman 50 Ti rotor. The pellets were then resuspended to 5 mg/ml protein concentration in the same solution containing 0.8% sodium cholate. These extracts were shaken at 0°C for 60 min and sedimented for 30 min at 50,000 rpm in a Beckman 50 Ti rotor. The supernatants were used as "cholate extracts."

Marker Enzyme Assays

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) activity was measured as a marker for the endoplasmic reticulum (27-29) using a modification of the procedures described by Baginski et al. (30) and Swanson (31). Reactions were started by adding 100 μl of a mixture containing 0.25 M sucrose, 1 mM EDTA, 100 mM glucose-6-phosphate, and 100 mM cacodylic acid (pH 6.5) to assay tubes containing 300 μl of tissue sample. Incubations were carried out at 37° for 45 min. The reaction was terminated by the addition of 2.0 ml of 2% ascorbic acid and 10% TCA (w/v). Assay tubes were mixed and centrifuged in a Beckman J-6 centrifuge at 3,000 x g for five min. One ml of the resultant supernatant was mixed with 0.5 ml of 1% ammonium molybdate (w/v) and 1.0 ml of a 2% arsenite and 2% citrate solution. The mixture was allowed to stand at room temperature for 15 min and absorbance was read at 840 nm. KH_2PO_4 was used to generate a standard curve from 0.01 to 0.25 μmol PO_4.

Acid phosphatase (orthophosphoric-monoester phosphohydrolase, acid optimum, EC 3.1.3.2), was used as a marker for lysosomes (27-29). The enzyme activity was measured spectrophotometrically at a wave length of 420 nm using 4-nitrophenyl phosphate as the substrate essentially as described by Walter and Schutt (32). Assays were incubated for 30 min at 25° in the absence of sodium tartrate. A standard curve was generated with 4-nitrophenol.

Galactosyltransferase, (UDP galactose: 2-acetamido-2-deoxy-D-glucosylglycopeptide-galactosyltransferase, EC 2.4.1.3.8), was utilized as a marker for Golgi (27-29). The enzyme activity was measured by a modification of the method described by Bretz and Staubli (33). UDP-^{14}C-galactose was used as the substrate and N-acetylglucosamine was employed as the acceptor molecule. Specific galactosyltransferase activity was defined as the total hydrolysis and transfer (assays in the presence of N-acetylglucosamine) minus the non-specific substrate hydrolysis (assays in the absence of N-acetylglucosamine). Assays contained the following reagents (final concentrations) in a final volume of 200 μl: 40 mM cacodylic acid, pH 6.6; 40 mM β-mercaptoethanol; 0.4% (w/v) Triton X-100; 40 mM $MnCl_2$; 20 mM N-acetylglucosamine or H_2O; 1.05 mM UDP-^{14}C-galactose (~25,000 cpm/assay); and 1 mM ATP added to protect against nonspecific substrate hydrolysis (34). The reactions were carried out at 37° for 60 min and terminated with the addition of 100 μl of 0.25 M EDTA (pH=7.4) and immediate chilling in an ice bath. N-acetyllactosamine, the product of the transferase reaction, was separated from unreacted UDP-galactose, galactose-1-phosphate, and free galactose by chromatography on AG 2-X8 (200-400 mesh, Cl^- form) anion exchange columns. Washed resin was poured to a bed height of 2 cm in Kontes disposable columns (I.D.= 8 mm). Columns were washed with 5 ml of 5% (w/v) sodium borate and 3 ml of water before applying the 300 μl sample. Assay tubes were washed with 500 μl of water which also was applied to the columns before eluting with 5 ml of water. The entire effluent (5.8 ml) was collected in scintillation vials, mixed with 6 ml of scintillation fluid, and counted in a Tracor Model 6892 scintillation counter at an efficiency of approximately 30%.

The columns were reused 8–10 times by using the following regeneration procedure. UDP-galactose, galactose-1-phosphate and galactose were removed by elution with 5 ml of 1 M NaCl and the protein was removed by washing with 3 ml of 1.0 M NaOH, 3 ml of H_2O, 3 ml of 1.0 M HCl, and 3 ml of H_2O. The columns were converted to the borate form (34) by washing with 5 ml of 5% (w/v) sodium borate and washed to neutrality with water.

Protein Determination

Protein was determined by the method of Lowry et al. (35) using bovine serum albumin as the standard.

RESULTS

As we have previously reported (10), short-term (15 min) incubation of 1321N1 cells with isoproterenol results in an alteration in the distribution of β-adrenergic receptors on sucrose density gradients (Fig. 1). Rather than migrating principally as a single peak at a density equivalent to that of other markers of the plasma membrane, a large portion of the receptors migrate as a "light peak" at sucrose densities of 30% or less. The properties of these light peak receptors have been compared to those of receptors from the plasma membrane fraction and from cell lysates. Saturation binding isotherms for ^{125}I-PIN were generated with receptors from the light peak and from the plasma membrane fraction. Scatchard plots were consistent with a single binding site in the light peak and the plasma membrane fraction derived from both control and desensitized cultures (Fig. 2). The affinity (12–15 pM) of ^{125}I-PIN in each receptor-containing fraction was similar to that (14 pM) determined in cell lysates (data not shown).

The kinetics of ^{125}I-PIN binding also were examined for each receptor fraction. The rate constants for association (k_1) of ^{125}I-PIN were 8.4×10^8, 8.3×10^8, and 11.7×10^8 M^{-1} min^{-1} for the control lysate, desensitized light peak, and desensitized plasma membrane fraction, respectively. The rate constants for dissociation (k_2) were 0.015, 0.031, and 0.021 min^{-1} for the same three receptor-containing fractions. The kinetically determined dissociation constants (k_2/k_1) were in good agreement with the K_D values determined by equilibrium assay.

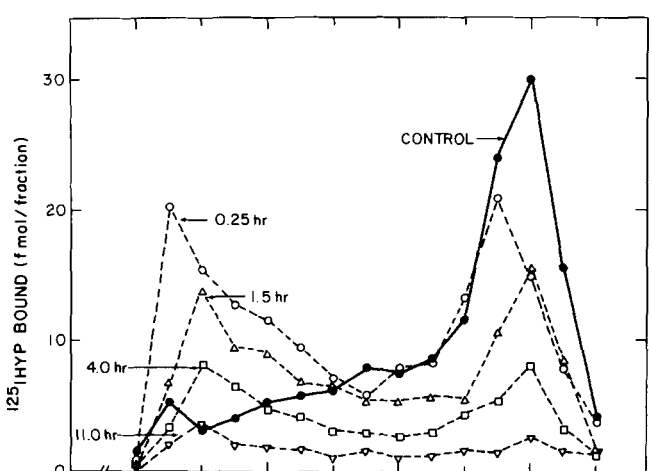

FIG. 1. **Catecholamine-induced alteration in the sucrose density gradient distribution of β-adrenergic receptors.** Cells were incubated in the absence of isoproterenol for 11 h (●) or in the presence of 1 μM isoproterenol for 0.25 (○), 1.5 (△), 4.0 (□), or 11.0 (▽) h. Following exposure to drug, the cells were treated with ConA and lysed as described under "Experimental Procedures." Lysates were layered on a sucrose density gradient and centrifuged for 1 h at 114,000 × *g*. The gradients used in this experiment (linear sucrose running from 30 to 55% (28 ml) above a 2-ml cushion of 60% sucrose) were somewhat different than the gradients used elsewhere (*e.g.* Figs. 4 and 7) for resolution of light peak receptors and the plasma membrane fraction (see "Experimental Procedures"). Two-ml fractions were collected from the top. Values were normalized to the amount of protein added to each gradient. The data are representative of three similar experiments. ^{125}*IHYP*, ^{125}I-hydroxybenzylpindolol.

