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Direct Insertion and Fluorescence Studies of Rhodamine-labeled β-Adrenergic Receptors in Cell Membranes

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Summary. Previous studies utilizing the fluorescence of propanolol as a probe for the beta-adrenergic receptor showed that this receptor is motionally constrained. To further study the beta-adrenergic receptor *in situ* we have reinserted rhodamine-labeled beta-receptors into cell membranes. This report presents documentation of their insertion and physiologic viability. Beta-receptors were purified by affinity chromatography (10,000-fold), then fluorescently labeled with tetramethyl rhodamine isothiocyanate, repurified (55,000-fold) and incubated with rat pancreatic islet cells. The binding of ^{3}H-dihydroalprenolol by these cells was increased from a B_{max} of 168 ± 2 to 309 ± 20 fmol/mg protein with no change in K_d. Various treatments which remove peripheral membrane proteins, e.g. NaOH, lithium diiodosalicylate, and trypsinization, did not alter binding by the cells with inserted receptors indicating that the receptors inserted into cell membranes. In islet cells treated with Koshland's reagent I, beta-adrenergic binding was completely abolished, but following incubation with isolated beta-receptors, the cells bound beta-adrenergic radioligand with a B_{max} of 100 fmol/mg protein, indicating functionality on the part of the inserted receptors. Furthermore, insertion of isolated receptors into frog erythrocytes resulted in increased production of cAMP in response to added isoproterenol. In pancreatic islet cells, incubation with labeled receptors caused the fluorescence to shift in wavelength with increased intensity indicating a shift from an aqueous to a lipid environment, probably into the membrane. Using fluorescence (P), it was found that the inserted receptors became motionally constrained to a P of 0.38 (limiting P_o = 0.42) during the first 15 min, remaining so for at least 2 hr. Colchicine (5 μg/ml) caused a decrease in P to 0.18 indicating release of constraint. Isoproterenol (10^{-5} M) caused a rapid decrease to P = 0.15. This effect was blocked by propranolol. Propranolol itself (10^{-5} M) had no effect. These results indicate that the labeled receptors rapidly insert into cell membranes and also support the view that agonist activation of the receptor causes an increase in receptor mobility, presumably due to release of constraint from cytoskeleton elements.

Key Words adrenergic · beta-receptor · catecholamines · adenylate cyclase · cyclic AMP · fluorescence

Introduction

Our recent studies have been directed at the influence of the beta-adrenergic receptor on several biologic systems, namely chloride transport (Cherksey & Zadunaisky, 1981) and insulin secretion (Cherksey, Zadunaisky & Altszuler, 1982). The interaction of the beta-receptor with its associated adenylate cyclase has been studied extensively and various models to explain the mechanism of the interaction have been proposed (Cuatrecasas, 1974; Tolkovsky & Levitski, 1978; Cherksey, Zadunaisky & Murphy, 1980; Rodbell, 1980; Cherksey & Zadunaisky, 1981; Cherksey et al., 1983). Although the models are based on premises which should be experimentally verifiable, such studies have been limited by the inability to observe the receptor directly or to monitor its behavior under varied conditions. Our previous studies demonstrated that the intrinsic fluorescence of the beta-adrenergic antagonist, propranolol, served as a useful probe for the beta-adrenergic receptor (Cherksey et al., 1980; Cherksey & Zadunaisky, 1981). This approach permitted the monitoring of receptor events under a variety of experimental conditions and revealed that the receptor is motionally constrained, presumably by elements of the cell cytoskeleton. More recent studies by others have strengthened this conclusion (Henis et al., 1982).

The use of propranolol as a probe does not allow monitoring of events induced by the beta-adrenergic agonists or by other antagonists as these would displace propranolol from the receptor. To circumvent these limitations, studies were undertaken to solubilize and purify the receptor protein, covalently label it at loci other than at the binding site, and to attempt to reinsert the labeled protein into cell membranes. Methods for the purification of the beta-adrenergic receptors to apparent homogeneity have been described (Shorr, Lefkowitz & Caron, 1981), and the purified receptor protein has been reinserted into *Xenopus* erythrocyte membranes (Cerione et al., 1983) via a vesicle fusion technique. Our approach differed from the previous studies in that we used a direct insertion technique.

In the present studies, it was our goal to insert only a limited number of receptors into the cell membrane so that the newly inserted fluorescent receptors would act as reporters of the larger native receptor population and also would not alter significantly the existing balance between alpha- and beta-adrenergic receptors.

The pancreatic islet cells were chosen as the substrate for our studies because their beta-adrenergic status has been extensively studied (Cherksey et al., 1982, 1983; Cherksey & Altszuler, 1984) and the present studies would subsequently provide a basis to study the relationship between adrenergic receptor status and a well-characterized physiologic function, namely insulin secretion. In a number of experiments, the frog erythrocyte, the cell origin of the isolated beta-receptors, was also studied.

The present data reveal that a purified beta-adrenergic receptor has been fluorescently labeled with tetramethyl rhodamine isothiocyanate with no alterations in its binding properties. Furthermore, these labeled receptors retained significant function following insertion into whole cells.

The fluorescence studies indicate that the inserted receptors become motionally constrained in the cell membrane. The constraint is released by cytoskeleton disruption and also by the beta-agonist, isoproterenol. These results confirm and extend our previous studies and further strengthen the concept that interaction of an agonist with a beta-adrenergic receptor results in mobility of the receptor.

Materials and Methods

Materials

DL-Propranolol, (+)-isoproterenol, (−)-isoproterenol, (−)-alprenolol, digitonin, collagenase, trypsin, lithium diiodosalicylate, oligomycin, cytochrome *c*, colchicine, bovine serum albumin, polyethylene glycol 6000 and DEAE-cellulose were obtained from Sigma Chemical Co.; D-propranolol and L-propranolol were obtained from Ayerst; vinblastine and vincristine from Eli Lilly; Sepharose CL-4B and Sephadex G-50 from Pharmacia; tetramethyl rhodamine isothiocyanate and dithiothreitol from Research Organics; Koshland's Reagent I (5-hydroxy 2-nitrobenzyl bromide) was obtained from Pierce; ^{3}H-dihydroalprenolol (specific activity 55 Ci/mmol) and cyclic AMP assay kits were from Amersham. All other chemicals were reagent grade or better.

Receptor Solubilization

Bullfrog (*R. catesbeiana*) erythrocyte membrane fragments were prepared as previously described (Cherksey et al., 1980). Erythrocytes were lysed in hypotonic buffer and centrifuged at 40,000 × *g* for 40 min. The pellet was resuspended in Ca^{++}-free Ringer's solution and recentrifuged at 40,000 × *g* for 40 min. This washing procedure was repeated three additional times. The membranes were then frozen until material from 20 to 40 frogs had been collected, representing 200-ml packed red cells starting material. The frozen membranes were thawed and pooled. The extent of subsequent purifications was assessed using the binding of ^{3}H-dihydroalprenolol to the membrane preparation.

