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Production and Characterization of the Polyclonal Anti-peptide Antibody for β-adrenergic Receptor

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Abstract—The analysis of membrane receptors for hormones and neurotransmitters has progressed considerably by pharmacological and biochemical means and more recently through the use of specific antibodies. Two kinds of antibodies could be produced, one is from synthetic peptides and the other from proteins such as purified receptor. Anti-peptide antibodies gave some advantages; epitope is evident and also receptor purification in quantity is not prerequisite. It can be also applied to the study of receptor structure-activity relationship. The purpose of the present study was 1) to produce and characterize a polyclonal antibody against a synthetic β2-adrenergic receptor peptide(Phe-Gly-Asn-Phe-Trp-Cys-Phe-Trp-Thr-Ser-Ile-Asp-Val-Leu) and 2) to determine the effects of this antibody on the β-adrenergic receptor ligand interaction. The peptide sequence contains an amino acid residue such as Asp-113 which was identified as one of important component for receptor-ligand interaction in site-directed mutagenesis studies. Production of antibody was performed by immunization of rabbits through popliteal lymph node with the peptide coupled with Keyhole Limpet Hemocyanin (KLH). The titer of antibody against this peptide was 1 : 1000. The anti-peptide antibody was able to detect a 67 kDa protein band in western blot corresponding to the molecular weight of the β-adrenergic receptor in partially purified receptor fraction derived from guinea pig lung. The antisera inhibited the specific binding of [^{3}H]dihydroalprenolol to β-adrenergic receptor in a concentration-dependent manner. The results from this study suggest that the peptide sequence selected in the present study is important for the receptor ligand interaction.

Keywords □ β-adrenergic receptor, a synthetic peptide, [^{3}H]dihydroalprenolol

The analysis of membrane receptors for hormones and neurotransmitters has progressed considerably by pharmacological(Dohlman *et al.*, 1991) and biochemical means(Ostrowski *et al.*, 1992) and more recently through the use of specific antibodies(Bahouth *et al.*, 1991). Antibodies against β-adrenergic receptor have been used for the wide range of studies including receptor structure(Moxham *et al.*, 1986, 1988), topography (Theveniau *et al.*, 1989; Wang *et al.*, 1989a) and distribution in the brain(Wanaka *et al.*, 1989) and cell(Wang *et al.*, 1989b).

Several means could be employed to obtain useful antibodies against receptor molecule. Generally, tissue contents of receptor molecules are very low, so the production of antibodies directed against affinity-purified β-adrenergic receptors appeared to be very difficult in the viewpoint of experimental hardwork, cost and time largely because of the limited availability of purified receptor in a large enough amount to induce desired immunologic reaction in the experimental animals. And also amino acid sequence homology among mammalian β-adrenergic receptor is so extensive that usually the antibody titer is extremely low.

In some cases, synthetic peptide have been used as an antigen to produce useful antibodies against receptor molecule. The two most important advantages of anti-peptide antibodies are that they can be prepared immediately after determining the amino acid sequence of a protein and that particular regions of a protein can be targeted specifically for antibody production. Occasionally by the careful examination of the amino acid sequence of the protein, specific antibodies

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against highly conserved protein can be easily prepared and these antibodies can be used as a useful biochemical tools for the investigation of functions mediated by the selected region in a given protein. By investigating the effect of antibodies directed against discrete region of the receptor to the function such as receptor-ligand interaction and G-protein coupling, it is possible to know the specific function mediated by that region without possible conformational change of receptor, in addition to obtaining useful biochemicl tool to pursuit the receptor. Even though this kind of approach for the determination of receptor structure-function relationship had been successfully applied to the receptor regions for the coupling of muscarinic and β-adrenergic receptors to their cognate G-proteins (Cheung *et al.*, 1991; Palm *et al.*, 1990), there is no clear demonstration of the importance of specific receptor regions for the receptor-ligand interaction except site-directed mutagenesis study.

The hydrophobic regions of the β-adrenergic receptor which might form transmembrane helices are thought to be important in receptor ligand binding(Dixon *et al.*, 1987). The prime candidates of amino acids which were important for ligand binding were considered as several hydrophilic groups in transmembrane region. Interestingly, substitution at Asp-113 to Asn or Glu dramatically affect the affinity of both agonists and antagonists for the β-adrenergic receptor(Strader *et al.*, 1988). Asp-113 was thought to act as the counterion for the cationic amino group of the adrenergic ligands.

