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Subtype Specific Recognition of Human α_2C2 Adrenergic Receptor Using Monoclonal Antibodies against the Third Intracellular Loop

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Monoclonal antibodies (Mabs) against human α_2C2-adrenergic receptor (α_2C2-AR) were raised in mice and characterized. Bacterially expressed fusion protein consisting a sequence from the putative third intracellular loop (amino acids 213-343) of human α_2C2 and glutathione-S-transferase (GST) was used as antigen. Results from mass spectrometry of purified thrombin cleaved α_2C2 polypeptide suggested that the epitope region would lie near the aminoterminal end of the 3rd intracellular loop of human α_2C2-AR.

Elevation of Mabs was detected with Western blotting from mouse blood samples. Three α_2C2 specific cell clones were expanded to *in vitro* production in hollow fiber systems. The specificity of the Mabs was further determined by immunoprecipitation and immunocytochemistry.

Scatchard analysis of thrombin digested, purified, Europium-labelled antigen (amino acids 213-343 of α_2C2) revealed binding affinity constants of 0.4×10^9, 0.7×10^9 and 1.6×10^9 M^{-1} and K_ds of 2.6, 1.4 and 0.6 nM for the three Mabs 2B1, 3G3 and 7G1, respectively.

α_2-Adrenergic receptors (α_2-ARs) belong to a superfamily of receptors that transmit their signals via guanine nucleotide binding proteins (G-proteins). Using molecular cloning techniques three human α_2-AR subtypes have been identified to date. These genetically defined subtypes were designated α_2-C10, α_2-C4 and α_2-C2 according to their chromosomal location (1-3). These cloned receptors represent pharmacologically specified subtypes α_{2A}, α_{2C} and α_{2B}, which were originally detected by their different ligand binding characteristics (4).

Structurally α_2-adrenergic receptors like all other G-protein coupled receptors are predicted to have a hydrofobic core consisting of seven α-helical membrane spanning domains. Their N-terminus is located extracellularly (5). The amino acid sequences within the seven transmembrane domains (TM) are well conserved in the three α_2-AR subtypes (amino acid similarity ~75%). In contrast, some other regions of the receptors show little similarity. Subtypes α_2-C10 and α_2-C4 are glycosylated but α_2-C2 is not (6). All three subtypes have a large and relatively divergent third intracellular loop (amino acid similarity ~20%) which consists of approximately 150 amino acids. The intracellular orientation of this region has been confirmed for the α_2-C10 subtype by Vanscheeuwijck et al. (1993).

Polyclonal antibodies have been prepared against bacterially expressed fusion proteins in which amino acid sequences from the third intracellular loop of the three α_2-adrenergic subtypes have been expressed together with glutathione S-transferase (GST) (7-9). So far, the specificity of these antibodies has been sufficient with respect to their specific recognition of target protein (10-12). However, the use of polyclonal antibodies has its restrictions. The source of antibodies is limited and batch to batch variation is common. Purification of serum containing normal immunoglobulins and controlling the activity of the antibody can cause problems. Polyclonal antibodies to adrenergic receptors

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Abbreviations: AR, adrenergic receptor; TM, transmembrane; F.i.a., Freund's incomplete adjuvant; i.p., intraperitoneally; Ig, immunoglobulin; TR-IFMA, Time Resolved ImmunoFluoroMetric Assay; Mab, monoclonal antibody; RAM, rabbit antimouse; EC, extra capillary; DMEM, Dulbecco's Modified Eagle's medium; IC, intra capillary; PVDF, polyvinyldifluoride; TFA, trifluoroacetic acid; BITC, 4-[2-(D-Biotinamido)-1-ethyl] phenylamine; IFMA, ImmunoFluoroMetric Assay; PMSF, phenylmethylsulphonylfluoride; SDS, sodium dodecyl sulphate; PBS, phosphate buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; pI, isoelectric point.

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have also been raised against synthetic peptides (13-19). Some of these antibodies have shown problems with respect to their sensitivity and receptor recognition properties. To study the anatomical localization, second messenger systems and physiological roles of the three α_2-adrenergic subtypes, monoclonal antibodies (Mabs) would be of great value. Mabs were produced earlier as ascitic fluid in mice against the adrenergic receptors (20, 21). However, stable monoclonal cell lines producing uniform quality Mabs *in vitro* in serum free media provide an unlimited source of antibodies to different types of assays. This type of production markedly facilitates the purification of these Mabs. Purification of the receptors would also benefit from subtype specific antibodies.

