Clin Exp Immunol 1994; 97:146–151

Characterization of anti-peptide antibodies directed against an extracellular immunogenic epitope on the human α_1-adrenergic receptor

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(Accepted for publication 28 March 1994)

SUMMARY

A synthetic peptide corresponding to amino acids 192–218 of the second extracellular loop of the human α_{1A}-adrenergic receptor was used to raise antibodies in rabbits. Affinity-purified antibodies specifically recognized main bands with a molecular weight of about 68, 40 and 37 kD on the electrotransferred membrane proteins of rat ventricle membranes. The incubation of these antibodies with rat myocardial membranes resulted in a decrease in the number of binding sites for the specific radiolabelled α_1-antagonist prazosin. These antibodies were also able to mimic the effects of agonist stimulation as demonstrated by a positive chronotropic effect on cultured cardiomyocytes. These results constitute the first immunochemical evidence of the presence of both the A and B subtypes of the α_1-adrenergic receptor in the heart. They also confirm that the second extracellular loop of the α_1-adrenergic receptors is an immunologically and functionally important domain.

Keywords α_1-adrenergic receptor immunogenicity peptide antibody cardiomyocytes

INTRODUCTION

Catecholamine responses are mediated by two main groups of receptors, the α- and β-adrenergic receptors. The α-adrenergic receptors are divided into two subtypes (α_1 and α_2). α_1-adrenergic receptors are involved in a variety of functions, including smooth muscle contraction [1] and the regulation of hepatic glycogen metabolism [2], and they are distributed in such different tissues as the brain, lung, spleen, liver and aorta smooth muscle [3]. Upon occupancy by an agonist, they exert their effect by activating phosphatidylinositol metabolism and mobilizing intracellular Ca^{2+} [4].

The α_1-adrenergic blockers are mainly used as anti-hypertensive agents which act on the postjunctional receptors present on the arteriolar and venous vasculature beds. They can also produce an anti-hypertensive effect by inhibiting the central α_1-adrenergic receptors [5].

Until now the α_1-adrenergic receptors have been classified into two subtypes: 1A and 1B. Both the human and rat 1A receptor subtypes have been cloned and sequenced [6,7], while only the primary structure of the hamster 1B subtype is known [8].

Previous studies have unambiguously demonstrated that G-protein-coupled receptors including β-adrenergic receptors, muscarinic acetylcholine receptors and α-adrenergic receptors share structural similarities, the most prominent being the presence of seven regions of hydrophobic amino acids that are thought to span the plasma membrane linked by extracellular and intracellular loops [9]. The second extracellular loops of the β_1-adrenergic [10] and the muscarinic acetylcholine receptors [11] were shown to be the targets of an autoimmune humoral response in patients with idiopathic dilated cardiomyopathy. Some patients with myasthenia gravis possess autoantibodies against the same loop of the β_2-adrenergic receptor [12]. Moreover, peptides corresponding to the second extracellular loops of the β-adrenergic receptors [13–15] and the muscarinic receptor M2 [16,17] were shown to induce antibodies with functional effects on their cognate receptors. We thus raised polyclonal antibodies directed against the second extracellular loop of the human α_{1A}-adrenergic receptor in order immunochemically to characterize these receptors in the heart and to confirm that antibodies directed against the chosen domain interfere functionally with the receptor function.

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MATERIALS AND METHODS

Peptide

A peptide corresponding to the sequence (residues 192–218) of

the second extracellular loop of the human α_{1A}-adrenergic receptor [7] (Table 1) was synthesized using the solid-phase method of Merrifield [18] using an Applied Biosystems 431A automated peptide synthesizer (Foster City, CA). The peptide was judged to be pure by high performance liquid chromatography (HPLC) analysis on a Vydac C-18 column and by amino acid analysis on an automated amino acid analyser (Beckman Instruments, Inc., Palo Alto, CA).

