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# A CARBENE-GENERATING PHOTOAFFINITY PROBE FOR BETA-ADRENERGIC RECEPTORS

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**A new radioiodinated (2.2 Ci/μmol) iodocyanopindolol derivative carrying a 4-(3-trifluoromethyldiazirino)benzoyl residue has been synthesized. The long-wavelength absorption of the diazirine permits formation of the carbene by photolysis under very mild conditions. [$^{125}$I]ICYP-diazirine binds with high affinity ($K_d$ = 60 pM) to β-receptors from turkey erythrocyte membranes. Upon irradiation, [$^{125}$I]ICYP-diazirine is covalently incorporated in a $M_r$ 40000 protein. Stereoselective inhibition of photolabeling by the (−)enantiomers of alprenolol and isoproterenol indicated that the $M_r$ 40000 protein contains a β-adrenergic binding site. The yield of specific labeling was up to 8.2% of total β-receptor binding sites. The $M_r$ 40000 protein photolabeled in the membrane could be solubilized at comparable yield with either digitonin or Triton X-100. Irradiation of digitonin-solubilized turkey erythrocyte membranes with [$^{125}$I]ICYP-diazirine resulted in specific labeling of two proteins with $M_r$ 40000 and 50000. In guinea-pig lung membranes, at least five proteins were photolabeled, of which one (with approximate $M_r$ 67000) was labeled specifically.**

## Introduction

Photoaffinity reagents [1,2] are efficient tools for biochemical characterization of ligand binding proteins in general and of hormone receptors in particular. They can be used to determine the molecular size of receptors in crude membrane preparations and to follow the binding protein through various stages of purification. Due to the extremely small number of β-adrenergic receptors, photoaffinity labeling requires probes which combine high affinity with high specific radioactivity and which give reasonable yields of covalent incorporation. Nitrene-generating aromatic azide derivatives of radioiodinated pindolol [3,4], cyanopindolol [5] and carazolol [6] have recently been shown to label specifically β-adrenergic receptors in avian and amphibian erythrocyte membranes. A nitrene-generating bifunctional reagent has been used for photoaffinity crosslinking of a carazolol derivative to β-receptors [7]. These studies [6] have revealed interesting differences in subunit molecular size of β-adrenergic receptors, presumably due to $\beta_1$ and $\beta_2$ subclasses.

The (3-trifluoromethyldiazirino)phenyl group has recently been introduced as a new carbene-generating photolabeling reagent [8]. Lipophilic compounds carrying this group have been successfully used for photolabeling of membrane compo-

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Abbreviations: ICYP-diazirine, (±)-1-[4-(3-trifluoromethyldiazirino)benzoyl]-3,3-dimethyl-6-hydroxy-7-(2-cyano-3-iodoindol-4-yloxy)-1,4-diazaheptane; ICYP-azide-1, (±)-1-(4-azidobenzimidyl)-3,3-dimethyl-6-hydroxy-7-(2-cyano-3-iodoindol-4-yloxy)-1,4-diazaheptane; ICYP-azide-2, (±)-1-(4-azidobenzoyl)-3,3-dimethyl-6-hydroxy-7-(2-cyano-3-iodoindol-4-yloxy)-1,4-diazaheptane; P40, P50, P67, proteins with $M_r$ = 40000, 50000, and 67000, respectively.

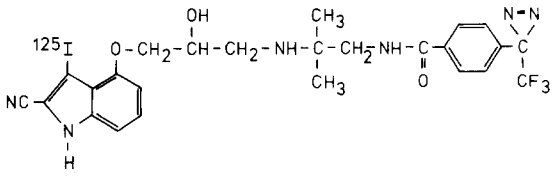

Fig. 1. Structure of [$^{125}$I]ICYP-diazirine.

nents [9,10]. We have synthesized a radioactive 4-(3-trifluoromethyldiazirino)benzoyl derivative of iodocyanopindolol, [$^{125}$I]ICYP-diazirine (Fig. 1). Photolabeling of $\beta_1$-adrenergic receptors of turkey erythrocyte membranes with this probe can be compared with results obtained with [$^{125}$I]ICYP-azide-2, an analogous nitrene-generating probe described previously [5]. Photoaffinity-labeling experiments were now extended to guinea-pig lung membranes containing predominantly $\beta_2$-adrenergic receptors and to detergent solubilized membrane preparations.

## Materials and Methods

*Materials.* 2-Cyano-4-(2,3-epoxypropoxy)indol was a generous gift from Drs. G. Engel and R. Berthold, Sandoz A.G. Basle, Switzerland. (−)-[$^3$H]Dihydroalprenolol (31 Ci/mmol), (±)[$^{125}$I]-iodohydroxybenzylpindolol (2 Ci/μmol) and Na$^{125}$I (2.2 Ci/μmol) were from Amersham, (+)- and (−)alprenolol hydrochloride from Hassle, (+)- and (−)isoproterenol bitartrate and bovine serum albumin from Sigma. Protein molecular weight standards for SDS-polyacrylamide gel electrophoresis were purchased from Boehringer Mannheim (aldolase, $M_r$ 40000) and Pharmacia (phosphorylase *b*, $M_r$ 97400; bovine serum albumin, $M_r$ 67000; ovalbumin, $M_r$ 45000; carbonic anhydrase, $M_r$ 30000; soybean trypsin inhibitor, $M_r$ 20100). Aprotinin and reagents for buffers and electrophoresis were obtained from Serva, Heidelberg. The buffers used were: buffer A: 10 mM Tris-HCl/90 mM NaCl (pH 7.4). Buffer B: 20 mM sodium phosphate (pH 7.4). Buffer C: 10 mM Tris-HCl (pH 7.4). Buffer D: 62.5 mM Tris-HCl/2% SDS/10% glycerol/5% 2-mercaptoethanol/0.001% Bromophenol blue (pH 6.8). Buffer E: 50 mM Tris-HCl/2 mM $MgCl_2$/1 mM $Na_2EDTA$ (pH 7.4). Buffer F: like buffer D but 5-times more concentrated.

