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Homologous Desensitization of the β-Adrenergic Receptor

Functional Integrity of the Desensitized Receptor from Mammalian Lung

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SUMMARY

Previous work has demonstrated that injection of rats with isoproterenol is rapidly (10 min) followed by the development of a homologous form of desensitization of the β-agonist-coupled adenylate cyclase in lung membranes. Half the receptor pool becomes sequestered in a light membrane fraction while the other half remains in the plasma membranes but becomes functionally uncoupled. In the present work we sought to assess whether "local sequestration" of the functionally intact receptor away from the effector adenylate cyclase in the plasma membrane contributes to the uncoupling of the β-adrenergic receptor observed in the plasma membranes. We tested the functionality of the desensitized β-adrenergic receptor in three different ways. We reconstituted the affinity chromatography purified control and "desensitized" receptors with pure N_s from human erythrocytes and assessed the ability to induce GTPase activity in N_s. Both control and desensitized β-adrenergic receptors stimulate similar levels of GTPase activity in N_s (852 ± 38 *versus* 738 ± 49 fmol of P_i released/30 min ($p > 0.05$, $n = 4$). To further assess the relative ability of control and desensitized β-adrenergic receptors to couple to another source of N_s we fused reconstituted β-adrenergic receptors to *Xenopus laevis* erythrocytes, which contain N_s and adenylate cyclase but essentially no β-adrenergic receptors. The functional interactions of control and desensitized β-adrenergic receptor with the adenylate cyclase system of the acceptor cells was assessed by measuring the β-agonist-stimulated adenylate cyclase activity and the agonist-induced formation of the high affinity state of the β-adrenergic receptor (R_H). Again both control and desensitized β-adrenergic receptors appeared to interact with N_s to the same extent.

To test if a local sequestration of the β-adrenergic receptor away from N_s within the plasma membrane might contribute to the uncoupling of the β-adrenergic receptors during desensitization, plasma membranes from control and desensitized lungs were treated with the fusogen polyethylene glycol to disrupt any compartmentalization of protein components within the plasma membrane. After polyethylene glycol treatment the previously uncoupled β-adrenergic receptors could be recoupled to N_s as assessed by the formation of R_H in agonist competition curves.

These data suggest that in marked contrast to the heterologous type of desensitization, homologous desensitization may involve a local sequestration of a functionally intact β-adrenergic receptor away from the adenylate cyclase effector system.

INTRODUCTION

Desensitization or tachyphylaxis is observed in many systems (1–3) and refers to the loss or blunting of cellular responsiveness to a hormone or drug after prolonged exposure to that hormone. In the adenylate cyclase system two major types of desensitization have been characterized. "Heterologous" desensitization results in a decreased responsiveness to the desensitizing hormone as well as to other hormones and in some instances nonhormonal stimulators including sodium fluoride and guanine nucleotides. cAMP analogs can mimic this process. An example of heterologous desensitization is seen in the turkey erythrocyte. Recent data indicate that in this

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system desensitization is associated with phosphorylation of the β-adrenergic receptor (4, 5). This stable modification of the receptor protein correlates with the functional alterations leading to the altered responsiveness of the adenylate cyclase to catecholamines (6).

Unlike the case for heterologous desensitization, in the "homologous" form of desensitization there is a loss or blunting of hormone responsiveness only to the desensitizing hormone. Homologous desensitization is observed in frog erythrocytes (2) and in mammalian lung (7) as well as in a number of cultured cell lines (1). In this form of desensitization a portion of the receptors (∼50%) appear to be sequestered out of the plasma membranes into an intracellular compartment (1, 7–9). These sequestered receptors are found in a "light vesicle" fraction which sediments at high centrifugal forces. Moreover, when the sequestered receptors from desensitized amphibian erythrocytes are directly fused to *Xenopus laevis* erythrocytes they can be shown to be functionally active (13). The remainder of the receptors copurify with the plasma membranes after desensitization. In the rat lung system in contrast to the frog erythrocyte these receptors are almost completely uncoupled as assessed by adenylate cyclase and ligand-binding experiments (7). However, it has not been known whether this uncoupling of receptors which copurify with the plasma membranes is due to an inherent decrease in their functionality or to a "microsequestration" within compartments of membrane contiguous with the plasma membranes (10–12). Moreover, the functional role of the recently discovered receptor phosphorylation which occurs in homologous desensitization (14) in causing either uncoupling or sequestration (or both) remains unknown.

Accordingly in the present study we sought to use an *in vivo* model of homologous desensitization, the rat lung, to probe the functional alterations of the "desensitized" β-adrenergic receptors which still copurify with the plasma membranes. To do this we have purified and reconstituted control and desensitized receptors derived from rat lung plasma membranes and assessed their functionality both by implanation into *X. laevis* erythrocytes as well as by direct co-reconstitution with the isolated guanine nucleotide regulatory protein of the adenylate cyclase system. As will be shown, the lack of any functional alteration of these receptors in these experiments is in striking contrast to the results previously obtained in the avian erythrocyte system, underscoring the differing mechanisms contributing to the various forms of desensitization.

EXPERIMENTAL PROCEDURES

Animals. Male Wistar Kyoto rats (150–200 g) obtained from Charles River Breeders (Boston, MA) were injected intraperitoneally with 0.5 ml of saline alone (controls) or containing (−)-isoproterenol (10 mg/kg). Ten minutes after injection the animals were sacrificed by decapitation and the lungs were excised rapidly (20–30 sec). Female *X. laevis* were obtained from Nasco (Fort Atkinson, WI).

Materials. [^{125}I]CYP,[5] [^{32}P]α-ATP, and [^{32}P]γ-GTP were from New England Nuclear. Extracti-gel and Sepharose 6CL were obtained from Pierce Chemical Co., and octyl β-D-glucopyranoside (octyl glucoside) was from Calbiochem-Behring. Alprenolol was a gift from Hassle Pharmaceuticals (Molndal, Sweden), and digitonin was from Gallard Schlesinger (Carle Place, NY). All other chemicals used were reagent grade.

