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Antigenic analysis of the second extra-cellular loop of the human beta-adrenergic receptors

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SUMMARY

Polyclonal antibodies were raised in rabbits by immunization with free peptides corresponding to positions 197–222 of the human β1-adrenergic receptor (β1 peptide) and the corresponding sequence (172–197) of the human β2-adrenergic receptor (β2 peptide). While the β2 peptide yielded antibodies that cross-reacted with the β1 peptide, the antibodies against the β1 peptide did not cross-react with the β2 sequence. Cross-reactivity of the anti-β2 peptide antibodies and the selectivity of the anti-β1 peptide antibodies were also revealed in the recognition by immunoblots of the β1- and β2-adrenergic receptors of different species or of the receptor gene products expressed in a bacterial vector. These antibodies could be used immunohistochemically to visualize the β-adrenergic receptors on rabbit heart. The anti-β2 peptide antibodies did not show any functional effect on the β-adrenergic receptors; the anti-β1 peptide antibodies were able to displace agonist affinity to higher values. Recognition of truncated peptides by the anti-β1 and anti-β2 peptide antibodies suggested that the cross-reaction of the anti-β2 peptide antibodies was due to the recognition of a common epitope on the C-terminal part of the peptides. The anti-β1 peptide antibodies recognized the N-terminal part of the peptide better than the C-terminal part. These results suggest that the second extracellular loop postulated in the structure of the human β-adrenergic receptor contains the T and B cell epitopes necessary for induction of an immune response. The selectivity and the functional properties of the antibodies raised against that loop in the β1 adrenergic receptor could have relevance in induction of auto antibodies in certain cardiomyopathic conditions.

Keywords β-adrenergic receptors epitope mapping immunogenicity peptides

INTRODUCTION

The existence of pathological states induced by an auto-immune response against membrane receptors is well documented in the case of myasthenia gravis (Almon, Andrew & Appel, 1974) and insulin resistant diabetes (Grünfeld *et al.*, 1980). Auto antibodies against the β-adrenergic receptors have been claimed to be involved in idiopathic cardiomyopathy (Limas, Goldenberg & Limas, 1989) the cardiomyopathic complications of Chagas' disease (Borda *et al.*, 1984) and in atopic allergic disorders (Venter, Fraser & Harrison, 1980). The successful cloning and sequencing of the human β1-adrenergic receptor (Frielle *et al.*, 1987) and of the human β2-adrenergic receptor (Kobilka *et al.*, 1987; Emorine *et al.*, 1987) allows a structural approach to identify a main immunogenic region on the β-adrenergic receptors similar to that found for the nicotinic acetylcholine receptor (Tzartos & Lindstrom, 1980).

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Apart from the putative extra-cellular N-terminal sequence of the β-adrenergic receptors, the only other extracellular and thus accessible loop that could be a candidate for an autoimmune reaction consists of the second extracellular loop. Its length and hydrophility are compatible with the potential presence of T and B cell epitopes (Fig. 1). Moreover, one of the cysteine residues in that loop has been reported to be important in the regulation of agonist affinity towards the receptor (Dixon *et al.*, 1987a). For these reasons, it was decided to synthesize peptides corresponding to the second extra-cellular loop of the human β1- and β2-adrenergic receptors and to study the antigenicity of these peptides by immunising rabbits with the free peptides emulsified in Freund's complete adjuvant. Using truncated peptides, a first approach was made to localize the functionally important epitopes found on the human β1-adrenergic receptor.