This report describes the preparation, production and characterization of monoclonal antibodies against the third intracellular loop of the human α_2C2 adrenergic receptor. Receptor subtype specific recognition was shown by specific binding of Europium-labelled α_2C2 peptide to Mabs produced, by immunoblotting, immunoprecipitation, and immunocytochemistry. No cross-reactivity with other human α_2-ARs could be detected.

MATERIALS AND METHODS

Production of anti α_2C2 monoclonal antibodies (Mabs) in Balb/c mice. An *Escherichia coli* strain harboring the plasmid pGEX-2T containing nucleotides 637-1029 of the human α_2-adrenergic subtype C2 cDNA was a gift from Dr. Kurose. Purification of the fusion protein GST (glutathione S-transferase)-α_2C2 was done according to manufacturer's specifications (Pharmacia).

Six months old male Balb/c mice were intraperitoneally immunized with 120 μg of the purified fusion protein GST-α_2C2 mixed with Freund's complete adjuvant. The mice were boostered twice at three to four week intervals with the same amount of this fusion antigen mixed in F.i.a. Level of α_2C2 specific antibodies in mouse serum was tested five days after the first booster with antigen specific ECL Western blotting (Amersham). The final booster dose (30 μg GST-α_2C2 fusion protein in PBS) was administered i.p. after six weeks and splenocytes were fused to mouse myeloma cells SP 2/0 three days after final booster. Details of the fusion and culturing of hybridomas in Optimem 1 with glutamax 1 based medium (Gibco) were described earlier (22). Two weeks after the fusion immunoglobulin (Ig) production of hybridomas was tested with ECL Western blotting. Positive clones were recloned, retested and expanded to *in vitro* production in Tecnomouse and AcusystR^{TM} systems (Cellex Biosciences Inc.) as described later.

The subclasses of Mabs were determined using Mouse Monoclonal Antibody Isotyping Kit (Dipstick format, 19663-012, Gibco BRL).

Ig-specific time-resolved immunofluorometric screening assay (TR-IFMA) for testing of cell clones. After recloning specific α_2C2 Mab production of cell clones was screened with TR-IFMA using microtitration wells (polysorb, Nunc) coated with rabbit antimouse Ig (RAM, Z 259, Dako). The DELFIA assay buffer (Wallac) was used as dilution buffer for cell culture supernatants and for control. The serum of the splenectomized mouse was used as a positive control in preliminary screening assays.

Samples were first shaken at room temperature (RT) for 5 min on Plate Shake (Wallac) and then incubated for approximately 18 hrs at +4°C. The frames were washed four times and Europium-labelled antimouse-Ig (Boehringer Mannheim Biochemica, cat. no 1097 105) (10 ng/well in 100 μL assay buffer) was added. Frames were incubated for one hour at RT. After washing as mentioned above the enhancement solution (200 μL/well, Wallac) was added and the frames were shaken for 5 min on Plate Shake at RT. The intensity of fluorescence was measured with a fluorometer (ARCUS-1230, Wallac).

In vitro production of anti-α_2C2 specific Mabs. The cell clones α_2C2/2B1, α_2C2/3G3 and α_2C2/7G1 were cultured in cell culture bottles (Falcon) in Optimem 1 containing 10% fetal calf serum (Hyclone). Clones α_2C2/2B1, α_2C2/3G3 and α_2C2/7G1 were injected into Tecnomouse Extra Capillary (EC) side (35, 39 and 45 $\times$ 10^6 cells, respectively). Cell clone α_2C2/3G3 (150 $\times$ 10^6 cells) was injected into EC side of AcusystR. In both hollow fiber systems Dulbecco's Modified Eagle's medium (DMEM, HyClone) containing 2 mmol/L L-glutamine (21051-024, Gibco BRL) and penicillin/streptomycin 5 IU/5 μg/mL (B-3001-D, HyClone) was used in the Intra Capillary (IC) side. Mabs were harvested in serum free medium: Optimem 1 with glutamax containing Nutridoma-SP (1:100, Boehringer-Mannheim).

Purification of Mabs. *In vitro* produced α_2C2 specific Mabs were purified by ProsepA chromatography (Bioprocessing Ltd.) using 0.1 M borate buffer, 0.75 M NaCl, pH 8.5 as binding buffer and 0.1 M glycine HCl pH, 3.0 as elution buffer. The fractions were neutralized immediately after elution with 1M Tris buffer, pH 9.0. Protein concentrations were determined by absorption measurements at 280 nm. The absorption coefficient used was $\epsilon_{1\ mg/mL} = 1.34$ for IgG.

