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Visualization of the Turkey Erythrocyte β-Adrenergic Receptor

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We have recently described the affinity chromatography purification of the turkey erythrocyte β-adrenergic receptor. The minute amounts obtained initially precluded extensive biochemical characterization. To improve the yield of the receptor, the erythrocyte membranes have been prepared by a new method. This procedure resulted in a 10-fold higher receptor density in comparison with the membrane preparation used previously. The new membranes also contained a catecholamine-sensitive guanine triphosphatase and an adenylate cyclase sensitive to Gpp(NH)p and ℓ-epinephrine. Solubilization by a double digitonin extraction resulted in a preparation containing 4–6 pmoles of ^{3}H-dihydroalprenolol binding sites per mg of membrane protein.

A single step of affinity chromatography on alprenolol-sepharose of the soluble digitonin extract resulted in an additional 1,000-fold purification of the receptor. The overall purification factor was 20,000 relative to the binding activity of the crude membrane preparations.

Electrophoresis in SDS-polyacrylamide of iodinated purified β-receptors revealed, after autoradiography, the presence of four major components. Three of these, corresponding to molecular weights of 170,000, 33,000, and 30,000, respectively, were not affected by reduction with β-mercaptoethanol and were not observed when the digitonin extracts were loaded on the affinity gel in the presence of an excess of ℓ-propranolol. A fourth 52,000-dalton component (60,000 daltons after reduction with β-mercaptoethanol) remained apparent even when affinity purification was prevented by addition of ℓ-propranolol.

Our results suggest that the β-adrenergic receptor is composed of at least three subunits that interact by noncovalent bonds.

Key words: turkey erythrocyte, β-adrenergic receptor, GTPase, adenylate cyclase

The development of radiolabeled β-adrenergic antagonists that bind to the receptors with high affinity and specificity [1–7] has considerably increased our understanding of the molecular structure and function of β-adrenergic receptors. It is now clear that the receptors are membrane proteins that undergo a conformational change upon binding of agonist molecules [8, 9] and that contain essential disulfide bonds, probably located near or at the hormone binding site [10–12].

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Detergent treatment of cell membranes results in the solubilization of the β-adrenergic receptors, with retention of pharmacological binding properties [13–15]. A substantial purification of the solubilized β-adrenergic receptors was achieved by affinity chromatography [15, 16].

Several groups have estimated the molecular weight of unpurified β-adrenergic receptors by techniques such as gel filtration [17], centrifugation in a sucrose density gradient [18], and SDS-polyacrylamide gel electrophoresis following the covalent labeling of the receptors with a radioiodinated β-adrenergic blocker [19].

These results are compared in this work with the data obtained with the β-receptor that we purified by affinity chromatography. For this purpose, we have improved our earlier published purification method by preparing plasma membranes with a 10-fold higher receptor density and by performing a double digitonin extraction. We now report the number and the molecular weight of several polypeptide constituents of the affinity purified receptor.

MATERIALS AND METHODS

Materials

The following were obtained as gifts: ℓ-propranolol (ICI, England); ℓ-isoproterenol, ℓ-norepinephrine, d-epinephrine (Sterling-Winthrop); dℓ-alprenolol (Ciba-Geigy Laboratories). Digitonin and polyethylene glycol 6,000 were obtained from Merck. Sepharose 4B and Sephadex G-50 were from Pharmacia. Bovine gamma globulin was from Sigma; cyclic AMP, phosphocreatine, creatine phosphokinase, ATP, GTP, App(NH)p, and Gpp(NH)p were purchased from Boehringer. α-^{32}P-ATP (16Ci/mmole), γ-^{32}P-GTP (21 Ci/mmole), 8-^{3}H-cyclic AMP (30 Ci/mmole), and Na^{125}I-iodide atom (1862 mCi/μ) were from the Radiochemical Centre, Amersham. ℓ-^{3}H-Dihydroalprenolol hydrochloride (45 Ci/mmole) was purchased from new England Nuclear Corp.

Methods

Membrane preparations. Turkey erythrocyte plasma membranes were prepared according to Caldwell [18] scaled up for large quantities of blood (200–400 ml). Modifications were as follows: 1) before hemolysis, the cells were washed three times with 145 mM NaCl to completely remove the upper layer of white cells, and 2) polytron treatment was followed by several centrifugations in 5 mM potassium phosphate buffer, pH 7.4, containing 2 mM $MgCl_2$ to obtain preparations free of nucleated ghosts and nuclei, as verified by phase contrast microscopy. The final pellet was suspended in 10 mM Tris-Hcl, pH 7.4, 145 mM NaCl, containing 2 mM $MgCl_2$, 1 mM EDTA and 10% (V/V) glycerol and stored under liquid nitrogen. An average of 50 mg of membrane proteins were obtained from 100 ml of blood. Membranes were stored for several months without loss of biological activities.

