Vol. 106, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1982 Pages 139-148

PHOTOAFFINITY LABELING OF ß-ADRENERGIC RECEPTORS: IDENTIFICATION OF THE ß-RECEPTOR BINDING SITE(S) FROM TURKEY, PIGEON, AND FROG ERYTHROCYTE

Abbas Rashidbaigi and Arnold E. Ruoho*

Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Received March 29, 1982

SUMMARY: The ß-adrenergic receptors in the erythrocyte membranes from turkey, pigeon, and frog have been identified in situ utilizing the photoaffinity label ±[^{125}I]-iodoazidobenzylpindolol, ±[^{125}I]IABP. The molecular weights determined by SDS-polyacrylamide gel electrophoresis are the following: turkey, 43,500; pigeon, 53,500, 46,000, and 45,000 [labeled in a ratio of 5 (53,500):2 (46,000 plus 45,000)]; and frog, a broad 60,000 to 67,000 dalton band. The data identify the binding site subunit(s) of these ß-adrenergic receptors and suggest that the receptor structure from different ß-receptor subtypes and different sources may be different. These biochemical differences may contribute to the pharmacologically observed distinction of ß-receptor subtypes.

The detailed molecular structure of the ß-adrenergic receptor is at present unknown. A considerable amount of effort is being directed at purification of this receptor from various sources (1,2). Estimates of the size of the native receptor extracted with Lubrol PX from wild-type S49 lymphoma cells have been reported to be in the range of 68,000 to 85,000 (3). The ß-receptor from cyc^- membranes showed similar hydrodynamic properties to the wild-type receptor (3). The size of the turkey erythrocyte receptor subunit, as analyzed by SDS-polyacrylamide gels, has been reported to be 70,000 dalton when eluted with propranolol from a monocolonal antibody affinity column (4). A wide range of molecular weights has been reported for the ß-receptor subunit(s) from various sources. These include 32,000 (5), 37,000 to 41,000 (6), 68,000, and 35,000 (7) for the turkey erythrocyte. The reason for these variations is not clear at present but may be due to inadequate methods for accurately determining small amounts of

*To whom all correspondence should be addressed.

0006-291X/82/090139-10$01.00/0

receptor in situ. The molecular weights which have been reported for other sources of ß-adrenergic receptor include 58,000 for frog erythrocyte (8) and 38,000 for mammalian lung (9).

Utilizing the high affinity and high specific radioactivity ligand ±[^{125}I]-iodoazidobenzylpindolol, ±[^{125}I]IABP (10), we have identified in situ the ß-adrenergic binding site polypeptide(s) from turkey, pigeon, and frog erythrocyte membranes. These results identify the binding site subunit(s) of the ß-receptor in these membrane preparations.

MATERIALS AND METHODS

Materials: The synthesis and characterization of ±[^{125}I]IABP was performed as described for nonradioactive (±)-IABP (11). The ±[^{125}I]IABP used in the experiments reported in this paper was greater than 99% radiopure. The specific activity of ±[^{125}I]IABP used in these photolysis experiments was 983 Ci/mmol. (-) Alprenolol D-tartarate, phenylmethylsulfonyl fluoride, α-chymotrypsinogen, fumarase, human transferrin, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) and aldolase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Acrylamide and N,N'-methylenebisacrylamide were obtained from Bio-Rad Laboratories (Richmond, CA), and N,N,N',N'-tetramethylethylenediamine (TEMED) was purchased from Eastman Kodak Co. (Rochester, NY). L-α-Lecithin ß,γ-dioleoyl was purchased from Calbiochem-Behring (La Jolla, CA).