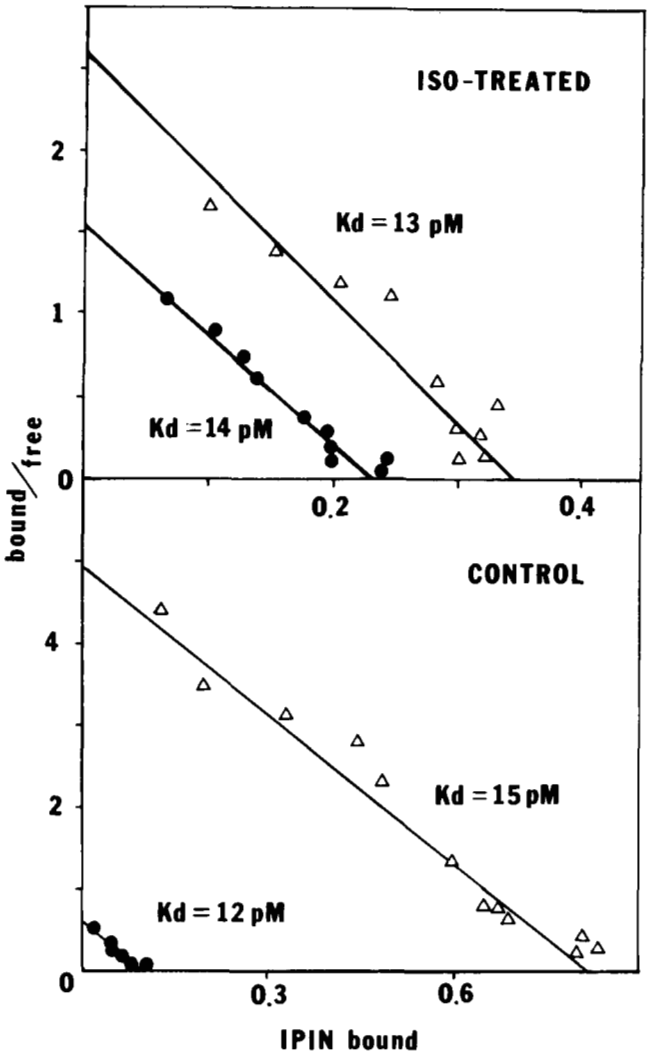

FIG. 2. **Scatchard analyses of ^{125}I-PIN binding to receptors in the light peak and plasma membrane fractions.** Cells were incubated in the absence or presence of 1 μM isoproterenol (*ISO*) for 15 min. Lysates from nine 150-mm dishes for each condition were then prepared and centrifuged on a continuous 30–60% sucrose gradient as described under "Experimental Procedures." The light peak (●) and plasma membrane (△) fractions of control and desensitized gradients each were pooled and diluted with 1 mM Tris (pH 7.4 at 4 °C) and pelleted by centrifugation for 60 min at 170,000 × *g* in a Beckman 42.1 Ti rotor. The pellets were diluted with 145 mM NaCl, 20 mM Tris (pH 7.5 at 25 °C), and 5 mM $MgCl_2$, and specific binding of ^{125}I-PIN was measured as a function of ligand concentration (3–300 pM) at a protein concentration of approximately 15 μg/assay.

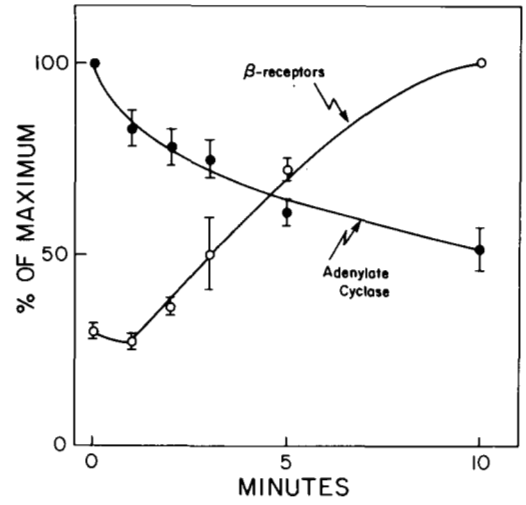

FIG. 3. **Temporal relationship of catecholamine-induced loss of hormone-stimulated adenylate cyclase activity to the conversion of β-receptors to the altered membrane form.** Four 150-mm dishes of 1321N1 cells were used per time point. Ten-ml aliquots of Hepes-buffered Eagle's medium containing 1 μM isoproterenol were warmed to 37 °C. The growth medium was aspirated from all dishes, and incubations were initiated by rapidly pouring the isoproterenol-containing medium onto the dishes and placing them in an incubator at 37 °C. Reactions were terminated by the rapid aspiration of the desensitization medium and immediate addition on ice of ice-cold Hepes-buffered Eagle's medium containing 0.25 mg/ml of ConA. Dishes were then placed on ice for 20 min, followed by hypotonic lysis as described earlier. Aliquots of the lysates were saved for determination of isoproterenol (10 μM)-stimulated adenylate cyclase activity (●), and the remaining lysate was layered on a 30–60% sucrose gradient and centrifuged as described under "Experimental Procedures." The specific binding of ^{125}I-PIN (○) in the light peak fractions was summed and plotted as a per cent of maximal activity. Isoproterenol-stimulated adenylate cyclase activity is plotted as the per cent of activity in control lysates. The data is the mean ± S.E. of four experiments.

Although the total number of receptors is only minimally reduced[3] during short-term (20 min) incubation of 1321N1 cells with isoproterenol (1–3), as we have previously described in detail, extended incubation results in a marked reduction in measurable receptors (2–4). Thus, the temporal relationship of receptors appearing in the light peak to those in the plasma membrane fraction was examined. Maximal conversion of receptors to the light peak form occurred within 10 min during incubation of cells with 1.0 μM isoproterenol. Once a maximal conversion of receptors to the light peak form occurred, receptors were lost from this and the plasma membrane fraction with similar time courses (Fig. 1). Thus, a precursor-product relationship could not be established directly for the light peak form of receptors in generating receptors that are lost from the cell.

