ELSEVIER

European Journal of Pharmacology 348 (1998) 123–133

ejp

An agonist-like monoclonal antibody against the human β_2-adrenoceptor

Diane Lebesgue [a], Gerd Wallukat [b], Alfredo Mijares [c], Claude Granier [d], Jorge Argibay [c], Johan Hoebeke [a,*]

[a] *Equipe d'Immunologie des Récepteurs, Immunologie des Maladies Infectieuses, CJF93-09 INSERM, Université François Rabelais, F-37200 Tours, France*

[b] *Max Delbrück Centrum für Molekulare Medizin, D-13125 Berlin, Germany*

[c] *CNRS UMR 6542 Physiologie des Cellules Cardiaques et Vasculaires, Université François Rabelais, F-37200 Tours, France*

[d] *Immunoanalyse et Innovation en Biologie Clinique, UMR 9921 CNRS, F-34060 Montpellier Cedex, France*

Received 27 November 1997; revised 9 February 1998; accepted 13 February 1998

Abstract

Monoclonal antibodies were produced against a peptide corresponding to the second extracellular loop of the human β_2-adrenoceptor. One of these monoclonals, inducing an agonist-like effect in neonatal rat cardiomyocytes, was used to define the structural and physiological basis of this activity. The epitope recognized by the antibody corresponds to the sequence Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu as determined by peptide scanning. Analysis by alanine modification of the peptide epitope showed the importance of the Trp, and Glu residues in antibody recognition The apparent affinity of the antibody assessed either by surface plasmon resonance or by functional titration on its agonist-like activity showed a similar value (10^8 M^{-1}). The antibody recognized the receptor in its native form as shown by immunofluorescence experiments on A431 cells but not in its denatured form as shown by its absence of staining in immunoblots. The positive chronotropic effect in vitro was specifically blocked by both the antigenic peptide and the specific β_2-antagonist ($\pm$)-1-[2,3-(Dihydro-7-methyl1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride (ICI118,551). This activity was mediated through activation of Ca^{2+} L-type channels as assessed in guinea pig cardiomyocytes. These results suggest that the epitope is located in an extracellular α-helix, whose recognition by the antibody could stabilize the receptor in its 'active' conformation. © 1998 Elsevier Science B.V.

Keywords: Anti-peptide antibody; β_2-Adrenoceptor; agonist, Surface plasmon resonance; Epitope mapping; Ca^{2+} channel, L type

1. Introduction

Increasing evidence shows that autoantibodies against G-protein-coupled receptors are present in several types of cardiovascular diseases and that the agonist-like activity of these autoantibodies could play a pathogenic role in, at least, some of the symptoms of these diseases (Hoebeke, 1995).

Structural knowledge on G-protein-coupled receptors was derived from the cloning and sequencing of the hamster β_2-adrenoceptor (Dixon et al., 1986), whose amphipathic profile corresponded to that of bacteriorhodopsin, whose three-dimensional structure was defined by electron-diffraction experiments (Henderson et al., 1990). These receptors are integral membrane proteins, consisting of seven membrane spanning α-helices linked together by extra- and intracellular loops, with the N-terminus at the extracellular side and the C-terminus at the intracellular site (Dohlman et al., 1987). Although the pharmacophore of the catecholamine receptors, belonging to this group, is localized in a hydrophobic pocket, encompassed in the seven trans-membrane α-helices (Strosberg, 1993), stabilization of the overall structure is mediated by a disulfide bridge that links the first and second extracellular loop together (Noda et al., 1994). Indeed, site directed mutagenesis studies have shown that the involved cysteines are essential for agonist recognition and for desensitization of the receptor (Dixon et al., 1987; Fraser, 1989; Liggett et al., 1989). The functional importance of the second extracellular loop, the existence of T cell epitopes on this loop and its hydrophilic character prompted us to consider it as one of the main targets for a functional autoimmune humoral response (Magnusson et al., 1989; Guillet et al.,

* Corresponding author. Present address: UPR9021 du CNRS, I.B.M.C., 15, rue Descartes, F-67084 Strasbourg, France. Tel.: +33-3-88-41-70-24; fax: +33-3-88-61-06-80; e-mail: hoebeke@ibmc.u-strasbg.fr

1991). This hypothesis was confirmed by the presence of autoantibodies with agonist-like activity against the β_1- and the β_2-adrenoceptor in patients with cardiac electrical disturbances of unknown origin (Chiale et al., 1995) and in patients with Chagas' disease (Sterin-Borda et al., 1988). Autoantibodies against the β_1-adrenoceptors and muscarinic M2 receptors were also found in patients with idiopathic dilated (Magnusson et al., 1990; Fu et al., 1993) or Chagas' cardiomyopathy (Elies et al., 1996) and against the α_1-adrenoceptor in patients with secondary malignant hypertension (Fu et al., 1994).

A specific B cell epitope in patients with Chagas' disease could be pinpointed to a polyanionic sequence in the second extracellular loop of the β_1-adrenergic and the muscarinic M2 receptor, cross-reacting with a ribosomal protein of the pathogenic parasite *Trypanosoma cruzi*. It corresponded to the sequence Ala-Glu-Ser-Asp-Glu situated at the N-terminus of the second extracellular loop of the β_1-adrenoceptor (Ferrari et al., 1995). The exact epitope recognized on the human β_2-adrenoceptor, however, could not be defined in the polyclonal autoimmune response. Another strategy was therefore used by preparing monoclonal antibodies against the second extracellular loop of the β_2-adrenoceptor and selecting one with agonist-like activity. The immunochemical characterization of the epitope recognized by this antibody has allowed us to define the amino acids on the receptor, involved in functional recognition.

2. Materials and methods

2.1. Peptides

Peptide β_2-H19C (His-Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu-Ala-Ile-Asn-Cys-Tyr-Ala-Asn-Glu-Thr-Cys) corresponding to the second extracellular loop (residues 172–190) of the human β_2-adrenoceptor (Emorine et al., 1987; Kobilka et al., 1987), peptide β_1-H19C (His-Trp-Trp-Arg-Ala-Glu-Ser-Asp-Glu-Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys) (Frielle et al., 1987) and other peptides (Table 1) used for this work were synthesized using the Fmoc (9-fluorenylmethyloxycarbonyl) procedure with an automated Applied Biosystems 431A peptide synthesizer. The peptides were purified by HPLC and checked by mass spectrometry.

2.2. Production of monoclonal antibodies

Balb/C mice were immunized with the free peptide β_2-H19C (100 μg) emulsified in complete Freund's adjuvant and injected subcutaneously. Four weeks later, a booster injection (100 μg in incomplete Freund's adjuvant) was given. Four weeks later 10 μg peptide dissolved in NaCl 0.9% was injected intravenously three days before harvesting the spleen cells for fusion.

Fusion was performed with polyethylene glycol 1500 (Boehringer, Mannheim, Germany) at 41% between splenocytes from the immunized mouse and myeloma SP20 cells, 10 splenocytes for 1 myeloma cell (Galfre and Milstein, 1981). Hybridomas were cultivated in 96 well plates precoated with peritoneal macrophages of Balb/C mice (5000 cells/well). 5×10^5 cells were distributed per well in RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum, 200-mM glutamine, 100-mM sodium pyruvate, 0.05 g of streptomycin/ml and 50 IU of penicillin/ml in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Secreting clones were screened by enzyme immunoassay and subcloned by limiting dilution (Oi and Herzenberg, 1980).