Membrane Preparation: Turkey erythrocyte membranes were prepared according to the method reported for the preparation of duck erythrocyte membranes (10). Pigeon erythrocyte membranes were prepared according to the method of Watts and Wheeler (12), and frog erythrocyte membranes were prepared according to the method of Stadel and Lefkowitz (13). All of the membranes were prepared in the presence of 0.1 mM phenylmethylsulfonyl fluoride and stored in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$) at -80°C with no loss of binding activity for several months. The concentration of membrane proteins was determined according to the method of Lowry et al. (14). The protein concentrations were as follows: turkey erythrocyte membranes, 0.74 mg/ml; pigeon erythrocyte membranes, 1.74 mg/ml; and frog erythrocyte membranes, 0.6 mg/ml.

±[^{125}I]IABP Binding Assay: Erythrocyte membranes (0.12-0.2 mg/ml in lysis buffer) in 0.1 ml aliquots were incubated in the presence and absence of 10^{-6} M (-)-alprenolol with various concentrations of ±[^{125}I]IABP. Incubation was performed at 30°C for 30 min, followed by dilution of 0.035 ml aliquots (in duplicate) into 10 ml of 35°C lysis buffer, and the membranes were collected on GF/A glass fiber filters (Whatman) with vacuum. The filters were then washed with 10 ml of lysis buffer at 25°C. The amount of ^{125}I was determined using a Packard 5230 gamma counter with 70% efficiency. The specific binding was determined by subtracting the total ±[^{125}I]IABP bound from the ±[^{125}I]IABP bound in the presence of (-)-alprenolol. Nonspecific binding was generally 10-30% of the total binding. A Scatchard (15) analysis was performed by plotting bound/free vs bound.

Preparation of Lecithin Vesicles: L-α-Lecithin ß,γ-dioleoyl (0.8 ml of a 37 mg/ml methanolic solution) was taken to dryness in a thick-walled glass tube under a nitrogen stream. Lysis buffer (150 ml) was added to this

residue, and the mixture was sonicated 4 times for 5 min each time with a 1-min rest interval between sonications at an intensity of 6 at 4°C. The sonicator used was a model W185F sonicator-cell disruptor (Heat Systems Ultrasonics, Inc., Plainview, NY). The vesicle suspension was then centrifuged at 33,000 x g for 10 min and the supernatant collected and adjusted to 1.33×10^{-9} M (-)-alprenolol prior to use. This vesicle-alprenolol mixture was called Buffer B.

Incubation and Photolysis Conditions: Erythrocyte membranes (0.1-0.12 mg protein) in 0.2 ml of lysis buffer were incubated with and without 10^{-6} M (-)-alprenolol and with $\pm$[^{125}I]IABP ($1-2 \times 10^{-9}$ M) in the dark for 30 min at 30°C in 8 ml Pyrex glass tubes. After incubation, the contents of the tubes were cooled quickly in ice water to 1°C, diluted by the addition of 3 ml of ice-cold Buffer B, and then centrifuged at 33,000 x g for 10 min. The membrane pellets were resuspended in 3 ml of ice-cold Buffer B and the washing procedure repeated 3 additional times. The final membrane pellets were resuspended in 2 ml of lysis buffer and photolyzed immediately with a 1-KW high pressure mercury lamp (AH-6, Advanced Radiation, Santa Clara, CA) for 3 sec at a distance of 10 cm from the lamp. After photolysis, the pellets were collected by centrifugation at 33,000 x g for 10 min. The pellets were then dissolved in 0.15 ml of a buffer containing 75 mM Tris-HCl, pH 6.8, 2.4% SDS, and 10% glycerin and heated at 98°C for 2 min. To each sample was added 5 µl of 2-mercaptoethanol and 10 µl of 0.05% bromophenol blue dye marker. Electrophoresis was performed on the entire sample on a 7-14% linear polyacrylamide gradient slab gel (16) in which the separating gel was composed of a linear gradient (16 x 14 x 0.15 cm) from 7% acrylamide and 0.19% N,N'-methylenebisacrylamide to 14% acrylamide and 0.38% N,N'-methylenebisacrylamide. The stacking gel (3 x 14 x 0.2 cm) contained 3% acrylamide, 0.08% N,N'-methylenebisacrylamide, 0.1% TEMED, 0.03% ammonium persulfate, 0.1% SDS, and 0.125 M Tris-HCl, pH 6.8. The electrode buffer contained 0.1% SDS, 0.19 M glycine, and 0.24 M Tris-HCl, pH 8.2. Separation was performed at a constant voltage of 30 volts for approximately 2 days with the cathode on top. The molecular weight of receptor polypeptide(s) was evaluated by the relation of log (M_r) vs log (%T) in the gradient gel (17). The gels were then stained with 0.1% (w/v) Coomassie Blue-G in 45.5% (v/v) methanol, 9% (v/v) acetic acid, and 54.5% (v/v) water, destained for 2 days in 7% acetic acid in water, and soaked in a methanol:acetic acid: water (7:3:30) solution containing 2% dimethylsulfoxide. The destained gels were then dried with a Bio-Rad model 224 gel slab drier under reduced pressure and subjected to autoradiography using Kodak XRA-5 film for 24 hr with a Dupont Quanta-III intensifying screen at -80°C. After autoradiography, the dried gels were sliced into 2-mm pieces, and the radioactivity in each piece was determined.