Prior to further purification, membranes were layered over a bed of 25% sucrose and centrifuged at 5000 × *g* for 20 min. Binding activity and protein content of the supernatant were determined and the supernatant was taken for further purification. The supernatant was centrifuged at 40,000 × *g* for 40 min and the pellet was resuspended in 150 ml of Ca^{++}-free frog Ringer's containing 1% digitonin and incubated for 4 hr with stirring at 23°C. The solution was then centrifuged at 40,000 × *g* for 1 hr. The supernatant was further purified using an affinity gel of alprenolol-Sepharose 4B (Caron et al., 1979). The digitonin solubilized material was first desalted on a column of Sephadex G-50 equilibrated with (mM): 100 NaCl, 10 Tris HCl, 0.5 dithiothreitol (pH 7.4). The desalted soluble extract was reacted batchwise with 50 to 60 ml of alprenolol-Sepharose gel and cycled twice through the affinity gel as described (Shorr et al., 1981). Samples were taken at each step for determination of protein and binding activity. At this point, the preparation exhibited an approximately 10,000-fold enhancement in binding activity. The protein solution was then concentrated to a volume of 3 to 5 ml by dialysis against polyethylene glycol 6000 flakes. Protein concentration ranged from 40 to 100 μg/ml as determined by Lowry assay (Lowry et al., 1951) using bovine serum albumin as the standard.

Receptor Binding Assays

The binding activity of the membrane preparation was determined via a filter binding assay previously described (Cherksey et al., 1982), utilizing ^{3}H-dihydroalprenolol as the radioligand. Incubations were performed in 1.0 ml volumes containing 0.5 ml of membrane suspension (0.5 to 1.0 mg/ml protein), 0.2 ml ^{3}H-ligand and either 0.3 ml of buffer or of a displacing ligand. Incubations were for 30 min at 23°C in triplicate.

After incubation, each tube was rapidly filtered under vacuum through Whatman GF/A glass fiber filters (24 mm) prewashed with insulin which was found to reduce nonspecific binding to the filters. The filters were washed five times with buffer, dried under vacuum and placed in scintillation vials containing 0.7 ml water and 10 ml Liquiscint® (National Diagnostics, Sommerville, N.J.). In each experiment, nonspecific binding of the ligand to the cells and/or membranes was determined by measuring the binding of the ligand to the cells in the presence of 10^{-5} and 10^{-6} M propranolol. Two concentrations of propranolol were utilized to assure the validity of the nonspecific binding measurement.

The binding of ^{3}H-dihydroalprenolol to the solubilized receptors was determined using a polyethylene glycol precipitation method (Venter, Fraser & Harrison, 1980). Protocols similar to those described for membranes and cells were utilized. After a 30-min incubation, 600 μg of gamma globulin in a volume of 50 μl was added to each tube, followed by the addition of 1 ml of 30% polyethylene glycol 6000, giving a final polyethylene glycol concentration of 15%. The tubes were incubated for 2 hr at 4°C with shaking. Each tube was then rapidly filtered under vacuum through Whatman GF/A glass fiber filters (24 mm) prewashed with insulin. The filters were washed twice with 2 ml volumes of

5% polyethylene glycol, dried under vacuum and placed in scintillation vials containing 0.7 ml water and 10 ml Liquiscint. Specific binding was determined in a similar manner as described for membrane binding.

Rhodamine Labeling

Tetramethyl rhodamine isothiocyanate (TRITC) was conjugated to the receptor protein using a dialysis method (Blakeslee & Baines, 1976). The receptor protein (1 ml, 40 to 100 μg, determined via Lowry assay) was added to 4 ml of 0.08 M borate buffer (pH 9.3) containing 0.4 M NaCl and 0.05% digitonin and placed in a dialysis bag. Three mg of TRITC in 0.2 ml DMSO were added to 100 ml 0.08 M borate buffer (pH 9.3) containing 0.1% digitonin. The receptor protein was dialyzed against this solution for 12 hr at 4°C with three changes of buffer. The rhodamine-labeled protein was then chromatographed on DEAE-cellulose (2.5 × 18 cm) at a flow rate of 5 ml/15 min. Five-ml fractions were collected and protein content, rhodamine fluorescence and binding activity were determined. Fractions containing both rhodamine fluorescence and binding activity (typically fractions 20 to 40) were pooled and the volume reduced to 2 ml by dialysis against polyethylene glycol 6000 flakes.

In order to verify that the rhodamine had reacted to form a covalent bond to the receptor protein, aliquots of the preparation were set aside and rechromatographed one week later. The elution profile obtained was identical to the original substantiating that the rhodamine label was covalently linked to the protein. Prior to further studies, the receptor preparations were further purified by a third step of affinity chromatography against the alprenolol-Sepharose gel, which resulted in a 55,000-fold purification of the receptor. Electrophoresis (Laemmli, 1970) on a uniform 10% SDS-polyacrylamide gel revealed a broad band with an apparent molecular weight (relative to the mobility of known standards) of 55,000 to 68,000 and a second minor band of 27,000. These results are similar to those previously reported (Shorr et al., 1981; Stiles et al., 1983).

Islet Cell Preparation

The pancreas was obtained from male Sprague-Dawley rats (225 to 300 g). The islets were obtained by the sedimentation method of Lacy and Kostianovsky (1967). The islets were cultured for 24 to 48 hr and cells obtained as previously described (Cherksey & Altszuler, 1984). The protein content of the cell suspension was determined by the method of Lowry et al. (1951) and ranged from 0.5 to 1 mg/ml. Cell viability was determined by the trypan blue dye exclusion test and only material in which at least 90% of the cells were viable was used for further studies.

Receptor Insertion

The receptor protein solution obtained from the third cycle of Sepharose-alprenolol gel affinity chromatography was dialyzed for 24 hr against four changes of 10 mM Tris HCl/100 mM NaCl (pH 7.4). The dialysate was then chromatographed on Sephadex G-50 followed by chromatography on DEAE-cellulose. The protein-containing fractions were pooled and the concentration reduced to 2 ml by dialysis against polyethylene glycol 6000 flakes. The protein concentration of this solution ranged from 0.1 to 4 μg/ml. The purified receptor preparation was then incubated with intact whole cells. Cells were prepared in Hank's solution at a protein concentration of 50 μg/ml. One ml of the purified receptor preparation was added to 10 ml of the cell solution and incubated for 1 hr at room temperature with gentle mixing. At the end of this time, the solution was centrifuged at 2000 rpm for 10 min to obtain the cells, which were resuspended in Hank's solution and recentrifuged. This procedure was repeated two additional times before the cells were used for further studies. In all experiments, cells carried through the centrifugation and washing steps but without added receptor protein were utilized as controls.