*Synthesis of [$^{125}$I]ICYP-diazirine.* To 69 mg (0.3 mmol) 4-(3-trifluoromethyldiazirino)benzoic acid [11] in 0.6 ml anhydrous dioxane, 41 mg (0.36 mmol) *N*-hydroxysuccinimide in 0.6 ml dioxane, finally 74.5 mg (0.36 mmol) dicyclohexylcarbodiimide in 0.6 ml dioxane were added. A crystalline precipitate formed and the suspension was stirred for 2.5 h at room temperature. The reaction mixture was then filtered through glass wool and the precipitate was washed with three times 0.8 ml dioxane. 272 mg (0.9 mmol) 3,3-dimethyl-6-hydroxy-7-(2-cyanoindol-4-yloxy)-1,4-diazaheptane [5] were dissolved in the combined filtrates. The solution was stirred for 15 h and evaporated to dryness in vacuo. The residue was dissolved in 1 ml chloroform/methanol (95 : 5, v/v) and purified by chromatography on a silica gel column (1.4 × 20 cm; Kieselgel 60 Merck) using the same solvent mixture. The eluted CYP-diazirine (155 mg, corresponding to 86% yield) was homogeneous and free of starting materials as shown by thin-layer chromatography. It migrated with $R_F$ 0.60 in 1-butanol/acetic acid/water (4 : 1 : 1, v/v). The ultraviolet light absorption of CYP-diazirine at 340–380 nm is shown in Fig. 2. Further details of synthesis and characterization of this compound are published elsewhere.

CYP-diazirine was radioiodinated by the chloramine-T method to theoretical specific radioactivity (2.2 Ci/μmol) following the procedure of Engel et al. [12] modified as follows: 5 nmol CYP-diazirine were reacted with 1 nmol Na$^{125}$I. [$^{125}$I]ICYP-diazirine was separated by paper chromatography from excess CYP-diazirine using 0.1 M ammonium formiate (pH 7.5), 0.01% phenol in water/methanol (7 : 3, v/v). With this solvent system, [$^{125}$I]ICYP-diazirine ($R_F$ 0.09) and CYP-diazirine ($R_F$ 0.38) can be separated well.

*Membranes and solubilized preparations.* Turkey erythrocyte membranes were prepared according to a published procedure for pigeon erythrocyte membranes [13], but dithiothreitol and phenylmethylsulfonylfluoride were omitted and aprotinin (1 mg/l) was included. Guinea-pig lung membranes were prepared as described [14]. Membranes were stored in liquid $N_2$. Pelleted membranes were solubilized either at a protein con-

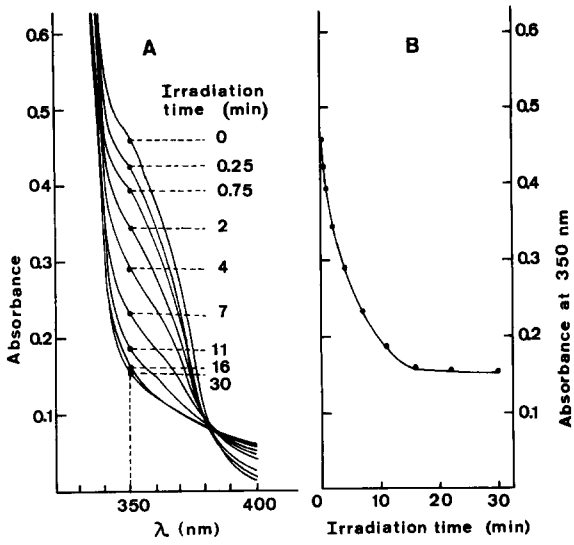


Fig. 2. Photolysis of CYP-diazirine. (A) Changes in ultraviolet light spectrum on irradiation. A 1 mM CYP-diazirine solution in 70 % ethanol/30% water (v/v) was irradiated at 4°C at 366 nm with a 6 W ultraviolet light lamp for the times indicated. (B) Decrease in 350 nm absorbance as a function of irradiation time

centration of 3 mg/ml in buffer A containing 1% digitonin or at a protein concentration of 6 mg/ml in buffer B containing 0.5% Triton X-100. The suspensions were mixed for 60 min and centrifuged for 30 min at 30000 × *g* and 4°C.

Protein was determined by the method of Lowry et al. [15] using bovine serum albumin as standard. In the case of solubilized preparations containing detergent, an equal amount of detergent was included in the protein standard.

*Measurement of β-adrenergic binding sites.* The concentration of β-receptor binding sites in membranes and digitonin-solubilized preparations was determined using established radioligand binding assays [16]. [$^3$H]Dihydroalprenolol binding to turkey erythrocyte membranes and [$^{125}$I]iodocyanopindolol binding to guinea-pig lung membranes was measured by filtration using Whatman GF/C filter disks [12,17]. [$^{125}$I]Iodohydroxybenzylpindolol binding to digitonin-solubilized preparations from turkey erythrocyte membranes was determined by a Sephadex G-50 gel filtration assay described for [$^3$H]dihydroalprenolol binding [17]. The concentration of β-adrenergic binding sites was 1.2 ± 0.25 pmol/mg protein in turkey erythrocyte membranes, 0.44 ± 0.1 pmol/mg protein in guinea-pig lung membranes, and 0.8 ± 0.2 pmol/mg protein in digitonin-solubilized preparations from turkey erythrocyte membranes.

