ELSEVIER

European Journal of Pharmacology 334 (1997) 115–126

ejp

Probing human β_1- and β_2-adrenoceptors with domain-specific fusion protein antibodies

Roland Jahns [a,*], Christian Siegmund [a], Valérie Jahns [a], Helmut Reiländer [b], Armin Maidhof [c], Werner Müller-Esterl [c], Martin J. Lohse [a], Fritz Boege [a]

[a] *Department of Pharmacology, University of Würzburg, Versbacher Straße 9, D-97078 Würzburg, Germany*
[b] *Max-Planck-Institute of Biophysics, D-60596 Frankfurt / Main, Germany*
[c] *Department of Pathobiochemistry, University of Mainz, D-55099 Mainz, Germany*

Received 23 May 1996; revised 7 August 1996; accepted 13 August 1996

Abstract

In order to generate antibodies suitable for immunological studies on β-adrenoceptors constitutively expressed at low levels in cells or tissues we have produced fusion proteins of the amino- and carboxy-terminus, and the second extracellular loop of the human β_1- or β_2-adrenoceptors with bacterial glutathione-*S*-transferase in *E. coli*. Rabbit antibodies raised against these fusion proteins strongly reacted with intact human β_1- or β_2-adrenoceptors in a subtype- and domain-specific manner. Antibodies directed against the second extracellular loop of the β_1-adrenoceptor reacted stronger with non-denatured receptors and decreased the affinity of the ^{3}H-labelled antagonist (−)-4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]CGP 12 177), indicating a specific interaction with the native receptor. In contrast, antibodies directed against carboxy- and amino-terminal receptor domains reacted strongly both with denatured and non-denatured receptors but did not interfere with binding of [^{3}H]CGP 12 177. Affinity purified antibodies were used for detecting the β_1- or the β_2-adrenoceptor subtype heterologously produced in Sf9 cells by enzyme-linked immunosorbent assay, Western blotting, immunoprecipitation, and indirect immunofluorescence microscopy. Moreover, we could demonstrate that avidity, titers, and specificity of these antibodies were high enough for studying β-adrenoceptors constitutively expressed in human A431 cells, where we observed a patched membrane distribution of the receptors. © 1996 Elsevier Science B.V.

Keywords: β_1-Adrenoceptor; β_2-Adrenoceptor; Antibody; Fusion protein

1. Introduction

Over the last two decades the analysis of β-adrenoceptors has progressed by pharmacological and biochemical means (Lohse et al., 1993). However, reports on immunological studies of β-adrenoceptors are rare, and sometimes contradictory. Thus, data obtained with receptor antibodies on the subcellular localization and ligand-regulated recycling of the receptors (Raposo et al., 1989) do not always agree with the results obtained with epitope-tagged receptors (Von Zastrow and Kobilka, 1992). Moreover, the molecular size and the shape of the protein bands of β_2-adrenoceptors of A431 cells, as demonstrated by immunoblotting (Kaveri et al., 1987) differed significantly from data obtained in the same cells by photoaffinity-labelling of the receptors (Boege et al., 1988). The generation of antibodies against these receptors appears to be unusually difficult (for review, see Malbon et al., 1991). This may in part be due to a lack of immunogenicity because mammalian β-adrenoceptors are very hydrophobic membrane proteins which have relatively small extracellular domains (Frielle et al., 1988). In addition, the protein structure of these receptors is highly conserved even among evolutionarily distant species (Moxham et al., 1986). Several attempts have been made to raise antibodies against purified β-adrenoceptors (Chapot et al., 1989), receptor-rich membrane preparations from native (Kaveri et al., 1987) and transfected cells (Chapot et al., 1990), or conjugated peptides corresponding to selected receptor domains (Aoki, 1992; Bahouth et al., 1991; Dunkel et al., 1989; Luxembourg et al., 1991; Wang et al., 1989a,b). It has also been attempted to raise anti-idiotypes to antibodies di-

* Corresponding author. Fax: (49-931) 201-3539; e-mail: jahns@wpxx02.toxi.uni-wuerzburg.de

rected against β-adrenergic ligands (Strosberg, 1989). A major problem of these antibodies seemed to be an extensive cross-reactivity between β-adrenoceptor subtypes or even other closely related receptors, such as cholinergic or muscarinic receptors (Bahouth et al., 1991; Couraud et al., 1983; Kaveri et al., 1987). Moreover, some of these antibodies appeared to be directed against epitopes not available on native membrane-bound receptors (Chapot et al., 1989).

A few immunohistological studies of β-adrenoceptor distribution in brain have been published (Aoki, 1992; Strader et al., 1983) but the immunological study of β-adrenoceptors constitutively expressed at low levels in mammalian cells or tissues still presents technical difficulties. As a consequence, the detailed analysis of the subcellular distribution of β-adrenoceptors required the use of heterologously expressed β-adrenoceptors tagged with N- or C-terminal epitopes (Von Zastrow and Kobilka, 1992, 1994), whereas data on the receptor distribution in tissues were largely derived from radioligand autoradiography (Dawidek and Robinson, 1995; Fernandez-Lopez et al., 1994; Woo and Leon, 1995) or in-situ hybridization of receptor-specific mRNAs (Meister et al., 1994). These techniques allow for the analysis of β-adrenoceptor distribution in various organs but they are less suitable for studying receptor localization on a cellular or subcellular level.

Immunization with recombinant proteins made by fusing selected domains of mammalian proteins with bacterial antigens appears to be a promising way of generating antibodies of predetermined specificity (Lerner, 1982). Here, we have chosen this approach for generating antibodies directed against selected extramembraneous domains of human β_1- and β_2-adrenoceptors. We report on the specificity of these antibodies and show their application in the study of the cellular disposal of β_2-adrenoceptors, constitutively expressed in human A431 epidermoid cells.

2. Materials and methods

2.1. Production and purification of fusion proteins

DNA fragments encoding the N-terminus, C-terminus or second extracellular loop of the human β_1- or β_2-adrenoceptors (see Fig. 1) were amplified by polymerase chain reaction (PCR) with an upstream *Bam*HI and a downstream *Eco*RI restriction site for subcloning. The PCR fragments were restricted, and inserted into the pGEX-1λT-vector (Pharmacia, Uppsala, Sweden) in frame with the 3′-end of the coding sequence of bacterial glutathione-*S*-transferase. The constructs were controlled by sequencing before transformation of *E. coli* XL-1 blue cells (Stratagene, Heidelberg, Germany).

Expression of fusion proteins was induced at 30°C with 1 mM isopropyl-1-thio-β-D-galacto-pyranoside for 3 h. Subsequently, the cells were harvested on ice, pelleted ($4000 \times g$, 4°C for 10 min), resuspended in a 1/10 volume of ice-cold PBS (phosphate-buffered saline: 140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), and lysed with a French Press (SLM Instruments, Rochester, NY, USA) at 12 000 psi in the presence of 20 μg/ml DNAse I (Sigma) and 2 mM $MgSO_4$. After addition of 0.2 mM phenylmethyl sulfonyl fluoride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100, the lysate was centrifuged ($10\,000 \times g$, 4°C for 15 min) and the soluble protein fraction was adsorbed to a glutathione-Sepharose 4B column (Pharmacia, Uppsala, Sweden). After washing with PBS, bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. The purity of the eluates was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. All the obtained products were essentially pure (80–90%); the only contaminant detectable was a split-product of 29 kDa, corresponding to bacterial glutathione-*S*-transferase. The yield of the purified fusion proteins varied from 2.5 mg to 15 mg per liter of induced bacterial culture.