Receptor Localization Experiments

To determine if the rhodamine-labeled receptors had actually inserted into the membrane environment or were associated with the membrane as peripheral proteins, both whole cell preparations and membrane preparations were subjected to treatments which are known to reveal peripheral membrane proteins. Pancreatic islet cells were initially incubated with the rhodamine-labeled receptors as described above and the cells were then lysed by placing the cells in a hypertonic buffer (10× Krebs-bicarbonate, pH 7.4) followed by homogenization with two strokes of a Teflon® pestle at 500 rpm. Lysis of the cells was confirmed by microscopic examination. The homogenate was then centrifuged 4000 × *g* for 20 min at 4°C. The pellet was discarded and the supernatant which contained the membrane fragments was placed on a bed of 25% sucrose and recentrifuged at 20,000 × *g* for 30 min. The supernatant from this step was recentrifuged at 37,000 × *g* for 30 min and the pellet was taken for study of its beta-adrenergic binding. Cells which had not been preincubated with rhodamine-labeled receptor were also subjected to the above procedure and served as controls.

The cells were also subjected to a number of chemical and enzymic treatments. Both control and rhodamine-labeled receptor-incubated cells were subjected to reaction with the enzyme trypsin at a concentration of 25 μg/ml in a Ca^{++}-free Tris-HCl buffer (50 mM Tris, pH 7.4) containing 5 mM EDTA. The preparations were incubated for 5 min at 37°C. The cells were then washed with the Tris buffer and binding of the beta-adrenergic radioligand ^{3}H-dihydroalprenolol was determined. In other experiments, cells were treated with the detergent lithium diiodosalicylate at a concentration of 40 mM at 37°C for 30 min. The cells were then washed five times with Tris buffer, resuspended in 50 mM Tris-HCl/5 mM $MgCl_2$ and ^{3}H-dihydroalprenolol binding determined. Additionally, cells were treated with 0.1 N NaOH for 30 min. This treatment was found to be particularly harsh and destroyed the integrity of the cells. In order to determine beta-binding to the NaOH-treated material, membranes were obtained. The preparation was first centrifuged at 37,000 × *g*, then resuspended into hypotonic buffer (5 mM Tris-HCl, pH 7.4), homogenized with five strokes of a Teflon® pestle at 500 rpm. The homogenate was centrifuged at 37,000 × *g* for 30 min and the resulting pellet was resuspended into 50 mM Tris-HCl/5 mM $MgCl_2$. ^{3}H-dihydroalprenolol binding to the preparation was then determined.

Lastly, cells were subjected to chemical modification of the membrane proteins by the reagent 5-hydroxy-2-nitrobenzyl bromide (Koshland's Reagent I) which is known to alter beta-receptor binding (Cherksey, Murphy & Zadunaisky, 1981). To chemically modify the beta-receptors, the cells were incubated, in a 1-ml volume, with 10^{-6} M Koshland's reagent for 30 min at 25°C, in the dark. The cells were then washed five times with 50 ml of 5 mM Tris-HCl to remove any residual reagent. The cells were

Table 1. Purification of frog erythrocyte β-receptor

Step	Yield for step	Overall yield	Binding activity (pmol/mg protein)	Purification (-fold)
Frog erythrocyte membrane fragments	100	100	0.14	1
Membranes after centrifugation on sucrose bed	90	90	0.3	2
Digitonin-solubilized protein	70	56	0.95	6.7
First alprenolol-gel batch-step	55	30	100	740
Second alprenolol-gel batch-step	40	12	1500	10,700
Final alprenolol-gel	25	3	9000	55,000

then incubated with the rhodamine-labeled receptors and radioligand binding was determined. In an additional series of experiments, cells were reacted with Koshland's reagent in concentrations ranging from 10^{-9} to 10^{-5} M, using the above protocol. One-half of the cells were then incubated with rhodamine-labeled receptors as previously described. Cyclic AMP accumulation with isoproterenol stimulation was determined as described below. Dose-response curves were then constructed. In all experiments, normal cells were carried through the procedures to serve as controls.

cAMP Determination

Cyclic AMP was determined in frog red blood cells and attempts were made to determine cAMP in pancreatic islet cells using our previously described procedures (Mendelsohn, Cherksey & Degnan, 1981). The levels of cAMP in the pancreatic islet cell preparation were below the detection limits of the assay. The cAMP levels were determined by suspending the frog red blood cells in buffer containing 5.6 mM glucose and 1-ml aliquots of the cell suspension were placed into easy assay tube containing 30 mM theophylline in a 0.5-ml volume giving a final theophylline concentration of 10 mM. The cells were incubated for 15 min following which 50 μl isoproterenol was added to give final concentrations ranging from 10^{-5} to 10^{-9} M and the incubation continued for an additional 30 min. The tubes were then placed in a boiling water bath for 10 min, centrifuged at 47,000 × *g* for 30 min and the supernatant saved for cAMP assay. Results were calculated on the basis of cell number, determined by counting the cell suspension in a hemocytometer.

Fluorescence Measurements

Fluorescence excitation and emission spectra were obtained for the rhodamine-labeled receptors in solution and during incubations with cells and membrane preparations. In each experiment the background fluorescence for the cells or membranes was determined and was subtracted from the experimental values obtained later for the same preparation. Rhodamine-labeled receptor protein (0.1 to 4 μg/ml) was then added directly into the quartz fluorescence cell and fluorescence excitation and emission spectra were obtained. Although it has been shown previously that correction for an inner-filter effect is not significant when the total optical absorbance of the sample in the cuvette is below 0.4 units at the excitation maximum of rhodamine (Cherksey et al., 1980), we chose to make all measurements at an optical absorbance of less than 0.1 units. In all experiments, appropriate corrections were made for inner-filter effects (Christmann et al., 1980).

Fluorescence spectra were obtained on a modified Aminco Bowman SPF spectrofluorometer. High-voltage was supplied by an auxiliary regulated power supply and output could be quantitated externally on a fast picoammeter (Keithly Instruments, Cleveland, Ohio). Steady-state fluorescence depolarization was examined with the aid of Glan prism polarizers mounted in both the excitation and the emission beams. Polarization corrections for the monochrometer were determined with a solution of N-phenyl-1-naphthylamine in spectrograde isooctane (Weber, 1972). Polarization spectra were examined for scattering by examination of serial dilutions of the whole cell and of the membrane preparations and extrapolation of all observed depolarizations to infinite dilution. The limiting polarization of the rhodamine-labeled beta-receptors was determined by examination of the depolarization of the preparation in glycerol/water solutions of known viscosity at 26°C. The observed polarizations were extrapolated to infinity viscosity (Vanderkooi et al., 1974). This procedure yielded a value for the limiting polarization P_o of the rhodamine-labeled receptors of $P_o = 0.42$.

Calculations

The results of the binding assays were expressed as Scatchard plots (Scatchard, 1949). Log dose-response curves were obtained for the cyclic AMP accumulation studies. Hofstee plots and Hill coefficients were obtained for the data. The data were tested by computer analysis of the curves using nonlinear least-squares curve fitting (Munson & Rodbard, 1980).

Fluorescence intensities were available as peak heights directly from the spectra. All spectra were corrected for background fluorescence and for inner-filter effects. Fluorescence depolarization was calculated in the manner described by Weber (Weber, 1953):

$$P = \frac{I_{\parallel} - I_{\perp}c}{I_{\parallel} + I_{\perp}c}$$

where P is the observed polarization, $I_{\parallel}$ is the fluorescence intensity with the polarizers parallel, $I_{\perp}$ is the fluorescence intensity with the polarizers perpendicular and c is the monochrometer correction factor.