The appearance of receptors in the light peak fraction occurs during a time when a major decrement in catecholamine-stimulated adenylate cyclase activity occurs. Thus, attempts were made to define in detail the temporal relationship between the change in the membrane form of β-adrenergic receptors and the uncoupling of adenylate cyclase as measured by a loss of isoproterenol-stimulated enzyme activity. As we have previously reported (3), catecholamine-stimulated adenylate cyclase activity is rapidly lost ($t_{1/2}$ = 2.5 min) during incubation of cells with isoproterenol to a level that is approximately 50% of control (Fig. 3). Although catecholamine-stimulated activity was reduced by 20–25% during the first 1–2 min of incubation with isoproterenol (Fig. 3 and Ref. 3), no significant change ($p > 0.05$) occurred in the number of receptors appearing in the light peak (Fig. 3). After this lag of 1–2 min, a conversion of β-adrenergic receptors to the light peak form occurred, with maximal levels of receptor appearing in this fraction within 10 min ($t_{1/2} \sim 4.5$ min).

Such kinetic experiments (Fig. 3) indicated that, at least at very short times of incubation, the uncoupling reaction as measured by isoproterenol-stimulated adenylate cyclase activity could be distinguished from the change in the membrane form of receptors. This distinction was pursued further by examining the effects of various chemicals on these two

[3] Over a 5-year period, some variability in the degree of receptor loss during short-term exposure of 132lNl cells to 1 μM isoproterenol has occurred. Although receptor levels in lysates from cells pretreated for 20 min with catecholamine are usually 90–100% of control, in some experiments receptor levels have been reduced to as low as 75% of control.

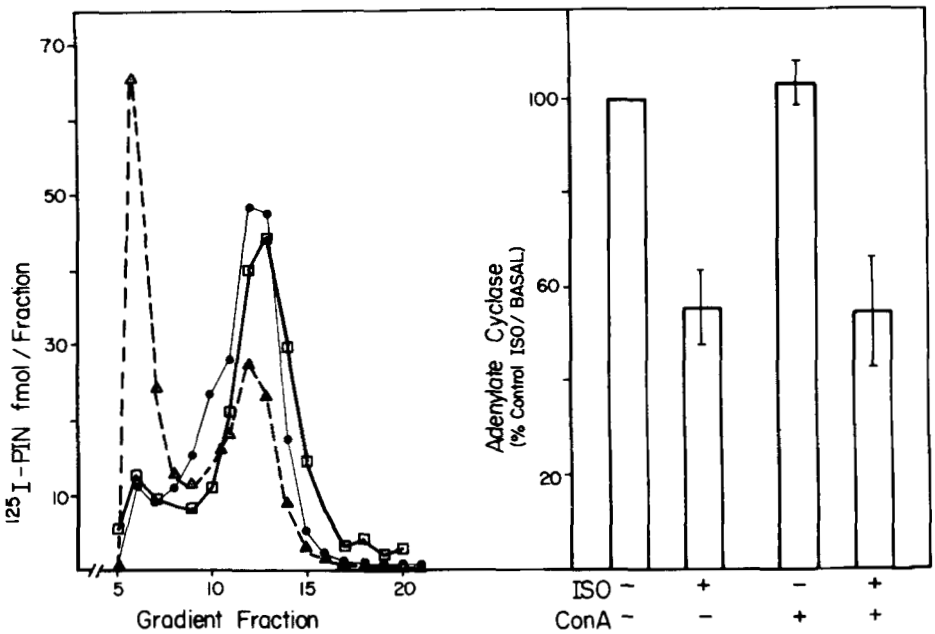

FIG. 4. **Effect of ConA on agonist-induced changes in catecholamine-stimulated adenylate cyclase activity and the membrane form of β-adrenergic receptors.** *Left*, cells were preincubated for 45 min at 37 °C with Hepes-buffered Eagle's medium with (□) or without (● and △) 250 μg/ml of ConA. Isoproterenol (1 μM) was then added to two sets (□ and △) of the cultures, and the incubation was continued for another 20 min. The medium was then aspirated, and all cultures were treated with ConA and gradients generated as described under "Experimental Procedures." The data are representative of six experiments. *Right*, isoproterenol-stimulated adenylate cyclase activity was determined in lysates from cells treated with ConA and/or isoproterenol (*ISO*) as described for the *left* panel of this figure. The data are expressed as the per cent of the value for isoproterenol-stimulated activity divided by basal activity for control cells. The data are the mean ± S.E. for four experiments.

processes. Preincubation of cells with 0.25 mg/ml of ConA[4] for 30–45 min at 37 °C prior to incubation with isoproterenol completely blocked the agonist-induced conversion of receptors to the light peak form (Fig. 4). In contrast, the loss of isoproterenol-stimulated adenylate cyclase activity in ConA-pretreated cells was the same as in control cells (Fig. 4). Thus, as suggested by the kinetic experiments, the agonist-induced uncoupling of the β-adrenergic receptor-linked adenylate cyclase apparently can occur without the change in the membrane form of β-adrenergic receptors. The effect of ConA on recovery from catecholamine-induced desensitization also was examined (data not shown). Cells were either desensitized for 30 min or desensitized for 15 min then treated with 0.25 mg/ml of ConA in the continued presence of isoproterenol for another 15 min. After incubation with isoproterenol and ConA, the cultures were washed three times with Hepes-buffered Eagle's medium at 37 °C with or without ConA and incubated for an additional 45 min at 37 °C. There was no effect of ConA on the capacity of cells to recover catecholamine-stimulated adenylate cyclase activity to control levels or on the conversion of receptors back to the plasma membrane form. A variety of other agents were examined for their activity as blockers of the agonist-induced modification of the membrane form of the β-adrenergic receptor. Methylamine (1–100 mM), colchicine (0.1 mM), di-5-dimethylaminonaphthalene-1-sulfonyl cadaverine (0.5 mM), and chloroquine (100 μM) all had no effect on the agonist-induced change in the gradient distribution of receptors.

The specificity of the change in the membrane form of β-adrenergic receptors was examined by determining the gradient distribution of adenylate cyclase activity of lysates from control and desensitized cells (Fig. 5). Basal and isoproterenol-, fluoride-, and fluoride + 0.03% Lubrol-PX-stimulated adenylate cyclase activities were measured in pooled fractions from the light peak and from the plasma membrane fraction. Although there was a 50% decrease in isoproterenol-stimulated adenylate cyclase activity in the plasma membrane fraction, no increase in any activity parameter of adenylate cyclase occurred in the light peak. However, assay of fluoride-stimulated activity in the presence of 0.03% Lubrol-PX[5] did

TABLE I

Activities of the components of adenylate cyclase in control and desensitized membrane fractions

Membrane fraction[a]	β-Receptor[b]	N_s[c]	C[d]
	fmol/mg	*nmol/min/mg*	*nmol/min/mg*
Control light peak	14	0.33	0.20
Control plasma membrane	118	1.59	2.66
Desensitized light peak	60	0.38	0.21
Desensitized plasma membrane	69	1.49	2.38

[a] Light and plasma membrane fractions were isolated from 27 plates each of untreated and desensitized (20 min at 37 °C with 1 μM isoproterenol) 1321N1 cells which had been grown to confluence. Pooled fractions were 1.39, 1.73, 0.89, and 1.24 mg/ml of protein for control light peak and plasma membrane and desensitized light peak and plasma membrane fractions, respectively.