Radioligand binding assays. [^{125}I]Iodocyanopindolol binding to β-adrenergic receptors in plasma membranes (lung and *X. laevis* erythrocyte membranes) was performed as previously described (7). Free and radioligand bound to soluble or reconstituted β-adrenergic receptor were separated by elution through Sephadex G-50 columns pre-equilibrated in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% digitonin (15).

Adenylate cyclase assays. Assays were performed as previously described (7).

Data analysis. Saturation and competition curves were analyzed by computer-assisted techniques utilizing nonlinear least squares curve-fitting techniques based on the law of mass action (16, 17). Statistical analysis of radioligand binding comparing the "goodness of fit" between two parameters was determined as previously described (17). Comparisons involving adenylate cyclase or GTPase determinations were done by an analysis of variance.

Preparations of β-adrenergic receptor. Purified rat lung membranes from control and desensitized animals (10 mg/kg isoproterenol, i.p.) were prepared 10 min after injection as described previously (7). The β-adrenergic receptors were solubilized in 5 volumes of 100 mM NaCl, 10 mM Tris, 1.25% digitonin, pH 7.2, for 45 min at 4° and purified (700–800-fold) using affinity chromatography on a Sepharose-alprenolol gel (15) with an overall yield of 55–70%. Partially purified receptor preparations were concentrated using an Amicon concentration cell with a YM-30 membrane prewashed in 100 mM NaCl, 10 mM Tris, 0.1% bovine serum albumin, pH 7.4. The concentrated receptor preparations (10–15 pmol/ml) were then reconstituted into phospholipid vesicles alone or together with pure nucleotide regulatory protein (N_s) (see below).

Preparation of the stimulatory nucleotide regulatory protein (N_s). N_s was purified from human erythrocyte membranes according to a modification (18) of the method of Sternweis *et al.* (19). The preparations used in this report were from step 8A (18) and were stored at −70° in 1 mM EDTA, 20 mM β-mercaptoethanol, 30% ethylene glycol, 20 mM Na HEPES, 150 mM NaCl, 150 μg/ml bovine serum albumin, 0.1–5% Lubrol-PX, pH 8.0. In the reconstitution experiments 8–12 pmol of N_s in 5-10 μl of the same buffer were used.

Insertion of β-adrenergic receptors into phospholipid vesicles. The insertion of partially purified β-adrenergic receptor with (0.45 μg/assay) or without pure N_s into phospholipid vesicles was performed as previously described (20). β-Adrenergic receptor (3–5 pmol in 300 μl, 100 mM NaCl, 10 mM Tris, 0.05% digitonin, pH 7.4) was incubated in a final volume of 0.5 ml with bovine serum albumin (2 mg/ml), sonicated soybean phosphatidylcholine (1.5 mM), octyl β-D-glucopyranoside (0.85%) with or (for fusion experiments) without N_s (8–10 pmol in 5–10 μl of 20 mM Na HEPES, 1 mM EDTA, 20 mM β-mercaptoethanol, 30% ethanol, 30% ethylene glycol, 150 mM NaCl, 150 μg/ml bovine serum albumin, and 0.1–5% Lubrol PX). After 30-min incubation on ice the detergent concentration was reduced using 1-ml Extracti-gel D columns which were prewashed with 100 mM NaCl, 10 mM Tris (pH 7.4), 2 mg/ml bovine serum albumin, and then pre-equilibrated with 1 ml of the same buffer without bovine serum albumin. The 2-ml eluates containing the protein lipid vesicle fraction were incubated with 0.5 ml of polyethylene glycol 8000 (final concentration, 12.5% w/v) in 100 mM NaCl, 10 mM Tris (pH 7.4) for 10 min at room temperature and then diluted (∼10-fold) with 100 mM NaCl, 10 mM Tris (pH 7.4), and centrifuged at 250,000 × *g* for 2 hr at 4°. The protein-lipid pellets were resuspended in 0.5 ml of 75 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, 1.5 mM EDTA, and assayed for radioligand binding (see above) or GTP hydrolytic activity. For fusion experiments the *X. laevis* erythrocytes were added directly to the protein lipid pellets. The reconstitution efficiency for the β-adrenergic receptors varied between 25–35% for

[5] The abbreviations used are: [^{125}I]CYP, [^{125}I]iodocyanopindolol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

both control and desensitized receptors as measured by radioligand binding.

GTP hydrolytic activity (GTPase). The GTPase activity was assayed by incubating 20 μl of the resuspended vesicles containing N_s from human erythrocytes and β-adrenergic receptors from control or desensitized rat lung (see above) in a total volume of 100 μl containing 75 mM Tris-HCl (pH 7.5), 12.5 mM MgCl, 1.5 mM EDTA, 0.2% BSA, 0.5 mM ascorbic acid, 1 mM adenyl-5′-yl-imidodiphosphate and ∼150 nM [γ-^{32}P]GTP (∼36,000 cpm/pmol) for 30 min at 30°. The GTPase activity was measured in the absence of any effector (basal) or in the presence of 10^{-4} M (−)-isoproterenol alone or with 10^{-3} M (+/−)-propranolol. The GTPase was stopped by the addition of 10 μl of 50% trichloroacetic acid. The mixture was centrifuged for 20 min at 1,000 × *g* (4°). 90 μl of the supernatant was assayed for ^{32}P-labeled inorganic phosphate by adding 4 ml of 1.25% (w/v) ammonium molybdate in 1.2 M HCl containing 40 μM potassium phosphate. 5 ml of isobutanol/benzene (1:1) was immediately added to this solution. The mixture was vortexed for 20 sec, and 2 ml from the top layer was counted in toluene-based liquid scintillation fluid.

