Characterization of the Human Lymphocyte β-Adrenergic Receptor by Photoaffinity Labeling

Alterations with Desensitization

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SUMMARY. Desensitization of the leukocyte β-receptor system has been associated with a functional uncoupling of the components of the β-receptor complex. In order to determine whether desensitization and uncoupling of the leukocyte β-receptor is associated with any structural alterations in the β-receptor, we studied labeling of lymphocytes using the photoactive β-adrenergic antagonist p-azido-m-[125I]iodobenzylcarazolol. Labeled peptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected using autoradiographic techniques. In broken cell preparations, specific labeling was demonstrated in two major peptide bands: mol wt = 68,000 and mol wt = 55,000. Inhibition of photolabeling was stereospecific and demonstrated an order of potency for agonists consistent with labeling of a β2-receptor. Preincubation of cells with the β-agonist, isoproterenol, resulted in a reduction in β-adrenergic-mediated adenylyl cyclase activity to 60% of control, but no change in total binding sites as determined by [125I]iodocyanopindolol binding. In photolabeling studies, desensitization was associated with a reduction in proportional labeling of the 55,000 mol wt band as compared to the 68,000 mol wt band to 58 ± 3% of control and a reduction in mobility of the upper band. These studies suggest that structural alterations in the human lymphocyte β-receptors occur with desensitization, analogous to changes in several other β-receptor model systems. Also, since the techniques described can identify alterations in human β-receptor structure, these methods may be exploited to determine whether structural alterations in lymphocyte β-receptors may occur in human disease states. (Circ Res 58: 384–388, 1986)

β-ADRENERGIC responsiveness is attenuated by persistent exposure to catecholamines. This phenomenon, referred to as desensitization, has been correlated with a reduction in β-receptor mediated cyclic adenosine monophosphate (cAMP) production. In some circumstances, this has been associated with β-receptor loss (cf Perkins et al., 1982; Harden, 1983; Insel, 1984; Stiles et al., 1984, for reviews).

It has become evident in many model systems that loss of β-receptors (“down regulation”) cannot explain solely the rapid reduction in β-adrenergic responsiveness which occurs upon desensitization. In many circumstances, this reduction in β-adrenergic-mediated adenylyl cyclase activity occurs without any change in β-receptor number or affinity for antagonists. However, desensitization in these systems has been associated with a reduction in β-receptor affinity for agonists (Perkins et al., 1982; Harden, 1983; Insel, 1984; Stiles et al., 1984).

The molecular explanation for this rapid loss of receptor-adenylate cyclase coupling without loss of β-receptor density is unclear. However, in several model systems, desensitization is associated with apparent structural alterations in the β-receptor. In some circumstances, these alterations have been detected by using photoaffinity labeling of the receptor and separation of β-receptor-associated polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Stiles et al., 1984; Rashidbaigi et al., 1983).

The human lymphocyte has been used to monitor β-receptor properties ex vivo since, in sever al circumstances, changes in lymphocyte β2-receptors have been shown to reflect alterations in β-receptors and β-adrenergic responsiveness in less accessible target tissues (e.g. heart, lungs, vascular smooth muscle) (Fraser et al., 1981; Colluci et al., 1981; Aarons and Molinoff, 1982). Consequently, we have studied lymphocyte β-receptor-associated polypeptides using photoaffinity labeling techniques and SDS-PAGE to determine whether functional desensitization of the human lymphocyte β-receptor is associated with any detectable structural alterations.

Methods

Lymphocyte Membrane Preparation

Lymphocytes were separated from whole blood samples (Boyum, 1968). Membranes were prepared according to a modification of previously described methods (Szamel...
and Resch, 1981). Mononuclear isolates consisting of at least 85% small lymphocytes were separated from the upper surface of a Ficoll-Hypaque gradient (6/10%) washed with phosphate-buffered saline, and sedimented at 500 g for 15 minutes. The cells were resuspended in a hypotonic buffer with 5 mM Tris, 3 mM EDTA (pH 7.6 at 4°C) and sedimented at 500 g for 15 minutes. The cells were resuspended in 140 mM KCl, 20 mM HEPES, 3 mM EDTA (pH 7.4) and subjected to nitrogen cavitation (Parr bomb, 600 psi for 15 minutes), following which cell membranes were disrupted but intact nuclei were still present. The lysate was centrifuged at 17,000 g for 20 minutes, the pellet was discarded, and the supernatant was centrifuged at 200,000 g for 45 minutes.

**Adenylate Cyclase Activity**

Assays of adenylate cyclase activity were performed according to previously described methods (Feldman et al., 1985). Maximally-activated and non-activated adenylate cyclase activities were determined by cyclic adenosine monophosphate (cAMP) generation with the addition of isoproterenol (100 μM) and guanosine triphosphate (GTP) (100 μM). Non-β-receptor-mediated activity was determined as the increase in activity with the addition of prostaglandin E1 (PGE1) (100 μM) and Gpp(NH)p (100 μM) a non-receptor specific activator of adenylate cyclase activity.