*Photoaffinity labeling of membranes.* Samples containing membranes and [$^{125}$I]ICYP-diazirine in 0.3 to 6 ml $N_2$-saturated buffer C were incubated for 60 min at 30°C in the dark. Nonspecific labeling was determined in the presence of nonradioactive β-adrenergic ligands (see legends to figures). When isoproterenol was used, 40 μM ascorbic acid was added as antioxidant. At the end of the incubation, samples were cooled on ice for 10 min, filled up to 6 ml with ice-cold buffer C and purged with $N_2$ for 15 s. Photolysis was routinely performed by irradiation at 366 nm for 60 min at 0°C using a 6 W ultraviolet light lamp (Fluotest, Original Hanau). The lamp was positioned 8 cm above the open sample tubes. Photolysis was also carried out for 10 min at 0°C at 15 cm distance using a 450 W high-pressure xenon lamp (Osram) equipped with a ultraviolet light filter (cut-off wavelength, 345 nm). After photolysis, the samples were centrifuged for 10 min at 30000 × *g* at 4°C. Membrane pellets were washed once with 5 ml buffer C and covalent incorporation of radioactivity was analyzed by SDS-polyacrylamide slab gel electrophoresis according to Laemmli [18]. Acrylamide concentrations were 4% in the stacking gel (1.5 × 14 × 0.2 cm) and 10% in the separating gel (11 × 14 × 0.2 cm). Samples ( 1 mg/ml) dissolved in buffer D were applied and electrophoresis was performed at room temperature for 16 h at 40 V. The gel was stained with Coomassie blue, destained and dried onto filter paper as described [19]. Autoradiography was carried out at −70°C using Kodak X-Omat film and DuPont Cronex Cassettes with intensifying screens. The dried gel tracks were cut into 2 mm slices and counted in a gamma-counter.

## Results

*Photolysis of CYP-diazirine*

Conditions for photolysis of [$^{125}$I]ICYP-diazirine were established using CYP-diazirine. As shown in Fig. 2, the absorption shoulder at 350 nm (ε = 460; 70% ethanol/30% water) decreased upon irradiation due to photolysis of the diazirine

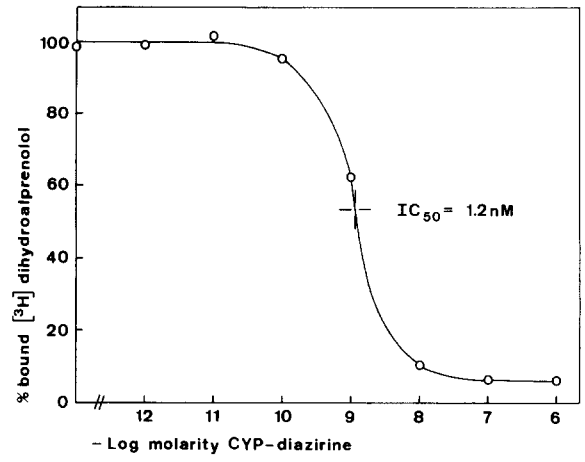


Fig. 3. Inhibition of [$^3$H]dihydroalprenolol binding by CYP-diazirine. Duplicates of samples containing 100 $\mu$g turkey erythrocyte membranes, 20 nM [$^3$H]dihydroalprenolol and 1 pM to 1 $\mu$M CYP-diazirine in 0.5 ml buffer E were incubated in the dark for 30 min at 30°C and cooled on ice for 5 min. Each sample was then diluted with 4 ml of ice-cold buffer E and filtered through a glass microfiber filter (Whatman GF/C, 2.5 cm diameter) under reduced pressure. The filter was washed four times with 5 ml buffer E, dried and counted at 50% efficiency.

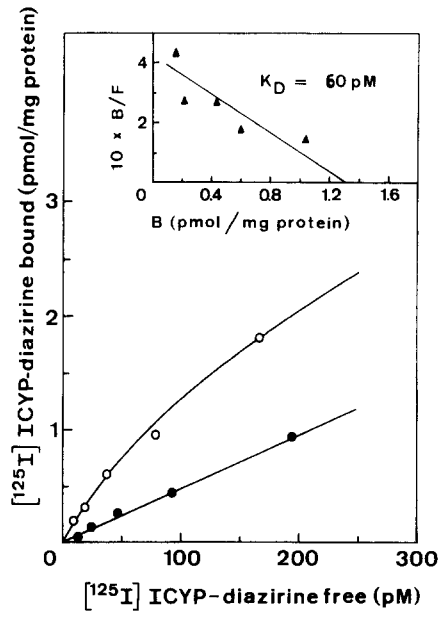


Fig. 4. Binding of [$^{125}$I]ICYP-diazirine. Samples in duplicate containing 10 $\mu$g turkey erythrocyte membranes in 0.5 ml buffer E were incubated for 60 min at 30°C in the dark with the indicated concentrations of [$^{125}$I]ICYP-diazirine in absence (○——○), and in presence of 2 $\mu$M (−)alprenolol (●——●). Each sample was then processed as described in the legend to Fig. 3 and counted at 78% efficiency. Insert: Scatchard plot of specific binding.

group [8]. Photolysis of a 1 mM CYP-diazirine solution was complete after 15 min irradiation at 366 nm with a 6 W ultraviolet light lamp (Fig. 2B). When filtered light of $\geq$ 345 nm wavelength from a 450 W high-pressure xenon lamp was used, photolysis was completed in 8 min.