Antibody production and purification

The synthetic peptide was used as an antigen to immunize two rabbits. Free peptide (1 mg) was emulsified in Freund's complete adjuvant (FCA) and injected subcutaneously at multiple points. Four weeks later, a booster injection (1 mg in Freund's incomplete adjuvant (FIA)) was given and the rabbits were bled 1 week after this injection. The immunoglobin fractions were prepared from the antisera and preimmune sera of rabbits by precipitation in 50% $(NH4)_2SO_4$, dialysed extensively against PBS (10 mM phosphate, 140 mM NaCl, pH 7·4). The immunoglobin fractions prepared from the antisera were then loaded on a Sepharose 4B cryanogen bromide (CNBr)-activated gel (Pharmacia, Sweden) to which the α_{1A}-adrenergic receptor peptide was covalently linked. After washing the immunosorbent with PBS, the specific anti-peptide antibodies were eluted with 3 M potassium thiocyanate (pH 7·4) followed by immediate extensive dialysis against PBS. The immunoglobin fractions prepared from the preimmune sera served as a control for ligand binding studies.

ELISA immunoassay

Fifty microlitres of peptide (50 μg/ml) in 100 mM Na_2CO_3 solution (pH 11·0) were coated for 1 h on Nunc (Kastrup, Denmark) microtitre plates.The wells were then saturated with PBS supplemented with 3% (w/v) of skimmed milk, 0·1% (v/v) of Tween 20 (E. Merck, Darmstadt, Germany), and 0·01% (w/v) of Thimerosal (Sigma, St Louis, MO) (PMT). The graded dilutions of rabbit antiserum or rabbit preimmune serum as a control were added to the saturated microtitre plates for 1 h. After washing the wells three times with PMT, an affinity-purified biotinylated donkey anti-rabbit IgG (H + L) antibody (Jackson Labs., Baltimore Pike, PA) (1:1000 dilution in PMT) was allowed to react for 1 h. After three more washings, the bound biotinylated antibody was detected by incubating the plates for 1 h with streptavidin-peroxidase (1 μg/ml) (Sigma) solution in PMT. This was followed by three washings in PBS and the addition of substrates H_2O_2 (2·5 mM) and 2,2′-azino-di-(ethyl-benzthiazoline) sulphonic acid (ABTS, 2 mM) (Sigma). Optical densities were read after 30 min at 405 nm in a microplate reader (Molecular Devices, Menlo Park, CA).

Immunoblotting assay

Rat cardiac membranes were used as the source of the α_1-adrenergic receptor for immunoblotting and radioligand binding studies, since heterologous systems have been previously used to demonstrate anti-receptor antibodies in human disease [7–10]. Moreover, the peptide we used corresponds to a sequence which is highly conserved in human and rat species with 93% identity [7] (Table 1).

Membrane proteins (75 μg), prepared from normal rat ventricles according to a previously described method [19], were subjected to electrophoresis on a 10% polyacrylamide gel in SDS and thereafter subjected to electrotransfer to nitrocellulose. After saturating the nitrocellulose strips with PMT buffer for 2 h at room temperature, the affinity-purified antibodies from rabbits (30 nM) were incubated at 4°C overnight with the nitrocellulose strips. After washing in PMT, the strips were incubated with a 1:500 dilution of affinity-purified biotinylated donkey anti-rabbit IgG (H + L) for 1 h at room temperature, followed by incubation with 1:500 streptavidin peroxidase solution in the same buffer. Strips were washed sequentially in PMT and in PBS containing 0·1% Triton X-100 before adding the chromogenic substrate H_2O_2-4-chloro-1-naphtol. As soon as protein bands were revealed, strips were washed extensively in water and stored at −20°C. The specificity was checked by the blocking effect of the peptide (15 μM) on protein revelation after preincubation with antibody overnight at 4°C.

Binding characteristics of α_1-adrenergic receptors

α_1-adrenergic receptors were characterized using ^{3}H-Prazosin (New England Nuclear, Boston, MA). Binding assays were carried out in 25 mM Tris buffer pH 7·4 containing 2·5 mM $MgCl_2$. Saturation-binding isotherms were obtained by incubating rat heart ventricular membranes for 1 h with varying concentrations of prazosin (0·125–4 nM) at 25°C. Specific binding was defined as that displaced by 10 μM phentolamine. The reaction was terminated by dilution with 5 ml of ice-cold buffer. The samples were then poured over GF/C 2·5 cm glass microfibre filters (Whatman International Ltd., Maidstone, UK) under reduced pressure followed by a wash with 15 ml of buffer. Filters were counted in a liquid scintillation counter (LKB, Uppsala, Sweden). The binding parameters