These membrane preparations resulted in 7- to 10-fold higher density of β-adrenergic receptors (1.6–2.3 pmoles/mg protein) compared with our earlier described preparation (0.2 to 0.3 pmoles/mg protein) [16]. We will therefore refer to the earlier and new preparations as "crude" and "purified" membranes throughout this text.

Solubilization procedure. Erythrocyte membranes (2.5–3 mg/ml) suspended in 10 mM Tris-HCl (pH 7.4) and 90 mM NaCl were treated with digitonin as described previously [20], but a solution was used instead of a suspension of 0.25% (W/V) digitonin

and two consecutive extraction procedures were made to increase the yield of solubilization. After centrifugation at 30,000g for 30 min at 4°C, the supernatant constituted the solubilized preparation.

Binding assays. β-adrenergic receptors in the membrane or solubilized preparations were identified by binding of ℓ-^{3}H-dihydroalprenolol (DHA) as described earlier [16]. Trapping and nondisplaceable binding were estimated by measuring the binding in the presence of an excess of unlabeled dihydroalprenolol (25μM). This value was substracted from the total binding to obtain specific binding. Results were expressed as pmoles of ℓ-^{3}H-dihydroalprenolol specifically bound per mg protein.

Adenylate cyclase assay. Adenylate cyclase activity was measured as published [20]. The assay medium contained 1 mM α-^{32}P-ATP (1.5×10^{6} cpm), 7 mM $MgCl_2$, 1 mM EGTA, 1 mM cyclic AMP, 50 mM Tris-HCl, an ATP-regenerating system (consisting of 25 mM phosphocreatine and creatine phosphokinase 1 mg/ml), and 60 μg of membrane protein in a final volume of 60 μl. Incubation was initiated by addition of the enzyme and performed for 20 min at 30°C. Results are the mean of triplicate determinations and are expressed in pmoles of cyclic AMP formed per 20 min per mg of protein.

GTPase assay. Liberation of $^{32}P_i$ from γ-^{32}P-GTP was measured as published [20] with small modifications. The assay medium contained 0.25 μM γ-^{32}P-GTP (1.4×10^{6} cpm), 5 mM $MgCl_2$, 1 mM App(NH)p, 2 mM creatine phosphate, 1 mg/ml creatine phosphokinase, 2 mM β-mercaptoethanol, 0.1 mM EGTA, 50 mM imidazole-HCl (pH 6.7), and 50 μg of membrane protein in a final volume of 100 μl. Incubation was initiated by addition of the enzyme, performed for 10 min at 37°C, and terminated by the addition of 100 μl of 2.5% SDS solution. $^{32}P_i$ was separated from nucleotide-bound phosphate on small charcoal columns wetted before use by the passage of 1 ml of 50 mM sodium phosphate buffer (pH 7.0). Eluates were collected in scintillation vials containing 10 ml of Aqualuma scintillation fluid (Lumac) and counted for 10 min in a Packard liquid scintillation spectrometer. The preparations of γ-^{32}P-GTP contained 3%–4% $^{32}P_i$. This blank was substracted from the determinations. Results are the mean of triplicate determinations.

Affinity chromatography. Affinity chromatography purification of the solubilized receptor was performed essentially as described earlier [15]. A column (3×0.5 cm) containing 0.75 ml alprenolol-agarose was first equilibrated with 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, then loaded with concentrated digitonin extract. The column was rapidly washed with equilibration buffer, followed by 10 mM Tris-HCl (pH 7.4), 1 M NaCl buffer. Elution of the receptor was achieved with a solution of 400 nM ℓ-^{3}H-dihydroalprenolol in the 10 mM Tris, 1 M NaCl buffer at a flow rate of 10 ml/h. All the buffers contained 0.05% (W/V) bovine immunoglobin (IgG), RNase, or digitonin. Presence of receptor in the eluted fractions was detected by binding of ℓ-^{3}H-dihydroalprenolol using the gel-filtration technique [16].