*Binding of CYP-diazirine and [$^{125}$I]ICYP-diazirine*

The affinity of CYP-diazirine for $\beta$-receptor sites was determined by competitive displacement of a $\beta$-adrenergic radioligand. CYP-diazirine at 1.2 nM inhibited specifically 50% of [$^3$H]dihydroalprenolol binding to turkey erythrocyte membranes ($K_d$ = 10 nM, at 20 nM concentration) (Fig. 3). $K_d$ of CYP-diazirine is 380 pM according to Ref. 16. Saturable binding of [$^{125}$I]ICYP-diazirine and nonspecific binding in the presence of (−)alprenolol are shown in Fig. 4. From the Scatchard plot (Fig. 4, insert), a $K_d$ value of 60 pM and a concentration of $\beta$-adrenergic sites of 1.3 pmol/mg protein were obtained with turkey erythrocyte membranes.

*Labeling of turkey erythrocyte membranes*

When mixtures containing membranes and [$^{125}$]ICYP-diazirine were irradiated, part of the radioactivity was incorporated covalently in a protein with $M_r$ 40000 (P40), as shown by electrophoresis and autoradiography (Fig. 5). Part of the radioactivity migrated below the dye marker, presumably representing [$^{125}$I]ICYP-diazirine free and/or bound to lipids. Labeling of P40 was dependent on time and intensity of photolysis (compare Fig. 2B). The intensity of P40 labeling increased between 10 pM and 100 pM concentrations approaching a limit at 500 pM. At this high concentration of photolabel, small amounts of radioactivity were also incorporated in proteins of higher molecular weight. P40 labeling was inhibited by 1 $\mu$M (−)alprenolol or 7.5 $\mu$M (−)isoproterenol. The same concentrations of the (+)enantiomers inhibited only very slightly.

For quantition, gel tracks were cut into 2 mm slices and counted. Fig. 6 shows histograms of $^{125}$I-radioactivity resulting from photolabeling with 100 pM [$^{125}$I]ICYP-diazirine. From the difference

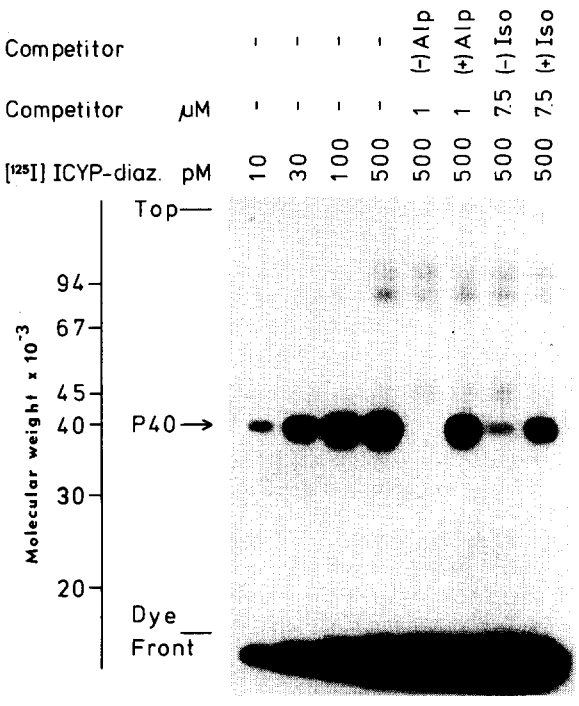


Fig. 5. Photoaffinity labeling of intact turkey erythrocyte membranes. Samples contained turkey erythrocyte membranes, 100 μg protein corresponding to 120 fmol β-adrenergic binding sites, and the indicated concentrations of [$^{125}$I]ICYP-diazirine and nonradioactive ligands. Sample volumes during incubation were 6 ml at 10 and 30 pM, 3 ml at 100 pM and 0.6 ml at 500 pM [$^{125}$I]ICYP-diazirine concentrations. The molar ratio of β-adrenergic binding sites to probe ranged only from 1:0.5 to 1:2.5. After photolysis and washing, 60 μg of membrane protein was applied to an electrophoresis gel. The autoradiograph was obtained after 16 h of exposure. The molecular weight calibration on the ordinate was obtained with marker proteins (see: Materials and Methods).

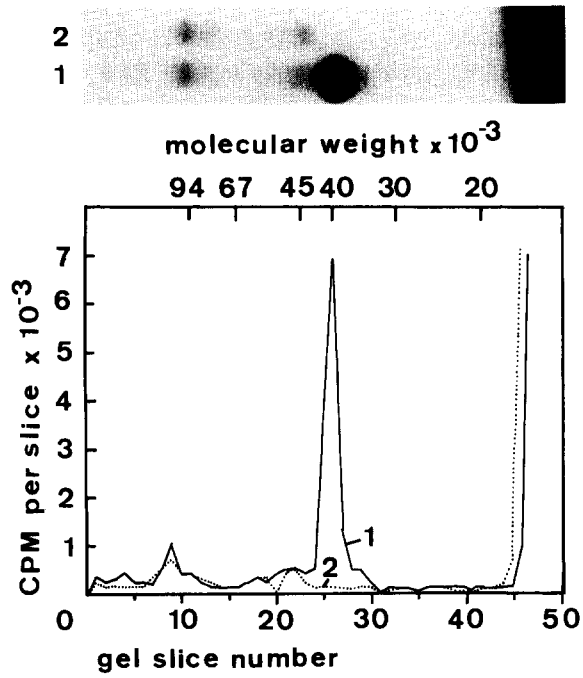


Fig. 6. Radioactivity patterns of photoaffinity-labeled turkey erythrocyte membranes in SDS-polyacrylamide gels. Top, autoradiographs from membranes (60 μg of protein) photolabeled at 100 pM [$^{125}$I]ICYP-diazirine in the absence (1) and presence (2) of 5 μM (−)alprenolol. Below, radioactivity profiles of the same gels.

of P40 labeling in absence and presence of (−)alprenolol, β-receptor specific covalent incorporation of [$^{125}$I]ICYP-diazirine was estimated. In the experiment in Fig. 6, 4.4% of the total β-receptor sites was covalently labeled. At a saturating concentration (2 nM) of [$^{125}$I[ICYP-diazirine, the yield of β-receptor specific covalent labeling was 8.2%.