2.2. Immunization

New Zealand White Rabbits were inoculated subcutanously with 50–100 μg of the purified fusion proteins in incomplete Freund's adjuvant, and boosted after 2, 4 and 8 weeks. Serum was drawn 6 and 12 weeks after immunization and assayed for reactivity with respective antigens by enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

2.3. Purification and preabsorption of the antisera

Immunoglobuline G (IgG) fractions were isolated by caprylic acid precipitation (Russ et al., 1983). Briefly, 400 μl of caprylic acid was added dropwise to 5 ml of crude rabbit serum. After 3-fold dilution with 60 mM sodium-acetate buffer, pH 4.0, the mixture was stirred for 30 min at 20°C and centrifuged ($12\,000 \times g$, 4°C for 15 min). The supernatant containing mostly IgG was filtered through glass wool, dialyzed overnight against PBS, and preabsorbed with bacterial glutathione-*S*-transferase. For this purpose, *E. coli* XL-1 blue cells were transformed with the wild type pGEX-1λT-vector, induced, lysed, and the soluble protein fraction was cross-linked to cyanogen bromide (CNBr)-activated Sepharose 6MB (Pharmacia) according to the manufacturer's instructions. IgG fractions were effectively preabsorbed for antibodies directed against bacterial gluthathione-*S*-transferase and other bacterial proteins by incubation with these Sepharose-coupled bacterial proteins for 2 h at room temperature.

2.4. Affinity purification of antibodies

Purified fusion proteins or purified bacterial glutathione-*S*-transferase were cross-linked to CNBr-activated Sepharose 6 MB according to the manufacturer's instructions. IgG fractions preabsorbed for bacterial proteins, as described above, were absorbed for a second time with purified bacterial glutathione-*S*-transferase and then bound to the immobilized fusion proteins for 3 h at 20°C. The column was washed with 50 volumes of PBS and 10 volumes of PBS, containing 1 M NaCl. Bound IgG were eluted with 4 M $MgCl_2$, pH 5.5, dialyzed against PBS, and, if necessary, concentrated by lyophilization. The average yield was 10–20% of the IgG loaded.

2.5. Heterologous expression of human β_1- or β_2-adrenoceptors in Sf9 insect cells

This was carried out as in principle described by Reiländer et al. (1991). The insect cell line Sf9 (*Spodoptera frugiperda*, ATCC accession number CRL 1711) was propagated at 27°C in TC-100 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 2 mM L-glutamine in 0.85% NaCl, and in the presence of penicillin 100 U/ml and streptomycin 100 μg/ml. The cells were infected by the recombinant *Autographa californica* nuclear polyhedrosis virus AcMNPV-β1AR (coding for the human β_1-adrenoceptor), AcMNPV-β2AR (coding for the human β_2-adrenoceptor) (Reiländer et al., 1991) or, for control purposes, with the wild type virus AcMNPV. 48–72 h after infection with recombinant baculovirus, cells were harvested and washed twice in ice-cold PBS buffer.

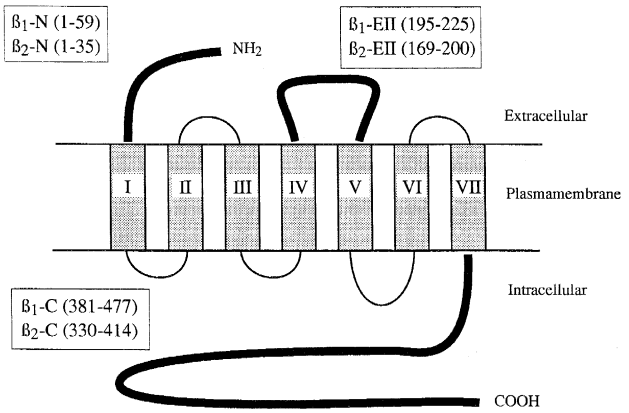


Fig. 1. Selection of receptor epitopes for construction of fusion proteins: the numbers in parentheses refer to the published amino acid sequences (Frielle et al., 1987; Kobilka et al., 1987) of the human β_1- and β_2-adrenoceptor receptor, respectively.

The expression of human β_1- or β_2-adrenoceptors was determined by specific binding of 1 nM (−)-4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]CGP 12 177) displaceable by 1 μM of D,L-CGP 12 177. Sf9 cells infected with the wild type baculovirus, which do not have β-adrenoceptors (Reiländer et al., 1991), served as a negative control. For coating of microtiter plates, cells were homogenized by douncing in 90 mM NaCl, 10 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM Pefablock, supplemented with 0.1 U/ml detergent-resistant endonuclease Benzonase (Merck, Darmstadt, Germany). Membrane proteins were solubilized by adding 0.1% Triton X-100 (Sigma, Deisenhofen, Germany). Solubilisates were centrifuged (1500 × *g*, 4°C for 10 min) to

Table 1
ELISA titers of preabsorbed immune-IgG for receptor portions of fusion proteins and intact heterogously expressed human β-adrenoceptors

Antigen	Subtype and domain specificity of the antibody					
	β_1-N	β_1-EII	β_1-C	β_2-N	β_2-EII	β_2-C
E.coli	–	–	–	–	–	–
bGST	–	–	–	–	–	–
FP β_1-N	**1:32 000**	1:1000	–	–	–	1:500
FP β_1-EII	–	**1:32 000**	–	–	1:500	–
FP β_1-C	1:500	1:500	**1:32 000**	–	–	1:1000
FP β_2-N	–	–	–	**1:32 000**	–	–
FP β_2-EII	–	1:500	–	–	**1:2000**	–
FP β_2-C	–	1:500	–	–	–	**1:32 000**
Sf9-WT	–	–	–	–	–	–
Sf9-β_1	1:32 000	1:4000	1:16 000	–	–	–
Sf9-β_2	–	–	–	1:32 000	1:1000	1:64 000

Microtiter plates were coated with 0.05 μg/well of fusion proteins (lines 3–8), or with membrane of baculovirus-infected Sf9 cells corresponding to 0.3 or 0.6 ng/well of β_1- or β_2-adrenoceptor protein, respectively (lines 9–10). For the heterogously expressed β-adrenoceptors, an equivalent amount of membrane proteins from Sf9 cells infected with wild type baculovirus (line 11) served as a control. Controls for fusion proteins were 0.05 μg/well of purified bacterial glutathione-*S*-transferase (bGST, line 2), or 0.05 μg/well of lysates of induced bacteria transformed with the wild-type expression vector (*E. coli*, line 1). Antigens and controls were probed with β-adrenergic antibodies preabsorbed with bacterial glutathione-*S*-transferase. Values given are the maximum dilution at which a statistically significant ($P < 0.05$) signal was obtained. Negative results at a dilution of 1:250 are indicated by a dash. Abbreviations are FP β_1-/FP β_2-N, FP β_1-/FP β_2-EII, FP β_1-/FP β_2-C, for fusion proteins of bacterial glutathione-*S*-transferase with the N-terminal portion, second extracellular loop, and C-terminal portion of the human β_1- or β_2- adrenoceptor (see Fig. 1). Sf9-β_1/Sf9-β_2/Sf9-WT, for membrane fractions of baculovirus-infected Sf9 cells.

