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Immunological Studies of β-Adrenergic Receptors

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Two types for antibodies have been raised against the β-adrenergic receptor: either by injection of highly purified receptor from turkey erythrocytes or by injection of anticatecholamine ligand antibodies, and induction of anti-idiotypic antibodies. Our data illustrate the interactions of the β-adrenergic receptor with these polyclonal antibodies. Preliminary results with monoclonal antibodies are also described. The redistribution of β-receptors on intact cells is visualized by the use of fluorescent antibodies. Immunoprecipitation of radioiodinated receptor by the antireceptor antibodies yields a single major 60,000 MW component.

Key words: antibodies, β-adrenergic receptors

The β-adrenergic catecholamine hormone receptor–adenylate cyclase complex constitutes a particularly favorable system to analyze the different stages of hormonal stimulation of cAMP production which involve the recognition of the hormone by the plasma membrane receptor, the transmembrane signaling through a regulatory GTP binding G/F protein, and the activation of adenylate cyclase [1–3].

Both the receptor and the regulatory protein have been studied by biochemical methods involving the binding of radioactive drugs, hormones, or nucleotides, and covalent affinity labeling by specific ligands. Classical purification techniques and affinity chromatography have led to the purification and extensive characterization of these multimeric proteins [4–8].

In recent years our group has also pursued the analysis of the β-adrenergic receptor by immunological methods [9, 10]. Two approaches have been utilized to prepare antireceptor antibodies: 1) affinity-purified receptor, obtained by chromatography over an alprenolol-agarose gel was used to immunize mice. The resulting poly- and monoclonal antibodies were used to analyze the receptor in a variety of conditions—on whole cells, in membranes, and in solution [9]. 2) Alternatively, antireceptor antibodies were obtained using antihormone antibodies as primary immunogens and analyzing the antiantibodies [10].

We report some of our recent data obtained with these types of antireceptor antibodies, and will compare these with similar results reported by other groups.

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MATERIALS AND METHODS

Immunological Methods

Preparation of the antibodies. Antibodies were raised in mice by subcutaneous injections of 1–2 pmol of affinity-purified receptor from turkey erythrocytes [11] in the presence of complete Freund adjuvant. Mice were boostered at 3 week intervals in the presence of incomplete Freund adjuvant and bled every week to prepare polyclonal antibodies [9]. For the preparation of monoclonal antibodies, mice were intravenously injected 3–6 weeks after the first booster and sacrificed 4 days after the booster immunizations; mouse spleen cells were then fused with mouse myeloma NS1, in the presence of 40% polyethylene glycol 1500, and supernates from the wells containing hybridomas were assayed 2–4 weeks later for anti-β-adrenergic receptor antibodies.

Anti-idiotypic antibodies were raised in rabbits by intramuscular injections of antialprenolol IgG from an allotype-matched rabbit [10].

Immunofluorescence technique. Turkey erythrocytes or S49 murine lymphomas were washed with MEM or Hanks medium and 10^6 cells incubated 1 hr at 30°C with each antiserum (30 μl) or normal serum as control. Cells were washed three times with 0.1 ml medium and then incubated for 30 min at 0°C with goat antimouse antibodies, labeled with fluorescein. After three washes, cells were screened for fluorescence.

Enzyme-linked immunosorbent assay (ELISA). Turkey erythrocytes or other β-receptor possessing or nonpossessing cells were coated on 96-well flat-bottom plates with poly-L-lysine and then fixed with glutaraldehyde, as described [12]. Cells were incubated with supernates from surviving hybridomas (100 μl). After washings, rabbit antimouse IgG antibodies were added, before a further incubation with sheep antirabbit-IgG antibodies, coupled to β-galactosidase. After addition of the enzyme substrate, absorbance in each well was read at 408 nm in an ELISA scanner (Artek).

Biochemical Methods

Binding assays. Turkey erythrocyte membranes were preincubated with dilutions of antibodies for 1 hr at 30°C in 75 mM Tris-HCl buffer, pH 7.4/25 mM $MgCl_2$, then tested for their ability to bind (−)-[^{3}H]DHA during a 10-min incubation at 30°C and finally filtered on glass-fiber filters [8].