*Solubilization of photolabeled membrane proteins*

After incubation of membranes with [$^{125}$I]-ICYP-diazirine and photolysis, labeled proteins were solubilized with digitonin or Triton X-100. Electrophoresis/autoradiography showed specifically labeled in P40 in preparations from turkey erythrocyte membranes solubilized with digitonin (Fig. 7A) or Triton X-100 (Fig. 7B). Noncovalently attached label, which migrates below the dye marker, was reduced to very low levels when the solubilized, labeled proteins were precipitated with acetone prior to gel electrophoresis (Fig. 7A).

*Labeling of digitonin-solubilized turkey erythrocyte membranes*

Solubilized preparations were incubated with [$^{125}$I]ICYP-diazirine and photolysed. Excess probe was removed either by acetone precipitation of proteins or by dialysis, as described in the legends to Figs. 7 and 8. Two proteins with $M_r$ 40000 (P40) and 50000 (P50) were labeled specifically, since labeling was completely inhibited by (−)alprenolol (Fig. 8). Radioactivity incorporated in P40 and P50, as well as unbound radioactivity, was considerably greater in the dialyzed samples (Fig. 8B) as compared to samples precipitated with acetone (Fig. 8A). This is apparent from the different

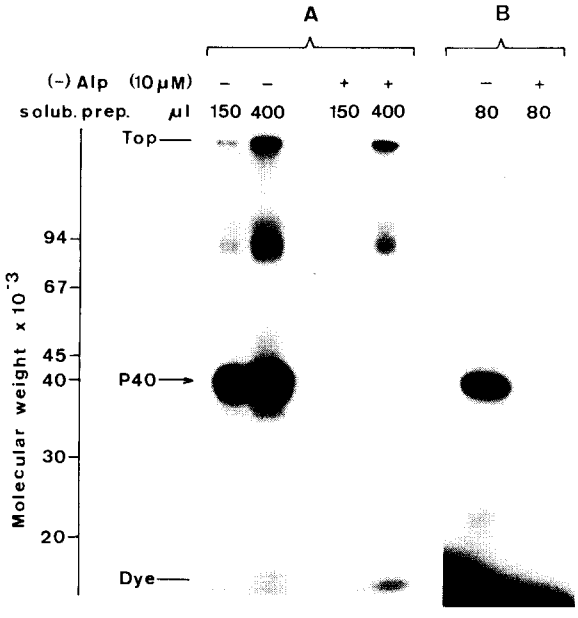


Fig. 7. Comparison of photoaffinity-labeled proteins from solubilized turkey erythrocyte membranes. (A) Solubilization with digitonin. (B) Solubilization with Triton X-100. Samples of 2 ml volume containing 3 mg membrane protein and 1 nM [$^{125}$I]ICYP-diazirine in absence and presence of 10 μM (−)alprenolol were incubated and photoaffinity labeled as described in Materials and Methods. The labeled membranes were washed with 10 ml buffer C and treated with 1 ml 1% digitonin in buffer A or 0.5 ml 0.5% Triton X-100 in buffer B. The proteins in the digitonin-solubilized preparation (A) were precipitated with 10 ml of ice-cold acetone. After 10 min on ice, the samples were centrifuged for 5 min at 30000 × *g* and 4°C. The precipitate was washed with 1 ml 5% trichloroacetic acid and 1 ml water, and dissolved in 200 μl buffer D. The Triton X-100 solubilized preparation (B) was mixed with one-fourth volume of buffer F. Samples corresponding to the indicated volumes of original solubilized preparation were applied to electrophoresis gels and autoradiographs were exposed for 14 h.

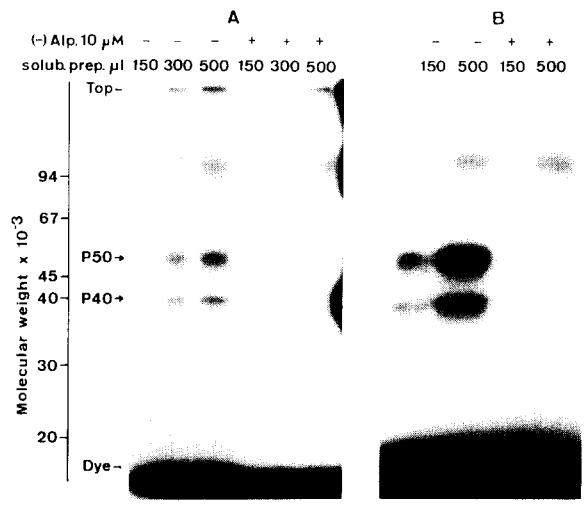


Fig. 8. Photoaffinity labeling of digitonin-solubilized turkey erythrocyte membranes. Solubilized membranes, 1 ml containing 220 μg protein and 180 fmol binding sites, were incubated in the dark with 500 pM [$^{125}$I]ICYP-diazirine for 60 min at 30°C and cooled on ice for 10 min. Nonspecific labeling was determined in presence of 10 μM (−)alprenolol. Following photolysis, unbound [$^{125}$I]ICYP-diazirine was partially removed in two ways: (A) Proteins were precipitated with acetone and the precipitate was washed and dissolved in 200 μl buffer D as described in the legend to Fig. 7. (B) The samples were dialyzed overnight at 4°C against 1 liter 5 mM Tris-HCl, 0.2% SDS (pH 6.8), lyophilized, and dissolved in 200 μl buffer D. Samples corresponding to the indicated volumes of original solubilized preparation were applied to electrophoresis gels. Autoradiographs were obtained after 120 h (A) and 12 h (B) of exposure.

autoradiographic exposure times stated in the legend to Fig. 8. Acetone precipitation of proteins is more efficient in removing excess [$^{125}$I]ICYP-diazirine than dialysis, but part of the labeled P40 and P50 was lost, presumably due to incomplete precipitation or resolubilization during washing. In contrast, dialysis gives good recovery of labeled P40 and P50, but it is quite inefficient in removing excess [$^{125}$I]ICYP-diazirine. This might be a consequence of micelle formation in the presence of digitonin, which could entrap the label and prevent its removal by dialysis.

