MOLECULAR PHARMACOLOGY, 12, 16-31

Structure-Activity Relationships of Adenylate Cyclase-Coupled *Beta* Adrenergic Receptors: Determination by Direct Binding Studies

CHHABIRANI MUKHERJEE, MARC G. CARON,[1] DEBRA MULLIKIN, AND ROBERT J. LEFKOWITZ[2]

Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

(Received May 26, 1975)

SUMMARY

MUKHERJEE, CHHABIRANI, CARON, MARC G., MULLIKIN, DEBRA & LEFKOWITZ, ROBERT J. (1976) Structure-activity relationships of adenylate cyclase-coupled *beta* adrenergic receptors: determination by direct binding studies. *Mol. Pharmacol.*, 12, 16-31.

Recently developed techniques for directly studying ligand binding to *beta* adrenergic receptors with (−)-[^{3}H]alprenolol have been used to delineate in detail the binding specificity of the adenylate cyclase-coupled *beta* adrenergic receptors in a model system, the frog erythrocyte membrane. The abilities of 60 *beta* adrenergic agents to compete for the binding sites and to interact with the adenylate cyclase (as agonists or antagonists) were quantitated and compared. The specificity of the receptors determined by direct binding studies or by adenylate cyclase studies was comparable. The K_D values of the agents as determined by inhibition of (−)-[^{3}H]alprenolol binding correlated well (r = 0.95) with their apparent dissociation constants determined by enzyme studies. The latter were determined as the concentrations of agonists necessary to cause 50% maximal enzyme stimulation, or the concentrations of antagonists necessary to produce a 2-fold rightward shift in the (−)-isoproterenol dose-response curve. Agonists and antagonists appeared to compete for the same set of receptor binding sites. Structure-activity relationships determined by the direct binding studies were in excellent agreement with those previously determined in more intact tissue preparations. For agonists the structural features which determined receptor affinity (assessed by direct binding studies) were distinct from those which determined intrinsic activity (maximum ability to stimulate adenylate cyclase). The affinity of agonists was increased by increasing the size of the substituent on the amino nitrogen, by a (−) configuration of the hydroxyl on the β-carbon, and by the presence of a catechol moiety. Methyl or ethyl substitution on the α-carbon had only a slight (generally inhibitory) effect on affinity. Intrinsic activity of agonists was determined primarily by the nature of the substituents on the phenyl ring. Full intrinsic activity requires the presence of hydroxyl groups on the ring at positions 3 and 4 as well as the β-carbon hydroxyl in the (−) configuration. Deletion of the β-carbon hydroxyl, as in compounds such as dopamine, dobutamine, and related agents, leads to substantial loss of intrinsic activity and affinity even in the presence of large amino nitrogen substituents. A methanesulfonamide group substituted for the hydroxyl in position 3 on the ring results in reduced intrinsic activity. Deletion of the

This work was supported by Grant HL 16037 from the National Heart and Lung Institute and a grant-in-aid from the American Heart Association, with funds contributed in part by the North Carolina Heart Association.

[1] Recipient of a postdoctoral fellowship from the Medical Research Council of Canada and the Province of Quebec.

[2] Established Investigator of the American Heart Association.

Copyright © 1976 by Academic Press, Inc.
All rights of reproduction in any form reserved.

ring hydroxyl at either position 3 or 4 or substitution by chlorine produces competitive antagonists. Structure-activity relationships of antagonists were similar to those of agonists, except that the catechol moiety was replaced by a single or double aromatic ring structure. Separation of this moiety from the ethanolamine side chain by an ether function significantly increased affinity. When a phenyl group was present, a single substituent at the *para* position was associated with reduced affinity.

INTRODUCTION

The receptors through which catecholamines exert their widespread physiological effects have been among the most thoroughly studied of all drug receptors (1). Two major classes of adrenergic receptors, *alpha* and *beta*, have been defined (2). Adrenergic receptors were originally classified on the basis of order of potency of certain agonist drugs (2). Subsequently the development of specific antagonist agents confirmed the validity of this classification (3). *Beta* adrenergic receptors are characterized by their much higher affinity for the agonist isoproterenol than for norepinephrine. Antagonists such as propranolol and dichlorisoproterenol also have high affinity for the *beta* receptors. By contrast, phentolamine and phenoxybenzamine, antagonists which are specific for *alpha* adrenergic receptors, do not interact with the *beta* receptors.