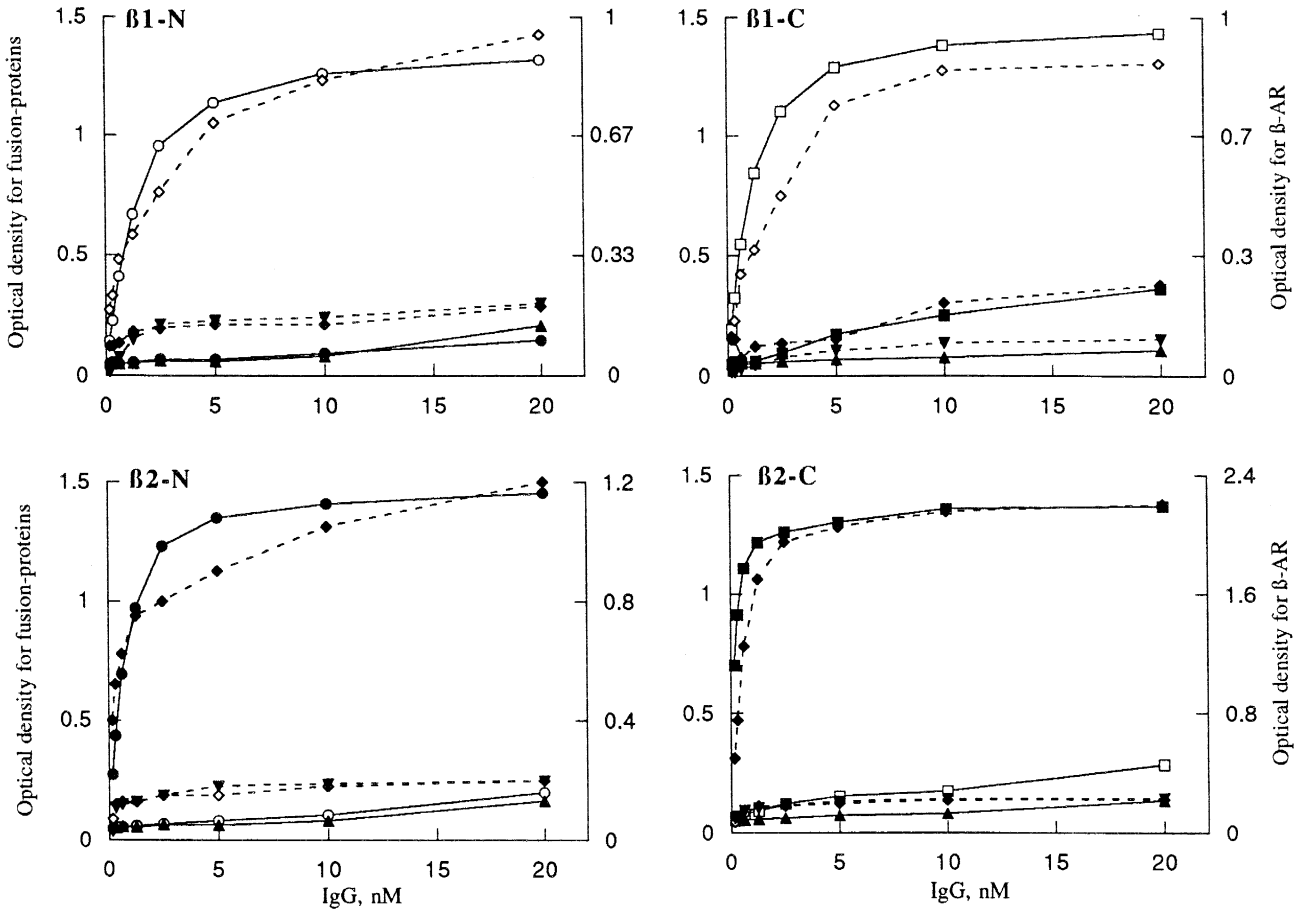


Fig. 2. Quantitative assessment of the binding of immune-IgG to fusion proteins or membranes of infected Sf9 cells by ELISA: microtiter plates were coated with 0.05 μg/well of fusion proteins of the aminoterminal (circles) or carboxyterminal portions (squares) of the human β_1- (open symbols), or β_2-adrenoceptor (closed symbols). Purified bacterial glutathione-*S*-transferase (0.05 μg/well) served as a control (▲). When using Sf9 membranes 0.3 ng of β_1- (◇) or 0.6 ng of β_2- (◆) adrenoceptor protein per well were coated (dashed lines). An equivalent amount of membrane proteins from Sf9 cells infected with wild type baculovirus served as a control (▼). Antigens were probed with immune-IgG raised against fusion proteins of the aminoterminal (β_1-/β_2-N) or carboxyterminal portions (β_1-/β_2-C) of the human β_1- or β_2-adrenoceptor at the indicated concentrations. Antibody binding was detected by peroxidase labelled secondary antibody and quantified by measurement of the optical density at 490 nm.

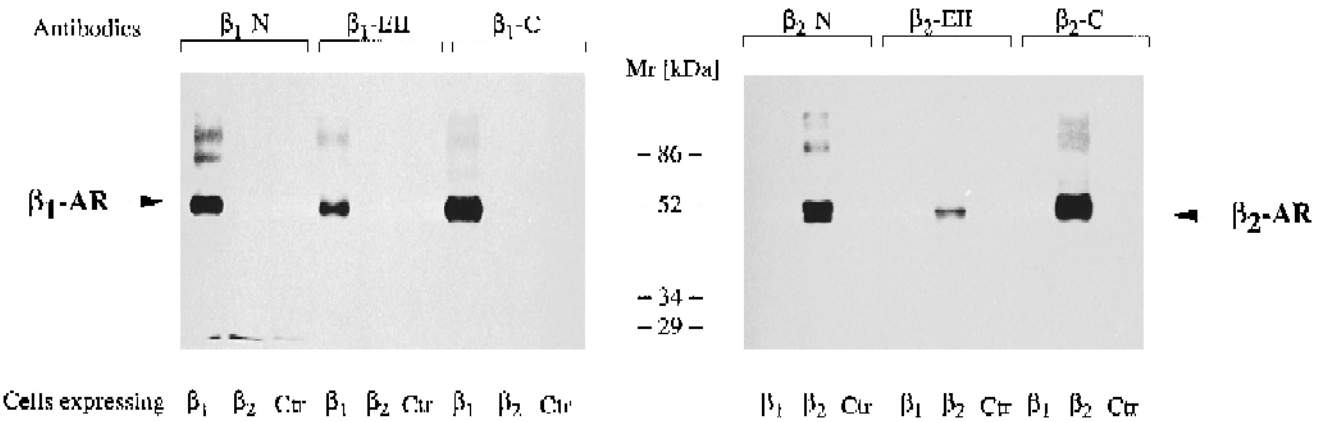


Fig. 3. Western-blots of recombinant human β-adrenoceptors probed with purified fusion protein antibodies: SDS-lysates of 5×10^5 baculovirus-infected Sf9 cells expressing β_1- or β_2-adrenoceptors were subjected to SDS-PAGE, and transferred to nitrocellulose. Sf9 cells infected with wild type baculovirus served as a control (CTR). Blots were probed with rabbit polyclonal anti-β-adrenergic antibodies diluted 1:5000. Bound secondary anti-rabbit IgG antibodies were visualized by ECL. Representative blots of multiple experiments are shown.

remove cell nuclei and debris. Protein concentration of the supernatants was measured according to Bradford.

2.6. Enzyme-linked immunoassays (ELISA)

These were carried out in flat bottom microtiter plates (Maxisorb, Nunc, Kastrup, Denmark). Purified fusion proteins, lysates of *E. coli*, or Sf9 cell membranes were diluted with 50 mM sodium carbonate buffer, pH 9.6 to a final protein concentration of 0.5 μg/ml. When fusion proteins or *E. coli* lysates were used, 100 μl of coating solution were applied to each well and proteins were allowed to adsorb at 4°C for 18 h. In the case of Sf9 cell membranes, coating corresponded to 0.7 and 1.4 ng of human β_1- or β_2-adrenoceptor protein per well, respectively, as deduced from radioligand binding determined prior to solubilization. After coating with antigens, the wells were washed 6 times with PBS, pH 7.4. Antibodies were serially diluted 250–32 000-fold (for fusion proteins and *E. coli* lysates) with PBS containing 0.05% Tween 20 and 2% bovine serum albumin. The different antibody fractions were adjusted to an equal IgG concentration determined by the Bradford assay. Then, the antibodies were serially diluted 250–64 000-fold in the same buffer. 100 μl of diluted antibodies were allowed to react with the immobilized antigens for 2 h at 37°C, followed by removal of the antibody solution and 6 washes with PBS in the presence of 0.05% Tween 20. Subsequently, 100 μl of horseradish peroxidase conjugated goat anti-rabbit IgG (Pierce, Rockford, IL, USA), diluted 1:2000 with PBS containing 0.05% Tween 20 and 2% bovine serum albumin, were added to each well and allowed to bind for 2 h at 37°C, followed by 6 washes with PBS/Tween 0.05%. Bound secondary antibodies were detected by the addition of 100 μl of substrate buffer (35 mM citric acid, 65 mM Na_2HPO_4, pH 4.5) containing 0.5 mg/ml o-phenylenediamine (Sigma) and 1.6 μl/ml H_2O_2. The reaction was carried out at 37°C for 30 min and stopped by the addition of 100 μl 3 M H_2SO_4 per well. Optical densities were read at 490 nm.