2.3. Enzyme immunoassay

Fifty microliters of peptide (10 μg/ml) in 100 mM Na_2CO_3 pH 9.6, were coated for 1 h on microtiter plates (Nunc, Kastrup, Denmark). The wells were then saturated with PMT (phosphate 10 mM NaCl 150 mM, pH 7.4 supplemented with 3% skimmed milk powder and 0.1%

Table 1
Sequences of synthetic peptides used

Receptor	Position	Peptide	Sequence
h β_2-Adrenergic	172–197	β_2-H26Q	His-Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu-Ala-Ile-Asn-Cys-Tyr-Ala-Asn-Glu-Thr-Cys-Cys-Asp-Glu-Phe-Thr-Asn-Gln
h β_2-Adrenergic	172–190	β_2-H19C	His-Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu-Ala-Ile-Asn-Cys-Tyr-Ala-Asn-Glu-Thr-Cys
h β_1-Adrenergic	197–222	β_1-H26R	His-Trp-Trp-Arg-Ala-Glu-Ser-Asp-Glu-Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys-Cys-Asp-Phe-Val-Thr-Asn-Arg
h β_1-Adrenergic	197–216	β_1-H19C	His-Trp-Trp-Arg-Ala-Glu-Ser-Asp-Glu-Ala-Arg-Arg-Cys-Tyr-Asn-Asp-Pro-Lys-Cys
h M2-Muscarinic	169–193	M2	Val-Arg-Tht-Val-Glu-Ser-Gly-Glu-Cys-Thr-Ile-Gln-Phe-Phe-Ser-Asn-Ala-Ala-Val-Thr-Phe-Gly-Thr-Ala-Ile
Control peptide		P23	Leu-His-Arg-Gly-His-Gln-Glu-Val-Glu-Leu-Glu-Lys-Leu-Pro-Thr-His-Asn-Asp-Pro-Glu-Ser-Leu-Glu-Ser

Position as found in the protein sequence.
Peptide nomenclature as found in the text.

Tween 20). Fifty microliters of hybridoma supernatants or monoclonal antibodies at different dilutions in PMT were added to the saturated plate for 2 h at 37°C. After washing the wells three times with PBS (phosphate buffered saline: phosphate 10 mM, NaCl 150 mM, pH 7.4) supplemented with 0.1% Tween 20, the monoclonal antibodies were revealed by incubation for 1 h at 37°C with a peroxidase conjugated anti-mouse IgG (H + L) (Interchim, France) diluted 1:1000 in saturation buffer. This was followed by three washings in the same washing buffer and the addition of the substrate 2.5 mM H_2O_2, 2 mM 2,2′-azino-di(ethyl-benzthiazoline) sulfonic acid. Absorbance was read after 30 min at 405 nm in a microplate reader.

2.4. Purification of the antibody

Anti-peptide monoclonal antibodies were purified from ascitic fluids on an affinity column made by coupling β_2-H19C peptide to CNBr-activated Sepharose-4B by the standard procedure (Pharmacia, Sweden). The ascitic fluid (2 ml) was extensively dialyzed against PBS, then adsorbed on the affinity column in PBS, eluted with 3 M $MgCl_2$ and immediately dialyzed against PBS.The concentration of the purified antibodies was calculated from the absorbance at 280 nm (optical density of 1.45 corresponding to 1 mg/ml). Purified anti-peptide monoclonal antibodies were aliquoted and stored at −80°C.

2.5. Mouse monoclonal antibody isotyping

Isotyping of the monoclonal antibodies was performed on hybridoma supernatant, ascitic fluids, and affinity purified monoclonal antibodies using the mouse monoclonal antibody isotyping kit from AMERSHAM.

2.6. Surface plasmon resonance

All measurements were performed on an upgraded Biacore instrument (Pharmacia). Streptavidin was immobilized on a CM5 chip, using the standard Pharmacia procedure at a density of 0.06 pmol/mm^2. The β_2-H19C peptide was allowed to react for 2 h at room temperature with a fivefold molar excess in a 0.1 M $NaHCO_3$ buffer at pH 8.5 of sulfosuccinimidyl-6-(biotinamido) hexanoate (Sigma, St. Louis, USA). The peptide was separated from the unreacted biotinylating agent by a PD10 desalting grade column (Pharmacia) and the subsequent solution (±1 mg/ml) allowed to interact with the immobilized streptavidin for 5 min (flow 5 μl/min). The molar ratio peptide/streptavidin as calculated from the increase in resonance units was 1.

Increasing concentrations of purified antibody diluted in 150 mM NaCl, buffered with 10 mM HEPES and supplemented with 3.4 mM EDTA and 0.001% Surfactant P20 (HBS) was allowed to react with the peptide during 12 min (flow 5 μl/min). The dissociation in the running buffer was followed for 5 min at the same flow rate. The biotinylated peptide–streptavidin complex was regenerated by a 4 min pulse in 3 M $MgCl_2$. Blanks were determined by studying the interaction of the antibody on a biotinylated irrelevant peptide of the same length.

2.7. Immunofluorescent analysis

The human epidermoid carcinoma cell A431 line, expressing a high amount of β_2-adrenoceptors, was grown at 37°C in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 200-mM glutamine, 100-mM sodium pyruvate, 0.05 g of streptomycin/ml and 50 i.u of penicillin/ml in a humidified incubator under an atmosphere of 5% CO_2. Cells were plated out into 175 cm^2 culture flasks until preconfluence. They were detached with a solution of 0.25% trypsin. After addition of 50 ml of RPMI 1640, they were centrifuged; the resulting pellet was resuspended in supplemented culture medium and subcultured into five 175 cm^2 flasks or 24 well plates.

Cells cultured in 175 cm^2 flasks were then harvested in PBS by scraping and centrifuged at 400 rpm in 4 min to sediment the aggregates. The resulting suspension was centrifuged and the pellet (10^6 cells) resuspended in 1 ml PBS containing the purified 6H8 monoclonal antibody (250 nM) and incubated for 2 h at 4°C. After washing by centrifugation (2 min at 7500 × g), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1/100 (v/v), was added to the cells. After 1 h of incubation, the cells were washed three times, fixed with 2% (v/v) of formaldehyde, and analyzed on a FACScan using the LYSIS program (Becton Dickinson).

2.8. Peptide and alanine replacement scanning

A set of 11 nonapeptides, overlapping by 9 residues, covering the sequence 172–190 of the human β_2-adrenoceptor was prepared by the Spot method of multiple peptide synthesis on cellulose membrane (Frank, 1992), as modified (Molina et al., 1996). The reactivity of the set of immobilized peptides was assessed by incubation with a dilution of monoclonal antibodies mAb6H8 or mAb5D9 (90 min at 37°C) followed by an alkaline phosphatase conjugated anti-mouse IgG (Sigma), diluted 1/1000 for 60 min at room temperature. Positive peptides were identified by the blue color developing on spots in the presence of 5-bromo-4-chloro-3-indolyl phosphate, as described (Frank, 1992).

By the same method a set of nonapeptides corresponding to the epitope recognized by the mAb6H8 was prepared by substituting an alanine for every residue. The alanine was replaced by a glycine. Recognition of the modified peptides by the monoclonal antibody was performed as described above.

2.9. Radioligand binding assays

Guinea pig lungs were minced and homogenized (polytron homogenizer, setting 6, 10 s twice) in ice-cold buffer

(pH 7.5) consisting of 20 mM Tris–HCl, 250 mM sucrose, 5 mM $MgCl_2$, 10 μM phenylmethylsulfonylfluoride (PMSF), 0.7 mg/ml pepstatin and 0.5 μg/ml leupeptin. The homogenate was centrifuged at $900 \times g$ for 10 min at 4°C, the supernatant was filtered through gauze, diluted with an equal volume of the same buffer used to homogenize but without sucrose and centrifuged for 10 min at $43\,000 \times g$. The resulting pellet (membrane particulate fraction) was resuspended in 600 mM KCl in the buffer mentioned earlier. It was placed on ice for 10 min, then recentrifuged at $43\,000 \times g$ for 20 min. The crude membrane preparation was resuspended in 20 mM Tris–HCl, 5 mM $MgCl_2$ buffer and aliquots were stored at −80°C until use.