The structure-activity relationships for occupancy of *beta* adrenergic receptors by agonists and antagonists have been studied in detail in various whole organ preparations (1). In subcellular membranes the stimulatory effects of catecholamines on the enzyme adenylate cyclase are almost invariably mediated by *beta* adrenergic receptors (4). Recent studies by several laboratories have indicated that the specificity of these "adenylate cyclase-coupled" *beta* adrenergic receptors is identical with that of the *beta* receptors determined in more intact preparations (5–10).

The assumption underlying all such structure-activity studies is that the structural requirements for stimulation or inhibition of a physiological or biochemical parameter are simple reflections of the binding specificity of some hypothetical structure called the *beta* adrenergic receptor. The most direct means of assessing such a binding specificity is to study the binding of a radioactive ligand to the receptors and to quantitate the ability of other agents to compete for occupancy of these sites.

We have recently developed methods for studying binding to *beta* adrenergic receptors in membrane fractions. These studies utilize (−)-[^{3}H]alprenolol, a potent competitive *beta* adrenergic antagonist labeled to high specific activity with tritium. Binding of this ligand to membranes from frog erythrocytes (11–15) and several mammalian tissues (16)[3] is rapid, reversible, and appears to display specificity and affinity appropriate to the *beta* adrenergic receptors (11–16). In this communication we describe detailed studies of the structure-activity relationships which determine the affinity of *beta* adrenergic agonists and antagonists for these receptor binding sites in frog erythrocyte membranes. The binding data are compared with data for stimulation or competitive inhibition of adenylate cyclase present in the same membranes. The direct binding data are in reasonable agreement with structure-activity relationships for *beta* adrenergic receptors previously delineated in membrane (adenylate cyclase) (5–10) or more intact preparations (1). These are the first studies to delineate in detail these relationships by direct binding techniques.

MATERIALS AND METHODS

Materials. The drugs used in this study were (+)- and (−)-alprenolol hydrochloride and (±)-*p*-alprenolol hydrochloride (Hassle Pharmaceuticals); (+)- and (−)-propranolol hydrochloride and practolol hydrochloride (Ayerst); (−)-isoproterenol hydrochloride and bitartrate, (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, (±)-octopamine hydrochloride, dopamine hydrochloride, tyramine hydrochloride, phenethylamine, (±)-dihydroxyphenylalanine, dihydroxymandelic acid, and (±)-normetanephrine hydrochloride (Sigma); pyrocate-

[3] Unpublished observations.

chol (Mann); (+)-isoproterenol bitartrate, (+)-epinephrine bitartrate, (+)-norepinephrine bitartrate, (±)-deoxyisoproterenol hydrochloride, (±)-chlorisoproterenol hydrochloride, (±)-isoetharine hydrochloride, (±)-*N*-cyclopentylbutanephrine hydrochloride, (±)-butanephrine hydrochloride, (±)-ethylnorepinephrine, (±)-Cobefrin hydrochloride, and (±)-phenylephrine hydrochloride (Sterling-Winthrop); (±)-metaraminol bitartrate (Merck Sharp & Dohme); (±)-Cc34 benzoate, (±)-Cc25 benzoate and (±)-ritodrine hydrochloride (Phillips-Durphar, Netherlands); (±)-protokylol hydrochloride (Lakeside); (±)-dichlorisoproterenol hydrochloride, dobutamine hydrochloride, (±)-ephedrine hydrochloride, 46868, and 46220 (Lilly); (−)-oxprenolol hydrochloride and phentolamine mesylate (Ciba); (±)-butoxamine hydrochloride (Burroughs-Wellcome); S-37260, S-40032-7, and 16483-6 (Aldrich); phenoxybenzamine (Smith Kline & French); and (−)-soterenol, (±)-soterenol hydrochloride, (±)-isoxsuprine hydrochloride, (±)-amidephrine hydrochloride, (±)-MJ 7963-1, (±)-MJ 8798-1, (±)-MJ 9184-1, and (±)-MJ 7434-1 (Mead Johnson). (±)-Hydroxybenzylpindolol was a generous gift from Dr. G. Aurbach.

cAMP,[4] ATP, phosphoenolpyruvate, and myokinase were obtained from Sigma Chemical Company. Pyruvate kinase was purchased from Calbiochem. [^{3}H]cAMP (1–5 Ci/mmole) and [α-^{32}P]ATP (10–20 Ci/mmole) were New England Nuclear products.