2.7. Western blotting

Western blotting was carried out with SDS lysates (2% SDS, 2% β-mercaptoethanol, 20% glycerol, 60 mM Tris-

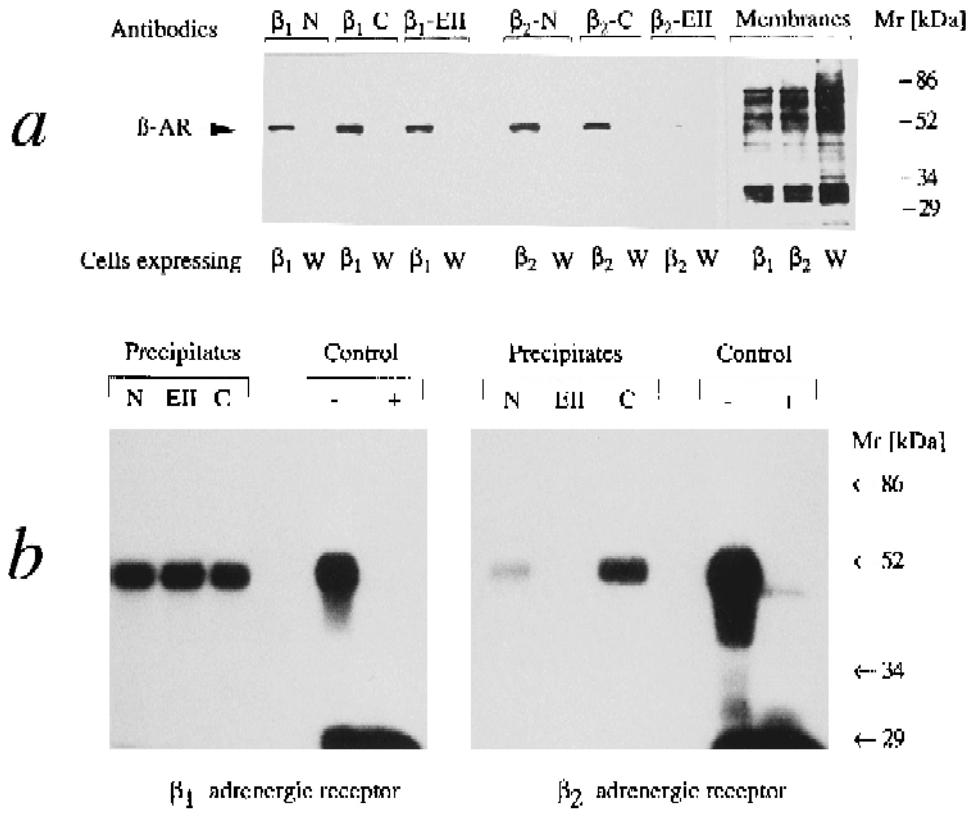


Fig. 4. Immunoprecipitation of recombinant human β-adrenoceptors. (a) Baculovirus-infected Sf9 cells, expressing human β_1- or β_2-adrenoceptors, or the wild type virus (W), were membrane-biotinylated, solubilised, and subjected to immunoprecipitation by antibodies, raised against the termini or the second extracellular loop of β_1- and β_2-adrenoceptors, respectively. Immunoprecipitates were blotted onto nitrocellulose and probed with peroxidase-labelled streptavidin. Aliquots of solubilisates of cells infected with the wild type baculovirus (W) served as a control. (b) Human β_1- or β_2-adrenoceptors produced in Sf9 cells were labelled with [^{125}I]cyanopindolol-diazirine, solubilised, and precipitated with polyclonal antibodies against the corresponding amino (N)- or carboxyterminus (C), or the second extracellular loop (EII). Precipitates were subjected to SDS-PAGE and autoradiography. Solubilisates of cells expressing β_1- or β_2-adrenoceptors labelled in the absence (−) or presence (+) of an excess of *l*-alprenolol served as controls.

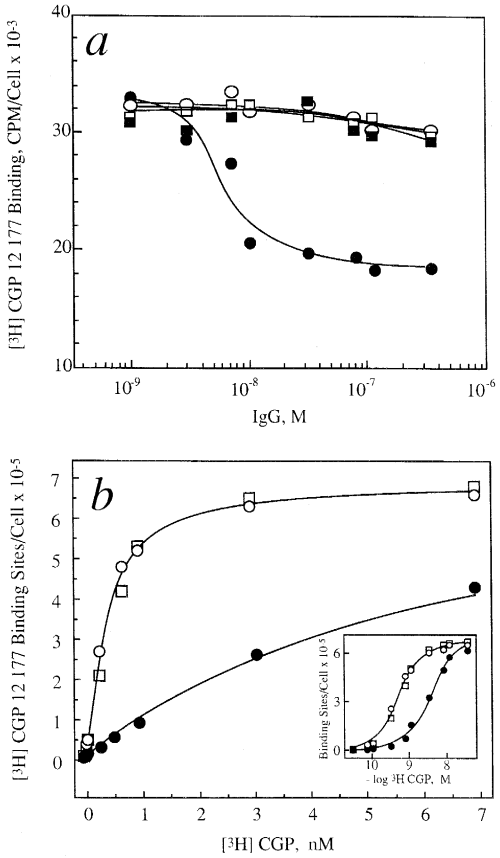


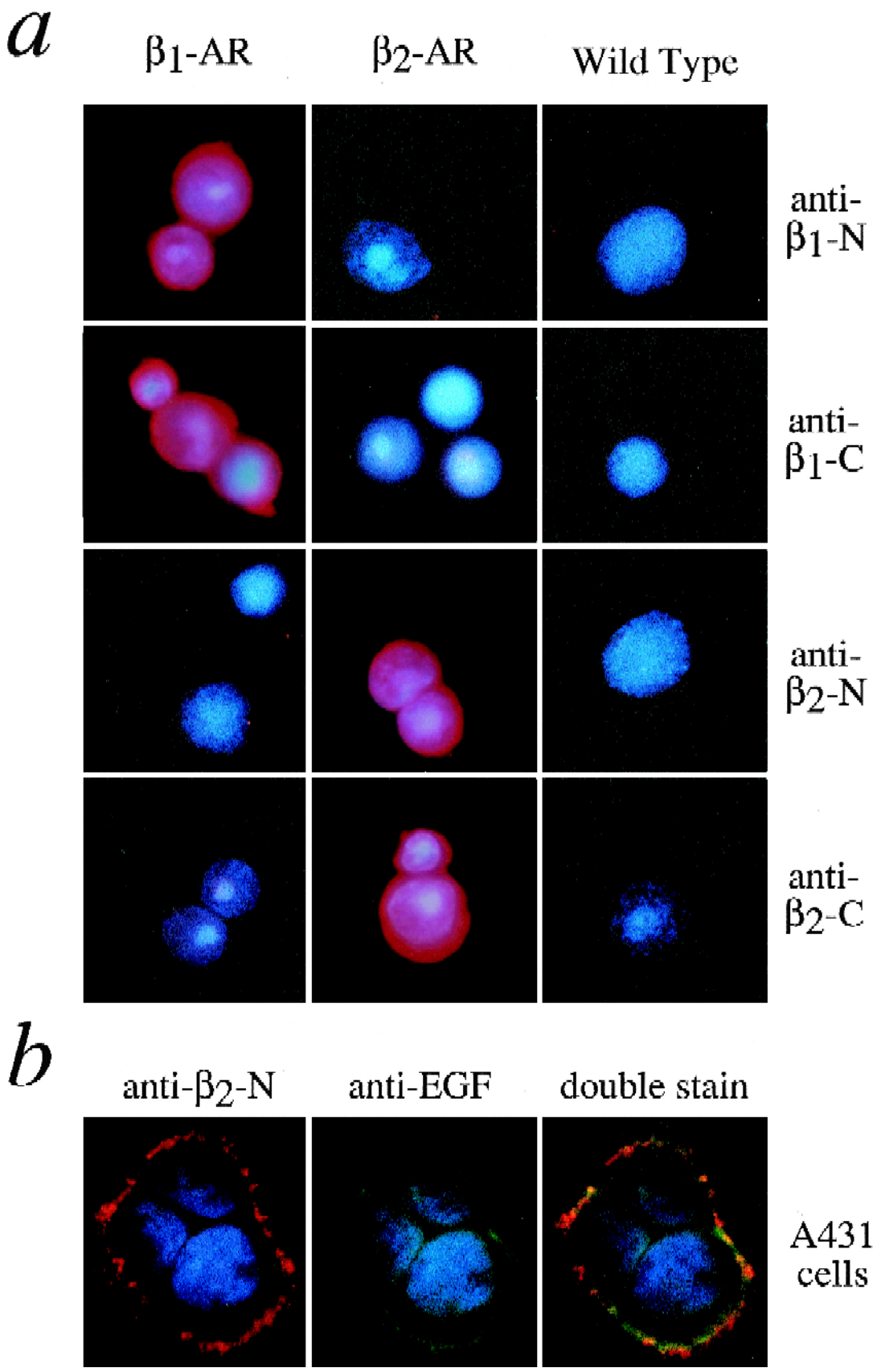
Fig. 5. Decrease in ligand affinity of human β_1-adrenoceptors bound to antibodies reacting with the second extracellular loop: (a) 10^3 Sf9 cells, expressing human β_1-adrenoceptors were incubated for 30 min at 30°C with various concentrations of purified immune-IgG directed against the second extracellular loop (●), the aminoterminus (■), or the carboxyterminus (□) of the human β_1-adrenoceptor, or with preimmune IgG of the rabbit immunized with the fusion protein of the second extracellular loop (○). Subsequently, 2 nM [^{3}H]CGP 12 177 was added and incubation continued for 30 min. After filtration, bound ligand was measured by liquid scintillation counting. Non-specific binding was determined in the presence of 1 μM cold CGP 12 177 and subtracted from the data. This is a representative of three experiments with similar outcome. (b) Cells were incubated with a fixed concentration (30 nM) of purified immune-IgG directed against the second extracellular loop of the β_1-adrenoceptor (●), or the corresponding pre-immune IgG (○), or in the absence of antibodies (□). Subsequently, binding of various concentrations of [^{3}H]CGP 12 177 was measured. Saturation curves were fitted to the data by computer-aided non-linear regression analysis, assuming binding to a single class of sites with uniform affinity. The insert shows a log-plot of the data.