Saturation binding isotherms were obtained by incubating lung membranes (10 μg of protein) for 1 h at room temperature with varying concentrations of 4-[3-(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one ([^{3}H]-CGP12,177) (0.0625–4 nM). Specific binding was defined as that displaced by 10 μM (−) propranolol. The reaction was stopped by dilution with 5 ml ice cold buffer. The samples were then poured over GF/C 2.5 glass microfibre filters (Whatman International, UK) under reduced pressure followed by two washes with 5 ml of buffer. Filters were counted in a liquid scintillation counter. The saturation binding variables B_{max} and K_d were determined from the commercially available computer assisted programs EBDA and LIGAND, of Munson and Rothbard as adapted for the Macintosh by MacPherson (1985).

2.10. Cardiomyocyte stimulation

Rat neonatal cardiomyocytes were prepared from ventricles of 1–2- days Wistar rats by a modified method according to Halle and Wollenberger (1970). The cells were cultured as monolayers for 4 days at 37°C in SM 20-1 medium supplemented with 10% heat-inactivated calf serum and 2 μM fluorodeoxyuridine and exhibited a spontaneous basal pulsation rate of about 160 beats/min.The cardiomyocyte cultures were washed with fresh medium containing serum and incubated for 30 min at 37°C with the same medium containing 10 nM arachidonic acid (Wallukat et al., 1994). The flasks were transferred to the heatable stage of an inverted microscope, and the basal beating rate was determined. Ten small circular fields (0.8 mm^2, 10 mm apart from each other) were inspected through the perforation of a metal template. Three to five clusters of synchronously beating cells in each of the 10 fields were selected, and the number of the contractions was counted for 15 s. This procedure was repeated for two to five identically treated culture flasks. The compounds to be tested were dissolved in the same medium used for the determination of the basal beating rate and incubated with the monolayers for the times indicated.

To study the signal transduction pathway of the antibody, cardiomyocytes were preincubated for 30 min with a phosphokinase A inhibitor, Rp isomer of adenosine-3′5′-monophosphorothioate (RPcAMPS 50 μM), or a phosphokinase C inhibitor (Calphostin C 100 nM) before adding mAbH68.

2.11. Electrophysiological measurements

Cardiomyocytes were isolated using a collagenase/protease digestion technique described elsewhere (Le Guennec et al., 1993). Dissociated cells were placed in a 1 ml chamber on the stage of an inverted microscope (Nikon, Diaphot 300, Japan). The chamber was continuously per-

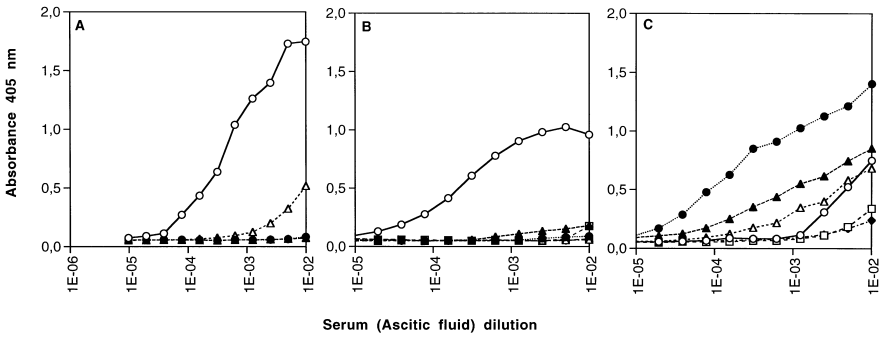

Fig. 1. Specificity of the anti-β_2-H19C response. (A) Titration of the serum of the mouse used for the hybridoma production. The enzyme immunoassay was performed on the free peptides, β_2-H19C (○-○), β_1-H19C (●-●), β_2-H26Q (△-△), β_1-H26R (▲-▲) directly absorbed on the microwell titre plates by subsequent incubation with the anti-β_2-H19C peptide mouse polyclonal antiserum and a peroxidase conjugated donkey anti-mouse IgG H + L. (B) Peptide specificity of monoclonal antibody mAb6H8. The titration curves for the ascitic fluid of mAb6H8 on peptides β_2-H19C (○-○), β_1-H19C (●-●), β_2-H26Q (△-△), β_1-H26R (▲-▲), M2 (□-□), P23 (■-■). The enzyme immunoassay was performed on the free peptides directly absorbed on the microwell titre plates by subsequent incubation with the ascitic fluid at different dilution and a peroxidase conjugated donkey anti-mouse IgG H + L. (C) Peptide specificity of monoclonal antibody mAb5D9. The titration curves for the ascitic fluid of mAb5D9 on peptides β_2-H19C (○-○), β_1-H19C (●-●), β_2-H26Q (△-△), β_1-H26R (▲-▲), M2 (□-□), P23 (■-■). The enzyme immunoassay was performed on the free peptides directly absorbed on the microwell titre plates by subsequent incubation with the ascitic fluid at different dilution and a peroxidase conjugated donkey anti-mouse IgG H + L.

fused at a rate of 1 ml/min with Tyrode's normal solution. The composition of this solution was (in mM): NaCl, 140; KCl, 5.4; HEPES, 10; $CaCl_2$, 1.8; $MgCl_2$, 1: NaH_2PO_4, 0.33 and glucose, 11. The pH was 7.3.

Whole-cell voltage clamp experiments were done in these isolated single cells at room temperature (22–25°C). During the experiments, cells were locally superfused with an extracellular solution containing (in mM): tetraethylammonium chloride (TEACl), 140; CsCl, 6; HEPES, 10; $CaCl_2$, 1.8; $MgCl_2$, 1 and glucose, 10. The pH was adjusted to 7.3 with tetraethylammonium hydroxide (TEAOH). This solution was superfused at a rate of 1 ml/min. All drug and antibody solutions were prepared daily and diluted as desired in the extracellular solution. The patch pipettes (2–4 MΩ) were fabricated with borosilicate glass. Pipettes were filled with (in mM): CsCl, 130; HEPES, 11; EGTA, 10; Tris–GTP, 1 and Mg^{2+}-ATP, 5. The pH was adjusted to 7.3 with CsOH. After the formation of a gigaseal (4–10 GΩ) between the cell membrane and the pipette and rupturing of the membrane patch, whole cell L-type Ca^{2+} currents (I_{Ca}) were elicited by 500 ms voltage steps applied from a holding potential of −80 mV to +10 mV every 7 s.

Cell capacity (proportional to cell size) was measured by integration of the current elicited by a 10 mV, 20 ms pulse and this value was used to evaluate I_{Ca} density (pA/pF). Currents were recorded using a RK300 Patch and Cell Clamp amplifier (Biologic, Meylan, France) con-