Grass frogs were obtained from Nasco-Steinhilber. Dowex AG50W-X8 was obtained from Bio-Rad, and alumina, neutral grade, from Nutritional Biochemicals.

(−)-Alprenolol was tritiated at New England Nuclear Corporation by catalytic reduction with tritium gas, using palladium as the catalyst, to a specific activity of 17–33 Ci/mmole. (−)-[^{3}H]Alprenolol is used here to identify the compound resulting from catalytic reduction of (−)-alprenolol with tritium. (−)-Alprenolol contains an unsaturated bond in the aliphatic chain at position 2 of the aromatic ring. The compound, in all likelihood, therefore, is (−)-[^{3}H]dihydroalprenolol. This material was homogeneous in each of the following thin-layer systems on silica gel plates: (a) ethanol–water, 88:12, R_F 0.31; (b) butanol–acetic acid–water, 25:4:10, R_F 0.68; (c) chloroform–methanol–ammonium hydroxide, 6:4:2 drops, R_F 0.67; (d) acetone–benzene–acetic acid, 70:25:5, R_F 0.24; (e) methanol–benzene–water, 15:2:3, R_F 0.36; (f) chloroform–acetone–diethylamine, 5:4:1, R_F 0.8. In each of these systems labeled and native alprenolol had identical R_F values.

When chromatography is performed on silica gel plates impregnated with 5% silver nitrate ("argentation" chromatography), compounds differing by only a single unsaturated bond may be separated (17). When labeled material was chromatographed on such plates and compared with native alprenolol, the following results were obtained: (a) acetone–benzene–acetic acid, 70:25:5, (−)-alprenolol R_F 0.65, (−)-[^{3}H]alprenolol R_F 0.83; (b) methanol–benzene–water, 15:2:3, (−)-alprenolol R_F 0.4, (−)-[^{3}H]alprenolol R_F 0.83.

When the tritiated material was chromatographed alone on silver nitrate-treated plates and the plates were examined under ultraviolet light, only a single spot was seen, corresponding to the R_F of the radioactivity. Thus no native, unreacted alprenolol was found contaminating the tritiated material.

The finding that the specific activity of the labeled material was less than "theoretical" (i.e., less than 60 Ci/mmole) was presumably due to tritium exchange with solvent during the labeling procedure, which could lead to reduction of some double bonds with hydrogen rather than tritium atoms.

We tested the biological activity of several lots of the labeled material with specific radioactivities ranging from 10–33 Ci/mmole as antagonists of isoproterenol-activated adenylate cyclase. In all cases the K_D obtained (approximately 5 nM) was the same, and was in turn identical with that of unlabeled native (−)-alprenolol. Since the tritiated material contained no unreacted native (−)-alprenolol, these as-

[4] The abbreviation used is: cAMP, adenosine cyclic 3′,5′-monophosphate.

says represent valid potency estimates of the tritrated compound.

Membrane preparation. Heparinized blood from southern grass frogs (*Rana pipiens*) maintained at 23° was collected by cardiac puncture, and the red cells were washed three times by centrifugation with a solution of 110 mM NaCl and 10 mM Tris-HCl, pH 7.4. The buffy coat was removed by aspiration. Erythrocytes were lysed in water and homogenized with a glass-Teflon homogenizer; the lysate was immediately adjusted to 5 mM Tris-HCl, pH 8.1, and 2 mM $MgCl_2$, and centrifuged at 4° in a Sorvall RC-2B instrument at 30,000 × *g* for 15 min. The pellet was washed three times, and the membranes were resuspended in 75 mM Tris-HCl buffer, pH 8.1, and 25 mM $MgCl_2$ by homogenization.

For some studies a more purified membrane fraction was obtained as follows. After the first centrifugation of the lysate, the pelleted material was resuspended by homogenization in 10 mM Tris-HCl, pH 8.1, and 10 mM $MgCl_2$, and centrifuged at 4° in a Sorvall GLC-1 (swinging bucket) centrifuge at 2000 × *g* for 10 min over a cushion (7 ml/30 ml of preparation) of the same buffer containing 50% sucrose. The material which sedimented through sucrose was discarded, whereas the supernatant was centrifuged at 30,000 × *g* for 15 min. The pellet was washed once with the same buffer and resuspended in 75 mM Tris-HCl, pH 8.1, and 25 mM $MgCl_2$. Adenylate cyclase assays were generally performed on the less purified preparations. Basal adenylate cyclase activity in these preparations (18 ± 5 pmoles of cAMP generated per minute per milligram of protein) was stimulated 8–15-fold by isoproterenol (100 μM).