HCl, 0.002% bromphenol blue, pH 6.8) of induced and non-induced cultures of transfected *E. coli*, or of baculovirus infected Sf9 cells. To each lane an aliquot of bacterial culture or the equivalent of 5×10^4 Sf9 cells were applied. The proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond C-extra, Amersham, Buckinghamshire, UK). Membranes were blocked with non-fat dry milk (5%) and incubated with the affinity-purified anti-β-adrenoceptor antibodies at dilutions of 1:5000–15 000 for 18 h at 4°C. Immunoreactive bands were visualized by enhanced chemoluminescence (ECL, Amersham) after incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:5000, Pierce, Rockford, IL, USA).

2.8. Immunoprecipitation of human β_1- and β_2-adrenoceptors

β-Adrenoceptors of baculovirus-infected Sf9 cells were specifically photoaffinity labelled with [^{125}I]iodocyanopindolol diazirine (Amersham-Buchler, Germany) as described in (Burgermeister et al., 1983), or membrane proteins were biotinylated with D-biotinoyl-ε-aminocaproicacid-*N*-hydroxysuccinimidester (biotin-7-NHS) according to the manufacturer's instructions (Boehringer-Mannheim, Germany). Sf9 cells infected with wild type baculovirus served as a negative control. Subsequently, cells were washed with PBS and solubilized with 1% Triton X-100 in 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 2 mM Pefablock, for 20 min on ice. Solubilisates were diluted 1:10 with PBS and 2–5 μl of undiluted antibodies were added to 1 ml of solubilisate, followed by incubation at 4°C for 14 h. Subsequently, 100 μl of packed protein A-Sepharose 6MB (Pharmacia, Uppsala, Sweden), preblocked with 2% non-fat dry milk in PBS for 14 h at 4°C, were added and incubation continued for 2 h at 4°C. Finally, protein A-Sepharose was sedimented, washed five times with ice-cold immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 2 mM Pefablock, 1% Triton X-100), and adsorbed receptors were eluted by vigorous shaking with 100 μl of non-reducing SDS-sample buffer (5% SDS, 20% glycerol, 60 mM Tris-HCl, 0.002% bromphenol blue, pH 6.8) for 1 h at 21°C. The

Fig. 6. Indirect immunofluorescence microscopy of fixed Sf9 cells or A_{431} cells expressing human β-adrenoceptors, stained with β-adrenergic antibodies. (a) Baculovirus-infected Sf9 cells, expressing human β_1- or β_2-adrenoceptors were spotted onto glass slides, fixed, permeabilized and stained as described under Methods. Fixed antibodies were detected with CY3-labelled goat anti-rabbit IgG Fab_2 fragments (red). Cell nuclei were counterstained with bisbenzimide (blue). Control cells infected with the wild type baculovirus and not expressing β-adrenoceptors were treated similarly. (b) A_{431} cells were grown on microscope slides, fixed and incubated with antibodies specific for the aminoterminus of the human β_2-adrenoceptor and with mouse monoclonal antibodies directed against the human epidermal growth factor receptor. Bound rabbit antibodies were detected by CY3-labelled goat anti-rabbit Fab_2 fragments (red), whereas monoclonal mouse antibodies were detected with CY2-labelled goat anti-mouse antibodies (green). Photos of a representative cell are shown with the staining pattern of β-adrenoceptors (anti-β_2-N), the epidermal growth factor receptors (anti-EGF), or double-staining of epidermal growth factor receptors and β-adrenoceptors. Fluorescence of the cells was inspected by microscope at a 400-fold magnification and red and blue fluorescence were documented by photography using identical exposure times.


a
β1-AR
β2-AR
Wild Type
anti-β1-N
anti-β1-C
anti-β2-N
anti-β2-C
b
anti-β2-N
anti-EGF
double stain
A431 cells

eluates were subjected to SDS-PAGE. For detection of photoaffinity labelled receptors, polyacrylamide gels were dried and autoradiographed. For detection of biotinylated receptors, proteins were transferred to nitrocellulose membranes by Western blotting. Blots were probed with horseradish-peroxidase coupled streptavidin (Boehringer-Mannheim, Germany). Bound streptavidin was detected by ECL. For control purposes streptavidin was subsequently stripped from the membranes by incubation with 10 M urea, 1% β-mercaptoethanol, 50 mM NaH_2PO_4, pH 6.5 and the blots were reprobed with receptor-antibodies, as described in the Western blotting section.

2.9. Radioligand binding assays

Binding of [^{3}H] CGP 12 177 to human β_1- or β_2-adrenoceptors expressed in baculovirus-infected Sf9 cells was carried out, as described in (Reiländer et al., 1991). Briefly, 10 000 Sf9 cells expressing $1–2 \times 10^6$ β_1- or β_2-adrenoceptors per cell, were incubated with [^{3}H]CGP 12 177 ($10^{-11}–10^{-8}$ M) (NEN, DuPont, Germany) in a final volume of 200 μl of PBS. Incubation at 30°C was stopped by rapid filtration (Whatman GF/C soaked in 0.3% polyethylenimine). Bound radioactivity was measured by liquid scintillation counting.