MATERIALS AND METHODS

Peptides

Immunogenic peptides H26R and H26Q (Fig. 2) were synthe-

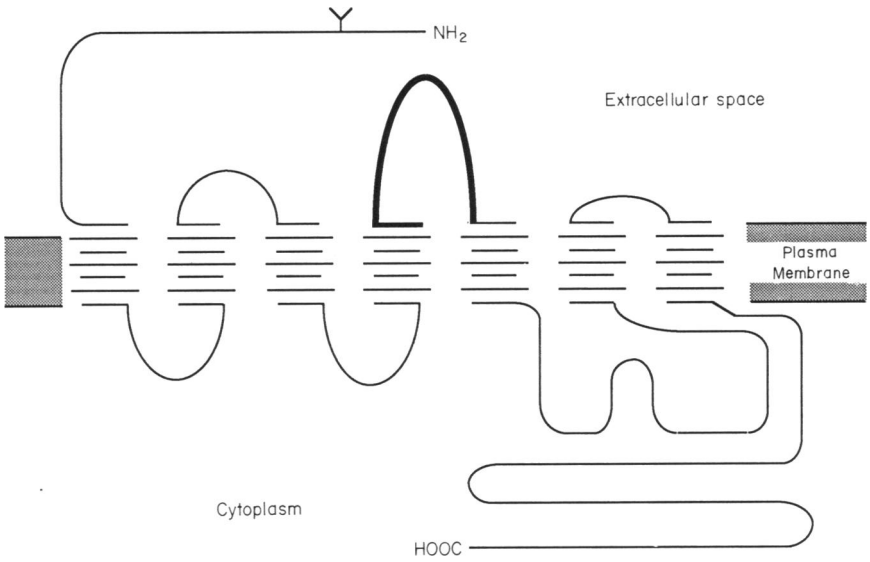

Fig. 1. Schematic representation of the bi-dimensional structure of the β-adrenergic receptors. The second extracellular loop whose sequence was used is marked in bold.

β1- receptor peptides (corresponding to position 197–222)	
H26R	H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-(C)
6H16D	H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D
4Y13R	Y-N-D-P-K-C-C-D-F-V-T-N-R
β2-receptor peptides (corresponding to position 172–197)	
H26Q	H-W-Y-R-A-T-H-Q-E-A-I-N-C- Y-A-N-E-T-C-C-D-F-F-T-N-Q-(C)
H16N	H-W-Y-R-A-T-H-Q-E-A-I-N-C- Y-A-N
Y13Q	Y-A-N-E-T-C-C-D-F-F-T-N-Q

Fig. 2. Peptides used for immunization and antigenic analysis. The peptides used for immunization were H26R and H26Q, respectively, in which a cysteine residue was added at the C-terminus and in which the cysteine residues were reduced. The peptides used for the enzyme immunoassays did not contain a C-terminal cysteine and the other cysteines were blocked with an acetamidomethyl group.

sized by the solid-phase method of Merrifield (1963) using an automated Applied Biosystems (Foster City, CA) 430A peptide synthesizer; they were desalted on a Biorad (Richmond, VA) P6 desalting grade molecular sieve using Na_2CO_3 0·1M as eluent, and stored in the same solvent at −20°C until use. The truncated peptides were synthesized by the same method using an automated Applied Biosystems 431A peptide synthesiser. They were desalted on a sephadex G25 column using CH_3COOH 1% as eluent and lyophilized until use. Composition of the purified peptides was tested in an automated Beckman (Palo Alto, CA) aminoacid analyser.

Immunization

Two rabbits were immunized with the peptide H26R and with the peptide H26Q, respectively, given intradermally after emulsification of 1 mg in Freund's complete adjuvant. Four weeks after the primary injection, a booster injection of the same amount of peptide was given subcutaneously in Freund's incomplete adjuvant. Bleedings started on week 5 and continued weekly for 7 weeks. Booster injections were given on weeks 9 and 14. Rabbits were bled to death 15 weeks after the first injection.

Antibodies

The enzyme immunoassays were performed using the antisera. Western blotting, immunohistochemical visualization of receptors and ligand binding experiments were performed with affinity-purified antibodies. The affinity column was made up by coupling the peptides to CNBr activated sepharose (Pharmacia, Uppsala, Sweden) by the standard procedure. After purification of the gamma-globulin fraction by 35% $(NH_4)_2SO_4$ saturation, the anti-peptide antibodies were adsorbed on the affinity column in phosphate-buffered saline (PBS) (phosphate 10 mM, pH 7·4), eluted with a 0·2 M glycine buffer, pH 2·6, and immediately after elution dialysed extensively against PBS.

The purified anti-peptide antibodies were stored at −20°C until use.