The purpose of the present study was to produce and characterize a polyclonal antibody against a synthetic peptide whose region seems to be important for the β2-adrenergic receptor ligand interaction, and also to determine the effect of this antibody to the receptor ligand interaction.

Materials and Methods

Materials

(−)-[^{3}H]Dihydroalprenolol([^{3}H]DHA, 76 Ci/mmol) was purchased from Amersham(Aylesbury, UK). Digitonin was obtained from Gallard-Schlessinger(Carle Place, NY). Whatman GF/B filters were purchased from Fisher(Pittsburgh, PA). Sepharose CL-4B was obtained from Pharmacia(Uppsala, Sweden). Electrophoresis reagents were obtained from Bethesda Research Laboratories(Gaithersburg, MD). Freund's Adjuvants and other immunochemical reagents were purchased from Pierce(Rockford, IL). All other reagents were obtained from Sigma Chemicals(St. Louis, MO) and were of the highest commercially available grade.

Solubilization and Partial purification of the Guinea Pig β-Adrenergic Receptor

Guinea pig lung crude membrane particulate fraction was pre-solubilized with 5 m*l* of buffer containing 0.25 % digitonin, 10 mM Tris, 100 mM NaCl, 5 mM EDTA, 10 mM benzamidine, 10 U/m*l* trasylol, leupeptin(10 μg /m*l*), pH 7.2. After 40 min incubation on ice, the suspension was centrifuged at 48,000×g, 40 min to yield the pre-solubilized particulate preparation. The resulting precipitate derived from 1 g lung, wet weight, was again solubilized by the homogenation and centrifugation procedure described above with 5 m*l* of buffer containing 1.5% digitonin. The solubilized preparations typically contained 1.7 pmol [^{3}H]DHA binding/mg protein. In some instances, soluble β-adrenergic receptor was partially purified by alprenolol-Sepharose CL-4B affinity chromatography to a specific activity of 1,100 pmol [^{3}H]DHA binding activity/mg protein.

Production of Antibody

The peptide used to construct the immunogen was the residues(102-115) of the human β-adrenergic receptor(Dixon *et al.*, 1986). The peptide sequence was Phe-Gly-Asn-Phe-Trp-Cys-Phe-Trp-Thr-Ser-Ile-Asp-Val-Leu. The peptide was synthesized by the Laboratory of Protein Chemistry, KIST Genetic Engineering Institute and was purified by HPLC. The peptide was conjugated to keyhole limpet hemocyanin(KLH) by glutaraldehyde method and the peptide-KLH conjugate was dialyzed overnight against excess PBS. New Zealand White rabbits(8 weeks of age) were immunized with an emulsion of 1 m*l* of the peptide-KLH or peptide alone in an equal volume of Complete Freund's Adjuvant(CFA). An aliquot of emulsion(0.1 m*l*) was introduced to popliteal lymph node which was exposed from popliteal cavity of right hind leg and the rest of emulsion(0.9 m*l*) was administered via intramuscular, subcutaneous and intradermal route. Booster immunization were performed at 4 weeks interval with Incomplete Freund's Adjuvant(IFA). One week after the final injection, rabbits were bled from the ear artery and serum was prepared. Serum was stored in aliquots at −70℃.

Dot Blot Assay

Antigens(Peptide-KLH, KLH, Bovine Serum Albumin (BSA), Peptide-BSA and Peptide, 4 μg each) were blotted on nitrocellulose membrane. Strips were blocked with 5% nonfat dried milk, 0.2% Tween 20 in PBS. Sera diluted 1 : 200 in blocking solution were added to the strips and incubated for 1 hour. After rinsing,

strips were incubated with peroxidase-labeled goat anti-rabbit IgG antibody diluted 1 : 500 in blocking solution. After rinsing, strips were then visualized with substrate solution(0.6 mg/ml 3,3'-diaminobenzidine in 50 mM Tris, pH 7.6, 0.05%(v/v) H_2O_2).

Enzyme-linked Immunosorbent Assay(ELISA)

Peptide-BSA congugates(50 ng) diluted in PBS was added to wells of microtiter assay plate(Falcon) and allowed to adsorb for 2 hour at room temperature. After Blocking, the wells were then incubated 2 hour at room temperature with 50 μl of serum which was serially diluted in blocking solution. The wells were then probed with 50 μl of peroxidase-labeled goat anti-rabbit IgG antibody diluted 1 : 500 in blocking solution. Each well was developed with 50 μl of soluble substrate solution(0.01 mg Tetramethylbenzidine/ml 0.1 M sodium acetate, pH 6.0, 0.01% H_2O_2) and the absorbance was read at 405 nm.