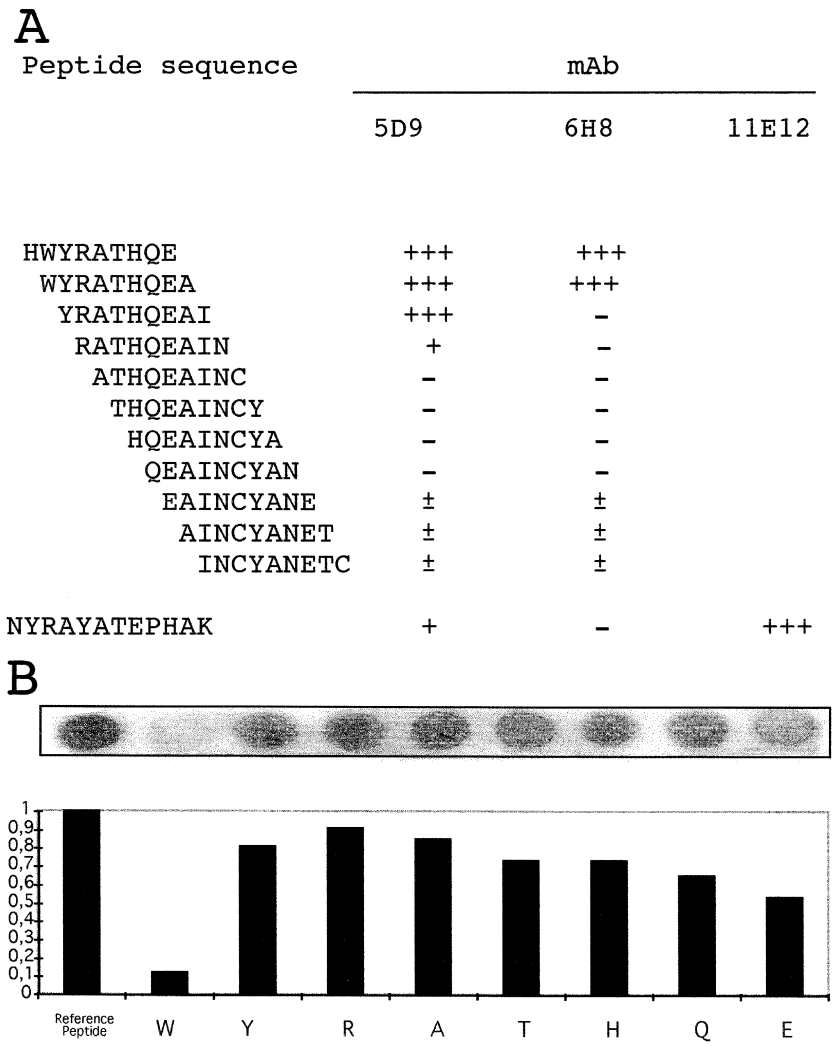

Fig. 2. Peptide mapping of the epitopes. (A) Identification of the peptide epitopes of mAb5D9 and mAb6H8. Overlapping nonapeptides covering residues 172–190 from the sequence of the human β_2-adrenergic receptor were prepared according to the Spot method of peptide synthesis on cellulose membranes (Frank, 1992, Molina et al., 1996) and reacted with mAb6H8 or mAb5D9, or mAb 11E12 as a control. The intensity of the coloration developed on reactive spots is indicated by the number of '+'; a faint coloration was observed for some peptides (indicated by '±') which was due to cross-reactivity with the alkaline phosphatase conjugated anti-mouse antibody used as a secondary antibody. (B) Identification of residues important for recognition of peptide Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu by mAb6H8. A set of analogs of the sequence in which each residue was in turn changed into alanine was prepared by the Spot method and reacted with 5 μg/ml mAb6H8. After the colored reaction had developed; the membrane was scanned (upper panel), and the intensity of the color on each spots was measured as described (Molina et al., 1996). The lower panel is a diagram of the intensity of each spot relative to the color of the reference peptide.

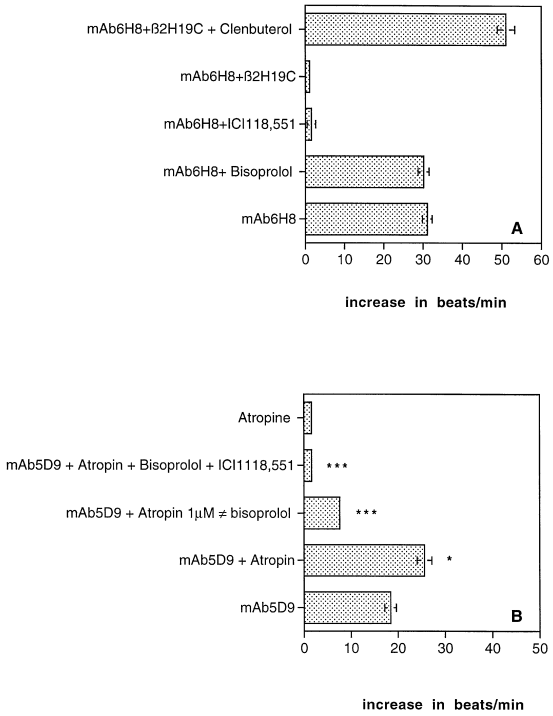

Fig. 3. Chronotropic effects of the two monoclonal antibodies mAb6H8 and mAb5D9 on cultured neonatal rat cardiomyocytes. Basal rate beating frequency of neonatal rat cardiomyocytes in culture was determined as described in Section 2. Substances indicated (see *Y*-axes) were added to neonatal rat cardiomyocytes cumulatively. The increase or decrease in beating frequency was determined as the difference between beating frequency 15 min after treatment and the spontaneous beating rate. Means ± SEM of 16 to 30 different measurements were used for calculation of the values. (A) β_2-adrenoceptor agonist-like chronotropic effect of the monoclonal antibody mAb6H8 on cultured neonatal rat cardiomyocytes. The β_2-adrenergic response is demonstrated by the effect of the selective β_2-agonist clenbuterol (1 μM). The effect of mAb6H8 was tested using the ascitic fluid at a dilution of 1:1000.The beating frequency was measured 15 min after the addition of the antibody. This effect was completely blocked 5 min after the addition of 10 nM ICI 118,551. The effect of the monoclonal antibody (1:1000) was neutralized by the preincubation of mAb6H8 with the peptide β_2-H19C (50 μg/ml) for 1 h at 37°C. (B) Effect of the monoclonal antibody mAb5D9 on cultured neonatal rat cardiomyocytes mAb5D9 (ascitic fluid at a 1/1000 dilution) shows a global positive chronotropic effect which had a muscarinic receptor component (as shown by its increase in beating rate after blocking of the muscarinic component with 1 μM atropine), a β_1-adrenergic component (as shown by the partial blocking effect of the β_1-antagonist 1 μM bisoprolol) and a β_2-adrenergic component (as shown by the blocking effect of the β_2-antagonist 10 nM ICI118,551. $* p < 0.05$ mAb5D9 in presence and absence of atropin. $* * * p < 0.001$ mAb5D9 in presence of atropine compared to presence of bisoprolol; mAb5D9 inpresnce of atropin and bisoprolol compared to presence of ICI118,551.

nected to a PC 486 computer, which was equipped with pClamp software (version 6.0.2, Axon Instruments). The currents were stored in the computer for subsequent analysis with pClamp software. The calcium currents were measured as the difference between inward peak current and the current at the end of the depolarization pulse.

To study the transduction pathway, cardiomyocytes were perfused with the phosphokinase A inhibitor RPcAMPS (1 mM) until reaching of equilibrium previously to addition of 13 nM mAb6H8 in the extrapipette medium.

3. Results

3.1. Peptide specificity of monoclonal antibodies

As shown in Fig. 1A, the serum of the mouse used for hybridoma production showed a highly specific and highly pronounced response against the immunogenic peptide β_2-H19C. Although the polyclonal response was high, only two clones could be conserved until subcloning and amplification (mAb6H8 and mAb5D9). While the specificity of mAb6H8 corresponded to the polyclonal response, mAb5D9 showed a heteroclitic response towards β_1-H19C which is the peptide equivalent to β_2-H19C from the β_1-adrenoceptor (Fig. 1B). Interestingly, mAb6H8 did not recognize the non-truncated β_2-H26Q peptide corresponding to the second extracellular loop of the β_2-adrenoceptor but was monospecific for the immunizing peptide β_2-H19C. In contrast, the mAb5D9 similarly recognized the truncated (β_1-H19C and β_2-H19C) and non-truncated (β_1-H26R and β_2-H26Q) peptides corresponding to the second extracellular loop of the β_1- and the β_2-adrenoceptors respectively (Fig. 1B). The non-specificity of mAb5D9 was confirmed by its ability to recognize the peptide corresponding to the second extracellular loop of the muscarinic M2 receptor (Fig. 1C). Its lack of specificity is compatible with the fact that it is an IgM while mAb6H8 is an IgG_1.