Enzyme immunoassay

Nunc (Roskilde, Denmark) microtitre plates were coated with solutions of 50 μg/ml peptide in Na_2CO_3 0·1M, mercaptoethanol 1% for 1 h at room temperature. After saturation of the wells with PBS supplemented with 3% skimmed milk powder and 0·1% Tween-20, antiserum dilutions in the same buffer were

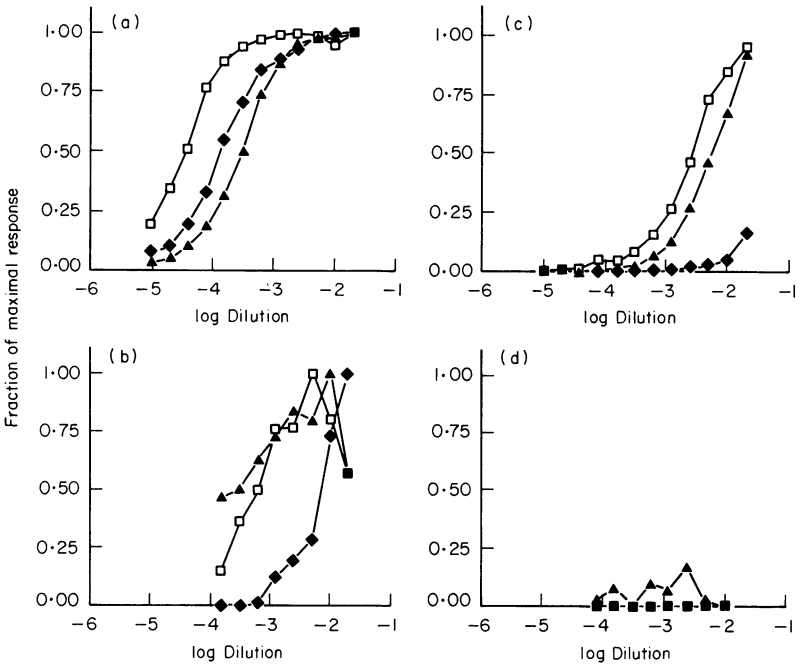

Fig. 3. Enzyme immunoassays of the anti-peptide sera on the peptides shown in Fig. 2. The assays were performed on the free peptides directly adsorbed on the microwell titre plates by subsequent incubation with the anti-peptide antisera, a biotinylated donkey anti-rabbit IgG antibody and streptavidin–peroxidase. For comparative reasons, the titres were normalized for the maximal response to each peptide. Maximal OD at 405 nm for H26R was 1·50; for 4Y13R, 1·62; for 6H16D, 1·20; for H26Q, 0·80; for Y13Q0·79; and for H16N, 0·83. Panels a and c show, respectively, the titration curves for the anti-β1 peptide and anti-β2 peptide antisera on H26R (□), 6H16D (◆) and 4Y13R (▲). Panels b and d show, respectively, the titration curves for the anti-β2 peptide and anti-β1 peptide antisera on H26Q (□), H16N (◆) and Y13Q (▲).

allowed to adsorb on the peptides for 1 h at room temperature. The rabbit antibodies were revealed by successive 1-h room temperature incubations with donkey biotinylated anti-rabbit IgG antibodies (Immuno Research Laboratories, West Grove, PA) diluted 1:1000 in saturation buffer and a streptavidin–peroxydase conjugate (Sigma Chemical Co., St Louis, MO) at a 1:800 dilution in the same buffer. After washing the wells in PBS, H_2O_2–ABTS (Boehringer Mannheim, Marburg, FRG) substrate was added and OD was read at 405 nm in a Titertek (Flow, Irvine, UK) ELISA reader.

Immunoblots

Membrane proteins from A431 cells and C6 glioma cells were actively transferred to nitrocellulose (Towbin, Staehelin & Gordon, 1979) after electrophoresis in a 10% polyacrylamide sodium dodecyl sulphate (SDS) gel under reducing conditions. After saturation in PBS supplemented with 5% skimmed milk powder and 0·1% Tween-20, nitrocellulose strips were incubated with 5 μg/ml purified anti-peptide antibodies in the saturation buffer overnight at 4°C. The rabbit antibodies were revealed by successive 1-h room temperature incubations with donkey biotinylated anti-rabbit IgG antibodies (Immuno Research Laboratories) diluted 1:1000 in saturation buffer and a streptavidin–peroxydase conjugate (Sigma) at a 1:500 dilution in the same buffer. The strips were washed in PBS supplemented with 0·1% Triton X-100 before incubation with the H_2O_2–4-chloro-1-naphtol substrate. As soon as blue bands appeared, strips were washed in distilled water and photographed. A similar procedure was used to test the specificity of the anti-peptide antibodies on membrane proteins from *Escherichia coli*, expressing the human β1- and β2-adrenergic receptors (Guillet *et al.*, manuscript submitted) but the overnight incubation with the anti-peptide antibodies were preceded by a 2-h preincubation at room temperature in the presence of 50 μg/ml peptide.