**Radioligand-Binding Studies**

β-Receptor-binding studies were performed according to previously described methods (Feldman et al., 1985) using [125I]-Iodocyanopindolol (ICYP) (2.2 Ci/μmol, New England Nuclear). ICYP and other drugs were prepared in 1.25 mM ascorbic acid as an antioxidant with 10 μg/ml bovine serum albumin.

Aliquots of the lymphocyte preparations containing 5–10 μg of protein were incubated with various concentrations of ICYP in a final volume of 250 μl containing 0.5 mM ascorbic acid, 1.0 μg bovine serum albumin, 12 mM Tris-HCl (pH 7.9, 25°C), 60 mM NaCl, 9 mM MgCl2, and 1.8 mM EDTA. Samples were incubated in disposable polypropylene tubes at 37°C for 100 minutes, at which time steady state had been reached at all concentrations. Incubations were stopped by the addition of 10 μM tris-HCl, 15 mM MgCl2 (pH 7.9 at 25°C) to each assay tube followed by separation of membrane bound and “free” ICYP by rapid filtration through Whatman GF/C filters. Each filter was washed with an additional 10 ml of buffer, and radioactivity was determined with a Beckman Gamma 4000 counter. Protein concentration was determined (Lowry et al., 1951) using bovine serum albumin as a standard. Estimates for Bmax and KD were derived from saturation-binding curves, using a computerized nonlinear curve-fitting analysis (SCATFIT) as previously described (Feldman et al., 1983).

Initial studies demonstrated that ICYP binding in lymphocyte homogenates is characterized by a physiological β2-receptor. Binding is saturable, reversible, and has appropriate stereoselectivity and affinity for β-receptor agonists viz. isoproterenol > epinephrine > norepinephrine.

**Photoaffinity Labeling with p-Azido-m-[125I]-Iodobenzylcarbazololozolol (pABC)**

Photoaffinity labeling with pABC was performed according to modification of the previously published methods (Stiles et al., 1983). Lymphocyte membranes (at a β-receptor concentration of approximately 50 pm) were incubated in 75 mM Tris, 3 mM EDTA (buffer A, pH 7.9, at 25°C) with 15 mM MgCl2 and pABC (80–120 pm) for 60 minutes at 30°C in the dark. All steps prior to photolysis were performed under dim light at 4°C. After incubation, samples were diluted with the addition of 0.1% ovalbumin in buffer A. Samples were pelleted at 175,000 g for 45 minutes and resuspended in buffer A with 0.1% ovalbumin. The lysate was centrifuged as described above. The resulting pellet was then resuspended in buffer A without ovalbumin and recentrifuged.

The pellet was resuspended in 5 ml of buffer A and transferred to a petri dish. The petri dish was placed 12 cm from a medium pressure mercury lamp (Ace-Hanovia 450 watt arc lamp) and irradiated for 60 seconds. The lamp is equipped with a 6-mm Pyrex filter and is air cooled. After exposure to light, membranes were collected by centrifugation at 175,000 g for 45 minutes. Samples were resuspended in SDS sample buffer with 10% SDS, 5% 2-mercaptoethanol, 1.5 mM EDTA, 10% glycerol, 25 mM Tris, 1.5 mM EDTA (pH 7.9 at 25°C). Samples were incubated 1 hour at 25°C; then, proteins (~100 μg/lane) were separated by SDS-PAGE (3.2% polyacrylamide stacking gel and 12.5 or 15% polyacrylamide running gel) (Laemmli, 1970). Dry gels were irradiated at ~80°C, and labeled polypeptides were detected by autoradiography.

**Results**

**Photoaffinity Experiments**

pABC was shown to label selectively two major polypeptide bands with mol wt ≈68,000 and mol wt ≈55,000 (Fig. 1). The addition of other protease inhibitors during membrane preparation including phenylmethylsulfonyl fluoride (100 μM), leupeptin (5 μM/ml), pepstatin (5 μg/ml), benzamidine (100 μM), and soybean trypsin inhibitor (5 μg/ml) did not alter the pattern of binding seen when EDTA (3 mM) alone was used. pABC labeling was inhibited by β-agonists with an order of potency of isoproterenol > epinephrine > norepinephrine (Fig. 2). Addition—

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Specific β-receptor labeling of human lymphocyte lysate-autoradiograph. Lysates were incubated with pABC in either the absence (−) or presence (+) of 1 μM propranolol and polypeptides separated by SDS-PAGE. The arrows indicate the mobility of standard protein markers.
FIGURE 2. β-Receptor photolabeling. Differential protection from labeling by β-adrenergic agonists—densitometric tracing. pABC labeling in the absence of agonists (1 μM solid line) is compared to the effect of coincubation with isoproterenol (1 μM dotted line) epinephrine (1 μM dashed line) and norepinephrine (1 μM dash-and-dotted line).