Adenylate cyclase assay. Turkey erythrocyte membranes were preincubated with dilutions of antibodies for 90 min at 0°C and then tested for adenylate cyclase activity by conversion of (α-^{32}P)ATP into cyclic[^{32}P]AMP. Assay conditions were the same as described [10].

Affinity purification of the receptor. β-Adrenergic receptor was solubilized with digitonin from turkey erythrocytes membranes and then purified on an alprenolol-Sepharose affinity gel [4, 5].

RESULTS

Polyclonal Antibodies Obtained Through Immunization With Receptor

Affinity-purified β-adrenergic receptor isolated from turkey erythrocyte plasma membranes contains two major 60- and 70-kilodalton and three smaller 30-, 33-, and 42-kilodalton polypeptide chains which can be well characterized in two-dimensional

polyacrylamide gel electrophoresis [11]. This material was injected in microgram quantities in mice. Polyclonal antibodies were shown to hemagglutinate β-receptors bearing erythrocytes (turkey, duck), whereas no agglutination was shown with red blood cells devoid of such receptors (rabbit, human). Immunofluorescence studies confirmed these results and permitted to extend these conclusions to other types of cells regardless of the β1 or β2 character of the adrenergic receptors. Thus the fluorescent antibodies stained turkey erythrocytes (β1), murine mastocytoma (β2), and wild-type S49 murine lymphoma cells (β2), whereas only background labeling was observed with a variant of these lymphoma cells, βd, severely deficient in β2 receptors [13].

Binding of the antibodies was also used to characterize the distribution of the β-adrenergic receptors at the cell surface. Thus the P815 mastocytoma cells were stained with fluorescent antibodies, patches of fluorescence were clearly discernable at 30°C (Fig. 1). These cells were much more intensively stained at 25°C or 30°C than at 37°C, in accordance with the observation that the number of binding sites is higher at the former temperature [14].

Binding of β-adrenergic ligands has been well characterized in the literature. Polyclonal antireceptor antibodies interfered only weakly with this specific recognition: Although hemagglutination of receptor-bearing erythrocytes could be decreased

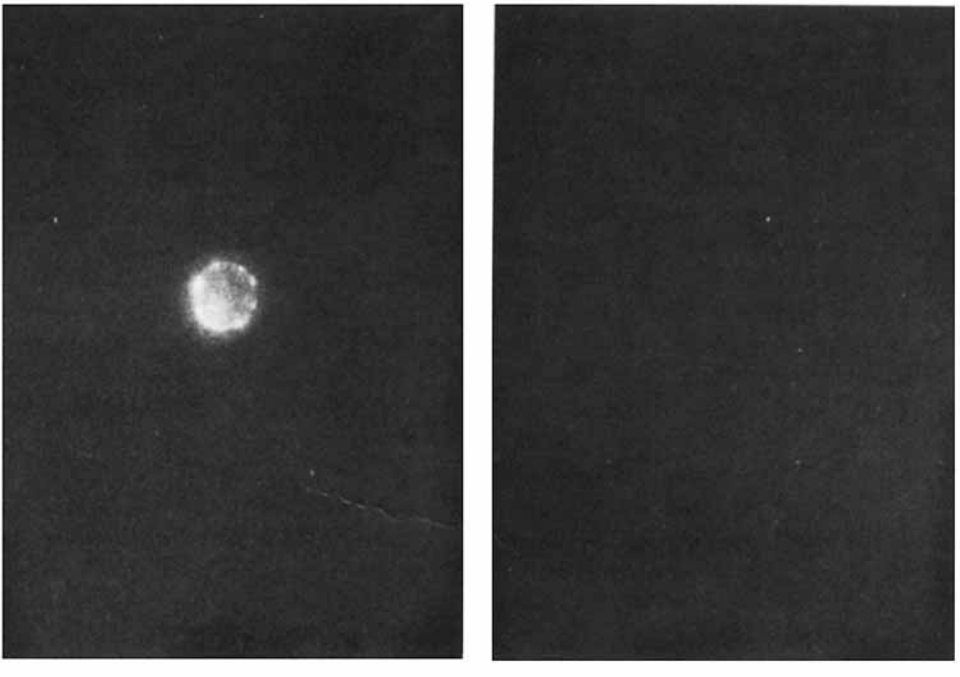

Fig. 1. Visualization of the binding of antireceptor antibodies to P815 mastocytoma β-receptor bearing cells. P815 mastocytomas (10^6 cells) were incubated 1 hr at 30°C with antireceptor IgG in Hank's medium. After three washings, fluorescently labeled rabbit antimouse IgG were added for 1 hr at 30°C. Cells were washed in PBS and then fluorescence was observed in a Leitz microscope. Patches of fluorescence were clearly discernible. Control was made with normal mouse IgG (right).

in some instances, binding of radiolabeled ligands was only partially prevented on whole cells, and not at all on purified membranes.