The fine specificity of the two antibodies was assessed by epitope mapping using overlapping peptides from the sequence of β_2-H19C. MAb6H8 recognition was restricted to the two first nonapeptides, therefore mapping the epi-

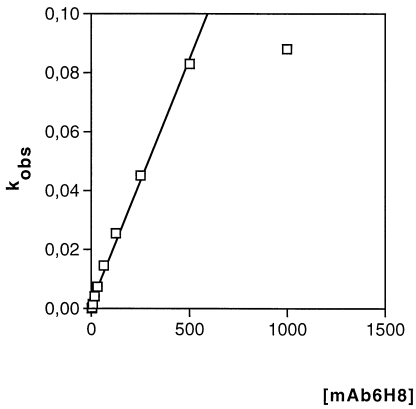

Fig. 4. Physico–chemical parameters of antibody-peptide interactions as measured by surface plasmon resonance. Observed association rates (k_{obs}) as a function of antibody concentration. The k_{obs} were calculated from the sensorgrams using model 4 in the BIAevaluation procedure. The slope of the linear fit corresponds to the bimolecular association rate constant (k_{on}); the scission point with the *Y*-axis to the dissociation rate constant (k_{off}).

tope to residues Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu while the mAb5D9 recognized four overlapping peptides thus indicating that this mAb binds to epitope Arg-Ala-Thr-His-Gln-Glu. It also weakly recognized a control peptide (Asn-Tyr-Arg-Ala-Tyr-Ala-Thr-Glu-Pro-His-Ala-Lys) (Fig. 2A).

Differences in specificity of the two mAb are also reflected in the agonist-like effects of ascitic fluids of both monoclonals. While Mab6H8 induced a specific β_2-adrenergic response in neonatal rat cardiomyocytes in culture, Mab5D9 exerted a positive chronotropic effect, recognizing the β_1-adrenoceptor (as shown by the partial blocking effect of the β_1-antagonist bisoprolol) and the β_2-adrenoceptor (as shown by the blocking effect of the β_2-antagonist ICI118,551) which was influenced by a moderate muscarinic effect (as shown by its increase after blocking of the muscarinic M2 receptor with atropine) (Fig. 3). In view of the monospecificity of mAb6H8, it was decided to analyze further its properties using affinity purified monoclonal antibody mAb6H8.

The fine epitope specificity of mAb6H8 antibody was assessed by alanine scanning of the sequence Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu identified by peptide scanning, (Fig. 2B). When the N-terminal tryptophane was replaced with an alanine, the modified peptide was no longer recognized, indicating that the tryptophane side chain was essential for the antibody recognition. The replacement of the C-terminal glutamic acid resulted in a slight decrease of antibody recognition (Fig. 2B), suggesting that the epitope has a non-sequential structure.

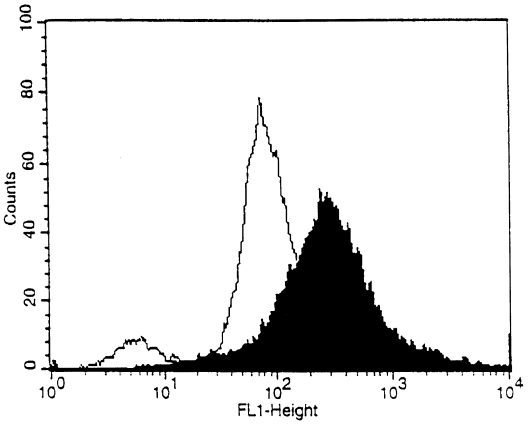

Fig. 5. FACS scan analysis of the A431 cell line immunostaining by the affinity purified mAb6H8. Intact A431 human epidermoid cells expressing the β_2-adrenoceptor were incubated in suspension for 1 h at 4°C in PBS containing (black) or not (white) the affinity purified monoclonal antibody mAb6H8 (260 nM). After washing by centrifugation, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1/100 (v/v), was added to the cells. After 1 h of incubation, the cells were washed three times, fixed with 2% (v/v) formaldehyde, and analyzed on a FACScan using the LYSIS program (Becton Dickinson).

Table 2
Radioligand binding assay of [^{3}H]-CGP12,177 on guinea-pig lung membranes

	B_{max} (fmol/protein mg)	K_d (pM)
control	158 ± 9	230 ± 27
MAb6H8	151 ± 8	234 ± 20

Saturation binding isotherms were obtained by incubating lung membranes, preincubated for 5 h at 4°C with 260 nM of affinity purified Mab6H8 or without (control), during 1 h at room temperature with varying concentration of [^{3}H]-CGP12,177 (0.0625–4 nM).
Specific binding was defined as that displaced by 10 μM (−) propranolol.
The saturation binding variables B_{max} and K_d were determined from the commercially available computer assisted programs EBDA and LIGAND, of Munson and Rothbard as adapted for the Macintosh by MacPherson (1985).

3.2. Physico–chemical parameters of the peptide–antibody interaction

The kinetic equilibrium constants were determined using the biotinylated peptide immobilized on a streptavidin coated on a CM5 chip in the BIAcore sensor. As shown in Fig. 4, the interaction corresponded to a pseudomonomolecular interaction yielding a k_{obs} (observed association constant) linearly with the antibody concentration, except at the highest dose were a saturation process starts (Altschuh et al., 1992). The slope of the plot yielded a bimolecular association rate constant (k_{on}) of 1.7×10^5 M^{-1} s^{-1} and the scission point with the *Y*-axis a dissociation rate constant (k_{off}) of 0.0017 s^{-1}. The affinity constant K_a calculated as the ratio k_{on}/k_{off} was 0.96×10^8 M^{-1}.

3.3. Receptor recognition by Mab6H8

The antibody did not recognize the denatured receptor in immunoblots either on A431 cells or on membranes of

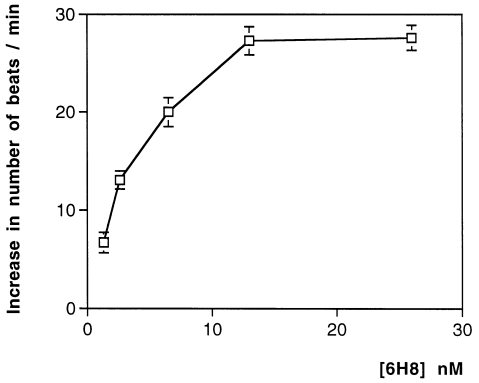

Fig. 6. Dose dependence of the β_2-adrenergic stimulation by the monoclonal antibody mAb6H8. Beating frequency of neonatal rat cardiomyocytes in serum free medium culture was determined as described in Section 2. Each point represents the mean beating rate of 10 different cardiomyocytes clusters. This figure is representative of three different experiments. The affinity purified monoclonal antibody mAb6H8 was added cumulatively at various dilutions. Every 15 min after the addition of a new dilution, the increase in beating rate was counted.

rat neonatal cardiomyocytes. In contrast, a specific immunofluorescence could be observed on A431 cells, which express a high amount of β_2-adrenoceptors (Fig. 5).

To study the pharmacological effect of mAb6H8, membranes of guinea pig lungs were used in a radioligand binding assay with ^{3}[H]-CGP12,177. The antibody did not change either the affinity of the radioligand or the maximal binding sites even at concentrations which were 10 times higher than the affinity constant calculated for the peptide–antibody interaction (Table 2). In contrast, the antibody exerted a dose-dependent agonist-like effect on the spontaneous beating rate of neonatal rat cardiomyocytes (Fig. 6). The EC_{50} (the dose resulting in 50% of the maximal response) calculated from the curve was 8 nM, a concentration similar to the dissociation constant calculated for the peptide–antibody interaction. As shown in Fig. 3, the agonist-like effect of the antibody is specific for the combining site (since it can be blocked by preincubation with the target peptide) and for the β_2-adrenoceptor (since it can be blocked with the β_2-antagonist ICI118,551).