2.10. Indirect immunofluorescence microscopy

Baculovirus-infected Sf9 cells were transferred onto glass slides (Cytofuge, Shandon, UK), whereas A431 cells were grown on the slides in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. Cells were fixed with 4% formaldehyde for 10 min at 4°C, permeabilized with 0.1% Triton X-100 for 30 s at 4°C, washed with isotonic buffer (buffer I: 150 mM NaCl, 5 mM Tris, 5 mM Hepes, pH 7.5), and blocked for 30 min at 20°C with 2% human and 2% standard goat serum. Subsequently, cells were incubated with affinity purified β-adrenoceptor antibodies, diluted 1:100 with buffer I in the presence of 0.5% bovine serum albumin for 1 h at 20°C. A431 cells were also stained with mouse monoclonal antibodies against human epidermal growth factor receptor (Pierce, Rockford, IL, USA) diluted 1:500 in buffer I. After 5 washes with Buffer I (5 min each at 20°C) CY3-conjugated goat anti-rabbit Fab_2 fragments (Jackson, IL, USA) diluted 1:1000 and CY2-labelled goat anti-mouse

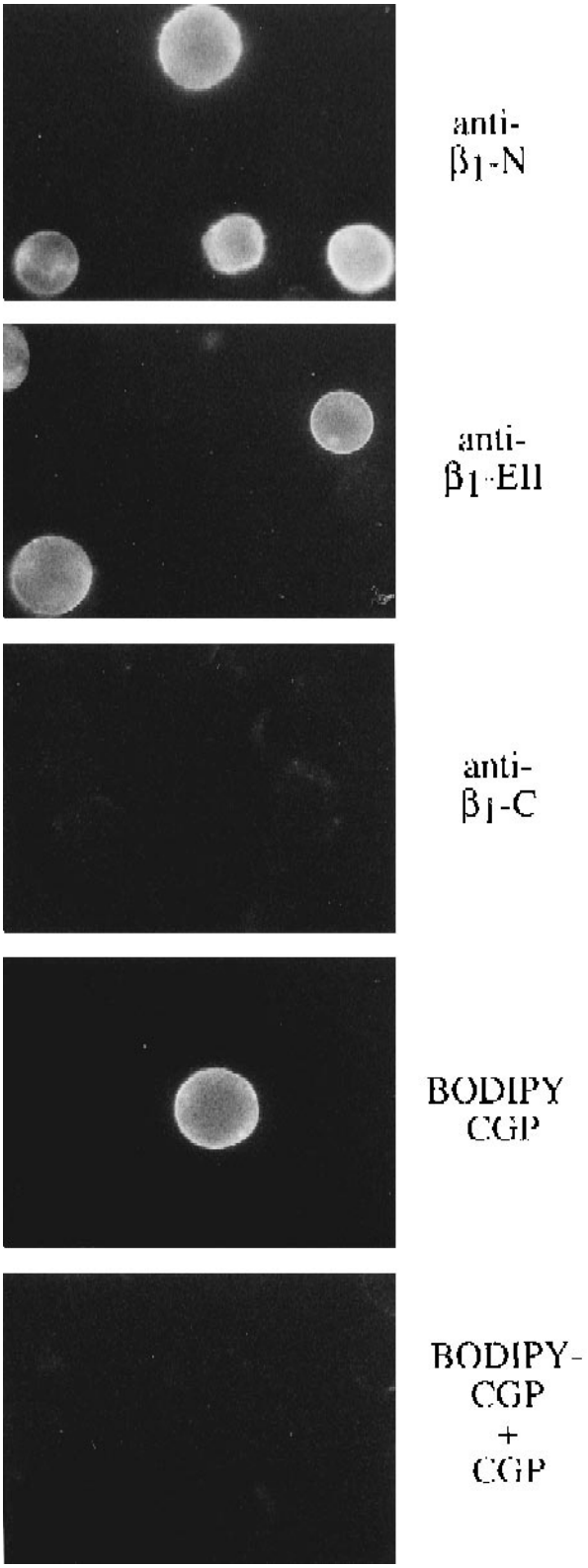


Fig. 7. Fluorescent images of non-fixed Sf9 cells expressing β_1-adrenoceptors. Cells were incubated with purified antibodies directed against N- and C-terminus or the second extracellular loop of the human β_1-adrenoceptor and CY3-labelled goat anti-rabbit IgG Fab_2 fragments. As a control, we used 5 nM of the fluorescent β-adrenergic ligand BODIPY-CGP either with or without an excess (5 μM) of non-labelled CGP 12 177 under the same conditions. Subsequently, the cells were sedimented, resuspended in buffer, spotted onto a glass slide, and fluorescence was inspected with a multi-immersion objective. Photographic images were taken with identical exposure times.

IgG (Rockwell, IL USA) diluted 1:300 in buffer I, containing 2% bovine serum albumin and 2% human serum, were applied together with 0.01 mg/ml bisbenzimide (Hoechst, Germany) and incubated with the cells for 20 min at 20°C. Cells were washed again (3 times for 5 min at 20°C with PBS followed by a single wash with H_2O), embedded in 1 M *N*-propyl gallate, and fluorescence of the cells was inspected in a Zeiss Axioplan fluorescence microscope with a Neofluar 63 NA oil immersion objective (Carl Zeiss, Jena, Germany). When native receptors were analyzed, cells were suspended in PBS, containing 2% bovine serum albumin. Primary and secondary antibodies were added simultaneously, or 5 nM of the fluorescent β-adrenergic ligand BODIPY-CGP (Heithier et al., 1994) was added to the cell suspension and incubation continued for 30 min at 20°C. Subsequently, cells were sedimented, washed 3 times with ice-cold PBS and a drop of the cell suspension was applied to a glass slide. Fluorescence of the cells was inspected with a Neofluar 40 multi-immersion objective. Photographic images were documented with AGFA 1000 RS color diapositive or KODAK TMAX 400 black and white negative films.

3. Results

Fig. 1 shows the segments of the human β_1- and β_2-adrenoceptors that were selected for construction of the fusion proteins with bacterial glutation-*S*-transferase used for immunization. Preabsorption of the sera with bacterial glutathione-*S*-transferase followed by affinity purification efficiently removed antibodies directed against glutathione-*S*-transferase and other bacterial proteins (Table 1, lines 1–2, and Fig. 2). Highly specific polyclonal rabbit antibodies were obtained against all of the chosen receptor domains, which recognized their respective antigens in an ELISA at dilutions up to 1:32 000 (Table 1). The sensitivity and specificity of the antibodies was high enough to obtain an at least 10-fold signal to noise ratio at IgG concentrations between 3 and 20 nM (Fig. 2). The only antibodies of lower sensitivity were those directed against the second extracellular loop of the β_2-adrenoceptor (titer 1:2000, see Table 1), possibly because this fusion product was less hydrophilic. None of the antibodies obtained showed a significant cross-reactivity with any other portion of the same receptor subtype (Table 1, lines 3–8). Moreover, the antibodies had a much lower sensitivity for the structurally related domain of the other receptor subtype (Fig. 2). These antibodies bound also strongly to native intact β-adrenoceptors, the β_1-/β_2-subtype selectivity in ELISA being even better than that observed with fusion proteins (Table 1, lines 9–11 and Fig. 2). Antibodies directed against the N- and C-terminal portions of the β_1- or β_2-adrenoceptor did also strongly react in a subtype-specific manner with the receptor proteins blotted onto nitrocellulose (Fig. 3), showing a strong band with a molecular mass of about 47 kDa. This is in good agreement with results published earlier (Reiländer et al., 1991) and with the expected molecular mass based on the gene sequences of the β_1- and β_2-adrenoceptor subtypes, which have been shown to be less glycosylated when expressed in insect cells (Reiländer et al., 1991). The double band seen in blots with the β_2-adrenoceptor might, thus, represent two different states of glycosylation. In addition, some bands of high molecular mass (e.g., about two times the molecular mass of the respective receptors) were observed in the Western blots, possibly corresponding to receptor aggregates, which are frequently observed in SDS-PAGE of G-protein-coupled receptors.