RESULTS

$\pm$[^{125}I]IABP bound to the ß-adrenergic receptor of turkey, pigeon, and frog erythrocyte membranes with high specificity. Binding isotherms and Scatchard plots (Fig. 1) indicated a K_D of 1.95×10^{-10} M for the ß-adrenergic receptor from turkey erythrocyte membranes with a 1662 fmol/mg receptor binding capacity (Fig. 1A). The K_D for pigeon erythrocyte membrane ß-receptor was calculated to be 1.15×10^{-10} M with a 1292 fmol/mg receptor binding capacity (Fig. 1B), and the K_D for frog erythrocyte membrane ß-receptor was calculated to be 1.64×10^{-10} M with a 667 fmol/mg

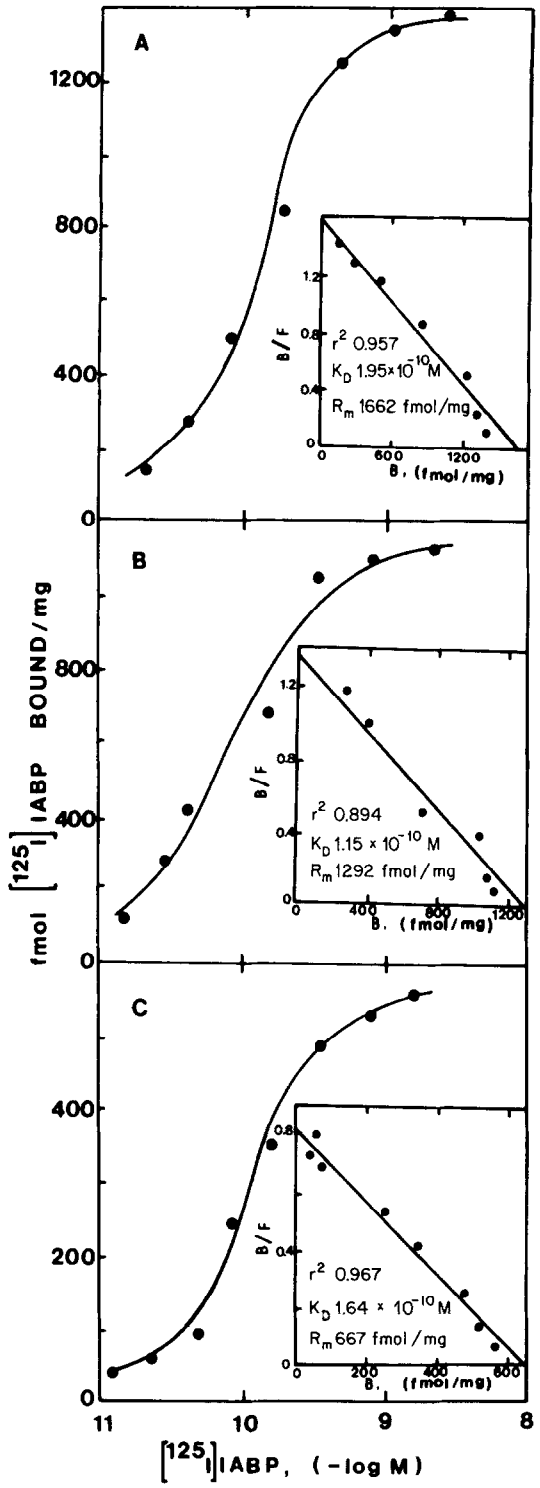


Fig. 1. Determination of ±[^{125}I]IABP binding isotherms to turkey (A), pigeon (B), and frog (C) erythrocyte membranes. Membranes, 0.1 ml (0.12-0.2 mg/ml), were incubated in lysis buffer with increasing