[b] The density of β-adrenergic receptors was determined from Scatchard analyses of ^{125}I-PIN binding to the fractions.

[c] The activity of N_s was determined in extracts of the membrane fractions prepared as described under "Experimental Procedures." Extracts were diluted with a solution containing (final concentrations) 20 mM Tris (pH 8), 1 mM sodium/EDTA, 1 mM dithiothreitol, 0.1% Lubrol-12A9, 10 mM $MgCl_2$, and 10 μM GTPγS and incubated for 60 min at 30 °C to activate N_s. cyc$^-$ membranes were reconstituted with three dilutions of each extract, and the specific activity of N_s was determined from these linear reconstitutions of adenylate cyclase with added extract. The activities are expressed per mg of the original membrane protein.

[d] The activity of the catalytic component of adenylate cyclase was determined by stimulation with a saturating concentration of purified rabbit liver N_s which had been activated with GTPγS. Resultant adenylate cyclase activity was measured as described under "Experimental Procedures."

[4] This effect of *preincubation* with ConA prior to incubation of cells with catecholamine should not be confused with the routine use of ConA in these experiments. That is, in all experiments, ConA treatment of cells *subsequent* to agonist-induced desensitization is used as a means of more effectively separating the light peak and plasma membrane fractions (10, 16, 17).

[5] A concentration-effect curve for the effects of Lubrol-PX on adenylate cyclase activity was generated for the light peak and plasma membrane fraction of gradients. An approximately 8-fold increase in basal or NaF-stimulated adenylate cyclase activity occurred in the presence of detergent in the light peak fractions. The optimal concentration for this effect was 0.03% Lubrol-PX. Only minor (~2-fold increase) effects of Lubrol-PX on enzyme activity were observed in assays with the plasma membrane fraction.

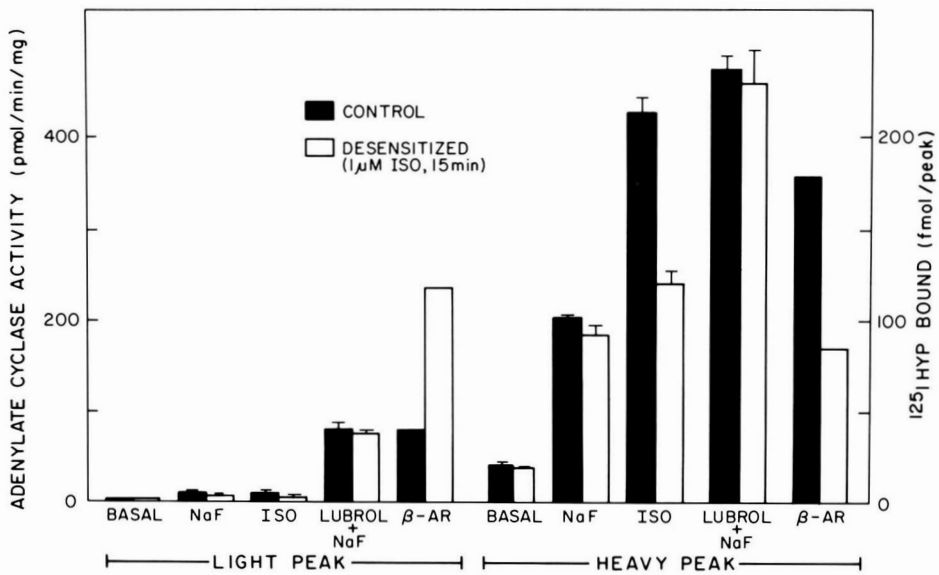

FIG. 5. **Adenylate cyclase activity in sucrose density gradient fractions generated from control and desensitized cells.** Peak regions from two control (*solid bars*) and two desensitized (*open bars*) gradients were prepared as described in the legend to Fig. 2. Basal, isoproterenol (*ISO*)-, NaF-, and NaF + Lubrol-PX (0.03%)-stimulated adenylate cyclase activities were measured in both the light peak and the plasma membrane (heavy peak) preparation. Samples were treated in the absence or presence of detergent for 20 min on ice immediately prior to assay. The specific binding of ^{125}I-PIN was determined as described under "Experimental Procedures. *β-AR*, β-adrenergic receptors.

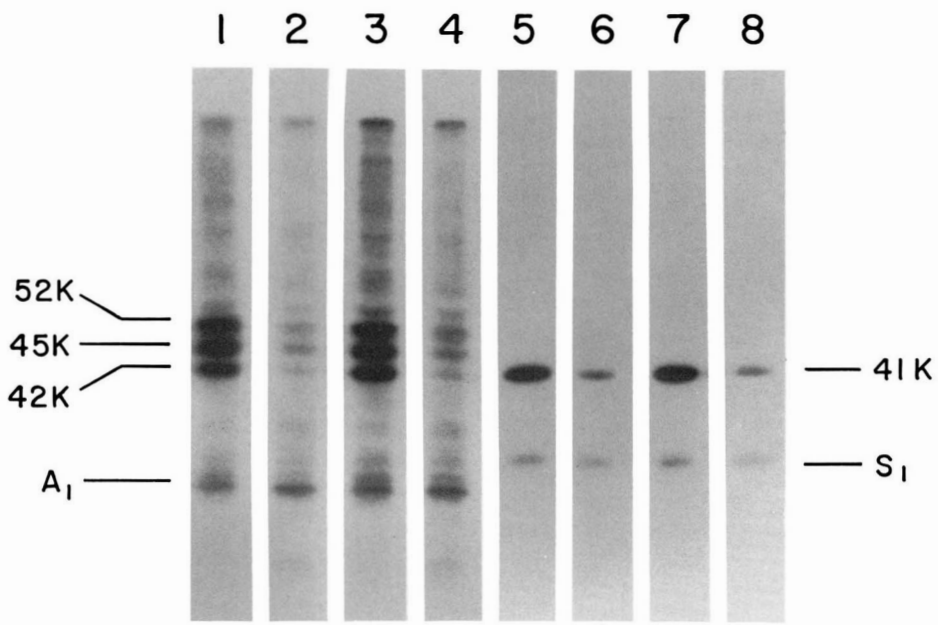

FIG. 6. **ADP-ribosylation of 1321N1 membrane fractions.** Light peak and plasma membrane fractions prepared from control or desensitized cells were ADP-ribosylated with [α-^{32}P]NAD and IAP or cholera toxin as described under "Experimental Procedures." A_1 and S_1 are the active subunits of cholera toxin and IAP, respectively. *Lanes 1–4* are autoradiograms of cholera toxin-labeled membranes exposed for 60 h (desensitized plasma membranes, desensitized light peak, control plasma membranes, and control light peak, respectively). In *lanes 5–8*, the samples also were labeled with IAP and autoradiograms were exposed for 6 h (desensitized plasma membranes, desensitized light peak, control plasma membranes, and control light peak, respectively). *52K*, for example, 52,000 daltons.

uncover a significant amount of latent enzyme activity in the light peak fraction from both control and desensitized cells (Fig. 5).