Cell fusion experiments. The fusion experiments were performed according to a modified procedure by Schramm (21) and Strulovici (13). *X. laevis* erythrocytes were washed three times in 100 mM NaCl, 10 mM Tris (pH 7.5). 6×10^7–1×10^8 packed erythrocytes were added directly to the protein-lipid pellets (obtained after the reconstitution experiments). In other experiments plasma membranes alone were incubated (5 min on ice) in 50 μg of phosphatidylcholine and 5 μg of lysolecithin type IV which had been sonicated in 10 mM Tris, 1 mM $MgCl_2$, pH 7.5. 10 μl of 100 mM $MgCl_2$, 5 mM Tris, pH 7.5, was added to the incubation mixture. The incubation was continued on ice for 5 min. After 1-min incubation at 30°, 500 μl of a 50% w/v polyethylene glycol solution (M_r ∼ 8000) was added. After 90 sec and then at 90-sec intervals gradual dilution of the cell suspension with increasing volumes (0.2, 0.35, 0.5, 1.5, 3.5, 7.5 ml) with buffer (100 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 10 mM Tris-HCl, pH 7.5, at 30°) was performed. The fusion was stopped by adding 20 ml of ice-cold buffer. The cells were centrifuged (48,000 × *g* for 10 min), resuspended in 1 ml of buffer (75 mM Tris-HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5, at 25°), and lysed by freeze-thawing in liquid nitrogen. The lysate was then washed 3 times with the same buffer. The final pellet was resuspended in 300 μl of the same buffer for measuring adenylate cyclase activity. This final suspension was further diluted 1:20 with 75 mM Tris-HCl, 25 mM $MgCl_2$, pH 7.4, at 25° prior to binding studies.

RESULTS

It has recently been shown that in the rat lung the adenylate cyclase system becomes rapidly desensitized after *in vivo* administration of isoproterenol (10 mg/kg, i.p.) (7). 10 min after injection of isoproterenol there is an almost complete loss of the β-agonist-sensitive adenylate cyclase activity with no change in basal, prostaglandin, or sodium fluoride-stimulated cyclase activity (Fig. 1). Two different processes contribute to this desensitization. About 40% of the β-adrenergic receptors become temporarily translocated into a light membrane (possibly intracellular) fraction (7). Moreover, the ability of those receptors remaining in the plasma to form the physiologically active high affinity state for agonists decreases.

The high affinity state of the receptor can be assessed by radioligand-binding techniques using agonist competition curves with an antagonist ligand such as [^{125}I] iodocyanopindolol to distinguish the high and low affinity states. In the absence of guanine nucleotides, the agonist competition curve is shallow and complex indicating that a portion of the receptor population binds

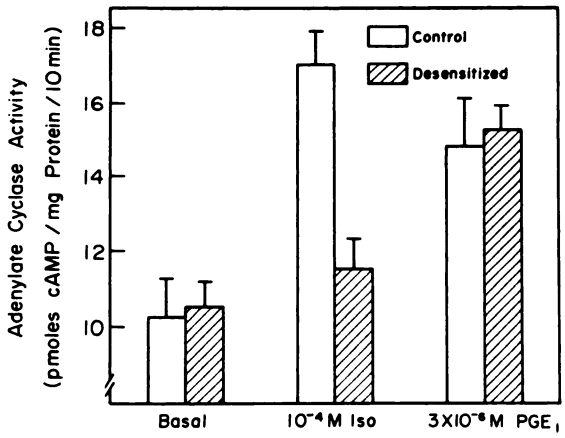

FIG. 1. *Adenylate cyclase activity in plasma membranes from rat lung after in vivo desensitization*

Adenylate cyclase activity was measured in lung plasma membranes of controls and of isoproterenol-injected (10 mg/kg) animals 10 min after injection. The β-agonist-sensitive adenylate cyclase activity assayed by stimulation with 10^{-4} M (−)-isoproterenol in the assay was significantly ($p < 0.01$) decreased. Basal, prostaglandin E_1 (PGE_1, 3×10^{-6} M)-stimulated, and sodium fluoride (10 mM)-stimulated (1468 ± 124 *versus* 1654 ± 182 pmol/mg/10 min, not shown) were not altered ($p < 0.05$) during densensitization. The data represent the mean ± SE of five experiments with triplicate determinations.

the β-agonist with high affinity (∼2 nM), while the remaining receptors are in a low affinity (∼200 nM) agonist-binding state (Fig. 2). In the presence of guanine nucleotides the agonist competition curves are shifted to the right and become steep and uniphasic indicating that all the receptors now bind the agonist with low affinity. Previous data have demonstrated that alterations in the high affinity agonist binding correlate with an impaired coupling of the receptor and the guanine nucleotide regulatory protein (for review see Refs. 1–3). As revealed by agonist competition curves in control membranes 50–60% of the receptors are apparently in the high affinity state indicating that these receptors can effectively couple to the guanine nucleotide-binding stimulatory protein (N_s) (Fig. 2). After desensitization, the ability of those receptors remaining in the plasma membrane to form the agonist-induced high affinity state decreases from 50–60% to 0–20% indicating that the desensitized receptors are greatly uncoupled from the N_s-protein (Fig. 2).

Receptors from the lung plasma membranes of control and desensitized animals were purified using an alprenolol Sepharose affinity chromatography column. The concentrated receptor (10–15 pmol/ml) was reconstituted together with the purified N_s protein (N_s) from human erythrocytes. The purified N_s protein has been shown to have a GTPase activity elicited by its interaction with the β-adrenergic receptor, which presumably terminates hormone and GTP-induced adenylate cyclase stimulation (20, 22), The isoproterenol stimulation of the hydrolysis of GTP by N_s was evaluated as a quantitative measure of the effective interaction of the control and desensitized receptor with the N_s protein. Equal amounts of receptors were reconstituted with equivalent efficiencies (25–30%). Under basal conditions equal amounts of P_i are released from N_s coreconstituted with