Immunoblot Assay

After SDS-polyacrylamide gel electrophoresis of soluble or partially purified β-adrenergic receptor, electrophoretic transfer of the proteins was performed in methanol/glycine/Tris buffer as described(Towbin *et al.*, 1979). After blocking, nitrocellulose strips were incubated for 2 hour at room temperature with sera diluted 1 : 200 in blocking solution. After washing, they were incubated with peroxidase-labeled goat anti-rabbit IgG diluted 1 : 500 in blocking solution for 2 hour at room temperature. After washing, strips were then visualized with diaminobenzidine substrate solution.

Effect of Peptide and Anti-Peptide Antibodies on Ligand Binding to Soluble β-Adrenergic Receptors

Serial dilutions of BSA coupled peptide or antiserum were preincubated with soluble β2-adrenergic receptors from guinea pig lung for 60 min at 23℃ in 0.5 ml aliquot. The mixture typically contained 0.1 ml of guinea pig lung soluble fraction, 0.1 ml of serial dilutions of antiserum, 0.1% digitonin, 10 mM Tris, 100 mM NaCl, 5 mM EDTA, pH 7.2. To determine receptor ligand binding 2 nM of [^{3}H]DHA was added and incubated for 30 min at 23℃. Receptor ligand complex was separated from free ligand by vacuum filtration through 0.5% polyethylenimine(PEI) pretreated Whatman GF/B filters. Nonspecific binding was defined as that binding not inhibited by 10 μM *dl*-propranolol.

Determination of Amount of Protein

Content of protein was estimated in all samples by the procedure of Bradford(1976), using bovine serum albumin as the standard.

Results

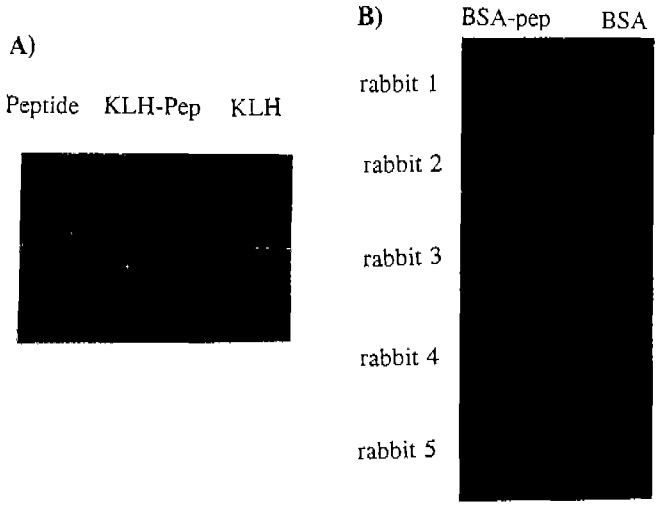

Fig. 1. Dot blot assay of antiserum. a) 4 μg of peptide, KLH-peptide and KLH were blotted to nitrocellulose membrane filter. After blocking with 5% blotto/0.2% Tween 20/PBS, antisera diluted 1 : 200 were incubated with strips for 1 hr. The antiserum incubated with strip B is from rabbit injected peptide alone. After washing, strips were incubated with peroxidase-labeled goat anti-rabbit IgG antibody diluted 1 : 500. Strips were visualized by 3,3'-diaminobenzidine. b) 4 μg of BSA-peptide and BSA were used as antigens. Row 1~3 were incubated with antiserum obtained from rabbit injected with KLH-peptide, and row 4, 5 were incubated with antiserum obtained from rabbit injected with peptide alone.