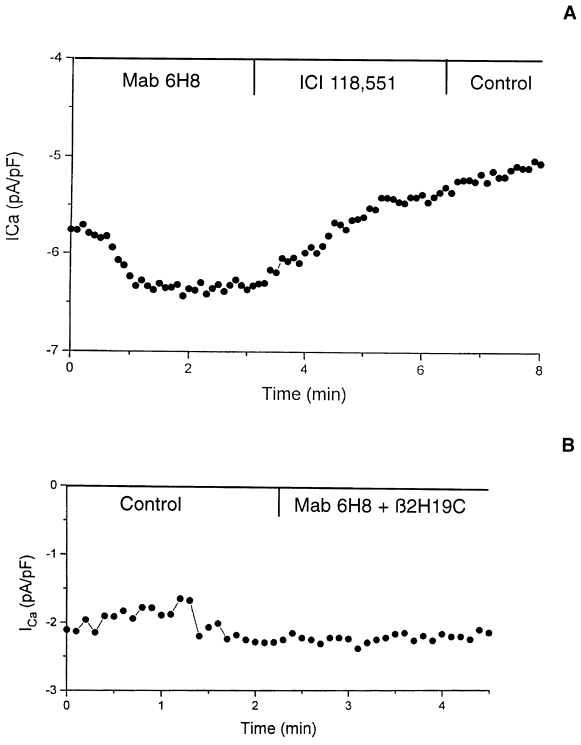

Fig. 7. Effects of affinity purified monoclonal antibody mAb6H8 on calcium currents (I_{Ca}) of ventricular guinea-pig cardiomyocytes. (A) Time course of I_{Ca} density in the same cell after the successive application of (1) the affinity purified mAb6H8 (13 nM),(2) the β_2-antagonist (ICI 118,551 10 nM) and (3) under control condition (control): The increase in I_{Ca} observed during mAb6H8 application is completely reversed by the application of ICI 118,551. (B) Time course of I_{Ca} density in the same cell under control conditions (control) and after the application of the affinity purified monoclonal antibody mAb6H8 13 nM preincubated for 1 h at 37°C with the peptide β_2-H19C 50 μg/ml (6H8 + β_2-H19C). The preincubation of the mAb6H8 with the peptide completely abolishes the specific increase of I_{Ca} mediated by the antibody alone as seen in Fig. 6.

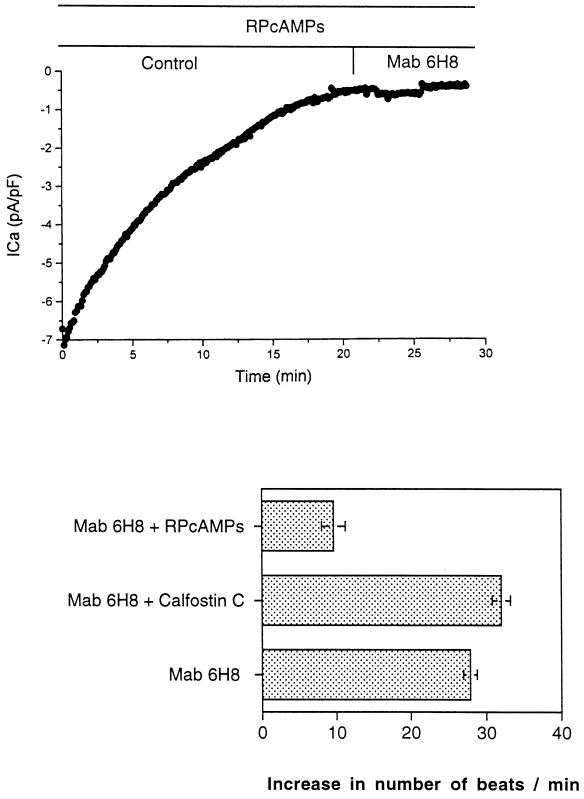

Fig. 8. cAMP dependency of the agonist-like effect of mAb6H8. Upper panel: Inhibition of the antibody effect on activation of Ca^{+2} channels in isolated guinea-pig cardiomyocytes. Cardiomyocytes were preincubated with 1 mM of RPcAMPS to equilibrium before extracellularly adding the antibody. No activation could be demonstrated. Lower panel: Inhibition of the antibody effect on neonatal rat cardiomyocytes. Increase in beating frequency with the antibody was assessed in presence of the phosphokinase A inhibitor RPcAMPS (50 μM) or the phosphokinase C inhibitor Calphostin (100 nM). RPcAMPS showed an inhibitory effect while Calphostin did not significantly change the antibody effect.

The positive chronotropic effect of β-adrenoceptor agonists may be explained by the entry of Ca^{2+} ions after cAMP dependent activation of L-type Ca^{2+} channels (Hartzell, 1988). Using a whole cell patch-clamp technique on adult guinea-pig cardiomyocytes, it could be verified that the antibody was able to activate L-type Ca^{2+} channels by means of the β_2-adrenoceptor, as measured by the inhibitory activity of the β_2-antagonist ICI118,551 (Fig. 7).

That cardiomyocyte stimulation by mAb6H8 was cAMP dependent could be demonstrated by the inhibitory effect of the phosphokinase A inhibitor RPcAMPS both on beating frequency of neonatal rat cardiomyocytes and Ca^{2+} channel activation in guinea-pig cardiomyocytes (Fig. 8).

4. Discussion

Autoantibodies against G-protein-coupled receptors with agonist-like activity have been shown to occur in several

cardiovascular diseases and are mainly but not exclusively directed against the second extracellular loop of those receptors (Hoebeke, 1995). The induction of cardiomyopathic changes in rabbits immunized with peptides corresponding to this domain of the β_1-adrenoceptor and the muscarinic M2 receptor suggests that these autoantibodies could play a role in the pathogenesis of this disease (Matsui et al., 1997). Monoclonal antibodies, sharing the agonist-like properties of those autoantibodies could be used to define the structural basis of the antibody effects as well as to study their in vivo effects by passive transfer. We describe here the structural and physiological characteristics of such a monoclonal antibody directed against the β_2-adrenoceptor.

In the same fusion, a hybridoma was obtained secreting an IgM recognizing structurally and functionally the β_1- and β_2-adrenoceptor together with the muscarinic M2 receptor. These results are compatible with the existence of autoantibodies reacting with multiple receptors in sera of patients with Chagas' cardiomyopathy (Elies et al., 1996) or in mice infected with the causative parasite, *T. cruzi* (Mijares et al., 1996a). The heteroclitic IgM response reflects the plasticity of the primary immune response against the highly antigenic second extracellular domain of G protein-coupled receptors. The multivalency of this antibody and its relative non-specificity excluded it for further structural studies.

Interestingly, the IgG1 mAb6H8 was specific for the truncated peptide corresponding to the 19 N-terminal amino acids of the second extracellular loop of the β_2-adrenoceptor and did not recognize the peptide corresponding to the whole loop. This suggests that the recognized epitope, which was defined as Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu by peptide scanning, has a different conformation in the short compared to the long peptide. The conformational nature of the epitope recognized is also suggested by alanine replacement studies in which the N-terminal Trp was shown to be essential and the C-terminal Glu important. These results suggest that the amino acids Trp, Ala, Thr and Glu belong to the same continuous surface when the peptide is constructed as an α-helix (Fig. 9). Modification of Ala to Gly or Thr to Ala has only a minor effect due to the size of the side-chain. This is in accordance with the results of Kobilka and colleagues, showing that the α-helix content of the β_2-adrenoceptor is much higher than that expected by the seven transmembrane helices alone (Lin et al., 1996).