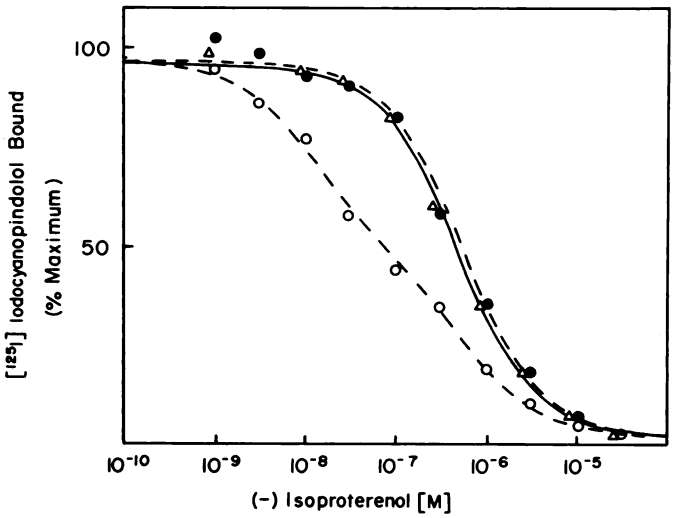

FIG. 2. *Competition binding of (−)-isoproterenol for [^{125}I]CYP in plasma membranes derived from control and in vivo desensitized rat lung 10 min after a single injection of saline (controls) or 10 mg/kg (−)-isoproterenol*

Binding assays were performed as described under Experimental Procedures using 100 pM [^{125}I]CYP. Shown is the competition of (−)-isoproterenol with [^{125}I]CYP binding in lung plasma membranes from controls in the absence (○– – –○) and in the presence of 10^{-4} M Gpp(NH)p (●– – –●). In these experiments the competition curves of isoproterenol for [^{125}I]CYP binding in lung plasma membranes from desensitized animals (10 min after 10 mg/kg isoproterenol injection) were superimposable in the absence and presence of Gpp(NH)p. Shown is the competition curve in the absence of Gpp(NH)p (△——△). Total number of receptor sites was identical in the absence and presence of Gpp(NH)p with the specific activity being 1180 ± 65 fmol/mg of protein in the controls and 750 ± 90 fmol/mg of protein in the desensitized membranes. The data points represent the mean of three experiments determined in duplicate. The lines through the data points represent a computer-derived fit utilizing nonlinear least squares curve-fitting techniques based on the law of mass action (see Experimental Procedures and Refs. 16 and 17).

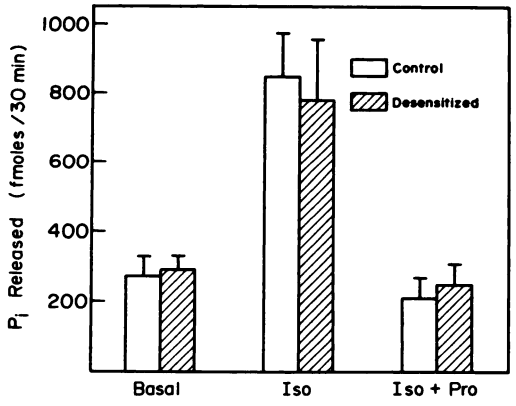

FIG. 3. *GTPase activity of phospholipid vesicles containing N_s and β-adrenergic receptor from control or desensitized animals*

Pure N_s from human erythrocytes and partially purifed β-adrenergic receptor from lung plasma membranes of control and desensitized animals were reconstituted and assayed for GTPase activity as described under Experimental Procedures in the absence (basal) or presence of the β-agonists (−)-isoproterenol (10^{-4} M) and/or the β-antagonist (±)-propranolol (10^{-3} M). Equal amounts of control or desensitized β-adrenergic receptor were reconstituted into the lipid vesicles (between 0.75–1.5 pmol) together with equal amounts of N_s protein (0.45 μg/assay). Basal GTPase activity was not different ($p > 0.05$). Both control and desensitized receptors are equally effective in mediating the β-agonist-stimulated GTPase activity (*Iso*) which can be completely blocked by the β-antagonist propranolol (*Iso + Pro*). As shown previously (20) propranolol in a concentration of 10^{-5} M could also completely block β-agonist-stimulated GTPase activity. Shown here is the mean ± SE of four experiments with triplicate determinations.

control or desensitized receptors (Fig. 3). In the presence of isoproterenol the GTPase activity is increased to the same extent in N_s reconstituted with control or desensitized receptor (852 ± 38 *versus* 738 ± 49 fmol of P_i/30 min, $p > 0.05$, $n = 4$). The agonist-induced stimulation of GTPase activity can be inhibited by simultaneous incubation with the β-antagonist propranolol. This indicates that receptors from control as well as from the desensitized animals are equivalent in their ability to transmit the stimulation by the β-agonist isoproterenol to the N_s protein.

To assess the ability of the isolated receptor proteins to stimulate the catalytic unit of the adenylate cyclase, the isolated receptors were reconstituted into lipid vesicles and then fused into the erythrocytes of *X. laevis*. These erythrocytes have been shown to possess the functional N_s protein and the catalytic unit of the adenylate cyclase; however, they contain few if any β-adrenergic receptors (13). The insertion of the reconstituted receptors into *X. laevis* erythrocytes ranged from 6–12% with equal efficiencies observed for the control and desensitized receptors. After fusion a total of 412 ± 26 fmol of control *versus* 404 ± 62 fmol of desensitized β-adrenergic receptors was associated with the plasma membrane fraction from the *X. laevis* erythrocytes as assessed by [^{125}I]iodocyanopindolol binding (data not shown). The basal adenylate cyclase activities measured in the membranes after fusion of the cells with control or desensitized receptors were similar (Fig. 4). Moreover, both control as well as desensitized receptors can effectively and equally mediate isoproterenol stimulation of the catalytic unit of the adenylate cyclase ($p > 0.05$, $n = 4$). This stimulation is β-adrenergic receptor mediated as can be shown by the inhibition with the β-antagonist propranolol. As would be expected stimulation by prostaglandin or sodium fluoride which are not mediated by β-adrenergic receptors were the same in the "control" and "desensitized" reconstitutions as well as in the plasma membranes from erythrocytes fused with lipid vesicles containing no β-receptors.