Table 1. Comparison of amino acid sequences of the second extracellular loops of α_1-adrenergic receptors between hamster, human and rat species

Species	Position	Sequences
α_{1A}		
Human	192–218	G-W-K-E-P-V-P-P-D-E-R-F-C-G-I-T-E-E-A-G-Y-A-V-F-S-S-V
Rat	228–254	G-W-K-E-P-V-P-P-D-E-R-F-C-G-I-T-E-E-V-G-Y-A- I-F-S-S-V
α_{1B}		
Hamster	183–209	G-W-K-E-P-A-P-N;-D-D-K-E-C-G-V-T-E-E-P-F-Y-A-L-F-S-S-L

The sequences are derived from references 6, 7 and 8. Amino acids differing from the human sequence are underlined. Conservative mutations are emphasized.

B_{max} and K_D were determined by the non-linear least squared fitting LIGAND algorithm as described previously [20].

Culture of neonatal beating cardiomyocytes

Hearts were removed aseptically from 1–2-day-old Wistar rats and cultured according to a previously described method [21]. Briefly, single cells were dissociated from the minced ventricles with a 0·2% solution of trypsin and were cultured at 37°C for 4 days as monolayers (9×10^4 cells/cm^2). On the day of the experiment, the medium was replaced and the cells were incubated at 37°C for 2 h as monolayers on a slowly moving rocker apparatus. The flasks were then transferred to the heated stage of an inverted Zeiss (Carl Zeiss, Jena, Austria) microscope, on which 10 small circular fields of the cell layer were inspected at 37°C through the perforations of a metal template. The number of beats of a selected isolated myocardial cell or a cluster of synchronously contracting cells in each of the 10 fields was counted for 15 s each time. The drugs (prazosin and phenylephrine) and antibodies were added cumulatively. As a rule, this procedure was repeated twice in different cultures to yield results representing a total of 30 cells or cell clusters. The basal rate of beating was in the range of 146·4 ± 16·2 beats/min.

RESULTS

Production and purification of anti-peptide antibodies

The two immunized rabbits produced high titres of anti-peptide antibody, as illustrated in Fig. 1. The ELISA test revealed plateau values at a dilution of 1:100 000 and, even at a dilution of 1:1000 000, a positive response remained. Affinity purification yielded 2·53 mg of antibody from 10 ml of serum, corresponding to approximately 2% of the total immunoglobulin fraction. However, an ELISA test revealed no immunoreactivity using preimmune serum (Fig. 1).

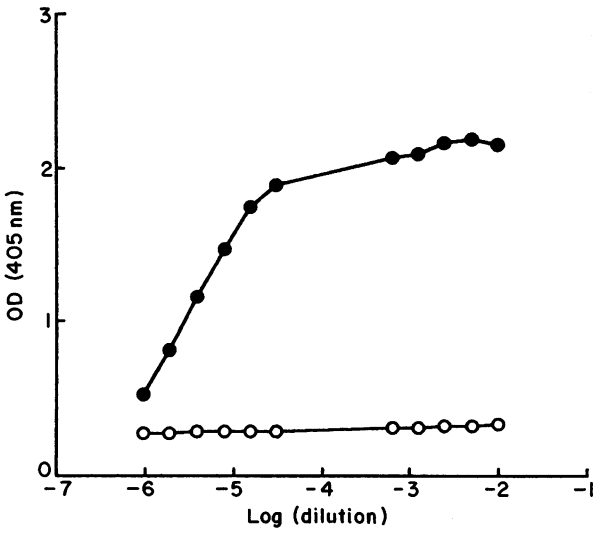

Fig. 1. Enzyme immunoassay of a rabbit anti-peptide antiserum. The absorption of the redox indicator ABTS was measured at 405 nm in a Molecular Devices ELISA reader after the revelation of the rabbit antiserum (●) or rabbit preimmune serum (○) fixed on the peptide, adsorbed on the plastic phase, with biotinylated donkey anti-rabbit IgG(H + L) antibodies and streptavidine-peroxidase. Values higher than 2 indicate the saturation of the absorption measurement.