Assays of (−)-[^{3}H]alprenolol binding were usually performed on the more purified membrane preparations, since specific binding of (−)-[^{3}H]alprenolol in these preparations was about 90% of total binding. Apparent dissociation constants of various adrenergic agents for adenylate cyclase and (−)-[^{3}H]alprenolol binding determined on the two types of preparations were the same.

Membranes were prepared fresh each day for each experiment, as were solutions of the agents tested.

Adenylate cyclase assays. These were performed as previously described (18, 19) by incubating membranes, agonists, and/or antagonists in a medium containing 25 mM Tris-HCl buffer (pH 8.1), 10 mM $MgCl_2$, 0.1 mM cAMP, 1.5 mM ATP, 1–2 μCi of [α-^{32}P]ATP, 5 mM phosphoenolpyruvate, 40 μg/ml of pyruvate kinase, and 20 μg/ml of myokinase in a volume of 50 μl for 10 min at 37°. At the completion of incubations, reactions were terminated by addition of 1 ml of a solution containing 100 μg of ATP, 50 μg of cAMP, and 15,000 cpm of [^{3}H]cAMP. [^{32}P]cAMP formed was isolated as described by Salomon *et al.* (20). Product recovery was monitored by following the recovery of [^{3}H]cAMP.

The affinity and activity of agonists were assessed by plotting dose-response curves for stimulation of enzyme activity. Typical results with several agonists are shown in Fig. 1A. K_D values of agonists were taken as the concentrations of each agent causing half the maximal enzyme stimulation observed with that agent. Isoproterenol dose-response curves were plotted in all experiments. The intrinsic activity (21) of each agent was taken as the maximum enzyme stimulation with the agent divided by maximum enyzme stimulation due to isoproterenol.

The affinity of antagonists for the adenylate cyclase-coupled *beta* adrenergic receptors was assessed as described by Mayer (7), by quantitating the ability of antagonists to cause a parallel rightward shift of the isoproterenol dose-response curve. Typical results are shown in Fig. 2A. All antagonists were tested at two or three different concentrations, generally selected to produce 5–100-fold shifts in the isoproterenol dose-response curve. Dissociation constants were then calculated from the equation K_B = [antagonist]/(CR−1) (22), where CR = ratio of equiactive concentrations of isoproterenol in the presence and absence of the given concentration of the antagonist. Equiactive concentrations of isoproterenol were generally assessed at the midpoints of the parallel portions of the stimulation curves

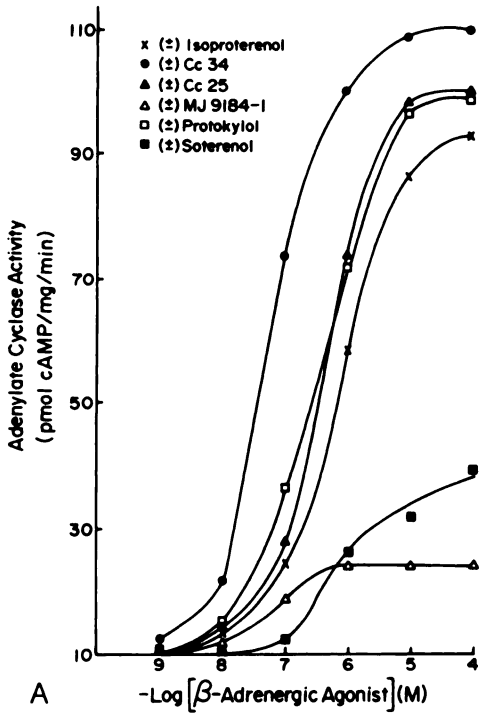


FIG. 1. *β-adrenergic agonists*

A. Stimulation of frog erythrocyte adenylate cyclase by potent *beta* adrenergic agonists. The results shown are the means of three experiments determined in duplicate, save for soterenol (two experiments).