*Labeling of guinea-pig lung membranes*

Irradiation of guinea-pig lung membranes in presence of 1 nM [$^{121}$I]ICYP-diazirine resulted in incorporation of radioactivity into several proteins with $M_r$ ranging from 50000 to 80000 (Fig. 9A). Most of these proteins were labeled unspecifically, that is, in the presence of various concentrations of (−) and (+)alprenolol. Labeling of a protein with approximate $M_r$ 67000 (P67) was, however, specific, since labeling was inhibited by 10 nM (−)alprenolol but not 10 nM (+)alprenolol. In contrast to the sharp bands of nonspecifically labeled proteins, the specifically labeled component migrated as a diffuse broad band. The fact that a low (10 nM) concentration of (−)alprenolol was sufficient for stereoselective inhibition of P67 labeling with 1 nM [$^{125}$I]ICYP-diazirine points to a high affinity of the lung $\beta_2$-adrenergic receptor

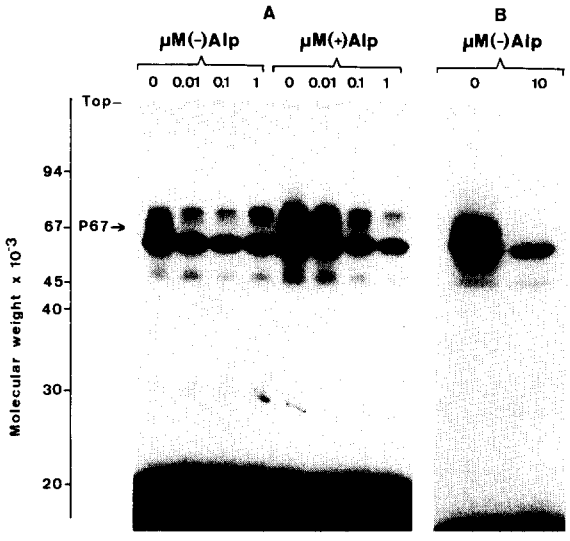


Fig. 9. Photoaffinity labeling of guinea-pig lung membranes. (A) Intact membranes, 335 μg protein corresponding to 145 fmol β-adrenergic binding sites, were incubated in 0.3 ml buffer C containing 1 nM [$^{125}$I]ICYP-diazirine in absence and in presence of the indicated concentrations of (−)- and (+)alprenolol, and photoaffinity-labeled as described in Materials and Methods. Samples containing 270 μg protein were applied to the gel. The autoradiographs were exposed for 72 h. (B) Intact membranes, 2.52 mg protein, were incubated in 2 ml buffer C containing 1 nM [$^{125}$I]ICYP-diazirine in the absence and presence of 10 μM (−)alprenolol and photoaffinity-labeled as described in Materials and Methods. Photolabeled membranes were washed with 10 ml buffer C and solubilized with 0.5 ml 0.5% Triton X-100 in buffer B. 80 μl of the solubilized membranes were mixed with 20 μl buffer F and applied to the electrophoresis gel. Exposure was for 14 h.

for (−)alprenolol. This is in agreement with results of radioligand binding experiments [20] which indicated a lower $K_d$ value (0.5 nM) for [$^3$H]dihydroalprenolol binding to $\beta_2$-receptors from rat lung membranes as compared with $\beta_1$ receptors from turkey erythrocyte membranes ($K_d$ = 8.2 nM) [21]. Solubilization of the photolabeled lung membranes with Triton X-100 yielded labeled P67 together with most of the other nonspecifically labeled proteins (Fig. 9B). However, one of the nonspecifically labeled proteins with $M_r$ 80000 was not recovered in the Triton X-100 solubilized preparation. This suggests that it might be tighly bound to detergent insoluble structures, perhaps the cytoskeleton.

## Discussion

The same cyanopindolol amine precursor used for the synthesis of CYP-azide-1 and CYP-azide-2 [5] was also used for the synthesis of CYP-diazirine. Moreover, the primary amine derivatives of alprenolol and carazolol have likewise been used for synthesis of a fluorescent β-adrenergic antagonist [22] and several β-adrenergic affinity adsorbents [23]. Thus, primary amines of β-adrenergic ligands have proved to be versatile synthetic intermediates. Since these compounds already contain the whole structure required for specific high affinity binding to β-receptors, derivatives of them are easily formed usually in one step. Various β-adrenergic probes may thus be obtained, all starting from the primary amine precursor, with a minimum of synthetic effort. The diazirine group absorbs at a higher wavelength (~ 350 nm) than the aromatic azides (~ 270 nm) used previously. This has the advantage that photolysis of [$^{125}$I]-ICYP-diazirine can be effected by low irradiation intensities and at wavelengths far above protein absorption. This should permit the labeling of β-receptors on intact cells with little or no damage to other proteins (including other components of adenylate cyclase).