The antibodies could also be used for immunoprecipitation. As shown in Fig. 4a, all of the receptor antibodies were able to specifically and selectively precipitate the receptor protein out of a lysate of biotinylated Sf9 cell membranes as revealed by detection with streptavidin. The immunoprecipitated band was clearly the receptor, because it was absent in Sf9 cells infected with the wild type baculovirus that were treated in the same way (Fig. 4a). Identity of the precipitated proteins was confirmed by reprobing the blots with antibodies directed against the N-terminus of the corresponding receptor subtype, after stripping of the streptavidin (not shown). Moreover, a similar result was obtained, when receptors were photoaffinity-labelled specifically with [^{125}I]iodocyanopindolol (CYP)-diazirine (Fig. 4b). Immunoprecipitation of the receptor by antibodies directed against the second extracellular loop of the β_2-adrenoceptor could not be obtained because of their low titers. In contrast, it should be noted that antibodies directed against the corresponding loop of the β_1-adrenoceptor were able to immunoprecipitate the receptor as effectively, as antibodies against the receptor termini. This is in contrast to the weak reaction obtained with these antibodies in immunoblots (Fig. 3) and might indicate that these antibodies preferentially recognize a native conformation of the receptor. This notion is supported by the observation that antibodies directed against the second extracellular loop of the β_1-adrenoceptor inhibited ligand binding to the receptor in a concentration-dependent manner (Fig. 5a), decreasing the ligand affinity by about 10-fold (Fig. 5b), whereas antibodies directed against the receptor termini did not interfere with receptor function. However, the total ligand binding capacity of β_1-adrenoceptors remained unchanged in the competition assay, indicating that some steric hindrance occurred by the binding of antibodies to the receptor or that antibody binding to the second extracellular loop stabilized a receptor conformation with lower ligand affinity. Thus, at low concentrations of [^{3}H]CGP 12 177, the accessibility of the corresponding binding site was hampered. This finding is also in agreement with the concept, that, besides the transmembrane domains forming the ligand binding pocket, the second extracellular loop of the β-adrenoceptor might

be involved in forming or stabilizing the ligand binding site (Dohlman et al., 1990; Noda et al., 1994). A similar effect could not be observed with antibodies directed against the second extracellular loop of the β_2-adrenoceptor, probably because the titers were too low (see above).

Making further use of baculovirus-infected Sf9 cells, we established procedures visualising β-adrenoceptors by indirect immunofluorescence microscopy. A bright fluorescent image of Sf9 cells expressing about $(1–2) \times 10^6$ of β_1- or β_2-adrenergic ligand binding sites was obtained after fixation and staining of the cells with antibodies directed against amino- or carboxytermini of the respective receptor subtypes (Fig. 6a). In contrast, Sf9 cells expressing the receptor subtype not matching the antibody were not stained significantly, and did not differ from the clearly negative result obtained with control cells infected with the wild type baculovirus. These observations confirm that the fusion-protein antibodies are specific for β-adrenoceptors and do barely cross-react between β_1- and β_2-subtypes.

Antibodies directed against the second extracellular loop hardly reacted with receptors of fixed cells (not shown). However, when native receptors of intact cells were probed with these antibodies, a bright image of the cell surface was obtained (Fig. 7). A similar image was also seen with antibodies directed against the aminoterminus of the receptor and with the fluorescent β-adrenergic ligand BODIPY-CG (Heithier et al., 1994). However, antibodies directed against the carboxyterminus of the receptor gave a negative result (Fig. 7). These observations confirm that antibodies raised against the fusion protein of the second extracellular loop are specific for a native conformation of the receptor. Antibodies specific for the intracellular carboxyterminal portion of the receptor did not recognize the native, membrane-bound receptor, unless the cells had been permeabilized. Thus, it appears that the domain specificity of the antibodies demonstrated in Table 1 is conserved when the antibodies react with native receptor proteins in a cell membrane.

Having, thus, obtained proof of the specificity of the antibodies for human β-adrenoceptors, we chose antibodies directed against the aminoterminus of the β_2-receptor subtype to visualize the cellular distribution of β_2-adrenoceptors expressed at physiological levels. For this purpose, we used human A431 cells, which constitutively express the β_2-adrenoceptor. Typical fluorescent images are shown in Fig. 6b. It can be clearly seen, that the β_2-adrenoceptors have an inhomogeneous and patchy distribution on the plasma membrane of the A431 cells, whereas epidermal growth factor receptors showed a much more diffuse distribution on the cell surface. Here again, carboxyterminal antibodies gave no detectable fluorescence signals when staining non-permeabilised A431 cells, whereas staining with carboxyterminal antibodies after permeabilisation with Triton 0.1% gave the same bright labelling as was obtained with the aminoterminal antibodies in both permeabilized and non-permeabilized cells.

4. Discussion

By using fusion proteins of extramembraneous segments of human β_1- or β_2-adrenoceptors with bacterial glutathione-*S*-transferase as an antigen, we raised polyclonal rabbit antibodies specific for human β-adrenoceptors. Titers and avidity of these antibodies were sufficient for their use in ELISA, Western blotting, immunoprecipitation, and indirect immunofluorescence microscopy. In all of these techniques the antibodies were shown to be selective for β_1- or β_2-receptor subtypes, allowing selective immunostaining of each subtype in cells and tissues expressing both β_1- and β_2-adrenoceptors. Moreover, these antibodies were suitable for probing the subcellular orientation of receptor domains. Finally, antibodies directed against the second extracellular loop appeared to recognize a native conformation of the receptor.

Although the characterization of these antibodies was mainly carried out with human β-adrenoceptors heterologously produced in Sf9 insect cells, visualisation of receptors constitutively expressed at much lower levels in human A431 cells was equally possible. So far, the most detailed studies on subcellular distribution and cycling of β-adrenoceptors have been done with epitope-tagged receptors heterologously expressed in mamalian cells (Von Zastrow and Kobilka, 1992, 1994). While these studies have confirmed the overall pattern of receptor dynamics derived from earlier observations made by indirect immunofluorescence microscopy of native receptors, certain differences were found. Thus, it should be noted that agonist promoted internalization of endogenous β-adrenoceptors of A431 cells as studied with a monoclonal antibody was found to involve non-coated vesicles (Raposo et al., 1989), whereas agonist-regulated redistribution of epitope-tagged β_2-adrenoceptors heterologously expressed in 293 cells appeared to involve the common pathway also used by transferrin receptors (Von Zastrow and Kobilka, 1994). Thus, it is not yet clear, whether heterologously expressed receptors are regulated and behave in the same manner as constitutively expressed receptors. Here, we show that in contrast to recombinant receptors overexpressed in Sf9 cells, receptors constitutively expressed in A431 cells are not uniformely distributed. They are clustered in confined regions of the cell membrane. This would argue in favour of an as yet unknown regulatory system, restricting the mobility of the non-stimulated receptors in the cell membrane, as has been proposed before on the basis of cellular receptor images obtained with a fluorescent ligand (Henis et al., 1982). A patchy distribution of β_2-adrenoceptors of A431 cells has similarly been observed by Wang et al. using peptide antibodies and high resolution indirect immunofluorescence microscopy (Wang et al., 1989a,b). However, in contrast with our data, the distribution of heterologously expressed β_2-adrenoceptors in HEK-293 cells (Von Zastrow and Kobilka, 1992, 1994) or in cardiomyocytes of mice transgenic for the human

β_2-adrenoceptor (Milano et al., 1994) was shown to be much more homogenous.

In summary, the receptor antibodies described in this paper appear to be valuable tools for studying the distribution of constutively expressed β-adrenoceptors in a subtype-specific manner and for checking the data obtained from genetically engineered model systems.

Acknowledgements

We would like to thank Ms Elisabeth Wilken for excellent technical assistance. We are indebted to Prof. D. Drenckhahn and Dr W. Ness, Department of Anatomy, University Würzburg, for help and advice with indirect immunofluorescence microscopy. Help and advice with immunization procedures was kindly provided by Dr G. Münscher, Behringwerke AG, Marburg, Germany.

References

Aoki, C., 1992, Beta-adrenergic receptors: astrocytic localization in the adult visual cortex and their relation to catecholamine axon terminals as revealed by electron microscopic immunocytochemistry, J. Neurosci. 12, 781.

Bahouth, S.W., H.Y. Wang and C.C. Malbon, 1991, Immunological approaches for probing receptor structure and function, Trends Pharmacol. Sci. 12, 338.