The antireceptor antibodies could be shown to immunoprecipitate the affinity-purified receptor (Fig. 2). Autoradiography revealed that it was mostly the 60-K polypeptide chain that was recognized by the antibodies.

Stimulation of adenylate cyclase of β-adrenergic agonists may augment the production of cAMP by up to 300%. Anti-β-receptor antibodies mimic the hormonal activation but at a lesser degree (50%) [9]. While the effect was not nearly so impressive as what was seen with the free hormones, it was specific for sera of immunized mice, and proportional to the amount of antibodies added. Moreover, the stimulation was not limited to the basal activity: Even in the presence of epinephrine one observed a clear-cut effect of the antibodies.

Monoclonal Antibodies Against the β-Adrenergic Receptor

Mice immunized against the purified β-adrenergic receptor were sacrificed after the polyclonal antibody response was analyzed. Fusion of the splenocytes with the

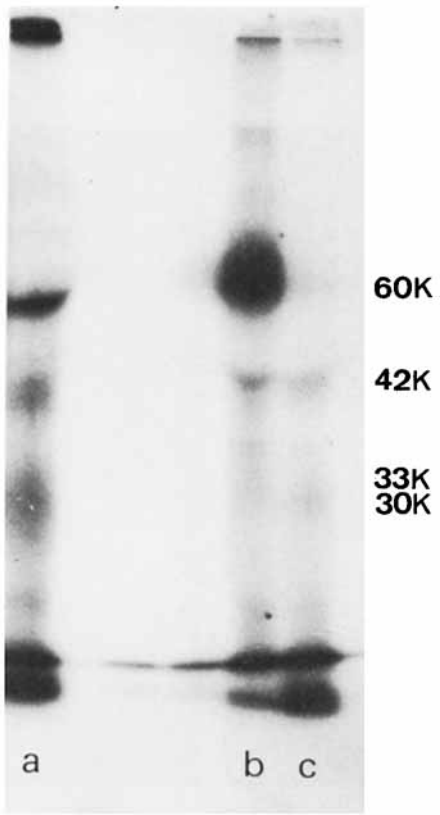

Fig. 2. Immunoprecipitation of radioiodinated purified receptor. Radioiodinated purified receptor was incubated overnight with mouse polyclonal antibodies (b) or normal mouse IgG (c). Rabbit antimouse antibodies were then added. The pellet was washed by centrifugation and submitted to electrophoresis on a SDS polyacrylamide gel. The figure represents an autoradiography of the gel. Line (a) is the receptor submitted to electrophoresis without incubation in the presence of IgG.

NS-I myeloma cell line yielded a number of clones of antibody-producing cells. Screening for antireceptor activity was performed in three stages: Supernatants of surviving clones were added to receptor-bearing cells and fluorescently labeled rabbit antimouse antibodies. Putative antibodies were also detected by an enzyme-linked immuno assay. Positive clones were subcloned and their supernatants were then used in inhibition-of-binding experiments, in the presence of radiolabeled alprenolol, a potent β-adrenergic antagonist.

Supernatants of 24 hybridomas out of 400 surviving hybrid cells were found to specifically recognize β-adrenergic-bearing turkey erythrocytes. None of these monoclonal antibodies inhibited the binding of catecholamine ligands to the receptor nor stimulated adenylate cyclase.

Polyclonal Anti-idiotypic Antibodies that Recognize the β-Receptor

Immunization of rabbits or mice with alprenolol–bovine serum albumin yielded antibodies that recognized not only free alprenolol but also other β-adrenergic ligands, both agonists and antagonists [15, 16]. These antibodies in turn were injected into genetically similar animals. The resulting antibodies were initially screened for binding to the anti-β-adrenergic ligand antibodies. The positive immunoglobulin fractions were shown to bind to receptor-bearing cells or membranes to block binding of alprenolol to β-adrenergic receptor, and to stimulate both the basal and the hormone-stimulated adenylate cyclase [10].