That the epitope could have an α-helix structure in the receptor is also suggested by the absence of recognition of the denatured receptor in immunoblot experiments while it is recognized as native on A431 cells, expressing a high amount of β_2-adrenoceptors. These results are in accordance with several studies, showing that antibodies directed against the second extracellular loop of G-protein-coupled receptors, are unable to recognize the receptor in immunoblot but recognize the native receptor in intact cells (Teufel et al., 1993; Guillaume et al., 1994; Verdot et al., 1995).

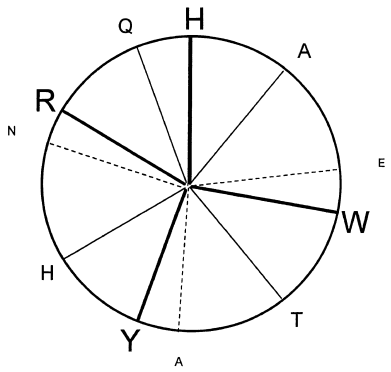

Fig. 9. α-helix structure of the recognized B cell epitope. The amino acids are situated at an angle of 100° as for a right-handed α-helix. The helix starts with the large-sized symbols (H,W,Y,R) continues with the medium-sized symbols (A,T,H,Q) and ends with the lower-sized symbols (E,A,N,Q). The amino acid most proximal to the essential W residue is the E residue. The two other residues situated in the same plane are A and T.

Further confirmation that the conformation recognized on the peptide is also present on the receptor is given by the comparison of the K_d of the peptide–antibody interaction which is approximately 10 nM and the effective concentration necessary to induce 50% of the agonist-like effect which is about 8 nM. Since, on the other hand, the association rate constant of the peptide–antibody interaction (1.7×10^5 M^{-1} s^{-1}) corresponds to values for such interactions, as measured with the BIAcore (Altschuh et al., 1992), induction of large conformational changes upon peptide–antibody and probably receptor–antibody interactions are unlikely.

The agonist-like effect of the antibody was further considered. Indeed, it was previously shown that polyclonal antibodies directed against the second extracellular loop of the β_2-adrenoceptor probably act through activation of L-type Ca^{2+} channels (Mijares et al., 1996b). This activation of L-type Ca^{2+} channels by a cAMP dependent kinase (Tsien et al., 1986; Hartzell, 1988) may result from the increase in cAMP produced by the activation of adenylyl cyclase by the antibody–receptor complex. mAb6H8 had a similar effect which could be blocked by the β_2-antagonist ICI118,551 excluding a non-specific effect on the channel itself or on the membrane of the target cell. The mechanism by which the mAb6H8 acts is thus likely the stabilization of an active conformation of the receptor, similar to what was found for the polyclonal antibodies (Mijares et al., 1996b). This conformation is able to stimulate the normal signal transduction pathway of the β_2-adrenoceptor, leading to cAMP dependent phosphokinase A activation, as shown by the inhibitory activity of the cAMP analog, RPcAMPS.

In view of a previous report, describing a monoclonal antibody against the second extracellular loop, which decreased in a non-competitive manner the binding of a

radioligand to the receptor (Guillet et al., 1992), the pharmacological properties of mAb6H8 were assessed upon radioligand binding to guinea pig lung membranes. No inhibition of binding could be observed. It is worthwhile to mention that the epitope, recognized by the pharmacologically active monoclonal antibody described by Guillet et al. (1992), was determined as Ala-Ile-Asn-Cys-Tyr (non-published data). As for antibodies with radioligand binding inhibitory properties against other G-protein-coupled receptors (Abd Alla et al., 1996), the cysteine seems an essential amino acid in the recognized epitope. Alternatively, the discrimination of epitopes recognized by antibodies with agonist-like activity without effect on radioligand binding has been described for the muscarinic M2 receptor (Fu et al., 1995). Further investigations are currently performed to study the effect of mAb6H8 on binding of inverse agonists, neutral antagonists, partial agonists and full agonists. These results will help to specify what active conformation is stabilized by the antibody, taking into account the multi-state receptor conformations proposed by Kenakin (1996).

To summarize, the complete characterization of mAb6H8, described here has increased our knowledge about the structure of the β_2-adrenoceptor and given us a highly specific tool to further analyze the mechanisms by which antibodies against G-protein-coupled receptors exert their effects.

Acknowledgements

This work was supported by BIOMED2 grant (BMH4-CT95-1008), and a grant from Biotechnocentre. D.L. is a recipient of a doctoral fellowship from the Région Centre (France).

References

Abd Alla, S., Quitterer, U., Grigoriev, S., Maidhof, A., Haasemann, M., Jarnagin, K., Müller-Esterl, W., 1996. Extracellular domains of the bradykinin B2 receptor involves in ligand binding agonist sensing defined by anti-peptide antibodies. J. Biol. Chem. 271, 1748–1755.

Altschuh, D., Dubs, M.C., Weiss, E., Zeder-Lutz, G., Van Regenmortel, M.H.V., 1992. Determination of kinetic constants for the interaction between a monoclonal antibody and peptides using surface plasmon resonance. Biochemistry 31, 6298–6304.

Chiale, P.A., Rosenbaum, M., Elizari, V., Hjalmarson, Å., Magnusson, Y., Wallukat, G., Hoebeke, J., 1995. High prevalence of antibodies against β_1- and β_2-adrenergic receptors in patients with primary electrical cardiac disturbances. J. Am. Coll. Cardiol. 26, 864–869.

Dixon, R.A.F., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Munford, R.A., Slater, I.S., Caron, M.G., Lefkowitz, R.J., Strader, C.D., 1986. Cloning gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. Nature 321, 75–79.

Dixon, R.A.F., Sigal, I.S., Candelore, M.R., Register, R.B., Scattergood, W., 1987. Structural features required for ligand binding to the β_2-adrenergic receptor. EMBO J. 6, 3269–3275.

Dohlman, H.G., Bouvier, M., Benovic, J.L., Caron, M.G., Lefkowitz, J.R., 1987. The multiple membrane spanning topography of the beta-2 adrenergic receptor. J. Biol. Chem. 262, 16655–16662.

Elies, R., Ferrari, I., Wallukat, G., Lebesgue, D., Chiale, P., Elizari, M., Rosenbaum, M., Hoebeke, J., Levin, M.J., 1996. Structural and functional analysis of the B cell epitope recognized by anti-receptor antibodies in patients with Chagas' disease. J. Immunol. 157, 4203–4211.

Emorine, L.J., Marullo, S., Delavier-Klutchko, C., Kaveri, S. V, Durieu-Trautmann, O., Strosberg, A.D., 1987. Structure of the gene for human β_2-adrenergic receptor: Expression and promotor regulation. Proc. Natl. Acad. Sci. USA 84, 6995–6999.

Ferrari, I., Levin, M.J., Wallukat, G., Elies, R., Lebesgue, D., Chiale, P., Elizari, M., Rosenbaum, M., Hoebeke, J., 1995. Molecular mimicry between the immunodominant ribosomal protein P0 of *Trypanosoma cruzi* and a functional epitope on the human β_1-adrenergic receptor. J. Exp. Med. 182, 59–65.

Frank, R., 1992. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron 48, 9217–9232.

Fraser, C.M., 1989. Site-directed mutagenesis of β-adrenergic receptor. Identification of conserved cystein residues that independently affect ligand binding and receptor activation. J. Biol. Chem. 264, 9266–9270.

Frielle, T., Daniel, K.W., Caron, M.G., Lefkowitz, R.J., 1987. Cloning of the gene for the β_1-adrenergic receptor. Proc. Natl. Acad. Sci. USA 84, 9494–9498.

Fu, L.X., Magnusson, Y., Bergh, C.H., Liljeqvist, J.A., Waagstein, F., Hjalmarson, Å., Hoebeke, J., 1993. Localization of a functional autoimmune epitope on the muscarinic acetylcholine receptor-2 in patients with idiopathic dilated cardiomyopathy. J. Clin. Invest. 91, 1964–1968.