Europium-labelling of anti-mouse Ig antibody and C2-peptide. GST-α_2C2 fusion protein was digested with thrombin according to manufacturer's instructions (Bulk GST Purification Module, Pharmacia). The thrombin cleaved fusion protein was subjected to SDS-PAGE and electroblotting onto polyvinyldifluoride membrane (PVDF, ProBlott, Perkin-Elmer) according to the procedure described elsewhere (23). The bands were visualised with Coomassie Brilliant Blue and N-terminal sequence analysis of the electroblotted sample was performed with an Applied Biosystems model 477A protein sequencer equipped with a model 120A phenylthiohydantoin amino acid analyzer.

Samples for mass spectrometry (0.5 μL) were mixed with varying amounts of α-cyano-4-hydroxycinnamic acid in 60% acetonitrile/0.1% trifluoroacetic acid (TFA)/H_2O and analyzed by matrix assisted laser desorption mass spectrometer (MALDI-MS, LASERMAT, Finnigan MAT Ltd., U.K.) according to the manufacturer's instructions. Micropreparative purification of the thrombin cleaved third intracellular loop was performed with chromatography on a Vydac C18, 2.1 $\times$ 150 mm reverse phase column (The Sep/a/ra/tions Group, Hesperia, CA, USA). The detection wavelength was 220 nm.

Anti-mouse Ig and the adrenergic receptor part of the fusion protein GST-α_2C2 (131 amino acids) were labelled with 150 and 125 times molar excess of Europium-chelate according to the Delfia Eu-labelling kit (Wallac), respectively.

Biotinylation of Mabs. Biotinylation of the α_2C2 specific Mabs (0.5 g/L) was performed in 0.5 M Na-carbonate buffer, pH 9.8, containing 100-fold molar excess of 4-[2-(D-Biotinamido)-1-ethyl]-phenylamine (BITC) diluted in N,N-dimethylformamide (10 mM). Mixture was incubated at least 4 h at RT (24). The proportion of biotinylation was determined on streptavidin and RAM plates and the intensity of fluorescence was measured as mentioned above. Purified Mab was used as a standard on RAM plate.

Affinity determination of antiα_2C2 Mabs with antigen specific IFMA. Affinity of the three α_2C2-specific Mabs to α_2C2 peptide was measured using Streptavidin Microtitration Strips (Wallac). Biotinylated Mab was incubated at concentration 0.3 pmol/well in 100 μL assay buffer for one h at RT on Plate Shake. Eu-labelled polypeptide was first diluted in assay buffer between 0.04 pmol/well and 3.4 pmol/well and then incubated to equilibrium at RT on Plate Shake. The frames were washed four times as described above and the enhancement solution was added (200 μL/well). The intensity of fluorescence was measured as mentioned earlier. The affinities of Mabs were determined by Rosenthal plotting (25).

Partial purification of receptors from whole yeast cells. Chelating Sepharose Fast Flow gel (Pharmacia) was first washed with 10 volumes of acetate buffer (0.1M Na-acetate, 1M NaCl, pH 4.5). Suspension was centrifuged, supernatant discarded and the gel was resuspended in 5 volumes of 50 mM $NiCl_2$ (diluted in acetate buffer). After centrifugation the Ni^{2+} matrix was washed again with 10 volumes of acetate buffer. Storage buffer (10mM HEPES, 1M NaCl, 10% glycerol pH 7.3) was applied after washing the gel with 10 volumes of dH_2O. Coupling was carried out at RT and the Ni^{2+} coupled gel was stored at 4°C.

Solubilization of *Saccharomyces cerevisiae* yeast whole cells expressing α_2-C2 adrenergic receptor subtype (26) was done by mixing gently with Rotamixer overnight at 4°C in solubilization buffer (20 mM HEPES, 0.1 mM EDTA, 2 mM n-methylmaleimide, 1mM 1,10-phenantroline, 0.1mM phenylmethylsulphonylfluoride (PMSF), 0.5% sucrose monolaurate, pH 7.4) at a total protein concentration of 5 mg/mL. Unsolubilized protein was pelleted 100 000 × g at 4°C for 1 h and NaCl and imidazole (Sigma) were added to final concentrations 0.5 M and 5 mM, respectively. Solubilized receptors were attached to Ni^{2+} coupled Sepharose (prepared as described above) rotating overnight at 4°C. Unbound material was washed with HEPES-buffer (20 mM HEPES, 0.5 M NaCl, 5% glycerol, pH 7.5) containing 10 and 20 mM imidazole. Receptor material was eluted with the HEPES buffer containing 150 mM imidazole. This eluate was used in screening of the hybridomas and cell clones with immunoblotting. Wild type *S. cerevisiae* cells were solubilized as described above and supernatant was used as negative control in screening studies.