Visualization of β-adrenergic receptors on rabbit hearts

Affinity-purified anti-peptide antibodies were used on formalin-fixed and paraffin-embedded tissue specimens from a normal non-immunized rabbit. Paraffin sections (5-μm thick) were deparaffinated in xylene and digested for 20 min with 0·05% trypsin (Sigma) at 37°C. Endogenous peroxidase activity was reduced by treating the sections with 0·5% H_2O_2 in methanol. The sections were incubated for 18 h at 4°C with affinity-purified anti-peptide antibodies at a concentration of 10 μg/ml.

The sections were subsequently incubated with biotinylated donkey anti-rabbit IgG antibodies (Immuno Research Laboratories) at a dilution of 1:500 and with a 1:500 dilution of a streptavidine–peroxydase conjugate (Sigma).

To visualize the immune complexes, the sections were treated with 0·05% diaminobenzidine hydrochloride and 0·02% H_2O_2 in tris-buffered saline for 2 min. Nuclear staining was performed with Mayer's solution at a dilution 1:2 for 3 min. Controls were designed by omitting the anti-peptide antibodies in the first step.

Ligand binding experiments on the β-adrenergic receptors

Cell membranes were prepared from A431 cells and C6 rat glioma cells as previously described (Jacobsson, Bergh &

Hjalmarson, 1983) but with omission of 1 mM dithiothreitol in the homogenizing buffer and of washing in 0·6 M KCl. Membranes were incubated overnight with affinity-purified anti-peptide antibodies (15 μg/ml). Binding experiments were performed by incubation with 30 pM (-)3-^{125}I-iodocyanopindolol (Amersham) in the presence of increasing concentrations of the agonist isoproterenol at 37°C for 45 min. After incubation, the membranes were filtered and washed on glass filters (Whatman GF/F, Maidstone, UK) and the residual radioactivity was counted. Blanks were set up in the presence of 10 μM propranolol as cold antagonist.

RESULTS

Enzyme immunoassay

Immunization of rabbits with the free peptides resulted in the production of anti-peptide antibodies at titres between 1:1000 and 1:10 000. The response against the peptide derived from the β1-adrenergic receptor showed a lower reactivity towards the C-terminal and N-terminal parts (Fig. 3a) with a higher titre for the N-terminal than for the C-terminal sequence. No cross-reactivity could be observed for the peptide derived from the β2-adrenergic receptor (Fig. 3d). The anti-β2 peptide antibodies showed a selective preference for the C-terminal sequence of the β2-adrenergic receptor derived peptide and only a minority of the polyclonal antibodies was directed against the N-terminal part of the sequence (Fig. 3b). In contrast to the anti-β1 peptide antibody response, the anti-β2 peptide antibodies cross-reacted with the β1 peptide; this cross-reactivity was completely localized in the C-terminal part of the peptide (Fig. 3c). The immunodominant epitope of the β2-peptide was thus localized mainly at the C-terminal part of the peptide, which also carried the common epitope responsible for the cross-reactivity of the anti-β2 peptide antibodies for the β1 peptide. The immunodominant epitope of the β1-peptide was less well localized, although the absence of cross-reactivity with the β2 peptide and the lower titre for the C-terminal part of the peptide suggested that it was localized on the N-terminal part of the peptide.

Immunoblots

To ascertain that the anti-peptide antibodies were able to recognize the receptor proteins, immunoblots were performed on membrane proteins from human epidermoid A431 cells having a high density of β2-adrenergic receptors (Delavier-Klutchko, Hoebeke & Strosberg, 1984) and C6 rat glioma cells carrying mainly β1-adrenergic receptors (Homburger *et al.*, 1981) (Fig. 4a, b).

The anti-β2 peptide antibodies recognized on the electrotransferred proteins of both cell membranes a protein band with a mol. wt of 60 to 65 kD, corresponding to the mol. wt of the β2-adrenergic receptors as determined by photoaffinity labelling (Stiles *et al.*, 1983) or by monoclonal anti-receptor antibodies (Kaveri *et al.*, 1987).