The final parameter of β-adrenergic receptor function evaluated in reconstituted systems was the ability of the control and desensitized receptors to form the coupled high affinity state of the receptors (R_H). As described elsewhere (17) under Experimental Procedures, the formation of the high affinity state of the receptors (presumed to be a ternary complex of agonist-receptor and guanine nucleotide regulatory protein) can be evaluated by computer modeling of agonist competition curves with radiolabeled antagonist ligands. In the original plasma membranes from control rat lungs about 50–60% of the receptors are able to form the high affinity state in the absence of guanine nucleotides (Fig. 2). After affinity purification the receptors are resolved from endogenous

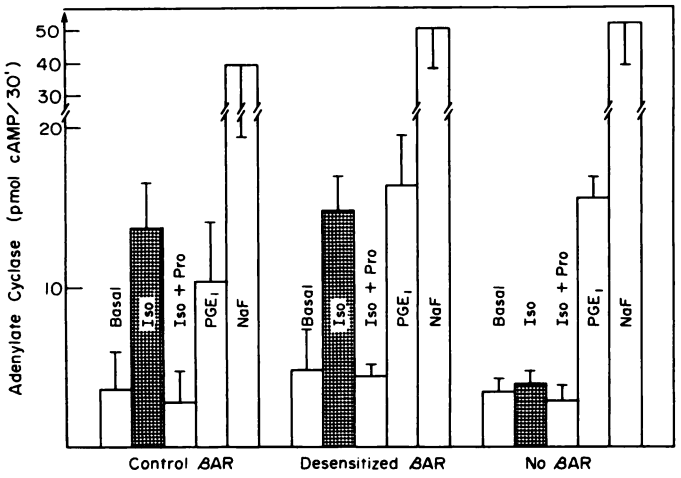

FIG. 4. *Adenylate cyclase activity in plasma membranes from Xenopus laevis erythrocytes hybridized with reconstituted affinity chromatography-purified β-adrenergic receptor from control or desensitized rat lung*

Reconstituted β-adrenergic receptor from control (*control βAR*) or desensitized (*desensitized βAR*) lung plasma membranes or reconstituted vesicles alone (*no βAR*) were fused to intact *X. laevis* erythrocytes and the plasma membranes prepared as described under Experimental Procedures. Equal amounts of control and desensitized β-adrenergic receptor were fused (412 ± 25 *versus* 404 ± 62 fmol, $n = 3$). Adenylate cyclase activity was measured in the absence (basal) or presence of various stimulators: 10^{-4} M (−)-isoproterenol (*Iso*), 10^{-4} M (−)-isoproterenol + 10^{-5} M (±)-propranolol (*Iso + Pro*), 3×10^{-6} M prostaglandin E_1 (*PGE_1*), 10 mM sodium fluoride (NaF). The β-agonist-stimulated cyclase activity was significantly ($p < 0.01$) increased in the plasma membranes from erythrocytes fused with β-adrenergic receptor as compared with vesicles containing no β-adrenergic receptor (*no β-AR*). Both control (*control βAR*) and desensitized β-adrenergic receptor (*desensitized βAR*) equally mediated the β-agonist-induced stimulation of cyclase activity ($p > 0.05$) which could be blocked by the β-antagonist propranolol. Basal, prostaglandin E_1, and sodium fluoride-stimulated activities were not different ($p > 0.05$). The data represent the mean ± SE of four experiments with triplicate determinations.

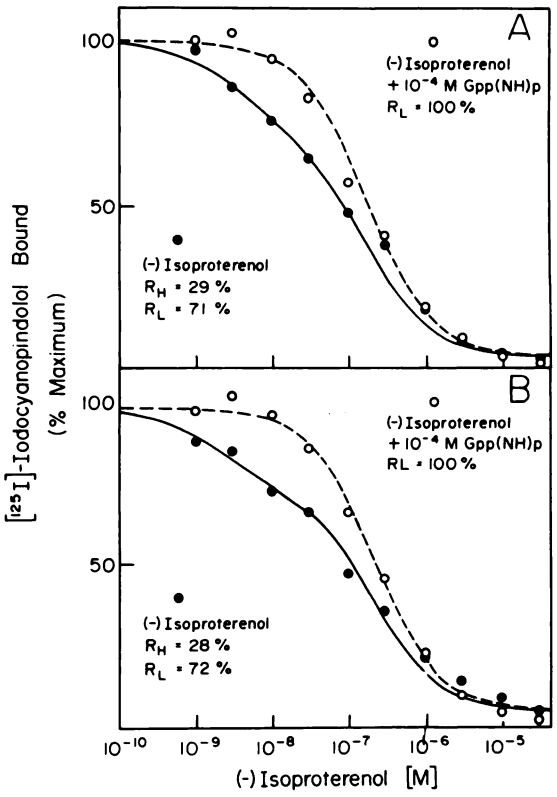

FIG. 5. *Competition binding of (−)-isoproterenol and [^{125}I]CYP in plasma membranes from Xenopus laevis erythrocytes hybridized with reconstituted affinity chromatography-purified β-adrenergic receptors from control (A) or desensitized (B) rat lung*

X. laevis erythrocytes were fused to reconstituted β-adrenergic receptor from controls or desensitized animals and plasma membranes prepared and binding assays performed as described under Experimental Procedures using 60 pM [^{125}I]CYP. Shown is the competition of (−)-isoproterenol with [^{125}I]CYP. Shown is the competition of (−)-isoproterenol with [^{125}I]CYP binding to the fused β-adrenergic receptor in *X. laevis* erythrocytes in the absence (●——●) or presence of 10^{-4} M Gpp(NH)p (O- - -O). Equal total amounts of control and desensitized β-adrenergic receptor were fused (412 ± 25 *versus* 404 ± 62 fmol). The data points represent the mean of three experiments with duplicate determinations. The lines through the experimentally determined data points represent a computer-derived fit utilizing nonlinear least squares curve-fitting techniques based on the law of mass action (see Experimental Procedures). Equal amounts of β-adrenergic receptor from controls and desensitized animals were reconstituted and fused (see Fig. 4). Identical amounts of β-adrenergic receptor inserted into the donor cells could form the high affinity state R_H (29 ± 6 *versus* 28 ± 4%) with equal high affinities for the agonist in the absence of guanine nucleotides (2.1 ± 0.8 *versus* 1.1 ± 0.4 nM). The dissociation constants for the low affinity site in the absence and in the presence of Gpp(NH)p were also not different (98 ± 16 *versus* 119 ± 15 nM).