Effects of Desensitization on Lymphocyte β-Receptors

To study the effects of desensitization on the lymphocyte β-receptor complex, we preincubated whole blood samples with isoproterenol (10 μM for 10 minutes at 37°C) (Motulsky et al., 1985). Lymphocytes then were separated, membranes were prepared as described, and adenylate cyclase activity was assayed.

With isoproterenol preincubation, maximum β-adrenergic-mediated adenylate cyclase activity (as determined by 0.1 nM isoproterenol-stimulated activity) was decreased to 60% of control cells (Table 1). In contrast, Gpp(NH)p- and PGE1-stimulated activity was not decreased (Table 1).

Radioligand-binding studies were performed after an identical treatment protocol. Saturation curves for specific ICYP binding comparing membranes from control and desensitized cells demonstrated no differences in Bmax or Kd for ICYP (Table 2).

In contrast to the ICYP-binding studies, significant alterations in the pattern of pABC labeling were noted in the membranes from cells exposed to isoproterenol (Fig. 2). The proportion of specific pABC labeling in the 55,000 mol wt band as compared to the 68,000 mol wt band was determined densitometrically. With desensitization, proportional labeling of the 55,000 mol wt band was reduced to 58 ± 3% of control (ratio of lower:upper bands control: 0.68 ± 0.05, desensitized: 0.40 ± 0.03; n = 5, P < 0.05). Additionally, an increase in the width of the 68,000 mol wt band was noted with desensitization which, in four of five studies, migrated as two bands with a new band appearing at mol wt = 90,000 (Fig. 3).

Discussion

Previous investigators have demonstrated that exposure of human leukocytes to β-agonists results in a functional desensitization of β-receptor-mediated responsiveness (Krall et al., 1980; Davies and Lefkowitz, 1983; Motulsky et al., 1985). This desensitization has been characterized as an uncoupling of the receptor-effector components of the β-receptor complex. Our data reconfirm these studies, demonstrating that preincubation with isoproterenol results in a specific reduction in lymphocyte isoproterenol-stimulated adenylate cyclase activity without corresponding reduction in β-receptor density, as determined by ICYP binding. These findings are consistent with a functional uncoupling of the lymphocyte β-receptor complex. The reduction in adenylate cyclase activity was β-agonist specific, suggesting a homologous mechanism of desensitization.

Structural receptor alterations associated with the
uncoupling process were studied using photoaffinity labeling with pABC. This technique has been used to study β-receptors in several systems (Rashidbaigi et al., 1983; Stiles et al., 1984). However, photolabeling has not previously been described for the human leukocyte β-receptor, a model frequently used for the study of regulation of β-receptor systems in man. β-Receptor-specific labeling in lymphocyte membranes was localized to two major polypeptide bands: mol wt = 68,000 and mol wt = 55,000. This pattern of two broad bands is similar to that seen with other mammalian β-receptors (Rashidbaigi et al., 1983; Stiles et al., 1984), and, in particular, the human myocardial β-receptor (Stiles et al., 1984). Upon desensitization, a selective loss in labeling of the 55,000 mol wt band was consistently demonstrated. A similar selective loss in β-receptor-specific photolabeling has been recently described with desensitization of the S49 lymphoma β-receptor using a different photoaffinity probe [125I]iodoazidopindolol (Rashidbaigi et al., 1983). Whether this represents an actual loss of β-receptor-associated polypeptide in the 55,000 mol wt band or simply a selective decrease in labeling by photoaffinity ligands has not been determined.

Previous studies of structural changes in the turkey erythrocyte β-receptor, a system which demonstrates a heterologous form of desensitization, have demonstrated that desensitization is associated with a small "upward shift" in mobility of the major pABC-labeled polypeptides (Stiles et al., 1984). A number of lines of evidence indicate that this shift results from phosphorylation of β-receptor polypeptides (Stiles et al., 1984). An increase in phosphorylation of β-receptor-associated polypeptides has also been demonstrated in the frog erythrocyte β-receptor complex, a system characterized by homologous desensitization to β-agonists (Sibley et al., 1985). Perhaps an analogous phosphorylation event occurs upon persistent exposure of human lymphocytes to β-agonists resulting in the pronounced desensitization of the β-receptor-adenylate cyclase system. However, regardless of the mechanisms involved, these studies suggest that the process of desensitization in a human β-receptor model may be associated with structural alterations of the receptor. Future studies will hopefully elucidate the basis for these changes and determine whether similar molecular changes characterize the alterations in β-receptor function seen in various disease states.

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Figure 3. Alterations in pABC labeling with desensitization. Panel A: autoradiograph. Des = desensitized, Con = control. A selective reduction in labeling of the lower band occurs with desensitization. In addition, the upper band is broader in Des and migrated as two bands (mol wt = 68,000, mol wt = 90,000). The autoradiograph shown is representative of the results from five identical experiments. Panel B: densitometric tracing from another identical study. Con = dashed line; Des = dotted line.
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