[$^{125}$I]ICYP-diazirine ($K_d$ = 60 pM) has a considerably higher affinity for β-receptors in turkey erythrocyte membranes than the noniodinated parent compound, CYP-diazirine ($K_d$ = 380 pM). This has also been observed to be the case with other iodinated indol-derived antagonists, such as pindolol [24] and CYP-azides (unpublished results). Thus, it seems plausible to assume that introduction of iodine in the cyanoindolyl moiety enhanced the affinity. The total concentration of specific binding sites for [$^{125}$I]ICYP-diazirine in turkey erythrocyte membranes was the same as that found with other radioligands. The specific covalent labeling with [$^{125}$I]ICYP-diazirine of a protein with $M_r$ 40000 (P40) in turkey erythrocyte membranes confirms earlier results with other photoaffinity probes [4–6]. The concentration dependence of P40 labeling closely paralleled saturation of specific binding sites. The maximal specific covalent labeling of 8.2% of total specific binding sites in turkey erythrocyte membranes may be compared with maximal specific labeling yields of

8.9 and 17.2% with [$^{125}$I]ICYP-azide-1 and -2, respectively [5].

Due to the very low $K_d$ values of these $\beta$-adrenergic photoaffinity probes, substantial dissociation of the receptor-ligand complex is unlikely to occur. Thus, the moderate, far-from-complete specific labeling is more likely a consequence of side-reactions of receptor-bound photoactivated probe with lipids, water or buffer substances. This would imply that the binding site on the receptor is not shielding the bound ligand effectively enough to prevent interactions with neighbouring molecules. Nevertheless, the lower labeling yield obtained with the carbene-generating probe is rather surprising, since [$^{125}$I]ICYP-diazirine and [$^{125}$I]ICYP-azide-2 have identical structures with exception of the photolabile trifluoromethyldiazirine and azide groups, respectively. Moreover, carbenes are considered to be more reactive than nitrenes [1].

The studies of Brunner and Richards [9], who have compared the labeling efficiency of phosphatidylcholine analogues carrying the same carbene- and nitrene-generating groups as described here, showed that the carbene labeled 10-times more lipid than the nitrene when the phosphatidylcholine analogs were incorporated into liposomes containing saturated phospholipid. But, when liposomes containing a mixture of a saturated and an unsaturated phospholipid were used, the unsaturated lipid was labeled with about equal efficiency by both probes. On the other hand, gramicidin A incorporated in liposomes containing saturated phospholipid was labeled at the nucleophilic tryptophan sidechain 2–3-times more effectively by the nitrene. It was reasoned that, due to the preference of carbenes for C-H insertion reactions with lipids, some of the carbene may have been used up, thus lowering the effective concentration available for labeling the peptide. The lower labeling efficiency of carbene versus nitrene towards the $\beta$-receptor could likewise be due to a greater reactivity of the carbene towards lipids. This is in keeping with the finding that more radioactive low molecular weight material was found on electrophoresis gels with [$^{125}$I]-ICYP-diazirine than with [$^{125}$I]ICYP-azide-2 (compare Fig. 6 with Fig. 5 of Ref. 5).

Solubilization of the $\beta$-adrenergic receptor is required for purification [7] and reconstitution [25]. Among the detergents tested, mainly digitonin has been used for receptor solubilization, since it has been found to solubilize the $\beta$-receptor in a form capable of binding radioligands [17,21]. However, digitonin, due to its poor solubility, low critical micelle concentration and large micelle size [26] and due to its interference with adenylate cyclase activity, is not an ideal detergent. We could show that photolabeled P40 can also be solubilized from turkey erythrocyte membranes with Triton X-100 with an efficiency comparable to that of digitonin treatment. We had shown before that in intact membranes P50 was specifically photolabeled but with lower yield than P40 and only at low concentrations of [$^{125}$I]ICYP-azides [5], whereas [$^{125}$I[ICYP-azides at high concentrations (like [$^{125}$I]ICYP-diazirine at all concentrations) labeled only P40. In digitonin-solubilized membranes, P40 and P50 were both specifically labeled by [$^{125}$I]ICYP-diazirine. Moreover, in the solubilized preparation, P50 was labeled by the carbene with even slightly higher efficiency than P40. Besides the P40 protein, a protein in the higher $M_r$ weight range ($M_r = 45\,000 \pm 3\,000$) was also labeled in turkey erythrocyte membranes by $^{125}$I-labeled *p*-azidobenzylcarazolol [27], whereas only the P40 protein was tagged by [$^{125}$I]iodoazidobenzylpindolol [4]. We have no explanation for the peculiar labeling pattern. One might, however, keep these differences in mind when one monitors receptor purification with photoaffinity reagents.

Photolabeling experiments with mammalian lung membranes have revealed differences between $\beta$-receptor containing mammalian and avian plasma membranes [6]. In guinea-pig lung membranes at least five proteins became labeled upon photolysis of [$^{125}$I]ICYP-diazirine, but only one of the proteins, with an approximate $M_r$ 67 000 (P67), was labeled specifically (Fig. 9). Although the nature of the proteins tagged nonspecifically in lung membranes by [$^{125}$I]ICYP-diazirine and their relationship to the $\beta$-adrenergic receptor are not known, one should take into consideration that the membrane preparation may originate from different types of cell, including smooth muscle cells [14]. It is possible, therefore, that $\alpha$-adrenergic receptors might be present in the preparation and account for 'nonspecific' labeling. Additional ex-