Boege, F., M. Ward, R. Jürss, M. Hekman and E.J. Helmreich, 1988, Role of glycosylation for beta 2-adrenoceptor function in A431 cells, J. Biol. Chem. 263, 9040.

Burgermeister, W., M. Nassal, T. Wieland and E.J.M. Helmreich, 1983, A carbene-generating photoaffinity probe for beta-adrenergic receptors, Biochim. Biophys. Acta 729, 219.

Chapot, M.P., P.O. Couraud, A. Schmutz, A.D. Strosberg and T.O. Durieu, 1989, A monoclonal antibody directed against the beta 1-adrenergic receptor from turkey erythrocyte membranes, Hybridoma 8, 535.

Chapot, M.P., Y. Eshdat, S. Marullo, J.G. Guillet, A. Charbit, A.D. Strosberg and C. Delavier Klutchko, 1990, Localization and characterization of three different beta-adrenergic receptors expressed in Escherichia coli, Eur. J. Biochem. 187, 137.

Couraud, P.O., B.Z. Lu, A. Schmutz, O. Durieu-Trautman, C. Klutchko-Delavier, J. Hoebeke and A.D. Strosberg, 1983, Immunological studies of β-adrenergic receptors, J. Biol. Chem. 21, 1840.

Dawidek, G.M. and M.I. Robinson, 1995, Beta-adrenergic receptors in human anterior optic nerve: an autoradiographic study, Eye 7, 122.

Dohlman, H.G., M.G. Caron, D.B.A., T. Frielle and R.J. Lefkowitz, 1990, Role of extracellular disulfide-bonded cysteines in the ligand binding function of the beta 2-adrenergic receptor, Biochemistry 29, 2335.

Dunkel, F.G., G. Münch, F. Boege, R. Cantrill and N.P. Kurstjens, 1989, Proteolytic degradation routes for turkey beta 1-adrenoceptor probed with antipeptide antibodies against the N-terminal sequence of the receptor, Biochem. Biophys. Res. Commun. 165, 264.

Fernandez-Lopez, A., V. Revilla, M.A. Candelas, M.I. Aller, C. Soria and A. Pazos, 1994, Identification of beta-adrenoceptors in rat lymph nodes and spleen: an autoradiographic study, Eur. J. Pharmacol. 262, 283.

Frielle, T., S. Collins, K.W. Daniel, M.G. Caron, R.J. Lefkowitz and B.K. Kobilka, 1987, Cloning of the cDNA for the human beta 1-adrenergic receptor, Proc. Natl. Acad. Sci. USA 84, 7920.

Frielle, T., B. Kobilka, R.J. Lefkowitz and M.G. Caron, 1988, Human beta 1- and beta 2-adrenergic receptors: structurally and functionally related receptors derived from distinct genes, Trends Neurosci. 11, 321.

Heithier, H., D. Hallmann, F. Boege, H. Reiländer, C. Dees, K.A. Jaeggi, D. Arndt-Jovin, T. Jovin and E.J.M. Helmreich, 1994, Synthesis and properties of fluorescent β-adrenoceptor ligands, Biochemistry 33, 9126.

Henis, Y.I., M. Hekman, E.L. Elson and E.J.M. Helmreich, 1982, Lateral motion of beta receptors in membranes of cultured liver cells, Proc. Natl. Acad. Sci. USA 79, 2907.

Kaveri, S.V., P. Cervantes-Olivier, C. Delavier-Klutchko and A.D. Strosberg, 1987, Monoclonal antibodies directed against the human A431-β2-adrenergic receptor recognize two major polypeptide chains, Eur. J. Biochem. 167, 449.

Kobilka, B.K., R.A. Dixon, T. Frielle, H.G. Dohlman, M.A. Bolanowski, I.S. Sigal, T.l. Yang Feng, U. Francke, M.G. Caron and R.J. Lefkowitz, 1987, cDNA for the human beta 2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor, Proc. Natl. Acad. Sci. USA 84, 46.

Lerner, R., 1982, Tapping the immunological repertoire to produce antibodies of predetermined specificity, Nature 299, 449.

Lohse, M.J., R.H. Strasser and E.J.M. Helmreich, 1993, The β-adrenoceptors, in: Neurotransmitter Receptors, ed. F. Hucho (Elsevier Science Publishers, Amsterdam) p. 137.

Luxembourg, A., M. Hekman and E.M. Ross, 1991, Immunologic mapping of the amino- and carboxy-termini of the turkey erythrocyte beta-adrenergic receptor: selective proteolysis of both domains, FEBS Lett. 283, 155.

Malbon, C.C., C.P. Moxham and H.J. Brandwein, 1991, Antibodies to β-adrenergic receptors, in: The β-Adrenergic Receptors, ed. J.P. Perkins (Humana Press, Baltimore, MD) p. 181.

Meister, B., A. Dagerlind, A.P. Nicholas and T. Hokfelt, 1994, Patterns of messenger RNA expression for adrenergic receptor subtypes in the rat kidney, J. Pharmacol. Exp. Ther. 268, 1605.

Milano, C.A., L.F. Allen, H.A. Rockman, P.C. Dolber, T.R. McMinn, K.R. Chien, T.D. Johnson, R.A. Bond and R.J. Lefkowitz, 1994, Enhanced myocardial function in transgenic mice overexpressing the β2-adrenergic receptor, Science 264, 582.

Moxham, C.P., S.T. George, M.P. Graziano, H.J. Brandwein and C.C. Malbon, 1986, Mammalian beta 1- and beta 2-adrenergic receptors. Immunological and structural comparisons, J. Biol. Chem. 261, 14562.

Noda, K., Y. Saad, R.M. Graham and S.S. Karnik, 1994, The high affinity state of the β2-adrenergic receptor requires unique interaction between conserved and non-conserved extracellular loop cysteines, J. Biol. Chem. 269, 6743.

Raposo, G., I. Dunia, C. Delavier Klutchko, S. Kaveri, A.D. Strosberg and E.L. Benedetti, 1989, Internalization of beta-adrenergic receptor in A431 cells involves non-coated vesicles, Eur. J. Cell. Biol. 50, 340.

Reiländer, H., F. Boege, S. Vasudevan, G. Maul, M. Hekman, C. Dees, W. Hampe, E.J.M. Helmreich and H. Michel, 1991, Purification and functional characterization of the human beta 2-adrenergic receptor produced in baculovirus-infected insect cells, FEBS Lett. 282, 441.

Russ, C., I. Callegaro, B. Lanza and S. Ferrone, 1983, Purification of IgG monoclonal antibody by caprylic acid precipitation, J. Immunol. Methods 65, 269.

Strader, C.D., V.M. Pickel, T.H. Joh, M.W. Strohsacker, R.G.L. Shorr, R.J. Lefkowitz and M.G. Caron, 1983, Antibodies to the β-adrenergic receptor: Attenuation of catecholamine-sensitive adenylate cyclase and demonstration of post synaptic receptor localization in brain, Proc. Natl. Acad. Sci. USA 80, 1840.

Strosberg, A.D., 1989, Anti-idiotypic antibodies that interact with beta-adrenergic catecholamine receptors, Methods Enzymol. 178, 265.

Von Zastrow, M. and B.K. Kobilka, 1992, Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the

plasma membrane and endosomes containing transferrin receptors, J. Biol. Chem. 267, 3538.

Von Zastrow, M. and B.K. Kobilka, 1994, Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization, J. Biol. Chem. 269, 18448.

Wang, H., L. Lipfert, C.C. Malbon and S. Bahouth, 1989a, Site-directed anti-peptide antibodies define the topography of the beta-adrenergic receptor, J. Biol. Chem. 264, 14424.

Wang, H.Y., M. Berrios and C.C. Malbon, 1989b, Localization of beta-adrenergic receptors in A431 cells in situ. Effect of chronic exposure to agonist, Biochem. J. 263, 533.

Woo, C.C. and M. Leon, 1995, Distribution and development of beta-adrenergic receptors in the rat olfactory bulb, J. Comp. Neurol. 352, 1.