Because the relationship of the rhodamine-labeled receptor and the membrane lipids is unclear, it would not be valid to reduce these data to microviscosities (Yguerbide, 1972). It is preferable to utilize the more phenomenological polarization ratio which has been demonstrated (Dale, Chen & Prand, 1977) to be a satisfactory indicator of microenvironment.

Results

The results obtained for the receptor solubilization using batch methods were similar to those obtained for this receptor as reported by other groups (Caron et al., 1979; Shorr et al., 1981). These results are summarized in Table 1.

Prior to any purification steps, ^{3}H-dihy-

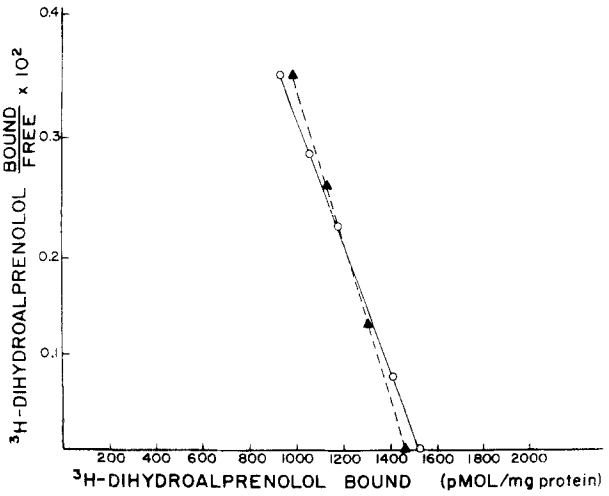

Fig. 1. Scatchard plot of the binding of ^{3}H-dihydroalprenolol to purified beta-adrenergic receptors. Solid line represents binding to affinity gel purified receptor preparation. Dotted line represents binding to rhodamine-labeled receptor preparation. Differences in Scatchard plots are not statistically significant indicating that rhodamine labeling of receptor has little effect on ligand binding

droalprenolol binding (B_{max}) to the frog red blood cell membrane fragments was determined to be 140 fmol/mg of membrane protein. Centrifugation of the membrane fragments over a bed of 50% sucrose at 15,000 × *g* for 20 min increased the binding to 300 fmol/mg protein. The digitonin extract was found to bind 950 fmol/mg protein. The most dramatic enhancement in the binding activity was found after the first stage of batch processing: the binding was 100 pmol/mg protein, which is a 100-fold greater binding activity than with the digitonin-solubilized material. The preparation from the second step of batch processing bound 1500 pmol/mg protein.

The Scatchard plot of the ^{3}H-dihydroalprenolol binding to receptor protein after the second stage of alprenolol-gel purification is shown in Fig. 1. As shown in Fig. 1, the preparation exhibited an affinity (K_d) of 0.4 ± 0.1 nM with a B_{max} of 1500 ± 97 pmol/mg protein representing an approximately 10,000-fold enhancement in binding activity (Cherksey et al., 1980). Also shown in Fig. 1 is the Scatchard plot for the specific binding of ^{3}H-dihydroalprenolol to the rhodamine-labeled receptors. The rhodamine label appeared to have little effect on either the K_d or the B_{max} of the receptors. As described in Materials and Methods, prior to further studies this preparation was subjected to an additional step of batch-processing against the alprenolol-gel, yielding a 55,000-fold final purification. This is comparable to the results previously reported for this preparation after three cycles of affinity chromatography using batch processing methods (Shorr et al., 1981).

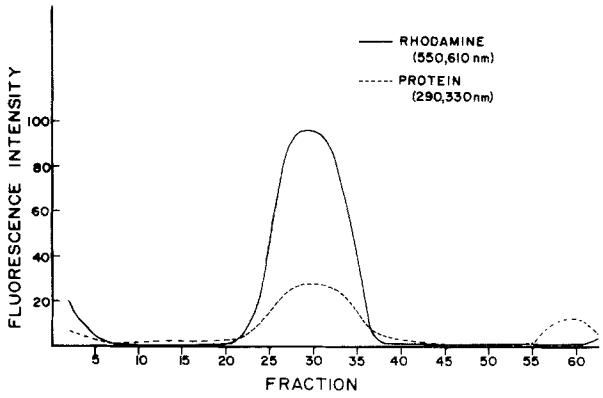

Fig. 2. Elution of rhodamine-labeled beta-receptors from DEAE-cellulose column (2.5 × 20 cm). Major peak (fractions 25 to 35) is due to rhodamine-labeled receptors. These fractions were pooled and concentrated for further studies. (Solid line, rhodamine fluorescence; broken line, tryptophan fluorescence)

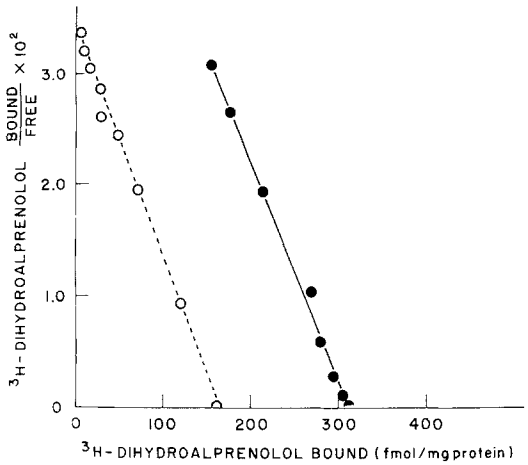

Fig. 3. Comparison of binding of ^{3}H-dihydroalprenolol to normal pancreatic islet cells (O----O) *vs.* islet cells incubated with isolated beta-receptors (●—●). Specific binding was found to increase from 168 to 309 fmol/mg protein. This additional binding was not removed by extensive washing, lysis, 0.1 N NaOH, or 40 mM lithium diiodosalicylate mild trypsinization

The elution profile of the rhodamine-labeled beta-receptors from the DEAE-cellulose column is shown in Fig. 2. Both the rhodamine and the protein co-elute in fractions 25 to 35, provided evidence supporting covalent labeling of the protein. Although the stoichiometry of the rhodamine labeling cannot be obtained with absolute certainty, it may be estimated from the quantum yields of the receptor tryptophan and from that of rhodamine. Assuming a value of 0.8 for the quantum yield of rhodamine and 0.15 for tryptophan, it is estimated that the stoichiometry of labeling is 0.75–0.85 to 1 versus a theoretical maximum of 1 : 1.