We also have examined the distribution of the components of adenylate cyclase in the light peak and plasma membrane fractions from both control and desensitized cells. Table I presents the results of the quantitative activities assayed for N_s and C. These results, in contrast to those for the β-adrenergic receptor, show that the specific activities of N_s and C are identical in membrane fractions prepared from control or desensitized cells. In both control and desensitized cells, the specific activities of N_s and C are 5–10-fold lower in the light peak fraction than in the plasma membrane fraction.

We also attempted to determine if desensitization involves a modification of N_s or N_i by examining the cholera toxin and IAP labeling patterns of membrane fractions prepared from control or desensitized cells. Fig. 6 presents an autoradiogram from such an experiment. All four membrane fractions display the same pattern of labeling with cholera toxin or IAP. Cholera toxin labels predominantly 52,000-, 45,000-, and 42,000-dalton substrates from these membranes, and the intensity of labeling correlates well with the N_s activities measured in reconstitution assays with cyc$^-$ membranes. In other preparations of 1321N1 cell membranes, the 42,000-dalton cholera toxin substrate was absent, suggesting that it may be a proteolytic product of the 52,000- or 45,000-dalton substrate. The predominant IAP substrate in all four membrane fractions was a 41,000-dalton peptide, as seen by Katada and Ui (36, 37) for C6 glioma cells. As with the cholera toxin substrates, the IAP substrate does not appear to be enriched in

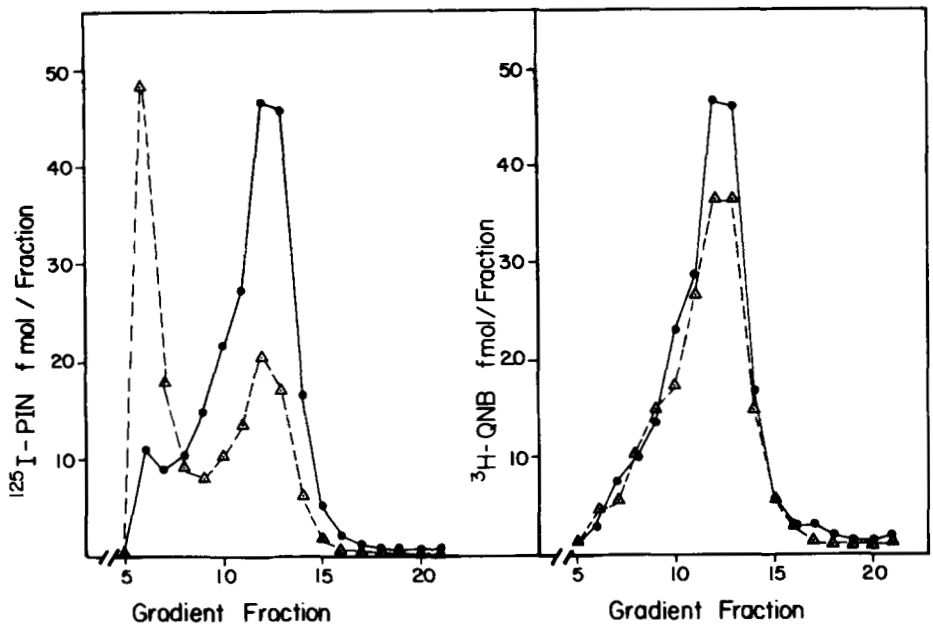

FIG. 7. **Comparison of sucrose density gradient profiles of β-adrenergic and muscarinic cholinergic receptors.** Sucrose density gradients were generated from lysates of control (●) and desensitized (△) 1321N1 cells (1 μM isoproterenol; 20 min) as described under "Experimental Procedures." The distribution of β-adrenergic receptors (*left*) was determined with ^{125}I-PIN, and the distribution of muscarinic receptors (*right*) was determined with [^{3}H]QNB. The data for [^{3}H]QNB are representative of three experiments.

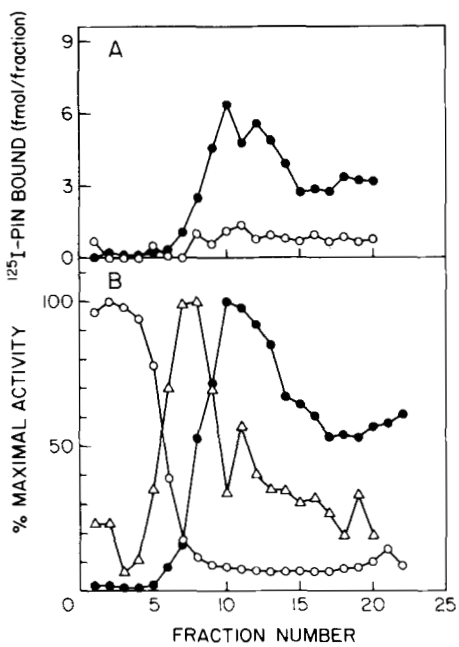

FIG. 8. **Gradient distribution of marker enzymes and β-adrenergic receptors.** *A*, the distribution of β-adrenergic receptors from control (○) *versus* desensitized (●) cells (15 min; 1 μM isoproterenol) was compared on a 15–30% sucrose continuous gradient topped with a step of 5% sucrose (see "Experimental Procedures"). *B*, cells were preincubated for 15 min with 1 μM isoproterenol, and the distribution of acid phosphatase (○), galactosyltransferase (△), and β-adrenergic receptors (●) was assessed on the gradient described above. The per cent of total activity recovered in the pellet fractions was 30–45% for galactosyltransferase and 20–30% for acid phosphatase.

the desensitized light peak fraction as compared with control cells.

Further evidence for the specificity of the isoproterenol-induced alteration in the gradient distribution of β-adrenergic receptors was provided by monitoring the fate of another membrane-bound receptor, the muscarinic cholinergic receptor. Under conditions where 40–50% of the β-adrenergic receptors were converted to the form appearing in the light peak, no change occurred in the density gradient profile of muscarinic receptors identified (38) by [^{3}H]QNB binding (Fig. 7).

One interpretation of the data indicating a change in the membrane form of β-adrenergic receptors during incubation of 1321N1 cells with isoproterenol is that an agonist-induced internalization of the β-adrenergic receptor has occurred. Thus, the extent to which β-adrenergic receptors in the altered membrane form co-migrated with markers for subcellular organelles was examined. The distribution on shallow sucrose density gradients of marker enzymes for Golgi, endoplasmic reticulum, and lysosomes was compared to that of β-adrenergic receptors appearing in the light peak. Lysates from cells previously incubated for 20 min with 1 μM isoproterenol were resolved on a 15–30% sucrose density gradient. No apparent association of ^{125}I-PIN binding activity with lysosomal enzyme marker activity (acid phosphatase) occurred (Fig. 8*B*). The marker for endoplasmic reticulum (glucose 6-phosphatase) exhibited a distribution similar to that of acid phosphatase on these shallow gradients and thus does not appear to be associated with the light peak of β-receptors (data not shown). While the majority of galactosyltransferase activity migrated at a different density, a small amount of this activity co-migrated with the light peak of β-adrenergic receptors (Fig. 8*B*).