Preparation and solubilization of membranes from control S115 cells. Membranes from Shionogi 115 (S115) cells expressing α_2-AR subtypes C2, C4 or C10 were prepared as described elsewhere (27,28). Membranes were used as such for screening of hybridomas. Solubilization was done as described previously (29). The soluble fraction was used as receptor material in immunoprecipitation studies.

Immunoblotting. Receptor samples prepared as mentioned above were supplemented with 2.5% sodium dodecyl sulphate (SDS) and electrophoresed on 12% polyacrylamide gels in Mini-PROTEAN II Dual Slab Cell (BioRad). Proteins were transferred from gels to nitrocellulose membranes (Schleicher and Schuell) using semidry blotting technique (BioRad). For nitrocellulose membranes prepared by slot blot technique 0.5 μg sample was diluted in phosphate buffered saline (PBS) (total volume 500 μL) and applied on MINIBLOTTER 20SL (Immunetics Inc.) apparatus in horizontal direction. Non-specific binding sites were saturated for one hour at RT in a blocking buffer (50 mM Tris buffer, 5 % nonfat dry milk, 2 mM $CaCl_2$, 80 mM NaCl, 0.2 % Nonidet P-40 and 0.02 % NaN_3, pH 8.0). Blots were probed with crude serum sample (immunized mouse), different cell culture supernatants (dilution 1:8) or after recloning with purified Mabs (at concentration 0.1-2 μg/mL) on MINIBLOTTER 20SL in vertical direction. All primary antibodies were diluted in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 0.2% Tween-20, pH 7.5). The bound specific antibody was detected using the ECL Western-blotting kit (Amersham). Protein concentrations were determined according to Bradford (30).

Immunoprecipitation. The capability of the antibodies in cell culture supernatants (250 μL) to recognize solubilized receptors of recombinant S115 cells was tested in Tris-buffer (50 mM Tris-HCl, 1mM EDTA) and 0.3% digitonin (Sigma) in a total volume of 500 μL using overnight incubation at 4°C. Serum of the immunized mouse was used as a positive control (20 μL) and serum of a non-immunized mouse (20 μL) was used as a negative control. Solubilized membranes from S115 cells expressing α_2C2 were used to detect specific binding. Solubilized membranes from S115 cells expressing adrenoceptor subtypes α_2C4 and α_2C10 were used to show the possible cross-reactivity of antibodies between the different subtypes. Protein A Sepharose (Sigma, cat no P-3391) was incubated with RAM. Uncoupled RAM was washed carefully with 0.1% bovine serum albumin (BSA) in PBS. The matrix was suspended in 0.1% BSA in H_2O. Soluble receptors coupled with α_2C2 Mabs were precipitated by adding RAM-protein A Sepharose and incubating for 2 h at 4°C. Immunocomplex was sedimented by centrifugation and the supernatants were assayed for binding. Binding assays were performed with 8 nM [^{3}H]rauwolscine (DuPont NEN) as described (29). Non-specific binding was determined in the presence of 10 μM phentolamine.

Localization of the receptors in cells by immunofluorescence. S115 cell lines expressing each on the human α_2-AR subtypes were cultured on coverslips and when confluent fixed with 4 % formaldehyde. In order to assure that the intracellular receptor antigen was reached, the fixed cells were permeabilized with 0.2 % Nonidet P-40 in a blocking buffer (5 % nonfat dry milk in 50 mM Tris-HCl, pH 7.6). Mabs 2B1, 3G3 and 7G1 were diluted to different concentrations (0.5 μg/mL, 1 μg/mL, 2 μg/mL and 5 μg/mL) and applied onto the cells in the same blocking buffer. Following 45 min incubation the cells were washed 4 times with PBS and incubated 30 min with fluorescein isothiocyanate (FITC)-labelled secondary antibody $F(ab)_2$ fragment of sheep anti-mouse IgG (Sigma) diluted to 1/500 in the same buffer. After additional 4 washes with PBS the specimens were mounted using anti-fade mounting medium on microscope slides. Immunofluorescence microscopy was performed using standard epi-fluorescence optics (Olympus BHS, Olympus 100×/DPlan Apo 100UV 1.30 objective).