Guanyl nucleotides considerably enhance the hormonal action on cyclase. This effect is reversed by the hormone-sensitive GTPase, and may be rendered permanent by the addition of a synthetic analogue: Gpp(NH)p. In the presence of this compound anti-idiotypic antibodies did not overstimulate the hormonal-activated cyclase (Fig 3).

A very high proportion of rabbits (four out of five) immunized with antihormone antibodies were indeed shown to produce antireceptor antibodies. However, the anti-idiotype antibodies did not always display similar properties: In two animals, unfractionated antibodies inhibited the binding of free ligands to both the antialpren-

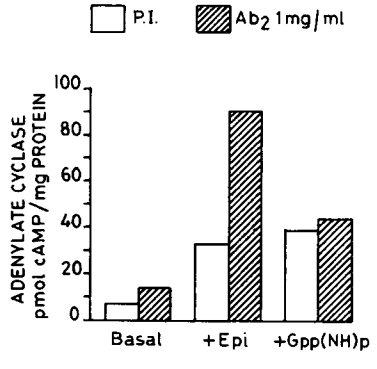

Fig. 3. Effect of anti-idiotypic antibodies on turkey erythrocytes adenylate cyclase activity in the presence of effectors. Turkey erythrocyte membranes were preincubated either with preimmune IgG (P.I.) or with anti-idiotypic IgG (Ab_2) at the same concentration (1 mg/ml). Adenylate cyclase activity was then assayed in the presence of buffer (basal), or epinephrine 10^{-4} M (+ Epi), or Gpp(NH)p 10^{-4} M (+ Gpp(NH)p). Results are means of three independent experiments in duplicate.

olol antibodies and to the membrane-bound receptor and also stimulated adenylate cyclase [10]. In two other animals, binding to receptor-bearing membranes could be revealed only after absorption on an antialprenolol antibody-agarose gel. It is likely that this step separated the idiotypic from the anti-idiotypic antibodies [16].

DISCUSSION

The data presented here, together with our previously published results, confirm that molecular characterization of β-adrenergic receptors can now also be approached by immunological methods. This progress was made possible for us by the obtention of affinity-purified adrenergic receptors from turkey erythrocytes [4, 5]. Conventional polyclonal antibodies were obtained from mice using microgram quantities of this material [9]. However, the new hybridoma methodology of somatic cell fusion has allowed Fraser and Venter [17] to bypass the receptor purification step and to raise monoclonal anti-β-receptor antibodies by immunizing with a preparation of solubilized β-receptor-bearing membranes. These antibodies did inhibit ligand binding to the receptor. Although the monoclonals we raised against the purified receptor specifically bind to β-adrenergic receptor-bearing cells, they are, in contrast to the polyclonals, unable either to inhibit ligand binding to the receptor or to stimulate adenyalte cyclase. Similar differences between monoclonal and polyclonal antibodies have been observed previously as we discussed before [18]; they have generally been ascribed to the much larger variety of specificities of antibodies for the various antigenic epitopes present on the β-adrenergic receptor. In addition, it is likely that negative selection might limit the development of antibodies that inhibit hormone binding or stimulate adenylate cyclase in the immunized mice themselves.

The alternative approach of raising antireceptor antibodies by immunizing animals with antiligand antibodies should in principle select positively for immunoglobulins that inhibit ligand binding. That this may be possible has been shown now for the insulin receptor [19], for the β-adrenergic receptor [10, 20] and for the thyrotropin receptor [21].

The anti-idiotypic antireceptor antibodies do not necessarily mimic similar properties of hormone or ligand. While we described stimulation of adenylate cyclase by anti-β-adrenergic ligand anti-idiotypic antibodies [10], Homcy et al [20] report the preparation of similar antibodies which inhibit the stimulation by catecholamine hormones of adenylate cyclase stimulation.

Whatever their properties, these new types of antireceptor antibodies will undoubtedly constitute valuable reagents, especially in those cases in which receptor purification still remains a remote possibility.

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