N_s, and no formation of the high affinity state is observed (2). However, after fusion of the partially purified receptors from control membranes, with *X. laevis* erythrocytes, about 30% of the receptors again bind the β-agonist with high affinity (∼2 nM) (Fig. 5A). This indicates that they can effectively couple to the N_s protein of the acceptor cells. This coupling efficiency is in good agreement with the coupling efficiency observed after reconstitution of the pure β-adrenergic receptor and pure N_s (20), where ∼30% of the receptors are also noted to form the high affinity state. Thus, as might be expected in the reconstituted systems the percentage of receptors forming the high affinity state is lower than in the original membranes where the receptors couple to the endogenous N_s protein (Fig. 2).

After desensitization the "coupling" of β-adrenergic receptors to the endogenous N_s in the original plasma membranes is markedly impaired (Fig. 2). However, after fusion, ∼30% of the desensitized affinity-purified and reconstituted β-adrenergic receptors can couple to the N_s protein of the acceptor cell (Fig. 5B). Thus the fusion of control and desensitized β-adrenergic receptors into *X. laevis* erythrocytes results in an indistinguishable percentage of receptors in the high affinity state (29 ± 6% *versus* 28 ± 4%). The dissociation constants for the high and low affinity states of the receptor were also indistinguishable after fusion in the two cases (K_H, 2.1 ± 0.8 nM *versus* 1.1 ± 0.4 nM; K_L, 98 ± 16 nM *versus* 119 ± 15 nM, $p > 0.05$). Moreover, these dissociation constants do not differ significantly from those observed in the original lung membranes (Fig. 2). Thus the control as well as the "desensitized" receptors from rat lung plasma membranes can be shown to effectively interact

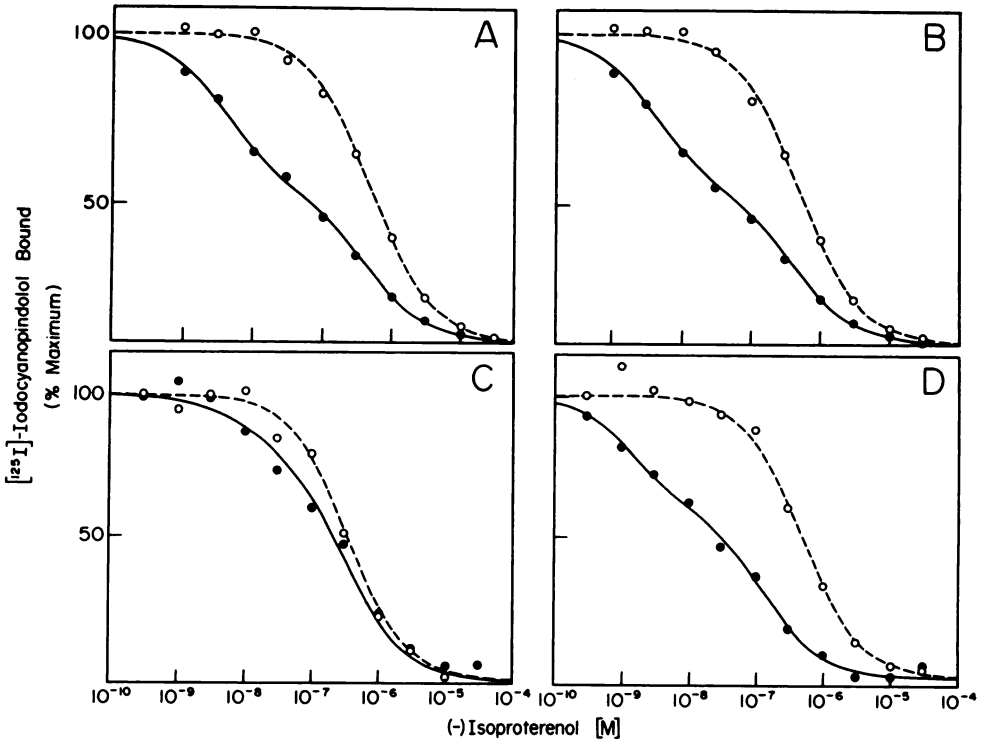

FIG. 6. *Effect of PEG treatment of rat lung plasma membranes from control (A and B) and desensitized (C and D) animals on competition of (−)-isoproterenol for [^{125}I]CYP binding to the β-adrenergic receptor*

Plasma membranes from control or desensitized rat lungs were incubated with lipids alone (A and C) or with the addition of polyethylene glycol (B and D) in the fusion procedure (see Experimental Procedures). The plasma membranes were then washed 3 times and binding assays performed using 100 pM [^{125}I]CYP. Shown is the competition of the β-agonist (−)-isoproterenol with [^{125}I]CYP in the absence (●——●) or in the presence (O– – –O) of 10^{-4} M Gpp(NH)p. Total number of binding sites was equalized in control and desensitized membrane suspensions (20 pM) and was unaltered before and after PEG treatment and in the absence or presence of Gpp(NH)p. Each data point represents the average of duplicate determinations. The data shown are representative of three experiments, with duplicate determinations at each point. The lines through the data points represent a computer-derived fit utilizing nonlinear least squares curve-fitting techniques based on the law of mass action (see Experimental Procedures).

with the N_s protein from two different sources (human erythrocytes and *X. laevis* erythrocytes) and even to mediate agonist stimulation of the adenylate cyclase.