Preparation and Characterization of Polyclonal Antibodies

As shown in Fig. 1, all three rabbits(1~3) which were immunized with an emulsion of the KLH coupled peptide in Complete Freund's Adjuvant(CFA) produced desired anti-peptide antibody, whereas two rabbits injected with only the peptide emulsified in CFA did not produce specific antibody(Fig. 1a). All the rabbits immunized with KLH-peptide produced antibodies against carrier protein(KLH) itself. To confirm the production of anti-peptide antibody, the peptide was coupled to another protein, Bovine Serum Albumin(BSA). As shown in Figure 1b, rabbit 1~3 showed immunoreactivity only to the BSA-peptide but not to unconjugated BSA, showing production of specific anti-peptide antibody. The immune sera produced in this study showed relatively high titer for the peptide antigen as shown in Fig. 2. In Fig. 3, the antibody preparation revealed visible band on the western blot using guinea pig lung soluble fraction(100 μg protein/lane) and recognized major band of MW 67,000 on the western blot of partially purified β-adrenergic receptor(2 pmol/lane) which was identical to the molecular weight of purified β-adrenergic receptor determined by silver staining fo-

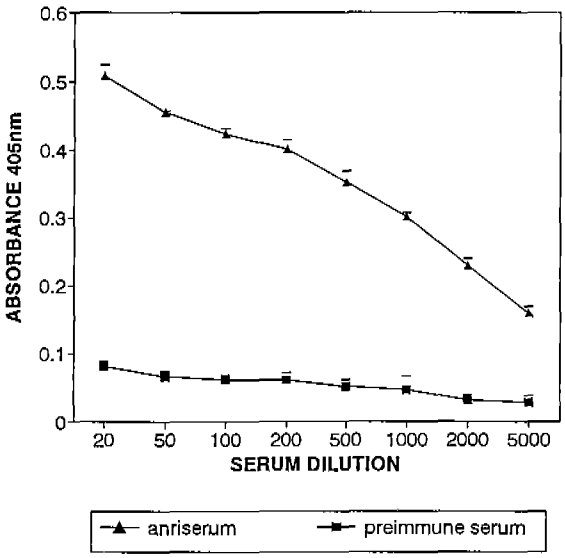

Fig. 2. Enzyme Linked Immunosorbent Assay(ELISA) of anti-peptide antisera. Assays were performed as described under Materials and Methods. The results are expressed as absorbance at 405 nm. The data shown are the means± S. E. of triplicate determinations from four separate experiments. Vertical line represents standard error.

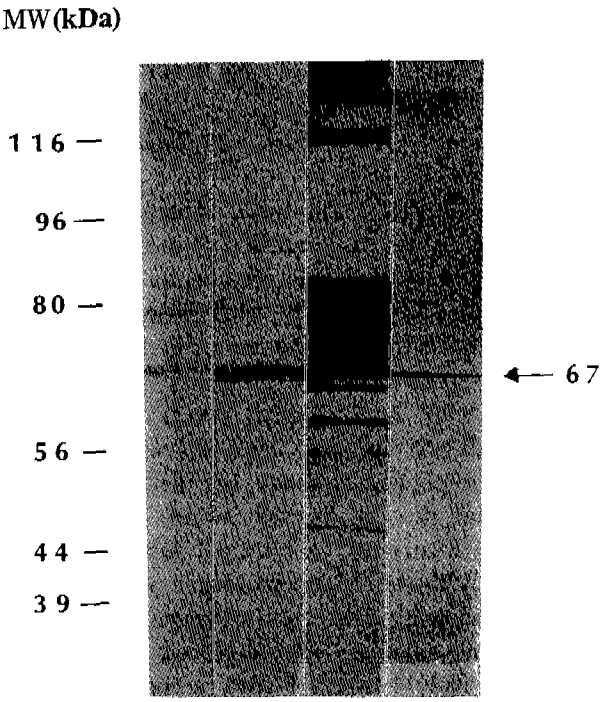

Fig. 3. Immunoblot assay of anti-peptide antiserum. 100 μg protein of soluble receptor preparation(lane 1) and 2 pmol of partially purified β-adrenergic receptor(lane 2) were electrophoresed and transferred to nitrocellulose membrane filter. Strips were incubated with antipeptide antiserum diluted 1 : 200. Second antibody was diluted 1 : 500. Control preimmune serum did not show any immunoreactive band. Lane 3 and 4 was partially purified guinea pig lung β-adrenergic receptor revealed by silver staining.