The specificity of the immunoblots was assessed by competition of the immunogenic peptides for the β-adrenergic receptor proteins expressed in *E. coli* transfected with a plasmid in which the receptor genes were embedded in a fusion protein (Guillet *et al.*, manuscript submitted). The anti-β2 peptide antibodies recognized a protein of 82 kD corresponding to the fusion protein and degradation products of, respectively, 65, 60, 32 and 30 kD which also disappeared by competition with the homologous peptide. The anti-β1 peptide antibodies revealed a major protein of 65 kD and one at 55 kD which completely disappeared by competition with the homologous peptide. Interestingly, the intact fusion protein could not be detected by the anti-β1 peptide antibodies.

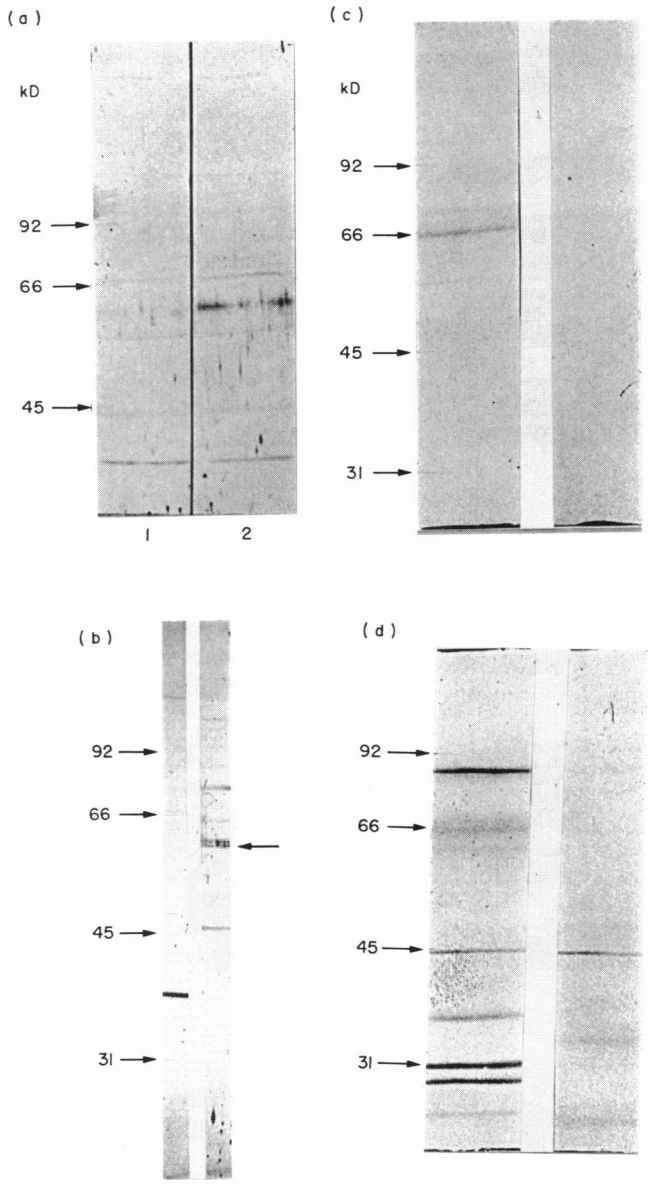

Fig. 4. Immunoblots of the affinity-purified antibodies on membrane proteins of C6 glioma cells (a) and A431 cells (b) with, respectively, anti-β1 peptide antibodies (1) and anti-β2 peptide antibodies (2). Immunoblots of the affinity-purified antibodies on membrane proteins from *E. coli*, expressing the β-adrenergic receptors. Panel c shows the immunoblot on membrane proteins from β1-adrenergic receptor expressing bacteria with anti-β1 peptide antibodies in the absence (left) and presence (right) of 50 μg/ml H26R peptide. Panel d shows the immunoblot on membrane proteins from β2-adrenergic receptor expressing bacteria with anti-β2 peptide antibodies in the absence (left) and presence (right) of 50 μg/ml H26Q peptide.