One possible concern in interpreting these results is that β-adrenergic receptors in an altered membrane form might exhibit different densities depending upon the point in a process of internalization and cellular processing at which they were examined. However, the peak of ^{125}I-PIN binding activity that occurred at 18–22% sucrose on the gradient illustrated in Fig. 8 migrated at the same density irrespective of the time (5–60 min) of incubation with isoproterenol (data not shown). As illustrated in Fig. 8*A*, the β-adrenergic receptors from control cells did not migrate as a distinct peak, but rather were broadly distributed throughout the 15–30% sucrose gradient. Adenylate cyclase activity also did not migrate as a distinct peak in these gradients (data not shown).

The results illustrated above were all obtained utilizing sucrose density gradients to resolve the altered membrane form of the β-adrenergic receptor from receptors associated with the plasma membrane. On the basis of this difference in density, the two receptor-containing fractions also can be resolved by differential centrifugation. As is illustrated in Fig.

9*A*, the majority of β-adrenergic receptors in control lysates were pelleted by a 10-min centrifugation at 15,000 × *g*. This *g*-force ranged from 10,000 to 15,000 × *g* in four experiments. In contrast, while greater than 50% of the β-adrenergic receptors in lysates from cells previously incubated (15 min) with isoproterenol were pelleted at the same low centrifugation forces, the remaining receptors were only pelleted at much greater forces (Fig. 9*A*). The force (30,000 to greater than 50,000 × *g*) necessary for sedimenting in a 10-min centrifugation all of the β-adrenergic receptors in lysates from desensitized cells was variable. This result is not surprising due to variations in the amount of tissue/ml in these experiments and inaccuracies inherent in this type of experiment with a preparative centrifuge. The data suggest that the altered membrane form of the receptors in lysates from desensitized cells can be resolved from the plasma membrane form simply by differential centrifugation. This point is more directly assessed in Fig. 9*B*. Receptors migrating in the light peak and plasma membrane fractions from desensitized cells were diluted to equivalent concentrations (~4%) of sucrose and subjected to differential centrifugation (Fig. 9*B*). Whereas receptors from the plasma membrane fraction were sedimented at between 20,000 and 30,000 × *g*, the majority of receptors in the light peak fraction were not sedimented by a 10-min centrifugation at 50,000 × *g*.

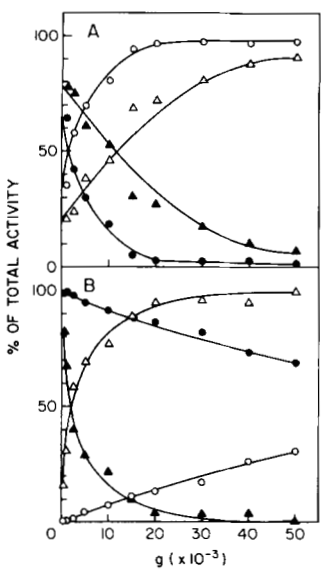

FIG. 9. **Differential centrifugation of β-adrenergic receptors.** *A*, differential centrifugation of lysates from control and desensitized cells. Five 150-mm dishes of 1321N1 cells were treated with (△ and ▲) or without (○ and ●) 1 μM isoproterenol for 20 min at 37 °C. Cells were then treated with ConA and lysed hypotonically as described under "Experimental Procedures." The lysate was made up to 11 ml with hypotonic buffer, and 1-ml aliquots were added to 10 centrifuge tubes. Paired control and desensitized samples were centrifuged for 10 min at various forces (500–50,000 × *g*) in a Beckman JA-21 rotor and J-21C centrifuge. ^{125}I-PIN binding was then measured in both pellets (*open symbols*) and supernatants (*closed symbols*). The data are plotted as the per cent of activity recovered under each condition and are representative of five similar experiments. *B*, differential centrifugation of receptors from the light peak and plasma membrane fractions from desensitized cells. Cells were desensitized for 20 min with 1 μM isoproterenol. The cells were treated with ConA and lysed, and sucrose density gradients were generated as described under "Experimental Procedures." Peak fractions from each gradient were combined and diluted to approximately 4% sucrose with the buffer used in ^{125}I-PIN binding assays. Aliquots were then added to centrifuge tubes and centrifuged as described in *A* of this figure. Plasma membrane pellet (△), light peak pellet (○), plasma membrane supernatant (▲), and light peak supernatant (●) are plotted as a per cent of specifically bound ^{125}I-PIN recovered in the supernatant and pellet at each centrifugation force.

DISCUSSION

The results of the present study provide evidence that the early phase of catecholamine-induced desensitization of adenylate cyclase can be separated into at least two different reactions. On a kinetic basis, "uncoupling" or loss of hormonal responsiveness occurs earlier than does formation of the light peak fraction of β-adrenergic receptors. This conclusion is supported by the observation that treatment of cells with ConA prevents formation of the light peak receptor fraction under conditions where neither the uncoupling reaction nor the recovery reaction are measurably altered. Thus, the following set of reactions can be proposed as a working hypothesis for the mechanism of receptor-specific, agonist-induced desensitization in 132 1N1 cells:

$$\beta AR_N \underset{-A}{\overset{+A}{\rightleftharpoons}} \beta AR_U \underset{-A}{\overset{+A}{\rightleftharpoons}} \beta AR_{LP} \underset{-A}{\overset{+A}{\rightleftharpoons}} \beta AR_L$$

where βAR_N, βAR_U, βAR_{LP}, and βAR_L are the native, uncoupled, light peak, and lost (1–4, 10) forms of the receptor, respectively, and A is a receptor agonist.

The observation that Reactions 1 and 2 are rapidly reversible (3) and that Reaction 3 is relatively slow in the forward direction and very slow in the reverse direction (3, 4) provide an explanation for the similar kinetics of loss of βAR_N and βAR_{LP} during continued exposure to isoproterenol (Fig. 1). In other words, conversion of a few molecules of βAR_{LP} to βAR_L would result in a rapid adjustment of the steady state levels of βAR_N, βAR_U, and βAR_{LP}; the consequence is the near parallel rates of loss of βAR_N and βAR_{LP} over an 11-h exposure to isoproterenol.

The results of the present study also provide further evidence for the specificity of the agonist-induced, receptor-specific desensitization process. Isoproterenol causes no movement of muscarinic cholinergic receptors to the light fractions of sucrose gradients. In addition, adenylate cyclase activity is not observed to increase in the light peak after exposure to isoproterenol nor are its components when they are assayed directly.

The presence of significant amounts of NaF-stimulated enzyme activity in the light peak region of both control and desensitized gradients is revealed by treatment of the samples with 0.03% Lubrol-PX. One interpretation of such results is that the light peak region is contaminated with right side-out vesicle fragments of the plasma membrane. If such were the case, these vesicles would not have measurable adenylate cyclase activity since ATP would have limited access to the active site of the enzyme within the vesicle. Lubrol would be expected to permeabilize the membrane to ATP. The fact that Lubrol caused a large increase (8-fold) in activity in the light peak but only a minor increase (2-fold) in enzyme activity in the heavy peak region (which contains primarily open sheets of plasma membrane) is consistent with this interpretation.