RESULTS

Follow-up of immunization. During immunization of the Balb/C mice, elevation of the α_2C2 specific antibodies was tested by ECL Western blotting with α_2C2-AR partially purified from *S. cerevisiae* expressing this subtype. Recombinant yeast cells expressing human α_2C2 was chosen as a host antigen for its higher α_2C2 expression compared to mammalian S115α_2C2 cell line. The expression levels in the recombinant cells were 45 pmol/mg total cellular protein in yeast-α_2C2 cells and 5 pmol/mg total cellular protein in S115-α_2C2 cells. Different dilutions of the primary antibody showed clearly the ability of the serum to recognize the α_2-C2 receptor subtype (data not shown).

Hybridoma screening. Cell culture supernatants (423/960) were tested for their α_2C2 recognition with Western blotting two weeks after the fusion was done. Recombinant human α_2-C2 receptors expressed in two different host systems were used as antigens in Western blots: recombinant S115 tumor cells (27, 28) and *S. cerevisiae* yeast cells (26). Flowthrough, 5 mM imidazole eluate from initial yeast α_2C2 purification and solubilized wild type *S. cerevisiae* yeast were used as negative controls. Membranes from S115α_2C2 cells, 100 and 150 mM imidazole eluates from yeast α_2C2 purification were used as positive controls and membranes from S115α_2C10 (1 pmol/mg total cellular protein)and S115α_2C4 (4 pmol/mg total cellular protein) cells were used to test the cross-reactivity between different subtypes. Approximately 10% of the hybridomas tested were α_2C2 positive in Western blots (44/423).

In preliminary screening ten α_2C2 antibody producing hybridomas were recloned and retested with mouse Ig specific TR-IFMA (data not shown). The cell clones were further characterized with Western blotting.

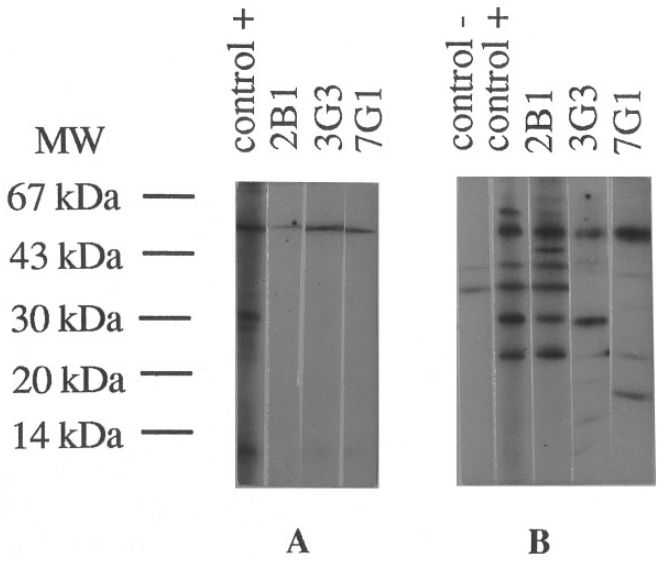

FIG. 1. Hybridomas positive in the preliminary screening were recloned and retested by immunoblotting. (A) Total membrane fraction (20 μg) was prepared from S115α_2C2 cells and resolved on 12% polyacrylamide gel. Sample preparation and immunoblotting was done as described under Materials and Methods. Supernatants from cell clone cultures were diluted 1:8 and used as primary antibodies (indicated on the top). Serum from the splenectomized mouse was used as positive control. (B) Partially purified α_2C2 (20 μg) from yeast whole cells (150 mM imidazole eluate) was run and immunoblotted under same conditions as blot A. Serum from a non-immunized mouse was used as a negative control and serum of the splenectomized mouse as positive control.

Samples were prepared as described in materials and methods and run on SDS-PAGE gels. Transferred proteins were probed with 1:5 dilutions of supernatants of the anti α_2C2 hybridomas. Using this technique one antigen (20 μg) and 20 supernatants from different hybridomas were tested on one membrane. Antigens for SDS-PAGE gels were prepared as described earlier. The ability of the antibodies to recognize α_2C2 receptors in the 49 kDa size range was confirmed by probing nitrocellulose filters with solubilized wild type *S. cerevisiae* yeast whole cells and solubilized wild type S115 membranes. Cross-reactivity with other α_2-ARs was ruled out by Western blotting nitrocellulose filters with membranes extracted from S115α_2C10 and S115α_2C4 cell lines. Serum of nonimmunized mouse was used as negative control and serum from the splenectomized mouse was used as positive control. Figure 1 shows the Western blot made with the three positive cell clones chosen for further studies.