Receptor protein recognition with the antibody

In order to ascertain that the affinity-purified antibodies were able to recognize the receptor protein, immunoblots were performed on membranes prepared from normal rat ventricles. The antibodies (30 nM) recognized, on the electrotransferred protein of rat cardiac membrane, main protein bands with a molecular weight of about 68, 40 and 37 kD. These bands were specific to the α_1-adrenergic receptors, since the staining of these bands was blocked after preincubation with the α_{1A}-adrenergic receptor peptide (15 μM) (Fig. 2).

Functional effect of antibodies on ligand binding to the α_1-adrenergic receptors

The affinity-purified antibodies (300 nM) induced a decrease in the number of binding sites (B_{max}) of ^{3}H-prazosin of 21% (58·2 ± 6·4 *versus* 73·7 ± 7·9 fmol/mg membrane protein) and an increase in K_D (0·31 ± 0·04 *versus* 0·17 ± 0·02 nM) in rat myocardial membranes (Fig. 3). Preincubation of the antibodies with the 192–218 peptide (10 μM) totally abolished the described effect. However, when IgG fraction prepared from rabbit preimmune sera was used, no effect was observed (Fig. 3).

Chronotropic effect on cultured cardiomyocytes

Spontaneously beating rat neonatal cardiomyocytes were used to study the functional effects of the antibodies. To assess the functional activity of the receptors in the cardiomyocytes, phenylephrine, an agonist of the α_1-adrenergic receptor, was used. It showed a dose-dependent (1 nM to 1 mM) stimulation of heart beating frequency, reaching a maximum at 100 μM

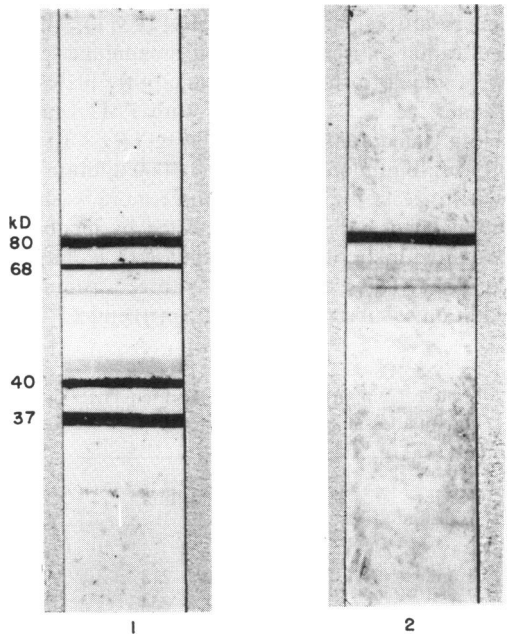

Fig. 2. Immunoblot on membranes from rat ventricles. Lane 1, proteins revealed by affinity-purified rabbit antibody (30 nM); lane 2, affinity-purified rabbit antibodies (30 nM) preincubated with the peptide 192–218 (15 μM) before incubation with the blotted proteins.

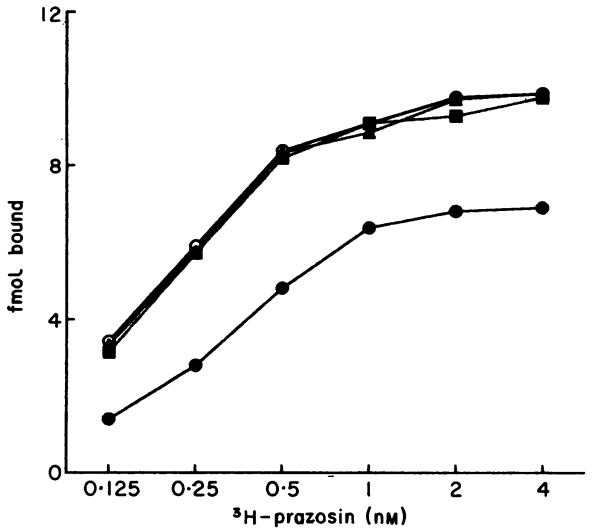

Fig. 3. Effect of antibodies on the saturation binding of cardiac α_1-adrenergic receptors. Membranes preincubated at 37°C for 1 h with affinity-purified antibodies (300 nM) (●) or without affinity-purified antibodies (○) or IgG fraction from rabbit preimmune sera (▲) were incubated with different concentrations of ^{3}H-prazosin. The blocking effect of peptide 192–218 (10 μM) (■) was obtained by preincubation with antibody at room temperature for 2 h before incubation with the membranes, and subsequent incubation with ^{3}H-prazosin.