Figure 3 shows the Scatchard plot obtained for the binding of ^{3}H-dihydroalprenolol to the pancre-

Table 2. Binding of β-adrenergic ligand[a] to incubated cells

Condition	Intact	Lysed[b]	LIS[c]	NaOH[d]	HNBB[e]	Trypsin[f]
Control (7)[g]	168 ± 2	250 ± 2	150 ± 8	250 ± 22	0	102 ± 9
Incubated with receptors (7)	309 ± 20	475 ± 5	265 ± 10	368 ± 15	100 ± 12	275 ± 15

[a] Radioligand-^{3}H-dihydroalprenolol; B_{max} in fmol/mg protein.
[b] Cells homogenized with two strokes of Teflon® pestil, at 500 rpm, lysis confirmed microscopically; homogenate then centrifuged at 20,000 × *g* for 30 min over 25% sucrose. Assay performed on membrane fragments in supernatant.
[c] 40 mM lithium diiodosalicylate; 30-min incubation.
[d] 0.1 N NaOH; 30-min incubation.
[e] Koshland's Reagent I, 10^{-6} M; 30-min incubation; cells washed five times prior to assay.
[f] Incubated in Ca^{++}-free medium, trypsin 25 μg/ml/EDTA 5 mM for 5 min at 37°C, centrifuged and resuspended in Krebs-bicarbonate.
[g] Number of experiments; Mean ± SD.

atic islet cells. Specific binding to the beta-receptors of this preparation was found to exhibit a K_d of 1.23 ± 0.19 nM and a B_{max} of 168 ± 2 fmol/mg protein, in agreement with previously reported values (Cherksey et al., 1982, 1983). The Hill coefficient obtained from these data was less than unity. This is in agreement with previous studies of binding to these cells (Cherksey & Altszuler, 1984). The second Scatchard plot (solid line) shown in Fig. 3 was obtained when 10^8 to 10^9 cells were incubated with the rhodamine-labeled receptors (0.1 to 4 μg/ml) in 25 ml with gentle stirring. Comparison of the plots indicates that the K_d for ^{3}H-dihydroalprenolol is essentially the same, while the cells incubated with the labeled receptors exhibit a significantly enhanced binding (B_{max}) of 309 ± 20 fmol/mg protein. The Hill coefficient calculated from these data was also less than 1. Similar results were also obtained when the receptors were incubated with frog erythrocytes (B_{max} = 320 fmol/mg protein). Washing of the cells four times after incubation with receptors did not significantly alter this result.

Additional procedures to determine the cellular location of the rhodamine-labeled receptors were performed. These included lysis of the cells followed by differential centrifugation, mild trypsinization, treatment with lithium diiodosalicylate, treatment with NaOH, and reaction with Koshland's reagent I.

Lysis of the cells would be expected to remove receptors which are not in the membrane, but would also cause a loss of total protein, with a resultant increase in B_{max}, since the latter value is expressed on the basis of tissue protein. Pancreatic islet cells were homogenized after incubation with receptors, with two strokes of a Teflon® pestle at 500 rpm, in hypertonic buffer (10× Krebs-bicarbonate) and the resulting homogenate was then centrifuged over 25% sucrose at 20,000 × *g* for 30 min. The supernatant was centrifuged at 37,000 × *g* for 30 min and the resulting pellet was resuspended in 50 mM Tris buffer. Binding of ^{3}H-dihydroalprenolol to the membrane fragments was then determined. As shown in Table 2, lysis followed by centrifugation increased binding in both control and incubated cells. The control value of B_{max} was 250 ± 2 fmol/mg protein while the incubated cells exhibited a B_{max} of 475 ± 5 fmol/mg protein.

Also shown in Table 2 are the results obtained when normal and receptor-incubated cells were treated with trypsin (25 μg/ml) and EDTA (5 mM) for 5 min at 37°C in Ca^{++}-free medium. The trypsinized normal cells exhibited a reduction in binding to 102 ± 9 fmol/mg protein from the control value of 168 ± 2 fmol/mg protein. The receptor-incubated cells exhibited a reduction from 309 ± 20 to 275 ± 15 fmol/mg protein. Although results for normal and incubated cells seem to differ, statistical analysis utilizing a two-tailed Student's *t*-test indicated no statistical difference at the 0.05 level, presumably due to the large standard deviations obtained for the binding to trypsinized preparations.

The physiological viability of the inserted receptors was tested using isoproterenol to stimulate cAMP production by receptors inserted into the normal red cell membrane. The cAMP response was determined as previously described. The erythrocytes were used because their cAMP response was sufficient for detection with our cAMP assay, whereas islet cell cAMP was below the limits of detection for the assay. As shown in Fig. 4, the added receptors enhanced the cAMP response to isoproterenol stimulation, the differences at all concentrations being statistically significant, using the two-tailed Student's *t*-test. It must be noted, however, that the curves for the control and cells with reinserted receptors are not identical in shape, possibly indicating altered behavior by the added receptors. To test the possibility that the enhanced responses of cAMP were due to a nonspecific per-

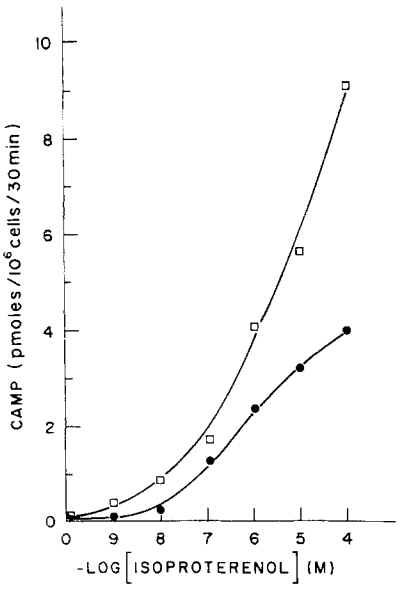

Fig. 4. Comparison of isoproterenol-stimulated cAMP production by normal frog red blood cells (●) and cells preincubated with isolated beta-receptors (□). Frog erythrocyte cells which contain added receptors exhibit a 1 to 2 order of magnitude greater sensitivity to isoproterenol and also higher peak levels of cAMP

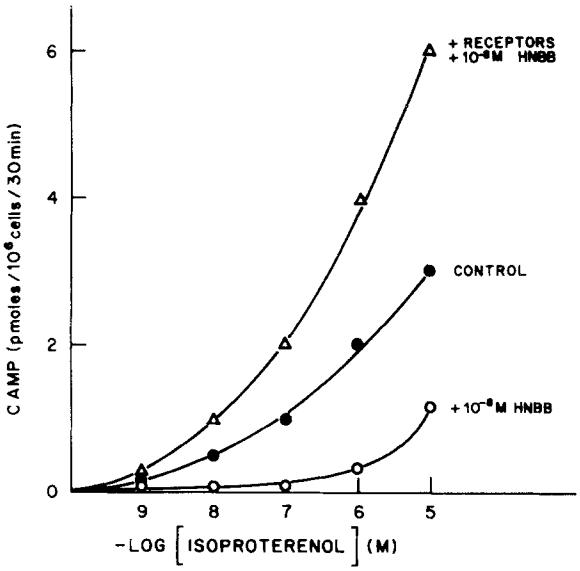

Fig. 5. Cells reacted with HNBB (Koshland's Reagent I) were incubated with rhodamine-labeled receptors as described in Materials and Methods, and isoproterenol-stimulated cAMP accumulation was determined. Reaction of cells with 10^{-8} M HNBB shifted the dose-response curve (○) two orders of magnitude to the right as compared to unreacted control cells (●). Incubation of reacted cells with receptor protein returned the responsiveness of the cells (△)

turbation of the cells, the effects of incubation of the cells with other proteins and with a less purified receptor preparation were tested. Neither albumin nor oligomycin had an effect on isoproterenol-stimulated cAMP production while preincubation with cytochrome *c* produced a small decrease in cAMP production. The solubilized membrane material taken from the first batch process step also enhanced the cAMP response, which on an equal weight basis was intermediate (but not proportional) between control and cells with reinserted purified receptors.