Production and purification of the α_2C2 specific Mabs. Three cell clones α_2C2/2B1, α_2C2/3G3 and α_2C2/7G1 were chosen for large scale production and further characterization. All the cell clones produced Mabs which belonged to mouse subclass IgG_1. The production rate for specific Mabs was 7.3, 4.6 and 2.3 μg/10^6 cells/day in cell clones α_2C2/2B1, α_2C2/3G3 and α_2C2/7G1, respectively. The growth curves of three clones were determined to facilitate the adaptation to *in vitro* production system. The glucose consumption and lactate production were measured as characteristics to optimize growth of clones and production on Mabs (Table 1). When cultures reached their logarithmic states, the cells were harvested and injected into EC side of the Tecnomouse bioreactor (surface area, 400 cm^2). The Mab production level of the clones was tested with TR-IFMA. When the productivity reached a stable level, the medium in the EC side was changed into serum free media to facilitate the purification of the Mabs. The batch type culture for α_2C2/3G3 could not be optimized (based on the growth curve) and the cell clone α_2C2/3G3 was also cultured in a larger bioreactor AcusystR (surface area 0.3 m^2) as a continuous flow culture. The *in vitro* production and characteristics of the Mabs for the three clones are shown in Table 1. The results show the ability of the cell lines to produce Mabs steadily over a long culture period. The purification of the Mabs was done with protein A chromatography as described in materials and methods. SDS-PAGE and IEF gels were run to confirm the purity of the Mabs. Isoelectric points (pIs) were approximately 7.3, 6.8 and 6.9 for the three Mabs 2B2, 3G3 and 7G1, respectively, as determined from the Isoelectric focusing (IEF) gel.

Characterization and affinity determinations of purified Mabs. In order to characterize the Mabs with respect to their binding properties, the antigen part of the GST-α_2C2 fusion protein was cleaved off with thrombin. N-terminal sequence analysis of the thrombin released third intracellular loop yielded a sequence Gly-Ser-Pro-Gly-Gln-Gly-Glu-Ser-X-Gln. This was consistent with the expected thrombin cleavage site, leaving two additional amino acid residues (Gly-Ser) at the N-terminus. However, mass spectrometry of the crude cleavage product revealed multiple ions with masses below the expected calculated mass of the 213-343 loop polypeptide in addition to the full-length product. This suggested extensive degradation of the polypeptide by *E. coli* proteases. The full-length product was isolated by reverse phase chromatography, and analyzed by mass spectrometry. The determined mass, 13789, was in agreement with the calculated mass, 13676. Purified polypeptide was labelled with Europium as described under materials and methods. The labelled product was analyzed again with mass spectrometry and the result showed that each peptide molecule contained one Eu molecule (determined mass 14249).

All three Mabs 2B1, 3G3 and 7G1 were biotinylated with 100 times molar excess of BITC. The level of biotinylation was approximately 31% for all three Mabs. The affinities of the three Mabs were assessed by specific saturable Eu-labelled C2 peptide binding. Affinity constants were determined by Scatchard plots (31).

TABLE 1

Characteristics of the Three Different Mab Productions

Characteristics of the Cell Clones in Cell Culture				Production of Mabs *In Vitro*			
Hybridoma	Species	Specific production rate $\mu g/10^6$ cells/day	Glucose consumption mmol/10^6 cells/day	Lactate production nmol/10^6 cells/day	Production type	Mab mg/m^2/day	Culture days
α_2C2/2B1	mouse/mouse	7.3	0.7	1.8	batch	65*	180
α_2C2/3G3	mouse/mouse	4.6	0.7	1.0	continuous flow	487**	72
α_2C2/7G1	mouse/mouse	2.5	0.4	0.8	batch	187*	150

Note. Quantitative analysis was done with TR-IFMA. Glucose consumption was measured with BM-Test strips (Boehringer Mannheim) on the Hypocount MX glucose detector (Design Center in London). Cultures were disrupted when the total amount of Mab reached desired level.

* Surface area of the bioreactor 400 cm^2 (0.04 m^2).

** Surface area of the bioreactor 0.3 m^2.

Scatchard analysis revealed association constants (K_a) 0.4×10^9, 0.7×10^9 and 1.6×10^9 M^{-1} and dissociation constants (K_d) 2.6, 1.4 and 0.6 nM for the three Mabs 2B1, 3G3 and 7G1, respectively.

Biphasic binding to Eu-labelled C2 peptide was detected for 2B1. This nonspecificity was found later also in Western blots. The binding of the other two Mabs 3G3 and 7G1 to Eu-labelled peptide was saturable and specific.