Protein. Protein determinations were performed by the method of Lowry et al [21], using bovine serum albumin as the standard. To determine the yield of protein after affinity chromatography, ^{125}I-labeled membranes were loaded on affinity gel, and the amount of iodinated, eluated proteins was determined.

Radioiodination. The solubilized membranes or the receptor were labeled with ^{125}I by the chloramine-T technique [22]. It was verified that the ℓ-^{3}H-DHA binding was not affected by iodinating reagents. Free ^{125}I was removed by passage of the iodinated material through a Sephadex G-25 column.

Electrophoresis. Slab polyacrylamide gel electrophoresis was performed as described by Laemmli and Favre [23] with a 4% stacking gel and a 10% separating gel. Purified,

radioiodinated receptor was dialysed overnight at 4°C in H_2O (containing 0.2% (W/V) of dodecylsodium sulfate (SDS) when receptor was eluted with buffer plus digitonin), lyophilized, and solubilized in sample buffer [25]. The slab gels were stained with Coomassie blue, photographed, dried, and then subjected to autoradiography. Molecular weight markers used were: fucose binding-protein, 25,000; *Crotalaria juncea* lectin, 33,000; actin, 45,000; IgG heavy chain, 52,000; serum albumin, 68,000.

RESULTS

Characteristics of ^{3}H-Dihydroalprenolol to Membrane-Bound and Solubilized Receptor

Table I presents a comparison of the ℓ-^{3}H-DHA binding characteristics with the earlier described (crude) and the present (purified) turkey erythrocyte membrane preparations. Whereas the β-adrenergic receptor density did not exceed 0.3 pmoles/mg protein in the crude membranes, densities of up 2–3 pmoles/mg protein are obtained by use of the purified membranes. Aside from this 7- to 10-fold increase in receptor density, there is no marked difference between the Hill coefficient (close to 1) and the equilibrium dissociation constant (K_d = 6–8 nM) for ℓ-^{3} H-DHA binding to both membrane preparations. The number of receptor sites per cell (about 900) is also the same when calculated on the basis of the receptor density and protein recoveries in the two preparations. Solubilization of the purified membranes by a double digitonin extraction procedure causes an additional 2- to 3-fold purification of the receptor (Table I). The K_d for ℓ-^{3}H-DHA is slightly higher but of the same magnitude in the digitonin extract obtained by the second method of preparation (9–13 nM vs 3–5 nM).

The other characteristics are similar in both preparations and remain typical of β-adrenergic receptor: the DHA binding is saturable, rapid, and reversible upon the addition of an excess of ℓ-propranolol (data not shown). Scatchard and Hill analyses indicate that DHA binds to a single class of noncooperative sites. β-adrenergic agonists inhibit the binding of DHA to membranes and to solubilized receptors with an order of potencies specific of β_1-adrenergic receptor: ℓ-isoproterenol > ℓ-norepinephrine > ℓ-epinephrine; the binding is dose dependent and stereospecific (Table II).

Adenylate Cyclase Activity in the Purified Membranes

The crude membrane preparations possessed a basal and a catecholamine-sensitive adenylate cyclase [16]. In Figure 1, we present the adenylate cyclase activities of the purified membranes: the basal adenylate cyclase activity is increased 7- and 49-fold by ℓ-epinephrine and Gpp(NH)p, respectively.

Gpp(NH)p and epinephrine added simultaneously stimulate 107-fold the adenylate cyclase activity; the effects of Gpp(NH)p and catecholamine on cyclase activation are synergistic. GTP has no effect on basal or on catecholamine-sensitive adenylate cyclase.

GTPase Activity

Whereas no catecholamine-sensitive GTPase was detected in the crude membrane preparation, a GTPase coupled to the β-adrenergic receptor was found in purified membranes. GTPase activity was measured in the presence of an excess of App(NH)p and a low concentration of GTP (0.25 μM) to decrease the rate of GTP hydrolysis by nonspecific NTPase [20]; NTPase was estimated in the presence of an excess of GTP (20 mM). Results, reported in Figure 2, showed that ℓ-epinephrine (100 μM) increased GTPase activity by 60%.