To further document that the increased sensitivity of the receptor-incubated cells was due to the added receptors, the native receptors in cell membrane were first reacted with Koshland's Reagent and then incubated with receptors. As is shown in Fig. 5, reaction of cells with Koshland's reagent at a concentration of 10^{-8} M shifted the dose-response curve for cAMP production by isoproterenol stimulation two orders of magnitude to the right and also reduced the maximal response of the cells. Addition of receptor cells after reaction with Koshland's reagent restored the responsiveness of the cell to isoproterenol stimulation.

In another series of experiments, the fluorescence intensity and steady-state depolarization of the rhodamine were monitored as the labeled receptors were incubated with islet cells. When 0.1 μg of

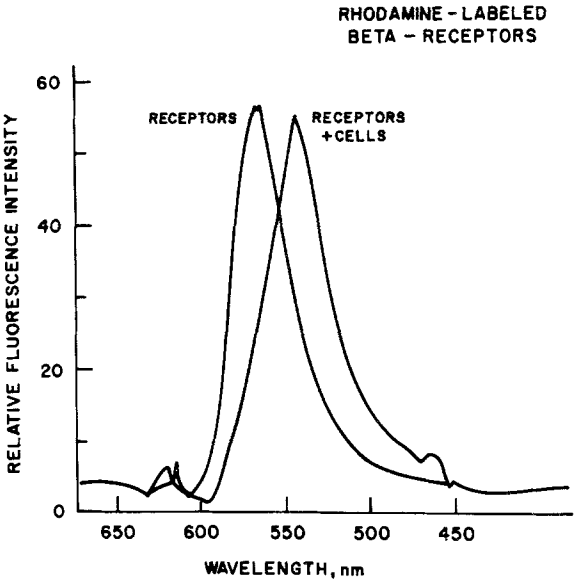

Fig. 6. Excitation spectra of rhodamine-labeled beta-receptors in absence and presence of cells. Addition of cells shifted the excitation maxima from 560 to 540 nm

the rhodamine-labeled beta-receptors were incubated with cells (50 μg/ml protein), the fluorescence was significantly intensified and shifted in wavelength. The excitation maximum shifted from 560 to 540 nm (Fig. 6) and the emission wavelength from

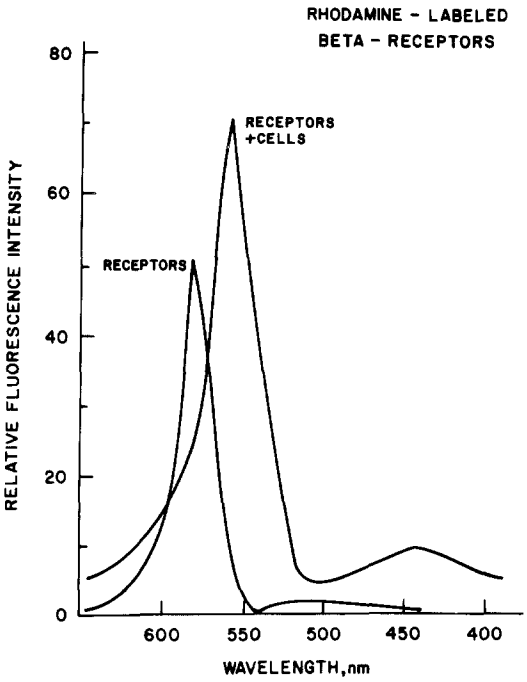

Fig. 7. Emission spectra of rhodamine-labeled beta-receptors in absence and presence of cells. Addition of cells cause a shift in the emission maxima from 585 to 565 nm with a significant increase in fluorescence intensity

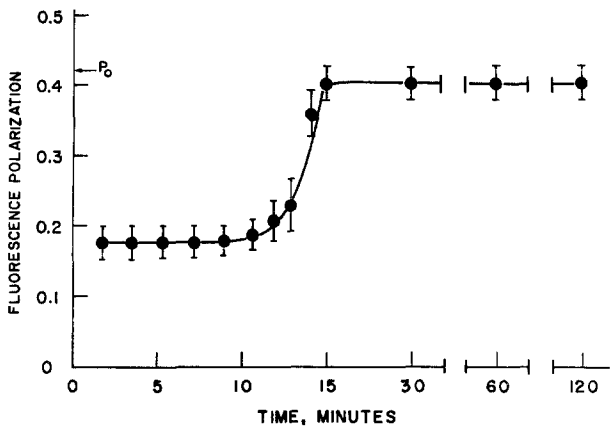

Fig. 8. Fluorescence polarization of rhodamine-labeled beta-receptors incubated with pancreatic islet cells. Immediately after addition of 0.1 μg rhodamine-labeled receptor protein, fluorescence polarization is minimal but is seen to increase with time. At 15 min the polarization has approached the limiting polarization (P_o) indicating virtually complete rotational constraint. Upon association of the receptor with the islet cells, the fluorescence intensity increased and underwent a blue shift

585 to 565 nm (Fig. 7). Figure 8 shows the fluorescence polarization when measured at an excitation wavelength of 540 nm and an emission wavelength of 565 nm. During the first 15 min after addition of the labeled receptors, the fluorescence polarization increased to 0.42,which is the limiting polarization value. This value was maintained during the 2-hr period of observation.

Addition of propranolol (10^{-5} M) had no effect on the fluorescence polarization (Fig. 9). Addition of colchicine (5 μg/ml) decreased the fluorescence polarization after a 30-min incubation period (Fig. 10), which is typical of this drug. Additional experiments utilizing the colchicine-like drugs vincristine and vinblastine produced similar results. Figure 11 shows that addition of 10^{-5} M (−)-isoproterenol produced an immediate, sharp decrease in the polarization, but this effect was not produced by (+)-isoproterenol. Addition of 10^{-6} M DL or L-propranolol blocked the decrease in polarization produced by (−)-isoproterenol, but 10^{-6} M D-propranolol did not.

Discussion

The ability to remove receptors from the cell membrane, label them and finally insert them into a physiologically or biochemically active system would provide a powerful tool to study receptor function. Isolation, solubilization and purification of receptor proteins has now been reported by a number of laboratories. The crucial subsequent step is the reinsertion of the receptors into a cell membrane and documentation of its physiological viability. In the case of the beta-adrenergic receptors, two approaches have been used, one involving the preinsertion of the receptor into lipid vesicles and then fusing the vesicles with the cell membrane (Eimerl, Neufeld, Korner and Schramm, 1980; Pederson & Ross, 1982; Cerione et al., 1983), and the other involving direct insertion of the receptors, as was done here. The vesicle fusion technique is highly efficient in adding receptor protein into the cell membrane. Such insertion of beta-receptors into the red blood cells of *Xenopus,* which do not normally possess beta-receptors, increased the capability of these cells to generate cyclic AMP (Cerione et al., 1983).