Immunohistochemical visualization of the β-adrenergic receptors on heart tissue

The possibility of the anti-peptide antibodies recognizing the β-

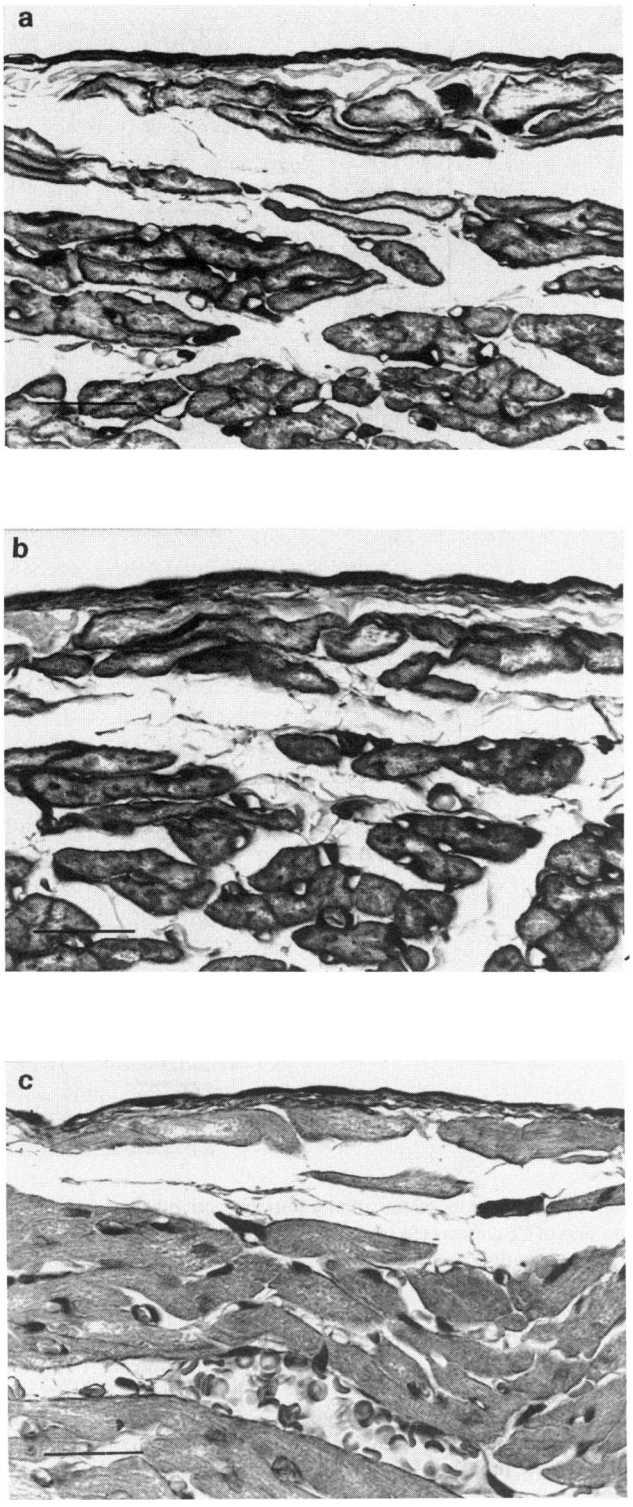

Fig. 5. Visualization of β-adrenergic receptors on rabbit heart tissue. Purified anti-peptide antibodies were used for immunohistochemical visualization of β-adrenergic receptors using biotinylated donkey anti-rabbit IgG antibodies and streptavidin–peroxydase conjugate. Fluffy membrane staining is due to the deposit of diaminobenzidine product of peroxydase activity. a, staining with anti-β1 peptide antibodies; b, staining with anti-β2 peptide antibodies; and c, control—in which the anti-peptide antibodies were omitted. Bar is 50 μm.