The data presented up to this point fail to document any stable functional alteration of the "desensitized" β-adrenergic receptors from the lung membranes. As noted above, another possible explanation for the reduced function of these β-adrenergic receptors is sequestration away from the effector units of the adenylate cyclase (N_s and C). That the receptors in the light membrane fraction are sequestered in this fashion in both rat lung and frog erythrocytes has been previously established ((7) for review see Ref. 2). We wished to test the hypothesis that even those "uncoupled" receptors still sedimenting with the plasma membranes were also functionally desensitized by virtue of some type of "sequestration" from the other components of the adenylate cyclase system. This was accomplished by treating isolated plasma membranes with phospholipids and PEG as would be done for a "fusion" experiment. Such treatment should lead to intermixing and reorientation of proteins and lipids of all membrane compartments of the "plasma membrane" fractions including the components of the adenylate cyclase system, β-adrenergic receptor, N_s, and C.

Due to the lability of the catalytic activity of adenylate cyclase in these membranes we could not use restoration of isoproterenol-sensitive enzyme activity as an end point for such experiments since sufficient enzyme activity

TABLE 1

Effect of PEG on agonist competition binding to β-adrenergic receptors of plasma membranes from control and desensitized lungs

The agonist (−)-isoproterenol competed for the binding of the β-antagonist [^{125}I]iodocyanopindolol in lung plasma membranes from controls and in lung plasma membranes from desensitized animals with or without PEG treatment. Computer modeling of agonist competition curves using Scatfit was performed as described under Experimental Procedures. Values shown are the computer-derived estimates ± standard error of the estimate of three experiments. R_H and R_L are percentages of β-adrenergic receptors in high and low affinity states. K_H and K_L are the dissociation constants of each receptor state for the agonist, (−)-isoproterenol. −PEG means that the membranes went through the same incubation as +PEG membranes except for the omission of PEG (See Experimental Procedures).

Membranes	Control	Control	Desensitized	Desensitized
PEG treatment	—	+	—	+
R_H (%)	56 ± 4	57 ± 5	15 ± 5[a]	48 ± 5
R_L (%)	44 ± 3	43 ± 5	85 ± 5[a]	52 ± 6
K_H (nM)	2.4 ± 0.9	3.4 ± 0.5	5.9 ± 3	4.5 ± 1
K_L (nM)	263 ± 55	255 ± 46	228 ± 35	216 ± 97

[a] Value differs from all other corresponding values, $p < 0.001$.

could not be measured in control or desensitized membranes after such treatments. Instead we quantitated the ability of isoproterenol to stabilize the high affinity (R_H) state of the receptor, previously documented to provide an index of receptor-N_s coupling in such systems.

As documented in Fig. 6, A and B, the treatment of control membranes with the "fusogen" mixture led to no change in "high affinity state" formation assessed by computer modeling of agonist competition curves. In both cases about ∼60% of the receptors were in the high affinity state, and guanine nucleotides converted all of these to the low affinity form. As the comparison of Fig. 6A to Fig. 2 shows, incubation alone did not change the percentage of receptors in the high affinity state. After desensitization (as described earlier, *e.g.* Fig. 2) only 0–20% of the receptors formed the high affinity state in the absence of guanine nucleotides. This percentage of R_H also does not change with incubation alone (Fig. 6C). However, after treatment with PEG and lipids this parameter increased to 62% high affinity state receptors which is essentially identical to the control situations (Fig. 6D). All of these high affinity state receptors were normally responsive to guanine nucleotides. In a series of experiments the actual K_H and K_L values were not different in any of these situations (Table 1); only the percentage of receptors in the high affinity state (R_H%) was altered by the treatment (Fig. 7). Thus the PEG/lipid treatment completely "recouples" the uncoupled desensitized receptors present in the plasma membranes.

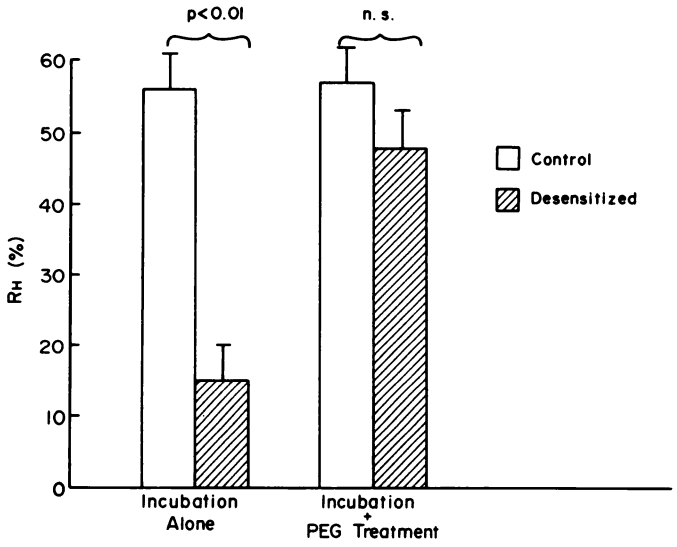

FIG. 7. *Proportions of the β-adrenergic receptor in the "high affinity state" in plasma membranes from control and desensitized rat lungs with or without PEG treatment*

The proportion of β-adrenergic receptor in the high affinity state (R_H %) was determined in a series of experiments with the agonist isoproterenol competing in the absence of Gpp(NH)p for the binding to the β-adrenergic receptors of control or desensitized lung plasma membranes after incubation alone or with additional PEG treatment. Indicated are the computer-derived estimates utilizing nonlinear least squares curve fitting techniques based on the law of mass action (see Experimental Procedures). Incubation alone had no effects on receptor proportions in the high affinity state or dissociation constants (Table 1). After PEG treatment the percentage of β-adrenergic receptors in the high affinity state in the plasma membranes from desensitized rat lungs markedly increased. R_H% from control lung membranes with or without PEG treatment and also from desensitized lung membranes with PEG treatment were not different ($p > 0.05$, $n = 3$). The dissociation constants did not change.