Characterization of the Mabs with immunoprecipitation and immunocytochemistry. To further characterize the three Mabs, immunoprecipitation of receptors solubilized from stable recombinant S115 cells expressing only one cloned human α_2-AR gene product was performed. Receptor preparations were incubated with 250 μL of each cell culture supernatant. Serum of the splenectomized mouse was used as a positive and serum of a nonimmunized mouse was used as a negative control. Receptor-Mab complexes were precipitated with the addition of RAM-Protein A Sepharose. As seen in fig. 2 only one Mab precipitated α_2-C2 gene product in the used assay conditions. Although Mabs 2B1 and 3G3 did not precipitate α_2C2 subtype in these conditions, they specifically recognized α_2C2 subtype in immunocytochemical studies. All three Mabs showed clear plasma membrane staining of S115 cells transfected with the subtype C2 at concentrations from 1 μg/mL to 5 μg/mL (Fig.3). Mabs 2B1 and 3G3 also stained well at 0.5 μg/mL. None of the Mabs stained the other two α_2-AR subtypes C10 and C4 expressed in the S115 cells in the same conditions.

DISCUSSION

In this work Mabs were raised in mice against the third intracellular loop of the human α_2-adrenergic subtype C2. Approximately 10% of the hybridomas were positive from which ten major hybridomas were recloned and retested. Three of the Mabs belonging to IgG_1 subclass were selected for large scale production and further characterization. All three Mabs specifically recognized the α_2-AR subtype C2 in immunoblotting studies and in indirect immunofluorescence. No cross-reactivity was detected to other α_2-AR subtypes C10 and C4. One of the Mabs, 7G1 immunoprecipitated solubilized receptors from S115α_2C2 cell membranes in used assay conditions. Affinity constants for the three Mabs were determined by binding to purified, Europium-labelled C2 polypeptide. The integrity of the polypeptide was confirmed by N-terminal sequencing and mass spectrometry. These observations support the conclusion that these antibodies specifically recognize the α_2-C2 adrenergic receptor. The three Mabs were proven to be conformation-specific, they recognized receptors poorly from thiol reduced samples after electrophoretic separation and transfer into nitrocellulose membranes (unpublished observations).

Cell clones α_2C2/2B1, α_2C2/3G3 and α_2C2/7G1 adapted well to the hollow fiber environment. Stable, high production level of Mabs was attained for all three cell clones over a long culture period although the clone α_2C2/3G3 did not adapt to produce Mabs as batch type culture. This technique enables the production of Mabs in large scale with no batch to batch variation. The purification of Mabs was markedly facilitated by secreting them into serum free media. Until now antibodies raised to α_2ARs have been polyclonal antibodies made in rabbits and chickens (7, 9). In addition to batch to batch variation, purification of the specific polyclonal antibodies cause difficulties due to unspecific binding in various assays. Total production level of antibodies in different animal sera can not reach the level of Mab production of stable cell clones. In addition, the specificity of Mabs is well defined when using hybridoma monoclonal technology that permits the production of antibodies to single antigenic sites of proteins.

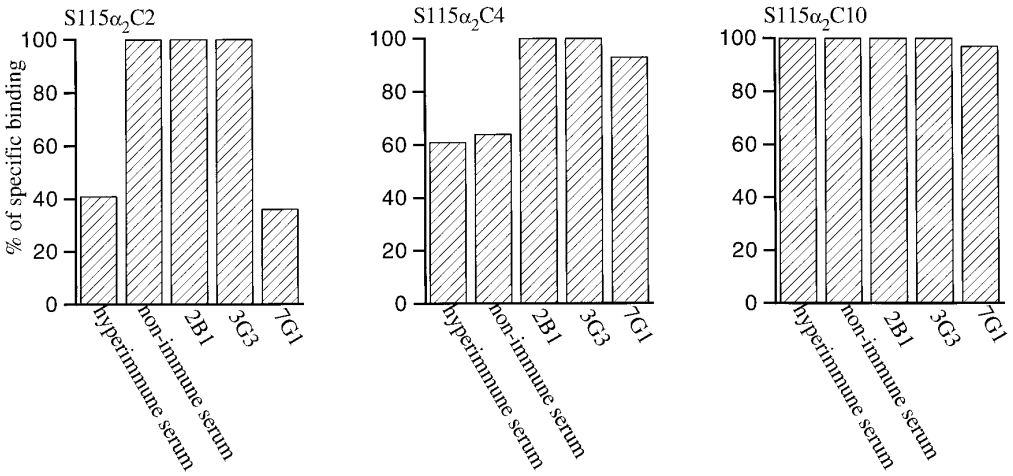

FIG. 2. Solubilized receptors prepared from the membranes of S115 cells expressing the three different subtypes were incubated with the serum of the splenectomized mouse, serum of non-immunized mouse and with the three Mabs 2B1, 3G3 and 7G1. Soluble receptors were precipitated by adding RAM-protein A complex and incubating for 2 h at 4°C. Immunocomplex was sedimented by centrifugation and supernatants were assayed for binding. Representative of three experiments with binding of 8 nM [^{3}H]rauwolscine done in duplicate. Receptor concentrations in the Mab incubations were determined to be 990 nM (S115α_2C2), 72 nM (S115α_2C4) and 220 nM (S115α_2C10).