The experimental results shown in Table I and Fig. 6 indicate that no detectable transfer of N_s or N_i into the light peak fractions occurs upon exposure of cells to isoproterenol. However, the results cannot exclude the possibility that a complex with a 1:1 stoichiometric ratio of N_s to β-receptor is shifted to these fractions. Based on our calculations, the N_s content of the light peak fractions of control samples (Table I) is about 580 fmol.[6] About 70 fmol of β-receptor were

[6] Northup *et al.* (48) have determined that the specific activity of

transferred to the light peak fractions during desensitization. In view of the potential variation in estimates of N_s, it seems unlikely that we could distinguish values for N_s of 580 and 650 fmol before and after desensitization. In fact, in the experiment shown (Table I), less N_s was found in the light peak fractions from desensitized cells. This probably is primarily a reflection of a difference in protein content since the percentage of total N_s in the light fractions was the same in control and desensitized preparations, and, as shown, the specific activity of N_s was slightly higher in the desensitized sample. Stadel *et al.* (13) have carried out similar experiments using frog erythrocytes. They concluded that N_s was not sequestered along with the β-receptor into a light vesicle fraction during desensitization. The conclusion was based on a reconstitution assay (22) for N_s similar to that used in our studies. Their results indicate that, after desensitization, the vesicle fraction contains about 11% of the N_s contained in the plasma membrane fraction of control cells. The N_s content of the vesicle fraction of control cells was not reported. Although Stadel *et al.* (13) did not report sufficient details to allow us to make the calculation described above, it is clear that the proportion of N_s in light and heavy peaks after desensitization in either frog erythrocytes or 1321N1 cells is low (11–14%) and that N_s is not transferred to the light vesicle fractions in proportion to the per cent movement of β-receptors. However, it is probable that the results from frog erythrocytes (13) need to be accepted with the same caveats as indicated for the results with 1321N1 cells.

The studies of marker enzyme distribution failed to demonstrate clear patterns of co-migration of the βAR_{LP} with the Golgi apparatus, endoplasmic reticulum, or lysosomes. However, these results cannot unequivocally exclude an association of the agonist-modified receptors with any of these organelles.

It is of interest to understand the nature of the β-receptors that appear in the light peak fraction of control gradients. Based on an estimate of plasma membrane contamination of the light peak, it can be predicted that no more than 10% of the total measurable β-receptors should appear in this fraction of gradients from control cells. In fact, we routinely find 8–12% of the total β-receptors in the light peak. This leads to the somewhat anomalous set of observations that on the one hand all of the β-receptors in the light peak from control gradients can be accounted for by plasma membrane contamination, *i.e.* native β-receptors, but on the other hand we previously observed that these receptors exhibited an uncoupled behavior in agonist competition binding studies (10). The lack of high affinity binding of agonist in the absence of GTP is a property we and others have attributed to the uncoupled state of the receptor during short-term desensitization (1, 8, 39). One possible explanation for this anomaly is that the contaminating plasma membrane fragments containing the receptor do not contain N_s, which is required for the expression of high affinity binding of agonist. Ross *et al.* (15) have calculated that if the β-adrenergic receptor and adenylate cyclase components of S49 lymphoma cells were randomly and independently distributed in the plasma membrane, then they both should not be found together in membrane fragments of the size observed in their studies. Also, Sahyoun *et al.* (40) observed that fragmentation by sonication of membranes containing catecholamine-responsive adenylate cyclase resulted in a fraction of smaller membrane fragments which contained both β-adrenergic receptors and adenylate cyclase activity, but hormone responsiveness was lost. Alternatively, receptors involved in transit to or from the plasma membrane could contribute to this fraction that no longer comigrates with markers for the plasma membrane. Indeed, in contrast to desensitized cells, the receptors in the light fractions from control gradients exist as a rather broad band with little evidence of a distinct peak of activity (Fig. 8*A*), suggesting that a heterogeneous set of receptor-containing structures contributes to this activity. Thus, the lack of high affinity binding of agonist and guanine nucleotide effects on binding in the light peak fraction from control cells could be due to any of several factors that would result in a lack of normal interaction of β-adrenergic receptors and N_s.

We and most other workers in this field have routinely utilized a 10–20-min centrifugation at 20,000–40,000 × *g* to prepare a membrane fraction for the determination of receptor levels during catecholamine-induced desensitization. In retrospect, this is a less than optimal practice that has a number of ramifications in relation to the data presented in Fig. 9. Thus, receptors discussed as lost (degraded?) due to their failure to be detected in a sedimented membrane fraction may in fact be present in a form such as that in the light peak that does not sediment under the given conditions for centrifugation. These receptors might indeed be "lost" in a functional sense but could rapidly reattain their functional association with adenylate cyclase upon removal of catecholamine from the medium and thus would be distinguishable from receptors that were degraded or modified in a long-lasting way. Variabilities in time and other conditions of centrifugation could lead to variabilities in the extent to which receptors were recovered in the sedimentable fraction. As pointed out earlier,[3] we have observed variabilities in the loss of receptors at early time points of desensitization. Stadel *et al.* (13) have reported recently that essentially all of the β-adrenergic receptors that are lost from a plasma membrane fraction during incubation of frog erythrocytes with isoproterenol can be recovered in a sedimentable form by centrifugation at 158,000 × *g*.

The catecholamine-induced conversion of β-adrenergic receptors to another membrane form that exhibits properties similar to those observed with 1321N1 cells has been reported for frog erythrocytes (11–13) and C6 rat glioma cells (41). Since the kinetics of occurrence of catecholamine-specific desensitization are remarkably similar in these three systems, as well as in a number of other cell types (5, 7–9), it is likely that the phenomenon studied in the present manuscript represents a generally occurring response of the β-adrenergic receptor system to catecholamines. In analogy with polypeptide receptor systems (42, 43), the source of the altered membrane form of the β-adrenergic receptor would be a process of catecholamine-induced receptor endocytosis. Indeed, the fact that catecholamines induce a rapid, selective conversion of the β-receptor to a form that sediments during centrifugation with characteristics that clearly differentiate it from receptors bound to large fragments of plasma membrane is difficult to explain by a known mechanism other than endocytosis. Chuang and Costa (11), Chuang *et al.* (12), and Stadel *et al.* (13) have presented arguments in favor of the occurrence of agonist-induced endocytosis of the β-adrenergic receptor of frog erythrocytes. Intact cell binding assays with desensitized 1321N1 cells also suggest that, during short-term incubation with catecholamines, receptors are rapidly sequestered in a

homogeneous preparations of rat liver N_s range between 7 and 17 μmol/min/mg of N_s when assayed according to standard procedures (22). Assuming a similar turnover number and molecular weight for the N_s of 1321N1 cells, we have used the data in Table I to calculate the amount of N_s in light and heavy peak fractions from sucrose gradients. These values (based on a specific activity of 12 μmol/min/mg of N_s) are: control light peak, 582 pmol; control heavy peak, 3475 pmol; desensitized light peak, 430 pmol; desensitized heavy peak, 2380 pmol.

localization that is inaccessible to membrane-impermeable β-adrenergic receptor radioligands.[7] Similar results have been obtained using glioma and lymphoma cells (44–46).