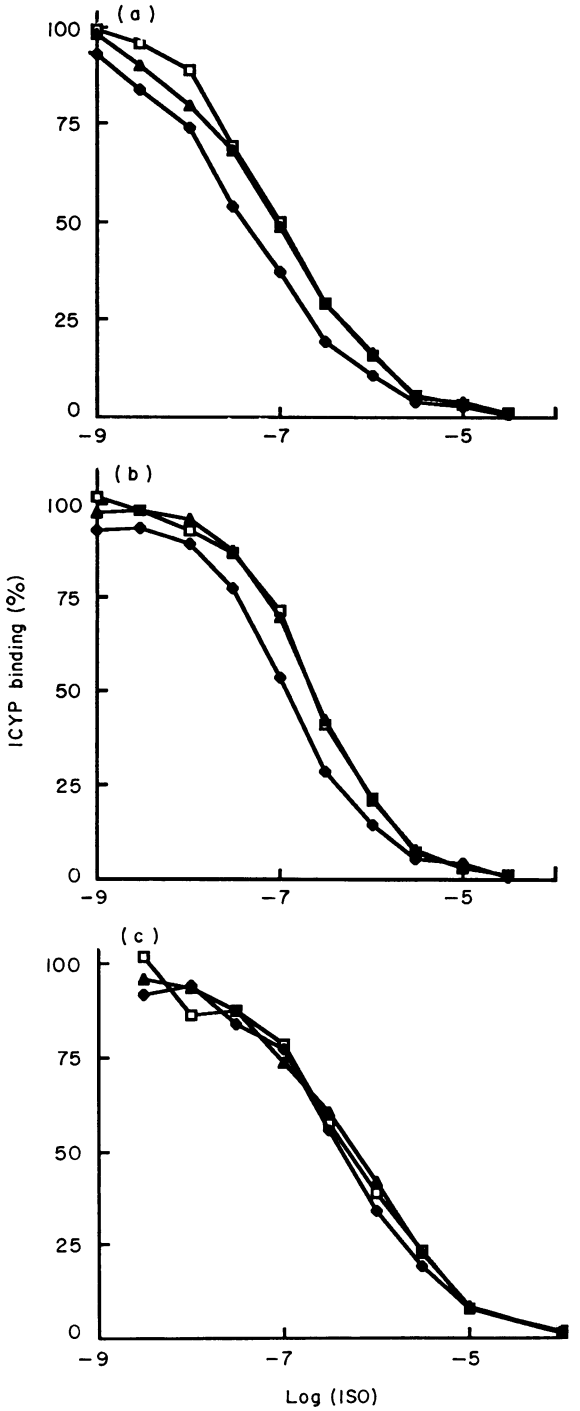

Fig. 6. Effect of the purified anti-peptide antibodies on agonist affinity of the β-adrenergic receptors. (a) Competition curves of the agonist isoproterenol for (-3)-^{125}I-iodocyanopindolol binding on membranes of C6 glioma cells, carrying 85% β1-adrenergic receptors. Curves are shown for control membranes (□), membranes preincubated with anti-β1 peptide antibodies (◆) and membranes incubated with anti-β2 peptide antibodies (▲). (b) Competition curves of the agonist isoproterenol for (-3)-^{125}I-iodocyanopindolol binding on membranes of C6 glioma cells in the presence of 1 mM of the non-hydrolysable GTP analog 5′-guanylylimidophosphate. □, ◆, and ▲ as in (a). (C) Competition curves of the agonist isoproterenol for (-3)-^{125}I-iodocyanopindolol binding on membranes of A431 cells carrying β2-adrenergic receptors. □, ◆, and ▲ as in (a).

adrenergic receptors *in situ* was assessed, using as target tissue the heart of a non-immunized rabbit. As can be seen in Fig. 5, both anti-β1 and anti-β2 peptide antibodies gave by immunoperoxidase staining a positive response that was mainly localized at the membrane level of the sarcolemma but also, to some extent in the endocardium and the smooth muscle cells of the vascular walls. The visualization with the anti-β1 peptide antibodies seemed to be more distinctly membrane bound than that with anti-β2 peptide antibodies (Fig. 5).

Functional properties of the anti-peptide antibodies

To test the potential effects of the anti-peptide antibodies on the ligand binding activity of the β-adrenergic receptors, membranes of A431 and C6 glioma cells were pre-incubated with the affinity-purified anti-peptide antibodies and subsequently tested for binding with the radiolabelled β-adrenergic antagonist (-)3-^{125}I-iodocyanopindolol. No inhibition of the binding of this radioligand on β2- or β1-adrenergic receptors could be shown (data not shown). When the affinity for the agonist isoproterenol was tested in competition experiments with the radioligand, the anti-β1 peptide antibodies induced a shift to higher affinities of the β1-adrenergic receptor of rat C6 glioma cells (Fig. 6a). They had no effect on the β2-adrenergic receptors on A431 cells (Figure 6c). The increase in affinity was only partially reversed by 1 mM of the non-hydrolysable guanosinetriphosphate analog, 5′-guanylylimidophosphate (Fig. 6b).