DISCUSSION

The findings reported here support a model for homologous desensitization in which a major mechanism is a sequestration of the receptors away from their physiological effectors. Two lines of evidence support this notion. First, when desensitized receptors from the "plasma membrane" fraction of the lung tissue were purified by affinity chromatography and their activity assessed in several reconstitution systems no functional alteration was observed. This despite the fact that in the original plasma membranes both adenylate cyclase and ligand-binding studies suggest almost complete uncoupling of the receptors. The normal functioning of these purified desensitized receptors was observed in three different ways: by receptor-mediated maximal stimulation of adenylate cyclase and formation of high affinity (HRN) receptor complexes upon insertion into *X. laevis* erythrocytes and by maximal stimulation of GTPase activity in the isolated N_s protein.

The second line of evidence involves the demonstration, using radioligand-binding techniques, that the ability of desensitized and uncoupled receptors in lung plasma membranes to form the high affinity HRN complex can be reestablished by intermixing plasma membrane components by treatment with the fusogen PEG. The notion is that β-adrenergic receptor and N_s units functionally separated by some form of sequestration into distinct membrane compartments are reunited by these procedures.

Several comments need to be underscored, however, in considering these findings. Desensitization in the original membranes was defined as a decrement in the maximal isoproterenol stimulation of cyclase. We used the same parameter in reconstitution experiments to assay the functionality of the receptors. Our results do not rule out the possibility that if complete isoproterenol dose-response curves had been examined in the reconstitution assays that small changes in K_{act} might have been found. Also it is obviously possible that the solubilization and purification procedures used as well as the reconstitution procedures utilizing polyethylene glycol might reverse some labile conformational or covalent alteration in the receptors which is responsible for their uncoupling in the plasma membranes. There is no definitive way to rule out this possibility, but similar procedures do *not* reverse the functional and structural (presumably phosphorylation) alterations of the β-adrenergic receptor which lead to desensitization in the turkey erythrocyte (6).

Another limitation of the present studies relates to the lability and low levels of catecholamine stimulation of adenylate cyclase in membranes from mammalian lung which is in keeping with numerous similar observations (Ref. 7 and references therein) reported in the past. This, together with the "toxic" effect of PEG treatment on enzyme activities prevented us from assessing catecholamine-stimulated adenylate cyclase in the desensitized

plasma membranes after PEG addition. Nonetheless, as documented by receptor-binding studies the desensitized receptors were recoupled to N_s following this treatment.

Finally it should be pointed out that none of the present results exclude the possibility that at some very early point in the desensitization process an alteration in receptor functionality might occur and explain the desensitization at that time. Recently several laboratories have presented data consistent with such a notion. Toews *et al.* (23) showed that desensitization of catecholamine-sensitive adenylate cyclase occurs prior to sequestration in human astrocytoma cells. Kassis and Fishman (24) used fusion studies to assess the functionality of desensitized β-adrenergic receptors. They fused membranes from control and desensitized cells (in which N_s and C had been inactivated by chemical modification) to Friend erythroleukemia cells which are devoid of β-adrenergic receptors. It was found that the receptors from the desensitized membranes were less able to activate adenylate cyclase.

Rather than ruling out a "functional" alteration in the receptors, our findings simply suggest that by 10–15 min, at which point maximum desensitization has been reached in this *in vivo* system, sequestration plays a major role in mediating the observed alteration. One possibility is that a very early occurring receptor alteration is reversed even as the receptors become sequestered. Although such a scheme is entirely speculative, recently evidence has been presented which suggests that, in frog erythrocytes, desensitized receptors can become sequestered in compartments of membrane which remain contiguous with the plasma membrane (25). Very recent data on intact S_{49} lymphoma cells suggest that this sequestration and the desensitization are both reversible with polyethylene glycol treatment (26).

Evidence for "sequestration" of desensitized β-adrenergic receptors has also been obtained in a number of cultured cell systems such as C6 glioma (27) and S49 lymphoma (28) and in isolated heart cells (29). In these systems the sequestration of the desensitized receptors has been documented in various ways. These have included a shift in the density of the membrane in which the desensitized receptors are found (1), a decrease in the binding affinity of hydrophilic agonists to the intact cells, and a decrease in the number of receptor-binding sites accessible at the cell surface to the hydrophilic antagonist [^{3}H]CGP 12177 (30).

Taken together our findings and previously reported data support the notion that different mechanisms of receptor alteration contribute to the *homologous* and the *heterologous* forms of desensitization. In the turkey erythrocyte, an extensively used model system for the study of heterologous desensitization, exposure to a β-agonist such as isoproterenol leads to a major loss of catecholamine responsiveness and less impressive losses in sodium fluoride-stimulated cyclase activity. Cyclic nucleotides can also produce this picture (2). There is no change in the number of receptors present at the cell surface. However, a covalent alteration of the receptor protein, due to phosphorylation, appears to correlate closely with the functional desensitization of the receptor protein (4, 5). This covalent alteration can be visualized as a change in the mobility of the photoaffinity-labeled receptor on sodium dodecyl sulfate gels. The stable covalent modification of the receptors by phosphorylation rather than sequestration appears to correlate with the desensitization process in avian erythrocytes (5). This stable modification of the receptors in the avian erythrocyte also can be documented by reconstitution and fusion experiments.

In contrast, in the *homologous* form of desensitization observed in the frog erythrocyte (2), the rat lung (7), or S49 lymphoma cells (31), the desensitized receptor proteins do not have an altered mobility on sodium dodecyl sulfate gels. Moreover, the different mechanisms of desensitization result in contrasting functional changes of the desensitized receptor proteins. This paper demonstrates that in the *homologous* type of desensitization observed in the mammalian lung the purified receptor proteins from plasma membranes have retained their functional integrity. Thus "sequestration" of functionally intact receptor molecules within some domain of the plasma membranes appears to play a major role in the homologous form of desensitization as documented here. This sequestration of receptors can be reversed by membrane fusogens and perturbants such as PEG. The nature of the underlying biochemical changes which regulate receptor sequestration remain to be elucidated. It could be speculated that phosphorylation of the β-adrenergic receptor might trigger the sequestration process, the uncoupling process, or both, in homologous desensitization.

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