periments with $\alpha$-adrenergic ligands are required to see whether that is the case. After this work was completed, Lavin et al. [6] reported that their photoaffinity reagent, $^{125}$I-labeled *p*-azidobenzylcarazolol, labeled specifically three proteins in rat and rabbit lung membrane preparations which contain mixtures of $\beta_1$- and $\beta_2$-adrenergic receptors in different proportions. These proteins had $M_r = 62\,000$–$65\,000$, $45\,000$–$47\,000$ and $36\,000$–$38\,000$. Guinea-pig lung membranes contain $\beta_2$- and $\beta_1$-receptors in an approximate ratio of 4 : 1 [12]. It is possible that in our experiments, specific labeling of additional $\beta$-receptor binding subunits might have been obscured by the presence of considerable amounts of nonspecifically labeled components. The differences in labeling patterns observed by us and Lavin et al. [6] could also reflect the different photoaffinity reagents used and/or result from species differences. Moreover, in the experiments of Lavin et al. [6], only one high concentration of unlabeled competitor, namely either 10 $\mu$M (−)alprenolol or 100 $\mu$M (−)isoproterenol, was used to verify specific labeling, whereas we have used a range of concentrations from 10 nM to 1 $\mu$M of (+) and (−)enantiomers of alprenolol for the assessment of labeling specificity. But, be that as it may, the information from photoaffinity labeling experiments reported so far indicates that the binding subunits of $\beta$-receptors from erythrocytes of turkey, pigeon and duck, usually, classified as $\beta_1$-receptors, have molecular weights in the range 39 000–52 500 [4–6]. In contrast, $\beta$-receptors from frog erythrocytes and rat reticulocytes classified as $\beta_2$-receptors [4] apparently have somewhat larger subunit molecular weights of 58 000 [7], 65 000 and 53 000 [6], respectively. Moreover, these $\beta_2$-receptor subunits migrate on electrophoresis gels as rather diffuse bands characteristic for glycoproteins [4,7]. Thus based on these criteria, the specifically labeled P67 in guinea-pig lung membranes which likewise migrates as a diffuse band would be classified as a $\beta_2$-receptor.

The photoaffinity labeling technique has thus proved itself as a useful tool for the molecular identification of $\beta$-receptors and their subtypes. Information obtained with this technique supplements and refines the classification of $\beta$-receptors based solely on pharmacological properties.

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After submission of this manuscript, Benovic et al. [28] reported evidence suggesting that the apparent heterogeneity of photoaffinity-labeled $\beta$-receptor peptides in mammalian lung membranes described earlier by the same group [6] was due to proteolysis. On the basis of these more recent experiments a protein with approximate $M_r$ 64 000 was specified as $\beta_2$-receptor in rat and hamster lungs. These findings strongly support our evidence that in guinea-pig lung membranes a protein with an approximate $M_r$ 67 000 (P67) represents a specific $\beta_2$-adrenergic receptor. The P67 protein was the only protein labeled specifically in the experiments reported above.

**References**

1 Baley, H. and Knowles, J.R. (1977) Methods Enzymol. 46, 69–114
2 Chowdry, V. and Westheimer, F.H. (1979) Annu. Rev. Biochem. 48, 293–325
3 Rashidbaigi, A. and Ruoho, A.E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1609–1613
4 Rashidbaigi, A. and Ruoho, A.E. (1982) Biochem. Biophys. Res. Commun. 106, 139–148
5 Burgermeister, W., Hekman, M. and Helmreich, E.J.M. (1982) J. Biol. Chem. 257, 5306–5311
6 Lavin, T.N., Nambi, P., Heald, S.L., Jeffs, P.W., Lefkowitz, R.J. and Caron, M.G. (1982) J. Biol. Chem. 257, 12332–12340
7 Shorr, R.G.L., Heald, S.L., Jeffs, P.W., Lavin, T.N., Strohsacker, M.W., Lefkowitz, R.J. and Caron, M.G. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 2778–2782
8 Brunner, J., Senn, H. and Richards, F.M. (1980) J. Biol. Chem. 255, 3313–3318
9 Brunner, J. and Richards, F.M. (1980) J. Biol. Chem. 255, 3319–3329
10 Brunner, J. and Semenza, G. (1981) Biochemistry 20, 7174–7182
11 Nassal, M. (1983) Liebigs Ann. Chem., in the press

12 Engel, G., Hoyer, D., Berthold, R. and Wagner, H. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. 317, 277–285
13 Puchwein, G., Pfeuffer, T. and Helmreich, E.J.M. (1974) J. Biol. Chem. 249, 3232–3240
14 Kleinstein, J. and Glossmann, H. (1978) Naunyn-Schmiedeberg's Arch. Pharmacol. 305, 191–200
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Williams, L.T. and Lefkowitz, R.J. (1978) Receptor Binding Studies in Adrenergic Pharmacology, Raven Press, New York
17 Caron, M.G. and Lefkowitz, R.J. (1976) J. Biol. Chem. 251, 2374–2384
18 Laemmli, U.K. (1970) Nature 227, 680–685
19 Fairbanks, G., Levinthal, C. and Reeder, R.H. (1965) Biochem. Biophys. Res. Commun. 20, 393–399
20 Barnett, D.B., Nahorski, S.R. and Rugg, E. (1977) Br. J. Pharmacol. 61, 459P
21 Vauquelin, G., Geynet, P., Hanoune, J. and Strosberg, A.D. (1979) Eur. J. Biochem. 98, 543–556
22 Henis, Y.I., Hekman, M., Elson, E.L. and Helmreich, E.J.M. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 2907–2911
23 Burgermeister, W. and Hekman, M. (1982) in Neuroreceptors (Hucho, F., ed.), pp. 111–124, Walter de Gruyter, Berlin
24 Ezrailson, E.G., Garber, A.J., Munson, P.J., Birnbaumer, L. and Entman, M.L. (1981) J. Cyclic Nucleotide Res. 7, 13–26
25 Eimerl, S., Neufeld, G., Korner, M. and Schramm, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 760–764
26 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
27 Shorr, R.G.L., Strohsacker, M.W., Lavin, T.N., Lefkowitz, R.J. and Caron, M.G. (1982) J. Biol. Chem. 257, 12341–12350
28 Benovic, J.L., Stiles, G.L., Lefkowitz, R.J. and Caron, M.G. (1983) Biochem. Biophys. Res. Commun. 110, 504–511