Fu, L.X.M., Herlitz, H., Wallukat, G., Hilme, E., Hedner, T., Hoebeke, J., Hjalmarson, Å., 1994. Functional autoimmune epitope on α_1-adrenergic receptors in patients with malignant hypertension. Lancet 344, 1660–1663.

Fu, M.L.X., Schultze, W., Wallukat, G., Hjalmarson, Å., Hoebeke, J., 1995. Functional epitope analysis of the second extracellular loop of the human heart muscarinic acetylcholine receptor. J. Mol. Cell. Cardiol. 27, 427–436.

Galfre, G., Milstein, C., 1981. Preparation of monoclonal antibodies: Strategies and procedures. Methods Enzymol. 73, 3–46.

Guillaume, J.L., Petitjean, F., Haaseman, M., Bianchi, C., Eshdat, Y., Strosberg, A.D., 1994. Antibodies for the immunochemistry of the human β_2-adrenergic receptor. Eur. J. Biochem. 224, 761–770.

Guillet, J.G., Hoebeke, J., Lengagne, R., Tate, K., Borras-Herreras, F., Strosberg, A.D., Borras-Cuesta, F., 1991. Haplotype specific homology scanning algorithm to predict T-cell epitopes from protein sequences. J. Mol. Recognit. 4, 17–25.

Guillet, J.G., Lengagne, R., Magnusson, Y., Tate, K., Strosberg, A.D., Hoebeke, J., 1992. Induction of a pharmacologic active clonotypic B cell response directed to an immunogenic region of the human β_2-adrenergic receptor. Clin. Exp. Immunol. 89, 461–467.

Halle, W., Wollenberger, A., 1970. Differentiation and behavior of isolated embryonic and neonatal heart cells in a chemically defined medium. Am. J. Cardiol. 25, 292–299.

Hartzell, H.C., 1988. Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. Prog. Biophys. Mol. Biol. 52, 165–247.

Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E., Downing, K.H., 1990. Model for the structure of bacteriorhodopsin based on hight-resolution electron cryo-microscopy. J. Mol. Biol. 213, 899–929.

Hoebeke, J., 1995. La seconde boucle extracellulaire des récepteurs couplés aux protéines G: une cible préferentielle pour des autoanticorps fonctionnels. Medecine/Sciences 12, 1661–1667.

Kenakin, T.P., 1996. Receptor conformation induction versus selection: all part of the same original landscape. Trends Pharmacol. Sci. 17, 190–191.

Kobilka, B.K., Dixon, R.A.F., Frielle, T., Dohlman, H.G., Bolanwski, M.A., Sigal, I.S., Yang-Feng, T.L., Franke, U.Y., Caron, M.G., Lefkowitz, R.J., 1987. cDNA for the human β_2-adrenegic receptor: a protein with multiple membrane spanning domains and encode by a gene whose location is shared with that of the receptor for platelet derived growth factor. Proc. Natl. Acad. Sci. USA 84, 46–50.

Le Guennec, J.Y., Peineau, N., Esnard, F., La Campagne, A., Gannier, F., Argibay, J., Gauthier, F., Garnier, D., 1993. A simple method for calibration of collagenase/protease E ratio to optimize heart cell isolation. Biol. Cell. 79, 161–165.

Liggett, S.B., Bouvier, M., O'Dowd, B.F., Caron, M., Lefkowitz, R.J., De Blasi, A., 1989. Substitution of an extracellular cysteine in the β_2-adrenergic receptor enhances agonist promoted phosphorylation and receptor desensitization. Biochem. Biophys. Res. Commun. 165, 257–263.

Lin, S., Gether, U., Kobilka, B.K., 1996. Ligand stabilization of the β_2-adrenergic receptor: Effect of DTT on receptor conformation monitored by circular dichroism and fluorescence spectroscopy. Biochemistry 46, 14445–14451.

MacPherson, GA. 1985. Kinetic, EBDA, LIGAND, Lowry (Manual). Biosoft. Elsevier Science, Amsterdam.

Magnusson, Y., Höyer, S., Lengagne, R., Chapot, M.P., Guillet, J.G., Hjalmarson, Å., Strosberg, A.D., Hoebeke, J., 1989. Antigenic analysis of the second extracellular loop of the human β-adrenergic receptor. Clin. Exp. Immunol. 78, 42–48.

Magnusson, Y., Marullo, S., Höyer, S., Waagstein, F., Anderson, B., Vahlne, A., Guillet, J.G., Strosberg, A.D., Hjalmarson, Å., Hoebeke, J., 1990. Mapping of a functional autoimmune epitope on the β_1-adrenergic receptor in patients with idiopathic dilated cardiomyopathy. J. Clin. Invest. 86, 1658–1663.

Matsui, S., Fu, M.L.X., Katsuda, S., Hayase, M., Yamaguchi, N., Teraoka, K., Kurihara, T., Takekoshi, N., Murakami, E., Hoebeke, J., Hjalmarson, Å., 1997. Peptides derived from cardiovascular G-protein-coupled receptors induce morphological cardiomyopathic changes in immunized rabbits. J. Mol. Cell. Cardiol. 29, 641–655.

Mijares, A., Verdot, L., Peineau, N., Vray, B., Hoebeke, J., Argibay, J., 1996a. Antibodies from *T. Cruzi* infected mice recognize the second extracellular loop of the β_1-adrenergic and M2-muscarinic receptors and regulate calcium channels in isolated cardiomyocytes. Mol. Cell. Biochem. 164, 57–62.

Mijares, A., Lebesgue, D., Argibay, J., Hoebeke, J., 1996b. Anti-peptide antibodies sensitive to the active state of the β_2-adrenergic receptor. FEBS Lett. 399, 188–191.

Molina, F., Laune, D., Gougat, C., Pau, B., Granier, C., 1996. Improved performance of Spot multiple peptide synthesis. Peptide Res. 9, 151–155.

Noda, K., Saad, Y., Graham, R.M., Karnik, S., 1994. The high affinity state of the β_2-adrenergic receptor requires unique interaction between conserved and non conserved extracellular loop cysteines. J. Biol. Chem. 269, 6743–6752.

Oi, V.T., Herzenberg, L.A., 1980. Immunoglobulin producing hybrid cell lines. In: Mishell, R.B., Shiji, S.M. (Eds.), Methods In Cell. Immunol. Freeman, San Francisco, p. 351.

Sterin-Borda, L., Perez-Leiros, C., Wald, G., Cremaschi, G., Borda, E.S., 1988. Antibodies to β_1- and β_2-adrenoceptors in Chagas' disease. Clin. Exp. Immunol. 74, 349–354.

Strosberg, A.D., 1993. Structure, function, and regulation of adrenergic receptor. Protein Sci. 2, 1198–1209.

Teufel, M., Pompejus, M., Humbel, B., Friedrich, K., Fritz, H.J., 1993. Properties of bacteriorhodopsin derivatives constructed by insertion of exogenous epitope into extramembranar loops. EMBO J. 12, 3399–3408.

Tsien, R.W., Bean, B.P., Hess, P., Lansman, J.B., Nilius, B., Nowycky, M.C., 1986. Mechanism of Ca channel modulation by β-adrenergic agents and dihydropyridine calcium agonist. J. Mol. Cell. Cardiol. 18, 691–710.

Verdot, L., Bertin, B., Guilloteau, D., Strosberg, A.D., Hoebeke, J., 1995. Characterization of pharmacologically active anti-peptide antibodies directed against the first and second extracellular loops of the serotonin 5-HT_1 receptor. J. Neurochem. 65, 319–328.

Wallukat, G., Morwinski, R., Kühn, H., 1994. Modulation of the β-adrenergic response of cardiomyocytes by specific lipoxygenase products involves their incorporation into phosphatidylinositol and activation of protein kinase C. J. Biol. Chem. 269, 29055–29060.