We have utilized the direct insertion approach for several specific reasons. Such direct insertion of receptors introduces only a small number of receptors into the cell membrane, allowing them to act as reporters of the larger native receptor population. Furthermore this approach allowed insertion of receptors with a minimum perturbation of the membrane environment. This is especially important due to the nature of the fluorescence studies which we conducted. Furthermore, minimal perturbation was deemed important since our ultimate interest is to study the physiologic status of adrenergic receptors in specific mammalian organs. In addition, it was

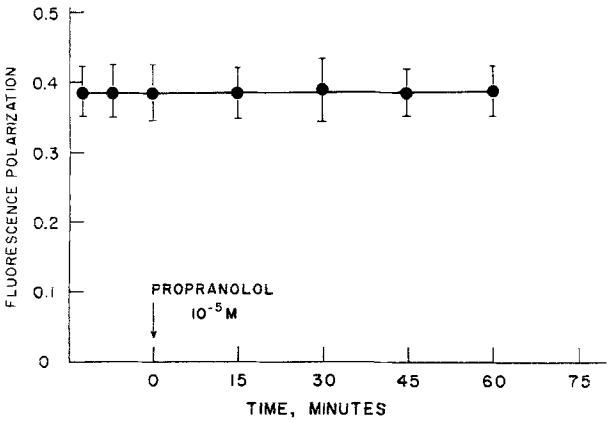

Fig. 9. Pancreatic islet cells were incubated with rhodamine-labeled beta-receptors as described for Fig. 8. Propanolol (10^{-5} M) was then added at $t = 0$. The addition of beta-antagonist was found to have no effect on polarization and thus on receptor constraint

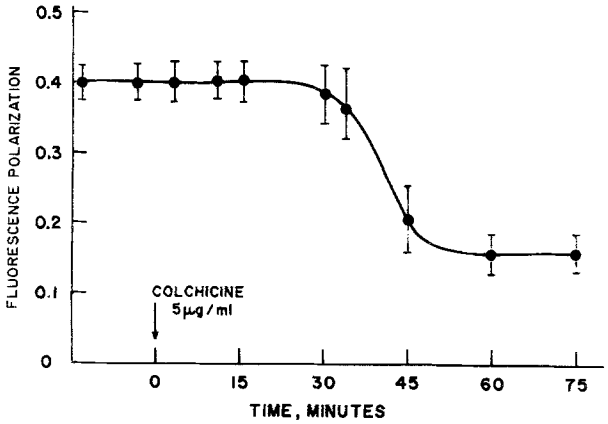

Fig. 10. Islet cells incubated with rhodamine-labeled beta-receptors were then treated with colchicine at a concentration of 5 μg/ml. After a lag period characteristic of this compound, the polarization was found to decrease indicating rotational mobility and release of constraint

also desirable not to distort the existing balance between the alpha- and beta-adrenergic receptors in the recipient membrane of the pancreatic beta-cells since this balance is important in modulating insulin secretion.

As evident from the experimental data, much effort has been devoted to document the insertion of the beta-receptor into the membrane and its localization. Firstly, it was demonstrated that the fluorescent label, rhodamine isothiocyanate, was attached by covalent bond to the highly purified beta-adrenergic receptor as indicated by the co-elution of the rhodamine and the protein on DEAE-cellulose (Fig. 2). The presence of the rhodamine molecule on the protein did not alter appreciably the binding activity of the receptor indicating that the attachment of the rhodamine occurred at loci removed from the ligand binding site. Although it is unclear that the proteins are uniformly labeled, the estimated stoichiometry of less than 1 : 1 suggests that the label is uniformly distributed.

Documentation of insertion was done in both frog erythrocytes and pancreatic islet cells, except when prevented by limitations of the system. Thus, fluorescence studies were not done in the erythrocytes because of the interference by hemoglobin, and cyclic AMP measurements were not performed on islet cells because the levels were below our limits of detection.

Incubation of the pancreatic islet cells with isolated beta-receptors increased the beta-adrenergic binding to the cells (Fig. 3). This is also supported by an increase in cAMP response to isoproterenol (Fig. 4). In order to more clearly demonstrate the contribution of the reinserted beat-receptors, the native beta-receptors of the cells were chemically

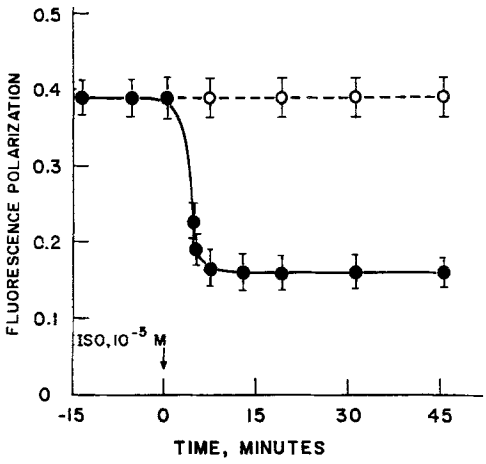

Fig. 11. Effect of isoproterenol on fluorescence polarization. Rhodamine-labeled beta-receptors were incubated with pancreatic islet cells as in Fig. 8. (−)-Isoproterenol (10^{-5} M) was added at $t = 0$. The polarization was found to decrease rapidly indicating that the beta-agonist caused a complete loss of rotational constraint (solid line). This effect was blocked by L-propranolol (dotted line) but not by D-propranolol. (+)-isoproterenol had no effect on the fluorescence polarization

modified with Koshland's reagent I which irreversibly reacts with the amino acid tryptophan. This reagent has been shown (Cherksey et al., 1981) to alter the structure of the beta-receptor binding site and prevent binding. Such treatment completely eliminated beta-adrenergic binding (Table 2) but, when these cells were then incubated with rhodamine-labeled beta-receptors, binding activity was returned to 100 fmol/mg. Since the effect of Koshland's is irreversible, this increased binding is attributed to the newly inserted receptors. Attempts

to alter the binding of the beta-adrenergic ligand to the cells using N-ethylmaleimide (NEM) were found to be ineffective, perhaps due to the fact that NEM was found to react not with the receptor protein itself but possibly with the nucleotide regulatory protein (Cherksey, 1984). The increased response to isoproterenol of red cells with added receptors (Fig. 4) indicates that the added receptors are physiologically viable. At the lower concentrations of isoproterenol, the control and receptor-incubated cells exhibited parallel curves for increased cAMP formation but the curves diverged at higher drug concentrations. The reason for this is not clear. The dose-response curves which we have obtained in the present study are similar to those previously reported (Cerione et al., 1983) for cAMP production by red cells from *Xenopus* when receptors had been reinserted using a vesicle fusion technique. The ability of the inserted receptors to return isoproterenol responsiveness to cells after treatment of the cells with Koshland's reagent (Fig. 5) provides further evidence that the newly inserted receptors are functionally incorporated into the membrane.