concentrations of ±[^{125}I]IABP in the presence and absence of 10^{-6} M (-)-alprenolol at 30°C for 30 min. The amount of specifically bound ±[^{125}I]IABP was then determined by filtering 35 µl aliquots (see Materials and Methods). The insets represent Scatchard analyses of the binding data. Results represent the mean of duplicate determinations.

Table I. Molecular weights of the beta-adrenergic receptor in different erythrocyte membranes

Species	M_r range[a]	Ratio of labeling
Duck[b]	48,500 ± 500	1
	45,000 ± 2,000	4
Turkey	43,500 ± 1,500	-
Pigeon	52,500 ± 1,500	5
	46,000 ± 1,000 plus 45,000 ± 1,000	2
Frog	63,500 ± 3,500	-
Rat[1]	62,500 ± 2,500	-

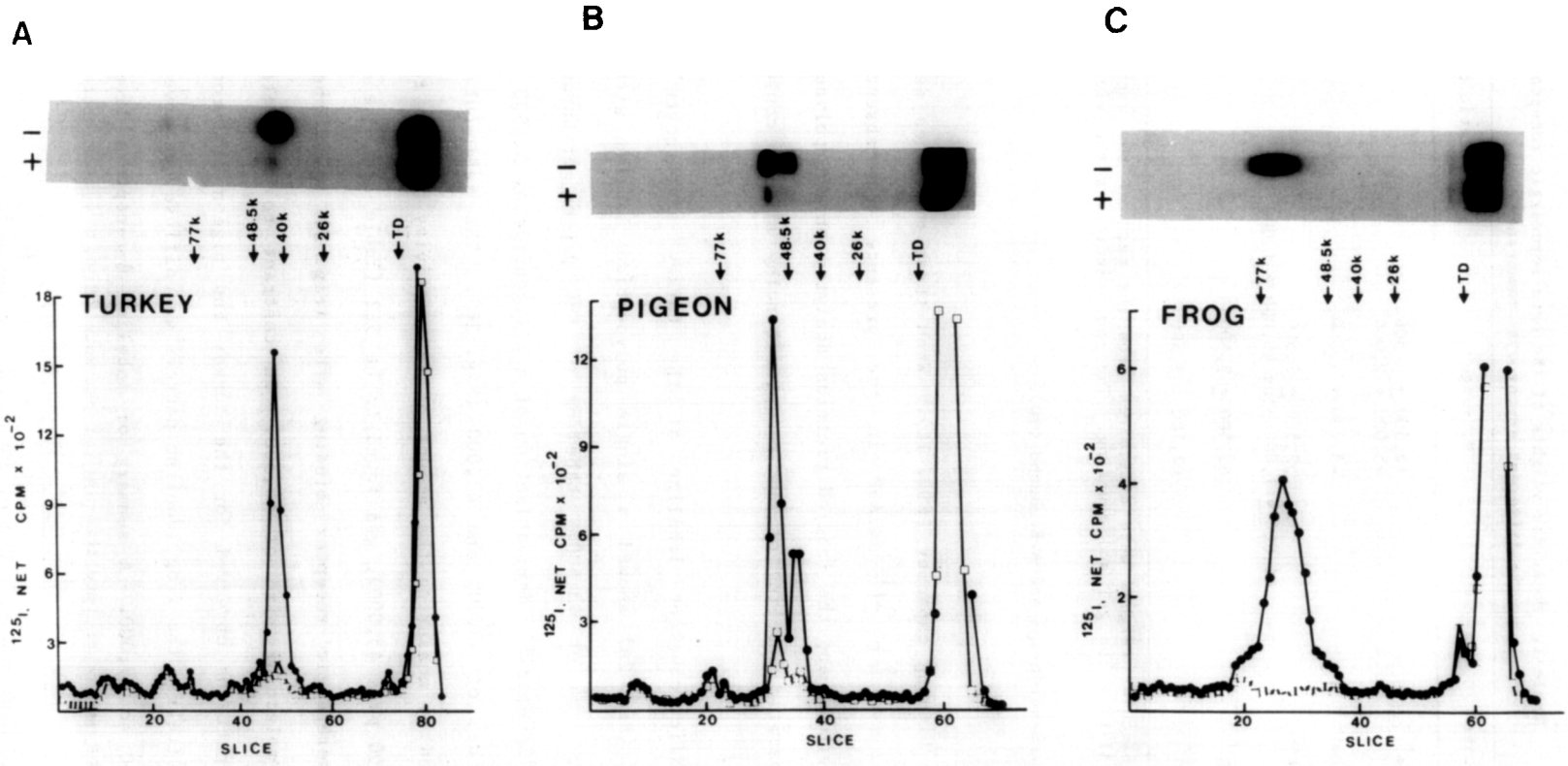
[a]Molecular weights were determined from the peak of the radioactivity after slicing the gel and counting. The range indicates the width of the band.