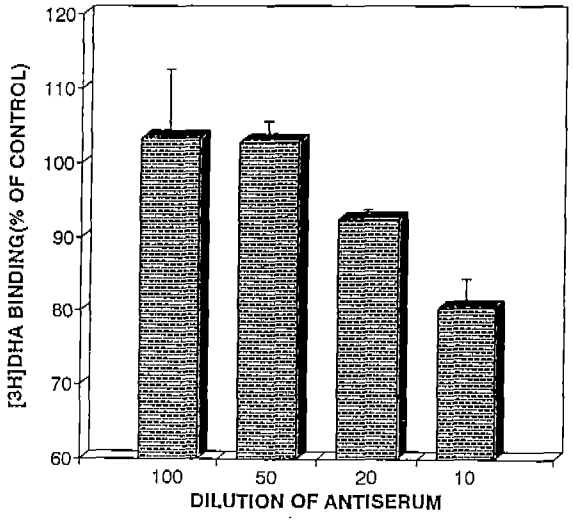

Fig. 4. Inhibition of [^{3}H]DHA binding to the soluble β2-adrenergic receptor from guinea pig lung by anti-peptide antiserum. Soluble receptor preparations were preincubated with serial dilutions of antiserum, and [^{3}H]DHA-specific binding to β-adrenergic receptors was determined with 2 nM[^{3}H] DHA(equal to K_d vaule). Bound ligand was separated by vacuum filtration using 0.3% polyethylenimine pretreated GF/B filters. The data shown are the means± S.E.M. of triplicate determinations from three separate experiments.

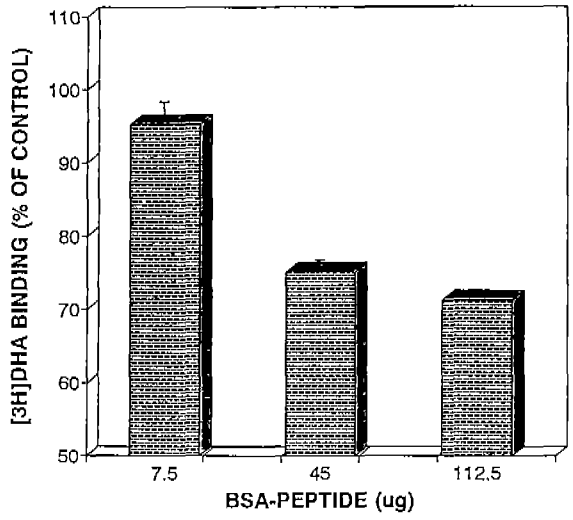

Fig. 5. Inhibition of [^{3}H]DHA binding to the soluble β2-adrenergic receptor from guinea pig lung by BSA-peptide. Soluble receptor preparations were preincubated with serial dilutions of BSA-peptide, and [^{3}H]DHA-specific binding to β-adrenergic receptors was determined with 2 nM [^{3}H]DHA (equal to K_d value). Bound and free form of radioligand was separated by vacuum filtration using 0.3% polyethylenimine pretreated GF/B filters. The data shown are the means± S.E. M. of triplicate determinations from three separate experiments.

llowing SDS-PAGE. In preparation solubilized with digitonin the antibody immunoprecipitated β-adrenergic

receptor ligand binding activity about 25% of control at 1 : 5 and 1 : 10 dilutions with the aid of excess goat anti-rabbit IgG(data not shown).

Effect of Peptide and Anti-peptide Antibody on Ligand Binding to Soluble β2-Adrenergic Receptor

To investigate the effect of antiserum on the β-adrenergic receptor ligand interaction, serial dilution of antiserum was added to the receptor [^{3}H]DHA binding mixture and the specific radioligand binding was determined. As shown in Fig. 4, antiserum containing anti-peptide antibody to β2-adrenergic receptors inhibited the specific binding of [^{3}H]DHA to guinea pig lung soluble β2-adrenergic receptors in a concentration-dependent manner. In case of 1 : 10 dilution, the antiserum inhibited the specific binding of [^{3}H]DHA about 20%. In addition to this, as shown in Fig. 5, synthetic peptide coupled to BSA also inhibited the specific [^{3}H]DHA binding to guinea pig lung soluble β2-adrenergic receptor in a concentration-dependent manner up to 30%, confirming the possible role of this particular sequence of the peptide as a constituent of ligand binding site in β-adrenergic receptor.

Discussion

In the present study the anti-peptide antibodies were produced only in rabbits injected with KLH coupled peptide, but not in rabbits injected with free peptide alone demonstrating the absence of immunogenecity of small molecular weight synthetic peptide. Several investigators have reported the production of anti-peptide antibodies against β-adrenergic receptors. Usually antibody titer to their cognate peptide was not so high indicating the poor immunogenicity of synthetic β-adrenergic peptide. To increase the antibody titer with minimal amount of antigen, we adopted the strategy of direct immunization into lymphoid organ(Sigel *et al.*, 1983). The antibody titer which was determined by indirect Enzyme-linked Immunosorbent assay (ELISA) was about 1 : 1000, which was higher compared with the results of other investigators(Weiss *et al.*, 1987; Wang *et al.*, 1989a).