Acknowlegements—We are indebted to Angie Hodgin for her efforts in preparing the manuscript. We extend our appreciation to Dr. Alfred G. Gilman for his interest in this work and his support of the experiments carried out by one of us (J. K. N.).

REFERENCES

1. Harden, T. K., Su, Y. F., and Perkins, J. P. (1979) *J. Cyclic Nucleotide Res.* **5,** 99–106
2. Su, Y.-F., Harden, T. K., and Perkins, J. P. (1979) *J. Biol. Chem.* **254,** 38–41
3. Su, Y.-F., Harden, T. K., and Perkins, J. P. (1980) *J. Biol. Chem.* **255,** 7410–7419
4. Doss, R. C., Perkins, J. P., and Harden, T. K. (1981) *J. Biol. Chem.* **251,** 12281–12286
5. Shear, M., Insel, P. A., Melmon, K. L., and Coffino, P. (1976) *J. Biol. Chem.* **251,** 7572–7576
6. Wessels, M. R., Mullikin, D., and Lefkowitz, R. J. (1978) *J. Biol. Chem.* **253,** 3371–3373
7. Homburger, V., Lucas, M., Cantau, R., Barabe, J., Penit, J., and Bockaert, J. (1980) *J. Biol. Chem.* **255,** 10436–10444
8. Iyengar, R., Bhat, M. K., Riser, M. E., and Birnbaumer, L. (1981) *J. Biol. Chem.* **256,** 4810–4815
9. Green, D. A., Friedman, J., and Clark, R. B. (1981) *J. Cyclic Nucleotide Res.* **3,** 161–172
10. Harden, T. K., Cotton, C. U., Waldo, G. L., Lutton, J. K., and Perkins, J. P. (1980) *Science (Wash. D. C.)* **210,** 441–443
11. Chuang, D.-M., and Costa, E. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 3024–3028
12. Chuang, D.-M., Kinnier, W. J., Farber, L., and Costa, E. (1980) *Mol. Pharmacol.* **18,** 348–355
13. Stadel, J. M., Strulovici, B., Nambi, P., Lavin, T. N., Briggs, M. M., Caron, M. G., and Lefkowitz, R. J. (1983) *J. Biol. Chem.* **258,** 3032–3038
14. Howlett, A. C., Sternweis, P. C., Macik, B. A., Van Arsdale, P. M., and Gilman, A. G. (1979) *J. Biol. Chem.* **254,** 2287–2295
15. Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L., and Gilman, A. G. (1977) *J. Biol. Chem.* **252,** 5761–5775
16. Scarborough, G. A. (1975) *J. Biol. Chem.* **250,** 1106–1111
17. Lutton, J. K., Frederich, R. C., Jr., and Perkins, J. P. (1979) *J. Biol. Chem.* **254,** 11181–11184
18. Barovsky, K., and Brooker, G. (1980) *J. Cyclic Nucleotide Res.* **6,** 297–307
19. Witkin, K., and Harden, T. K. (1981) *J. Cyclic Nucletoide Res.* **7,** 235–246
20. Harden, T. K., Scheer, A. G., and Smith, M. M. (1982) *Mol. Pharmacol.* **21,** 570–580
21. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem.* **58,** 541–548
22. Sternweis, P. C., and Gilman, A. G. (1979) *J. Biol. Chem.* **254,** 3333–3340
23. Sternweis, P. C., Northup, J. K., Smigel, M. D., and Gilman, A. G. (1981) *J. Biol. Chem.* **256,** 11517–11526
24. Schleifer, L. S., Kahn, R. A., Hanski, E., Northup, J. K., Sternweis, P. C., and Gilman, A. G. (1982) *J. Biol. Chem.* **257,** 20–23
25. Bokoch, G. B., Katada, T., Northup, J. K., Hewlett, E. L., and Gilman, A. G. (1983) *J. Biol. Chem.* **258,** 2072–2075
26. Laemmli, U. K. (1970) *Nature (Lond.)* **227,** 680–685
27. Hinton, R. H., and Reid, E. (1976) in *Mammalian Cell Membranes* (Jameson, G. A., and Robinson, D. M., eds) Vol. 1, pp. 161–197, Butterworth Inc., Boston
28. Wallach, D. F. H., and Lin, P. S. (1973) *Biochim. Biophys. Acta* **300,** 211–254
29. Fleischer, S., and Kervina, M. (1974) *Methods Enzymol.* **31,** 6–39
30. Baginski, E. S., Foa, P. P., and Zak, B. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed) Vol. 2. pp. 876–880, Academic Press, New York
31. Swanson, M. A. (1950) *J. Biol. Chem.* **184,** 647–659
32. Walter, K., and Schutt, C. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed) Vol. 2, pp. 856–860, Academic Press, New York
33. Bretz, R., and Staubli, W. (1972) *Eur. J. Biochem.* **77,** 181–192
34. Bergeron, J. J. M. (1979) *Biochim. Biophys. Acta* **555,** 493–503
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
36. Katada, T., and Ui, M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 3129–3133
37. Katada, T., and Ui, M. (1982) *J. Biol. Chem.* **257,** 7210–7216
38. Meeker, R. B., and Harden, T. K. (1982) *Mol. Pharmacol.* **22,** 310–319
39. Wessels, M. R., Mullikin, D., and Lefkowitz, R. J. (1979) *Mol. Pharmacol.* **16,** 10–20
40. Sahyoun, N., Hollenberg, M. D., Bennett, V., and Cuatrecasas, P. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 2860–2864
41. Frederich, R. M., Waldo, G. L., Harden, T. K., and Perkins, J. P. (1983) *J. Cyclic Nucleotide Res.* **9,** 103–118
42. Brown, M. S., and Goldstein, J. L. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 3330–3337
43. Pastan, I. H., and Willingham, M. C. (1982) *Annu. Rev. Physiol.* **43,** 239–250
44. Staehelin, M., and Simons, P. (1982) *Eur. Mol. Biol. Org. J.* **1,** 187–190
45. Staehelin, M., and Hertel, C. (1983) *J. Recept. Res.* **3,** 35–43
46. Hertel, C., Staehelin, M., and Perkins, J. P. (1983) *J. Cyclic Nucleotide Res.* **9,** 119–128
47. Perkins, J. P., Waldo, G. L., and Harden, T. K. (1982) *Fed. Proc.* **41,** 1327
48. Northup, J. K., Smigel, M. D., and Gilman, A. G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **257,** 11416–11423

[7] M. L. Toews, C. Hertel, T. K. Harden, and J. P. Perkins, unpublished observations.