Localization within the membrane of the inserted receptors in the present study was verified in a number of different ways. The results obtained upon lysis of the cells followed by centrifugation indicate that the labeled receptors remained in the membrane fractions and thus were not simply taken up by the cell into the cytoplasm or into cell organelles.

It is not surprising that the receptor protein would re-enter the lipid environment of the cell membrane upon reduction of the detergent concentration. The structure of the receptor protein undoubtedly includes lipophilic regions to stabilize normal lipid-protein interactions (Tanford, 1982). Here, reduction of the digitonin concentration would favor, thermodynamically, the reinsertion of the lipophilic protein into the membrane or out of the aqueous environment, although the existence of a kinetic (energy) barrier might alter this result. It is also of interest that the receptor protein has reinserted with its binding region facing the outside environment and hence has assumed a correct orientation both to bind adrenergic ligands and to activate adenylate cyclase. This result suggests that the lipid-protein interactions might be specific in nature thereby forcing the protein into its correct orientation. Alternatively, the binding region of the protein might represent a large enough hydrophilic region so as to dictate the proper functional orientation of the protein. The present results cannot distinguish between these possibilities.

The experiments involving NaOH, lithium diiodosalicylate, and mild trypsinization were undertaken to assess the possibility that rhodamine-labeled proteins had not actually inserted into the cell membrane but rather were loosely associated with the cell as peripheral rather than as integral membrane proteins. The results obtained (Table 2) clearly indicate that they behave as integral membrane proteins. Treatment with 0.1 N NaOH or 40 mM lithium diiodosalicylate which would remove peripheral proteins, had no significant effect on either the native cell beta-adrenergic receptors or on the reinserted rhodamine-labeled receptors. It is noteworthy, however, that the amount of binding removed by trypsin is greater in the normal cells than in the receptor-inserted cells. Although this difference was not statistically significant due to the large standard deviation, it is possible that the difference is real. Thus it is conceivable that the normal cells contain two populations of beta-receptors, one being more accessible to trysin. These could represent the high- and low-affinity states of the receptor (Kent, Delean & Lefkowitz, 1980), or active and inactive receptors. The new receptors may have inserted into the region of the less-accessible receptor population or perhaps into a less-accessible membrane region.

The present results using rhodamine-labeled receptors confirm and extend earlier findings which utilized the fluorescence of propranolol as a probe for the beta-adrenergic receptor (Cherksey et al., 1980). The rhodamine-labeled receptors when added to the islet cells became rotationally constrained and colchicine, vinblastine, and vincristine released the constraint. Isoproterenol also produced a rapid release of the constraint. This effect was found to be stereospecific in that only the physiologically active (−)-isomer produced the effect of fluorescent polarization while the (+)-isomer did not. The effect produced by (−)-isoproterenol could be blocked with both DL-propranolol and the active L-isomer, but was not blocked by D-propranolol.

It is not possible to determine if the change in fluorescence polarization produced by the adrenergic agonist and by colchicine represents a release of receptors back into solution (or cytoplasm) or if the receptors are free in the membrane lipid. The first possibility seems unlikely as a narrow bandwidth (2 nm) was used and if the receptors left the lipid environment the consequent shift in wavelength would cause a reduction in fluorescence intensity, which was not the case. The more likely explanation is that the receptors remain in the membrane when activated by agonists. The small difference between the initial polarization values for the receptors in

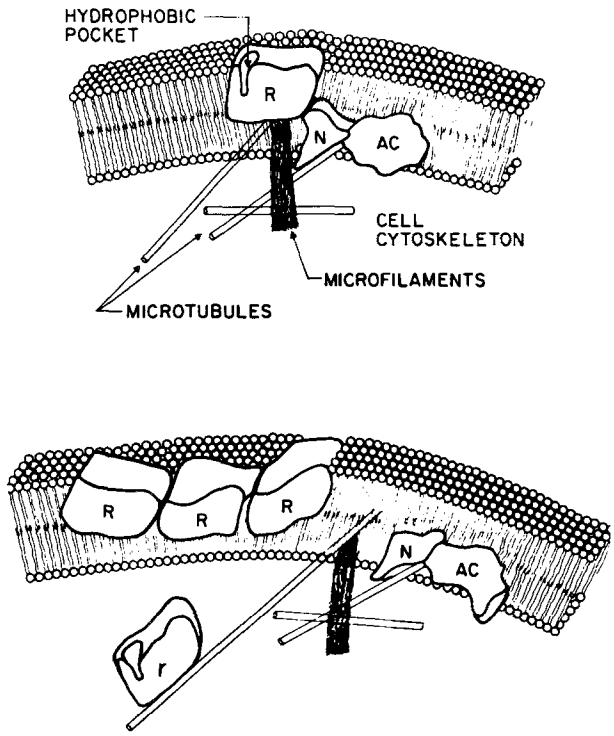

Fig. 12. (Upper) This figure summarizes the information that has been obtained using fluorescence methods and illustrates possible mechanism for agonist-induced activation of adenylate cyclase. The receptor, containing a hydrophobic pocket to which antagonists bind, is shown attached to the cytoskeleton either directly to microtubules or to microtubules via microfilaments. (Lower) After activation of the receptors by agonists, receptors (*R*) diffuse away from the adenylate cyclase (*AC*)-nucleotide regulatory site complex (*N*) releasing inhibition and form aggregates prior to internalization. A new receptor (*r*) replaces the activated receptor. Note that figure is not to scale

lipid (Fig. 6) and after release (Fig. 11) may represent an agonist-induced, and hence physiological, alteration in the lipids (Strittmatter, Hirata & Axelrod, 1979). Alternatively, it may represent a movement of the receptor protein in the membrane in a manner which permits more of the protein to be in the aqueous environment possibly due to release of cytoskeletal constraint.

A number of models have been suggested to account for the interaction of the beta-adrenergic receptor and its associated adenylate cyclase and nucleotide regulatory protein (Tolkovsky & Levitski, 1978; Rodbell, 1980; Braun, Tokovsky & Levitski, 1982; Swillens, 1982). The present study neither supports nor invalidates these models. However, as proposed earlier (Cherksey et al., 1980; Cherksey & Zadunaisky, 1981) and depicted in Fig. 12, an alternate, albeit speculative, model is suggested. The cytoskeleton normally maintains a fixed spatial relationship between the receptor and its effector subunits, forming a complex which is inactive in the basal state. Antagonists may bind at the hydrophobic site of the receptor and stabilize this inactive complex. Binding of agonist or hormone destabilizes the complex resulting in the release of the receptor from the cytoskeleton thus allowing activation of the adenylate cyclase by the nucleotide regulatory protein. Further studies will be necessary to determine the sequence of interactions between the specific components of cytoskeleton-receptor complex.

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