The anti-β2 peptide antibodies did not have any effect on the β2- or on the β1-adrenergic receptors (Fig. 6).

DISCUSSION

Peptides derived from the sequences of the β-adrenergic receptors have been widely used to raise antibodies for verifying the sequence of proteolytic fragments (Yarden *et al.*, 1986; Dixon *et al.* 1986), for studying the expression of genes in transfected cells (Dixon *et al.*, 1987b) or for topological studies (Rubenstein, Wong & Ross, 1987; Zemcik & Strader, 1988). In all these studies, peptides were coupled to carrier proteins so that no information was available about the intrinsic immunogenicity of these sequences. The purpose of this study was to investigate the immunogenicity of receptor sequences which could be involved in induction of auto-immune anti-receptor antibodies, and therefore we decided to use free peptides as immunogen. The only two regions of the β-adrenergic receptors whose accessibility into the extracellular space and whose lengths are compatible with the potential existence of T and B cell epitopes (necessary for the induction of an auto-immune response) are the N-terminal region and the second extracellular loop (Fig. 1). Since of those two regions only the second extracellular loop has been claimed to be important in agonist affinity regulation (Dixon *et al.*, 1987a), we decided to study the immunogenicity of the sequences corresponding to that domain. The high homology of these sequences in the human β1- and β2-adrenergic receptors also made them interesting tools to investigate the possible existence of a discriminative immune response against the two receptors.

The results described here clearly demonstrated that both for β1- and β2- adrenergic receptors, the sequences corresponding to the second extracellular loop had an intrinsic immunogenic potential. They resulted in the production of anti-peptide antibodies able to recognize the whole receptor protein by immunoblotting and the native membrane receptor in heart tissues. Recognition by the anti-peptide antibodies of β-adrenergic receptors of different species (human, rat and rabbit) suggested that the B epitopes should be well conserved.

The sequences of the β2-adrenergic receptors of four different species (Dixon *et al.*, 1986; Emorine *et al.*, 1987; Gocayne *et al.*, 1987; Allen *et al.*, 1988) show that the second extracellular loop was highly conserved (85% conservation). The equivalent sequences for the β1-adrenergic receptors are only available for humans (Frielle *et al.*, 1987) and turkey (Yarden *et al.*, 1986) and are more divergent than the loop in the β2-adrenergic receptor.

While the second extracellular loop of the human β2-adrenergic receptor appears to have an immunodominant B-epitope localized on the C-terminal part of the sequence and is responsible for the cross-reactivity with the β1-adrenergic receptor, the immune response against the same structure on the β1-adrenergic receptor is not so easily interpreted. An immunodominant epitope may be localized on the N-terminal part and does not show any cross-reactivity with the β2-adrenergic receptor (Fig. 3).

The monospecificity of the anti-β1 peptide antibodies is also shown to be of functional relevance, since only these antibodies were able to increase agonist affinity for the β1-adrenergic receptor. Although more studies are needed to give a mechanistic interpretation of the observed results, the only partial reversibility of the effect by 5′-guanylylimidophosphate suggests that the epitope is involved in the conformational changes associated with the coupling of the receptor to the signal transducing G_s protein.

The existence of a β1-adrenergic receptor specific immune response could be of value in the study of cardiac diseases for which an autoimmune response against the β-adrenergic receptors is reported (Borda *et al.*, 1984; Limas *et al.*, 1988). While in these studies the effect on the β-adrenergic receptors and especially antagonist binding could be mediated through alloimmune IgG (Sterin-Borda *et al.*, 1984; Solano *et al.*, 1988), the anti-β1 peptide antibodies had different properties: they did not inhibit antagonist binding on the receptor but only influenced the agonist affinity.

Using the β1 peptide as antigen, sera from patients suggested to contain antibodies interfering with the β-adrenergic receptor could be screened to detect antibodies whose effects may be hidden by the presence of alloimmune antibodies. Monoclonal antibodies could be used to define exactly the epitope recognized on the second extracellular loop of the β1-adrenergic receptor and to study the possibilities of inducing cardiomyopathic changes by passive transfer. Antibodies monospecific for the β1-adrenergic receptors constitute invaluable tools to study the distribution and fate of these receptors on cells and tissues which express both β-adrenergic receptor subtypes.

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