TABLE I. Comparison of ℓ-^{3}H-DHA Binding Characteristics of Membrane-Bound and Solubilized Receptors in Crude and Purified Turkey Erythrocyte Membrane Preparation

	Membranes[a]		Digitonin extracts[a] from:	
ℓ-^{3}H-DHA binding characteristics[b]	Crude	Purified	Crude membranes	Purified membranes
B_{max}(pmole/mg protein)	0.20–0.30	1.60–2.30	0.30–0.45	4–6
K_d (nM)	6–8	6–8	3.5	9–13
nH	0.92	0.98	0.98	0.98

[a]Membranes (about 1 mg of protein/ml) and digitonin extracts (about 0.5 mg of protein/ml) were incubated with increasing concentration of ℓ-^{3}H-DHA for 8 min at 30°C. Binding to the digitonin extract was measured by the polyethyleneglycol precipitation method.
[b]B_{max}: concentration of receptor sites. K_d: equilibrium dissociation constant for ℓ-^{3}H-DHA binding. nH: the Hill coefficient. These parameters were calculated from Scatchard and Hill representations of the saturation binding data.

TABLE II. Apparent Equilibrium Dissociation Constants of β-Adrenergic Agents for Binding to Membrane-Bound and Solubilized Turkey Erythrocyte Receptors*

	Apparent K_d for binding to:	
Compounds	Membranes (in μM)	Digitonin extract (in μM)
ℓ-Isoproterenol	0.10	0.023–0.043
ℓ-Norepinephrine	0.20–0.41	0.09
ℓ-Epinephrine	0.60–0.73	0.20
d-Epinephrine	8	15
ℓ-Propranolol	0.0023–0.0038	0.004

*Binding of ℓ-^{3}H-DHA was displaced by increasing concentrations of the various compounds. The apparent equilibrium dissociation constant K_d for the receptor was determined from the equation $K_d = IC50/1 + A/K_{dA}$, where IC is the concentration of the catecholamine required to inhibit specific binding of the DHA by 50%, A is the concentration of ℓ-^{3}H-DHA (10 nM), and K_{dA} is its equilibrium dissociation constant (given in Table I).

TABLE III. Purification of the β-Adrenergic Receptor of Turkey Erythrocyte Membranes *

	Crude membranes	Purified membranes	Digitonin extract	Purified receptor
Proteins (mg)[a]	10	1	0.2	0.00012
B_{max}(pmole/mg protein)	0.25	2.0	5.0	4900
Sites × 10^{-12} mg protein	0.15	1.2	3.0	2940
Total number of sites × 10^{-12}	1.5	1.2	0.6	0.30
Yield (%)		80	40	20
Purification		9	20	19600

*The results were referred to 2 × 10^9 cells (in 1 ml of blood). Values shown represent the means of duplicate determinations in four separate preparations.
[a]Proteins were determined as described in Materials and Methods. B_{max} was defined according to the legend of Table I.

Purification of the Receptor

Solubilized receptor was purified by affinity chromatography. The digitonin extract (3–4 pmoles of ^{3}H-DHA binding after concentration under vacuum) was loaded at 30°C on 0.75 ml of gel. About 90% of the binding activity and 5%–10% of the total proteins were retained by the gel. Elution with buffer containing free ligand released 35%–60% of the applied receptor and 0.06% of the applied proteins. Proteins were estimated using ^{125}I-labeled solubilized membranes: when 7×10^6 cpm were loaded on the affinity gel, 6.5×10^6 cpm passed through the column unretarded, and 4.2×10^3 cpm were eluted by DHA. A 1,000-fold purification over the soluble digitonin extract was obtained, thus resulting in a 16,000- to 25,000-fold purification compared with the initial binding activity of the crude membrane, with a 17%–25% yield (Table III). The specific binding activity of the purified receptor reached a value up to 6,000 pmoles per mg of protein.

Visualization

The eluate from affinity chromatography was iodinated and submitted to SDS-gel electrophoresis followed by autoradiography. The autoradiography (Fig. 3) showed four bands with apparent molecular weights of 170,000, 52,000, 33,000, and 30,000 daltons.