[b]A. Rashidbaigi and A.E. Ruoho (10).

receptor binding capacity (Fig. 1C). When these membranes were photolyzed with 1-2 x 10^{-9} M ±[^{125}I]IABP in the presence and absence of 10^{-6} M (-)-alprenolol and the labeled proteins analyzed on SDS-polyacrylamide slab gels, specific labeling of the receptor binding site(s) could be detected (Fig. 2).

Specific covalent labeling of the turkey erythrocyte ß-adrenergic receptor (Fig. 2A) showed a single polypeptide labeled with an average molecular size of 43,500. Labeling of the pigeon erythrocyte membrane resulted in specific derivatization of a polypeptide of 52,500 and two minor polypeptides of 46,000 and 45,000 (Fig. 2B). The ratio of labeling, as calculated by measuring the amount of radioactivity in each region (52,500 and 46,000 plus 45,000), was found to be 5:2 (Table I). The frog erythrocyte ß-adrenergic receptor binding site (Fig. 2C) was labeled with an average molecular size of 63,500. In contrast to the relatively sharp banding patterns observed for the turkey and pigeon erythrocyte receptor binding site(s), the frog labeling pattern was diffuse and covered the range of 60,000 to 67,000. A summary of labeling ß-receptors from erythrocyte membranes is presented in Table 1.

[1]A. Rashidbaigi and A.E. Ruoho, unpublished observations.


A
B
C
+
-
77k
48.5k
40k
26k
TD
TURKEY
PIGEON
FROG
^{125}I. NET CPM x 10^{-2}
SLICE

DISCUSSION

±[^{125}I]IABP specifically bound to the ß-adrenergic receptor in membranes prepared from turkey, pigeon, and frog erythrocytes with high affinity (Fig. 1). Following photolysis in the presence and absence of (-)-alprenolol, the average molecular weights of the binding site subunit(s) were found to be the following: turkey, 43,500 ± 1,500; pigeon, 52,500 ± 1,500, 46,000 ± 1,000, and 45,000 ± 1,000; and frog, 63,500 ± 3,500 (Fig. 2; Table I).