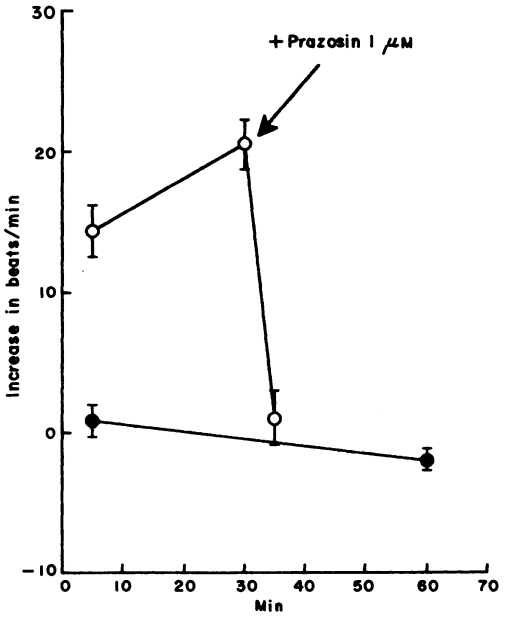

Fig. 5. Time-dependent chronotropic effect of the antibodies (418 nM) on cultured cardiomyocytes in the presence or absence of peptide 192–218 (380 nM). Results present the mean ± s.e.m. of 32–40 observations. ○, Time-dependent response in the absence of peptide. Prazosin (1 μM) was added after 30 min; ●, blocking effect of peptide on antibody activity. The antibodies were preincubated with peptide before being added to the cardiomyocytes.

(Fig. 4b). The antibody exerted a dose-dependent response (from 42 nM to 670 nM), reaching a maximum at 500 nM (Fig. 4a). This response was also time-dependent, rising slowly from 5 to 30 min, as shown in Fig. 5. This effect was totally abolished by the peptide (380 nM) used as an immunogen, thereby suggesting that it was specific to the antibody-combining site (Fig. 5). Prazosin (1 μM), an antagonist of the α_1-adrenergic receptor, was able completely to inhibit the positive chronotropic effect of the antibodies (Figs 4a and 5).

DISCUSSION

The peptide corresponding to the sequence of the second extracellular loop of the human α_{1A}-adrenergic receptor yielded antibodies of high titre (1:10 000). The amount of antibody in the serum (2% of the total immunoglobulin fraction) corresponds to the amount found after immunization with a carrier protein, thereby suggesting that the peptide contains a T cell epitope. Analysis by a predictive program [22] confirmed that the C-terminus of the peptide (residues 206–218) contained an immunogenic domain capable of recognition by mouse MHC class II molecules of the A^k and the E^d haplotype. The second extracellular loop of the α_{1A}-adrenergic receptor thus shares the immunogenic properties previously demonstrated for the β_1- [13] and β_2-adrenergic receptors [13,15].

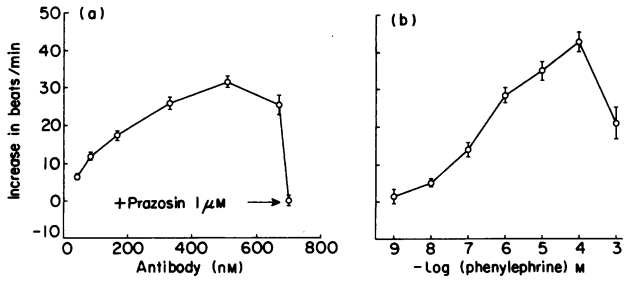

Fig. 4. Dose-dependent chronotropic effect of antibody or the α_1-adrenergic agonist, phenylephrine, on cultured cardiomyocytes. Results present the mean ± s.e.m. of 30 observations. (a) Dose-dependent chronotropic effect of antibody. The specificity of the antibody activity was determined by inhibition with prazosin (1 μM). (b) Dose-dependent chronotropic effect of phenylephrine.