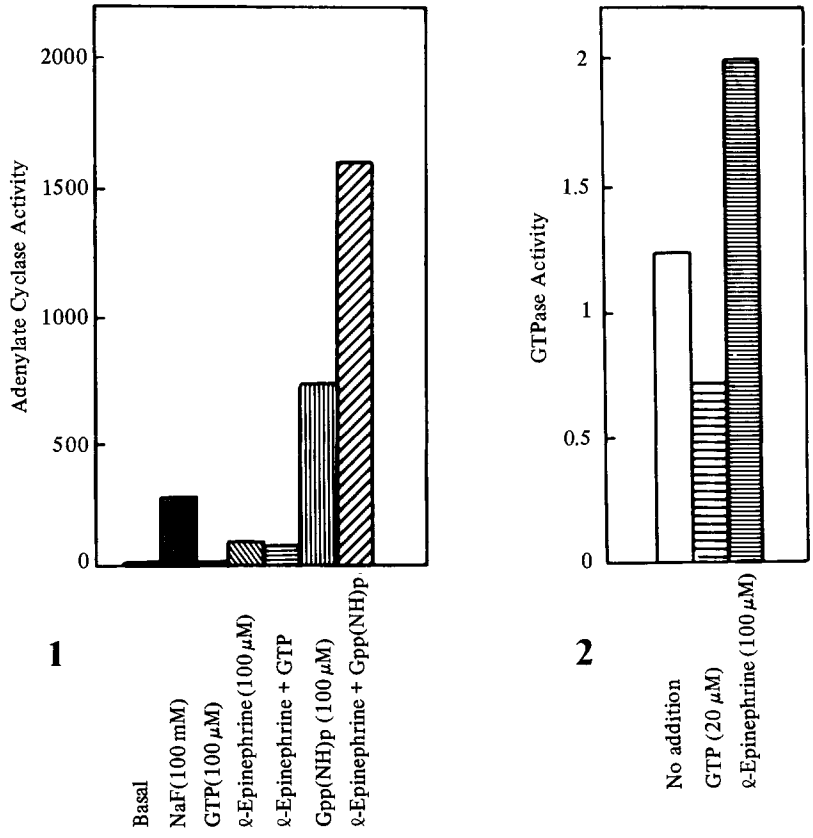

Fig. 1. Effect of NaF, GTP, Gpp(NH)p, and catecholamine on adenylate cyclase activity in purified turkey erythrocyte membranes. Adenylate cyclase activity is expressed in pmoles of cAMP produced per 20 min per mg of protein. Values are means of three determinations.

Fig. 2. GTPase activity in purified turkey erythrocyte membranes. GTPase activity is expressed as pmoles $^{32}P_i$ formed per mg protein per min. Values are means of three determinations.

These protein bands constituted a very small fraction of the total membrane proteins (Table III) and were not detected by Coomassie blue, except for the 170,000 dalton component. The autoradiography of the samples reduced by β-mercaptoethanol prior to electrophoresis (Fig. 3) was not significantly different from that obtained when this reduction was omitted; the 30,000, the 33,000, and the 170,000 dalton bands remained, but the 52,000-dalton component was replaced by a 60,000-dalton band. When the digitonin extract was loaded in the presence of an excess of ℓ-propranolol (10 mM), no receptor was retained on the column as evidenced by the binding studies; at the same time, the 170,000, 33,000, and 30,000 dalton components were not visible on the autoradiogram; the 52,000 or 60,000 dalton band was still slightly visible. The same results were obtained for every purified membrane preparation.