The experiments reported in this paper define a feature of using ±[^{125}I]IABP as a photolabel for the ß-receptor in membrane preparations. Since the ±[^{125}I]IABP-receptor complex is extremely stable at 1°C (t1/2 for dissociation is 160 min, 243 min, and 250 min for turkey, pigeon, and frog, respectively),[1] removal of excess ±[^{125}I]IABP was readily performed by centrifugation in the dark without appreciable dissociation of the receptor-photoligand complex prior to photolysis. Under these conditions, labeling of the specific ß-receptor subunit(s) with ±[^{125}I]IABP was demonstrated to be almost absolute (Fig. 2). Even after washing, however, approximately one-half of the photolabel remained in the membrane, associated primarily with lipid [see plus (-)-alprenolol conditions in Fig. 2]. This conclusion was based on the fact that, in the protected receptor preparation with cold ligand [i.e., plus (-)-alprenolol conditions], a radioactive band was always detected below the dye marker as

---

Fig. 2. SDS-polyacrylamide gradient patterns of the specifically labeled ß-adrenergic receptors of turkey (A), pigeon (B), and frog (C) erythrocyte membranes photolyzed with ±[^{125}I]IABP. Membranes (100-200 µg protein) were incubated in lysis buffer with ±[^{125}I]IABP (1-2 x 10^{-9} M) in the presence and absence of 10^{-6} M (-)-alprenolol, washed 4 times at 1°C with Buffer B, photolyzed in lysis buffer, and electrophoresed on 7-14% gradient slab gels (see Materials and Methods). Upper panels represent 24-hr autoradiograms of stained, destained, and dried gels. -, Membranes to which ±[^{125}I]IABP was added. +, Membranes to which ±[^{125}I]IABP and (-)-alprenolol were added prior to washing and photolysis. Lower panels represent the same gels sliced into 2-mm pieces after autoradiography and the amount of ^{125}I determined in each piece. ●, ^{125}I Profile obtained for the membranes incubated in the presence of ±[^{125}I]IABP alone prior to washing and photolysis. □, ^{125}I Profile obtained for the membranes incubated in the presence of ±[^{125}I]IABP and (-)-alprenolol prior to washing and photolysis. Standard proteins were: human transferrin, 77,000; fumarase, 48,500; aldolase, 40,000; α-chymotrypsinogen, 26,000.

either non-photolyzed ±[^{125}I]IABP or lipid-inserted products. This ±[^{125}I]IABP component could not readily be washed out of the membranes before photolysis. These data indicate that ±[^{125}I]IABP can be used to detect ß-receptor binding site(s) in membrane preparations by taking advantage of the high affinity and thus the very slow dissociation of the receptor-photolabel complex.

The diffuse nature of the frog ß-receptor binding site on SDS-polyacrylamide gel electrophoresis is reminiscent of the behavior of glycoproteins in this electrophoresis system (18). A similar profile for the purified frog receptor has been recently described (8,19) with a molecular weight estimated at 58,000 to 60,000.

The turkey ß-receptor, on the other hand, appeared as a distinct band (Fig. 2A) with a much lower subunit molecular weight. If there is carbohydrate present, perhaps there is less heterogeneity than in the case of the frog erythrocyte receptor, which has been characterized as a $ß_2$-subtype (20). Since the turkey ß-receptor has been reported as a $ß_1$-subtype (1), receptor structures from various subtypes may be different. The photolabeling pattern of the rat erythrocyte ß-adrenergic receptor binding site (Table I) appeared similar to frog.[1] This ß-receptor, which has been characterized as a $ß_2$-subtype (21), showed an average molecular weight of 62,500 on 9.2% SDS-polyacrylamide gel electrophoresis with a range of 60,000 to 65,000 occurring as a diffuse band. This striking similarity in electrophoresis behavior between frog and rat (both $ß_2$-subtypes) may indicate that $ß_2$-adrenergic receptors are similar.