The immune sera obtained in this study contained antibodies against carrier protein KLH itself, which complicated the screening procedures. Therefore, we used a second carrier protein, Bovine Serum Albumin, which showed no immunologic reaction with all the sera tested in this study to check that the antibodies are specific for the peptide itself and not the carrier protein. Immune sera displayed positive signal only to the BSA-peptide showing the production of specific anti-peptide antibodies. This screening procedure seems to apply other cases when the carrier protein coupled peptides are used as antigens(Harlow and Lane, 1988). The antibody fraction recognized a 67 kDa protein band in the western blot of the soluble and partially purified β-adrenergic receptor from guinea pig lung. The major problem encountered during preparation of anti-peptide antibodies is whether they can recognize the native protein. In most cases they recognize denatured proteins in western blot analysis, but it can not be predicted from these data whether they will recognize native proteins also. In the present study, the anti-peptide antibodies recognized native β-adrenergic receptor as evidenced by the concentration dependent indirect immunoprecipitation of β2-adrenergic receptor which was solubilized with digitonin from the guinea pig lung.

The peptide sequence selected in this study contained Aspartate residue which is the 113 rd amino acid in β2-adrenergic receptor and reported very important in receptor-ligand binding. In concord with the result obtained by site directed mutagenesis assay(Strader *et al.*, 1987), the antibodies produced in this study specifically inhibited 2-adrenergic receptor-[^{3}H]DHA binding in a concentration dependent manner. In addition to this, peptide coupled to BSA inhibited [^{3}H]DHA binding in a concentration dependent manner which again demonstrated the participation of the residues contained in the synthetic peptide in the receptor-ligand binding.

The maximum extent of inhibition of receptor-ligand binding achieved in this study with the addition of anti-peptide antibodies was about 20%. Several possibilities can explain this partial inhibition. First, the affinity of anti-peptide antibodies to the native β-adrenergic receptor did not seem to be so high to provide 100% inhibition. In indirect immunoprecipitation experiment, only 27% of receptor activity was precipitated. Other investigators also reported incomplete immunoprecipitation similar to the result obtained in the present study(Fraser and Venter, 1980; Theveniau *et al.*, 1989). The β-adrenergic receptors contain seven transmembrane helices, and its complex 3-dimensional structure could prevent access of antibody molecule to the discrete epitope. Second, there have been some reports regarding the participation of some other residues in the receptor molecule for the receptor-ligand binding as well as Asp113 which act as a counterion for the cationic amine group of ligands(Strader *et al.*, 1987, 1989). Pharmacological studies have suggested that disulfide bonds and perhaps a rearrangement of

disulfide bonds may be important for ligand binding and for agonist activation of the β2-adrenergic receptor (Moxham and Malbon, 1985; Pederson and Ross, 19 85). Some investigators suggested the role of conserved hydrogen bond donors and aromatic residues in ligand binding. Several serine residues with side chains that could donate a hydrogen bond to the ligand and several aromatic residues that could interact with catechol ring by hydrophobic interaction were reported to display a profound effect on isoproterenol binding when substituted by other amino acids(Strader *et al.*, 1989). Therefore it may be plausible to think that occupation of one of these residues by specific antibodies may inhibit the receptor ligand binding only in part. Finally, the effect of detergent, digitonin, used in this study on the antibody function did not defined rigorously. To minimize the possible deleterious effect of digitonin on the antibody function, it is necessary to use purified receptor fraction which contain only minimal concentration of digitonin. The exact nature of this inhibitory effect of peptide and/or anti-peptide antibodies could be resolved by the scatchard analysis of saturation binding assay, and also by employing the powerful technique of monoclonal antibody production and epitope mapping. Production of monoclonal antibodies is currently under way.

In summary, we produced anti-peptide antibodies against β2-adrenergic receptor using β2-adrenergic synthetic peptide. The high affinity and specificity of the antibodies seems to be satisfactory to the requirement for the specific antibodies as a useful biochemical tool for the study of β-adrenergic receptor. And also the anti-peptide antibodies produced in this study inhibited receptor-ligand binding which demonstrated that this particular peptide sequence has an important function on receptor ligand interaction.

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