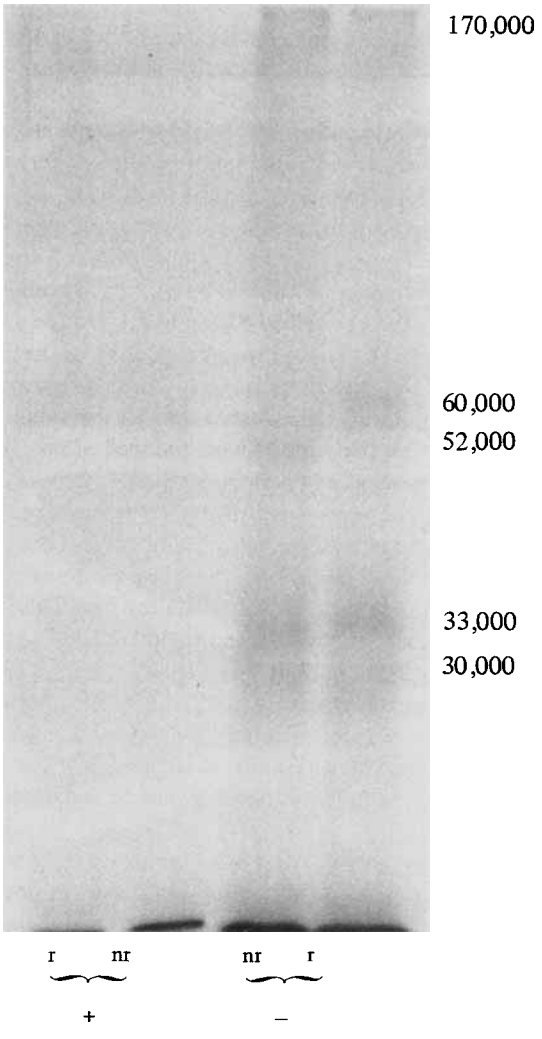

Fig. 3. Polyacrylamide gel electrophoresis of radioiodinated, purified β-adrenergic receptor from turkey erythrocyte membranes. Autoradiography. The digitonin extracts were loaded on the affinity gel in the absence (–) or in the presence of an excess (+) of ℓ-propranolol. The eluates were labeled with ^{125}I by use of chloramine T. They were electrophoresed on 10% SDS-polyacrylamide gel after reduction (r) by 5% (V/V) β-mercaptoethanol or without reduction (nr).

DISCUSSION

The aim of the present work was the visualization of the β-adrenergic receptor of turkey erythrocyte membranes and the characterization of its molecular properties. For this purpose, we used an improved technique of membrane preparation whose main difference from that previously described [16] was as follows: the erythrocytes were hemolyzed in a ten times greater volume; the hemolysate was homogenized in a five-times higher volume, using a polytron apparatus. The obtained membranes contained 8 to 10 times more sites per mg of protein when compared with previous preparations. Interestingly, the improved method of membrane preparation revealed the presence, in the conditions described by Selinger and Cassel [20], of a catecholamine-sensitive GTPase not seen in the earlier preparations. In addition, we were able to observe an enhancement by epinephrine of the Gpp(NH)p adenylate cyclase stimulation of the β-adrenergic system of the turkey erythrocyte. Finally, the second extraction with digitonin increases the yield of solubilization and also doubles the degree of receptor purification. The DHA binding of the preparations, before and after solubilization, exhibit all the pharmacological properties of β-adrenergic receptor.

The solubilized receptor was purified 1,000-fold in a single step by affinity chromatography with a 35%–55% yield of ^{3}H-DHA binding. Overall, the purification reached 16,000- to 25,000-fold when the activity was compared with the activity of the crude membrane preparation. Purified receptor is now available in pmole amounts: we obtained up to 7 pmoles when loading 20 pmoles of soluble preparation on the affinity gel.

To assess the purity of the receptor isolated by affinity chromatography and to determine its molecular weight, we performed SDS-polyacrylamide gel electrophoresis followed by autoradiography of the radioiodinated eluates from the alprenolol-sepharose.

In these denaturating conditions, three labeled bands, with a molecular weight of 170,000, 33,000, and 30,000 daltons, were observed when the column was loaded in the absence of ℓ-propranolol, whether the samples were reduced or not. The 33,000 dalton band is present as a major component and was the only one seen previously in less radiolabeled samples [24]. None of the bands were visible when ℓ-propranolol was applied simultaneously with digitonin extract. This suggests that these three components are related to the binding site of the receptor; it is unlikely that these proteins bound nonspecifically to the alprenolol sepharose. In contrast, the 52,000-dalton band, also present in the nonreduced samples, which remains weakly visible in the presence of ℓ-propranolol, is probably not involved directly in the specific DHA binding.

In earlier experiments, the molecular weight of the solubilized turkey erythrocyte membrane β-adrenergic receptor was estimated by gel filtration to equal about 200,000 daltons. The present results suggest that receptor is composed of three subunits: 170,000, 33,000, and 30,000 not covalently linked, since they can be separated by treatment with SDS.

Our findings are in agreement with those obtained in very different conditions by Atlas and Levitski [19]. In their study, two components were visualized after affinity labeling of intact turkey erythrocytes with a tritiated β-adrenergic ligand. Their molecular weights were higher than the one observed in our study, 42,000 and 38,000 instead of 33,000 and 30,000. Recent reports on the insulin [25] and on the acetylcholine receptors [26] clearly suggest the existence of various interacting components, which may or may not be directly involved in the binding of the ligand, the transmission of the signal to other components, and further metabolic changes in the cell.

We have recently [24] proposed a model for the turkey erythrocyte receptor cyclase complex that involves at least four different components: the β-adrenergic receptor, a guanine nucleotide binding site associated or part of the receptor, the adenylate cyclase, and a hormone-sensitive GTPase associated to GTP binding protein. It is likely that at least some of the subunits visualized in the present work correspond to the functional components of the receptor.

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