Because the immunoprecipitation with Mabs is often limited, this possible disadvantage of Mabs was avoided by using protein A Sepharose precoupled with rabbit antimouse Ig and using this as immunosorbent.

Though epitope mapping of the three Mabs was not done, some conclusions can be drawn from mass spectrometry results of the purified α_2C2 polypeptide and SDS-PAGE gels run with purified fusion protein GST-α_2C2 (data not shown). Degradation of the fusion protein by *E. coli* proteases suggests that the epitope

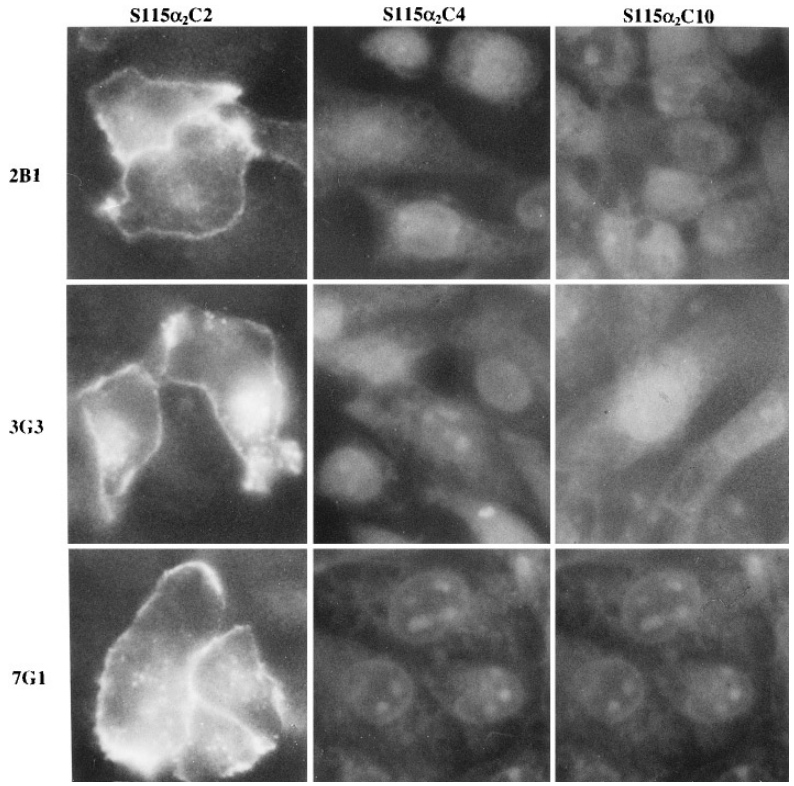

FIG. 3. Indirect immunofluorescence of α_2C2 receptors in the plasma membrane of S115 cells expressing the different subtypes of α_2-ARs.

within the receptor could lie near the beginning of the sequence (aminoacids 213-343) fused with GST.

Fusing splenocytes of hyperimmunized mouse with SP 2/0 myeloma cells has yielded cell clones producing specific, high affinity monoclonal antibodies to the human α_2C2-AR. Immunohistochemical mapping of α_2ARs is currently emerging as powerful approach to elucidate the distribution and physiological functions of receptor subtypes. For example an antiserum against the third intracellular loop of rat α_2C10 has been used to study the distribution of α_2C10 in rat brain (8). This subtype was shown to be expressed predominantly in areas involved in the control of autonomic functions like central cardiovascular control. The Mabs obtained in this study can be used equally to determine the localization of α_2C2 in human tissues. The affinity and specificity of these Mabs also enables to use them as affinity matrix in structural studies aiming to receptor purification and crystallization. We have produced large quantities of uniform quality Mabs in bioreactors and thus proved the capability of the cell clones to secrete Mabs over a long culture period. These cell lines provide a unlimited source of Mabs for different types of assays. The use of these Mabs should enlighten the *in vivo* role the α_2C2 subtype and better the understanding of the diversity of adrenergic receptors and their structure in general.

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