The fact that the pigeon ß-receptor binding sites appear as three polypeptides, with the 52,500 polypeptide predominant (Fig. 2B; Table I), could be due to proteolysis, even though these membranes were freshly prepared in the presence of the protease inhibitor, phenylmethylsulfonyl fluoride. On the other hand, these polypeptides in the pigeon erythrocyte membrane may compose the binding site together or exist as separate forms of the receptor in the membrane bilayer. Photoaffinity labeling of the duck

erythrocyte ß-adrenergic receptor with ±[^{125}I]IABP (10) also showed two polypeptides on SDS-polyacrylamide gel electrophoresis but labeled in a ratio of 1:4, with the lower molecular weight polypeptide predominating (Table I).

The data presented in this paper show that ß-receptors from different species contain binding site subunit(s) of varying sizes. Not only are the absolute sizes different, but the electrophoresis behavior of the binding site polypeptides is also different. This may indicate a fundamental biochemical difference in ß-receptor subtypes. Whether each subunit is a separate receptor molecule or whether the receptor exists as several oligomers of the basic subunit(s) reported here is not presently known. The precise structural correlation between ß-receptors which confer different drug specificities, such as $ß_1$ and $ß_2$, may depend on the biochemical characteristics of the individual protomers (e.g., degree of glycosylation), which may indirectly affect quaternary structure.

ACKNOWLEDGMENTS

This work was supported by Grant NS12392 from the National Institutes of Health.

REFERENCES

1. Vauquelin, G., Geynet, P., Hanoune, J., and Strosberg, D. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3710-3714.
2. Caron, M.G., Srinivasan, Y., Pitha, J., Kociolek, K., and Lefkowitz, R.J. (1979) J. Biol. Chem. 254, 2923-2927.
3. Haga, T., Haga, K., and Gilman, A. (1977) J. Biol. Chem. 252, 5776-5782.
4. Fraser, C.M., and Venter, J.C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7034-7038.
5. Strosberg, A.D., Vauquelin, G., Durieu-Trautmann, O., Delavier-Klutcho, C., Bottari, S., and Andre, C. (1980) Trends Biochem. Sci. 5, 11-14.
6. Atlas, D., and Levitski, A. (1978) Nature 272, 370-371.
7. Charlton, R.R., Soiefer, A.I., and Venter, J.C. (1980) Fed. Proc. 39, 1616.
8. Shorr, R.G.L., Lefkowitz, R.J., and Caron, M.G. (1981) J. Biol. Chem. 256, 5820-5826.
9. Soifer, A.I., and Venter, J.C. (1980) Fed. Proc. 39, 313.
10. Rashidbaigi, A., and Ruoho, A.E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1609-1613.
11. Rashidbaigi, A., and Ruoho, A.E. (1982) J. Pharm. Sci. 71, 305-307.
12. Watts, C., and Wheeler, K.P. (1978) Biochem. J. 173, 899-905.
13. Stadel, J.M., and Lefkowitz, R.J. (1979) Mol. Pharmacol. 16, 709-718.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Scatchard, G. (1948) Ann. N.Y. Acad. Sci. 51, 660-672.

16. Laemmli, U.K. (1970) Nature 227, 680-685.
17. Lambin, P., Rochu, D., and Fine, J.M. (1976) Anal. Biochem. 74, 567-575.
18. Markowitz, S., and Marchesi, V.T. (1981) J. Biol. Chem. 256, 6463-6468.
19. Levin, T.N., Heald, S.L., Jeffs, P.W., Shorr, R.G.L., Lefkowitz, R.J., and Caron, M.G. (1981) J. Biol. Chem. 256, 11944-11950.
20. Mukherjee, C., Caron, M.G., Coverstone, M., and Lefkowitz, R.J. (1975) J. Biol. Chem. 250, 4869-4876.
21. Dickinson, K., Richardson, A., and Nahorski, S.R. (1980) Mol. Pharmacol. 19, 194-204.