The affinity-purified polyclonal anti-peptide antibodies recognized three specific protein bands of 68, 40 and 37 kD, respectively. Photoaffinity studies of mammalian α_1-adrenergic receptors revealed two main protein species of 80–85 kD and 52–59 kD, respectively, according to the tissues used. Combining these results with the molecular weights derived from cDNA-derived amino acid sequences, the 80–85 kD protein would correspond to the α_{1B} subtype, which has 515 amino acids but four glycosylation sites at the N-terminus [8], while the 52–59 kD protein would correspond to the unglycosylated α_{1A} subtype, which is 548 amino acids long [7]. The lower molecular weight fragments revealed by the anti-peptide antibodies probably correspond to proteolytic fragments of both subtypes.

The affinity-labelled rat liver α_1-adrenergic receptor was shown to have a molecular weight of 43 kD by Guellaen *et al.* [23]. This molecular weight corresponds to the 40-kD protein revealed by the anti-peptide antibodies. It was later

shown that the receptor identified by Guellaen *et al.* was probably a proteolytic fragment of a protein of 58 kD [3]. This molecular weight corresponds to that predicted for the unglycosylated α_{1A} subtype. Since no protease inhibitors were used during the heart membrane preparation, we suggest that the 40-kD protein corresponds to a fragment of this receptor subtype.

The 68-kD protein revealed by the anti-peptide antibodies would then correspond to the proteolytic fragment obtained from the affinity-labelled α_{1B}-receptor subtype. Indeed, it has been shown that, in the absence of protease inhibitors, the 85-kD receptor of rabbit aorta vascular smooth muscle (corresponding to the α_{1B} subtype) produces a broad band at 65 kD [3]. In view of the high homologies between the second extracellular loops of both subtypes (Table 1), it is not surprising that the anti-peptide antibodies cross-react with the α_{1B} subtype. The narrowness of the revealed protein band suggests that the proteolytic fragment no longer contains the glycosylated N-terminus. Finally, the 37 kD could correspond to an unidentified proteolytic fragment which was also affinity-labelled in the spleen [3].

The interpretation of the immunoblot experiments is strengthened by the pharmacological properties of the anti-peptide antibodies on the α_1-adrenergic receptor. Ligand-binding studies of the affinity-purified antibodies revealed the ability *in vitro* to decrease the maximum amount of binding sites without any significant change in antagonist affinity compared with IgG fraction from rabbit preimmune serum (Fig. 3). This suggests a non-competitive inhibition, similar to that found for anti-peptide antibodies raised against the second extracellular loop of the β_1- [14] and β_2-adrenergic receptors [15]. These results thus confirm that the anti-peptide antibodies recognize not only receptor proteolytic fragments on immunoblots but also the native radioligand-binding receptor, and that these effects are not due to naturally occurring antibodies to cell surface receptors.

Functional antibodies directed against the second extracellular loop of the β_1-adrenergic [14] and the muscarinic acetylcholine M2 receptor [17] were shown to behave as agonists on neonatal rat heart myocytes *in vitro*. Since phenylephrine, an α_1-adrenergic specific agonist, was shown to have a positive chronotropic effect in the same experimental system [21,24], the effect of the anti-peptide antibodies was tested. The anti-peptide antibodies described here were able to induce a positive chronotropic effect in neonatal rat cardiomyocytes similar to that of the α_1-adrenergic agonist phenylephrine. The specificity of this effect was shown by its blocking with the immunogenic peptide or with the α_1-adrenergic receptor antagonist, prazosin.

The results presented here thus support the hypothesis that the second extracellular loop of the G-protein-coupled receptors is an immunologically and functionally important domain. As we have previously shown that this domain gives rise to an autoimmune response, resulting in autoantibodies which stimulate the cognate receptors, we suggest that autoantibodies against the α_1-adrenergic receptor may be found in certain diseases without known etiology. In view of the therapeutic efficacy of α_1-adrenergic antagonists in certain cases of essential hypertension, we are currently screening the sera of patients with this disease for recognition of the peptide, used here as an immunogen.

ACKNOWLEDGMENTS

The authors would like to thank Klas-Göran Sjögren and Eva Angwald for their technical assistance. This work was supported by the Swedish Heart and Lung Foundation (project 21021, to M.L.X.F.) and Biotechnocentre (to J.H.). M.L.X.F. has been awarded a postdoctoral scholarship by the Swedish Heart and Lung Foundation.

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