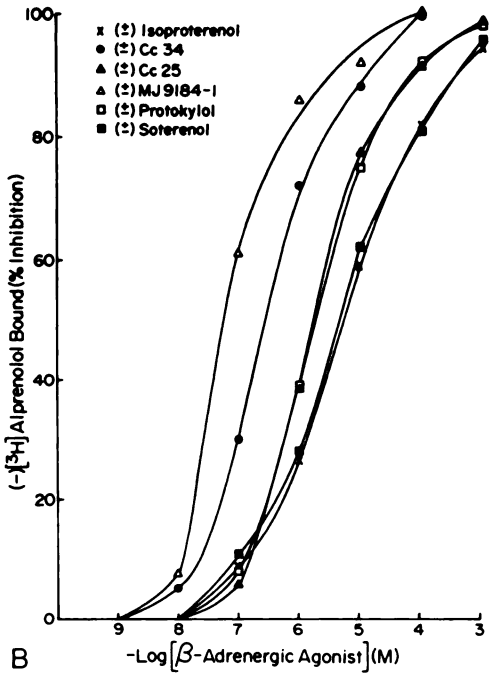


FIG. 1. *β-adrenergic agonists*

B. Inhibition of (−)-[^{3}H]alprenolol binding to frog erythrocyte membranes by potent *beta* adrenergic agonists. Values shown are the means of four determinations from two experiments.

(see Fig. 2A). The values shown in the various tables represent the means of values determined at all concentrations of the antagonist tested in two to six separate experiments.

In preliminary experiments we determined that comparable results were obtained in this system when antagonists were added 30–45 min prior to agonists and when agonists and antagonists were added simultaneously at the beginning of incubations. These findings are in agreement with those of Kaumann and Birnbaumer (6) in cardiac membranes and suggest that problems of diffusion and equilibration with receptors are less important in these subcellular membranes than in intact tissue preparations. In our experiments agonists and antagonists were generally added simultaneously.

Adenylate cyclase and binding assays were performed under identical conditions of incubation save for the presence of ATP in the enzyme assays. We have previously demonstrated (12) that ATP and other nucleotides (15) are without effect on (−)-[^{3}H]alprenolol binding.

Binding assay. (−)-[^{3}H]Alprenolol binding assays were performed essentially as previously described (12). (−)-[^{3}H]-Alprenolol, 25 nM, was incubated with 0.3–0.4 mg of membrane protein for 10 min at 37° in a medium containing 50 mM Tris-HCl (pH 8.1) and 15 mM $MgCl_2$ in a volume of 150 μl. At the completion of the incubations, duplicate 50-μl aliquots were placed over 300 μl of the incubation buffer in small polyethylene centrifuge tubes and centrifuged for 1 min in a Beckman microfuge 152. The membranes and bound (−)-[^{3}H]alprenolol were pelleted almost immediately. The surface of the pellet was washed once with the same buffer, and the pellet was solubilized overnight by shaking with 0.5 ml of 10% sodium dodecyl sulfate and 10 mM EDTA and then counted in a liquid scintillation spectrometer after addition of a Triton X-100 toluene-based

fluor. In all experiments the amount of (−)-[^{3}H]alprenolol "nonspecifically" bound and/or trapped in the membrane pellets was determined by incubating membranes and (−)-[^{3}H]alprenolol in the presence of 10 μM (±)-alprenolol or propranolol, which blocked all *beta* adrenergic receptor binding sites (12). "Nonspecific" binding was generally 10–15% of total binding and was subtracted from all experimental values. "Specific binding" in purified membrane preparations ranged from 2 to 2.5 pmoles of (−)-[^{3}H]alprenolol bound per milligram of protein.

This binding method was validated by equilibrium dialysis. In three experiments, with (−)-[^{3}H]alprenolol present at 10 nM, specific binding as assessed by the centrifugation method was 1.30 ± 0.2 pmoles/mg of protein and, by equilibrium dialysis (18 hr, 4°), 1.37 ± 0.14 pmoles/mg of protein (not a statistically significant difference). Thus binding as assessed by this simple centrifugation assay was a true reflection of equilibrium binding. It should be noted that the half-time for dissociation of (−)-[^{3}H]alprenolol from the receptors (about 2 min at 37°; about 5 min at room temperature, at which centrifugation was performed) was sufficiently slow relative to the time needed to pellet the membranes (a few seconds) that insignificant dissociation occurred during the procedure (23, 24).

In all tables the K_D values for binding listed for the various agents were calculated from the concentrations causing 50% inhibition of specific binding, using the following relationship as described by Cheng and Prusoff (25): $EC_{50} = (1 + S/K_m)K_D$ where K_m denotes the equilibrium dissociation constant of (−)-[^{3}H]alprenolol determined by direct binding studies, S is the concentration of (−)-[^{3}H]alprenolol present in the binding assays, EC_{50} is the concentration of a ligand causing 50% inhibition of specific (−)-

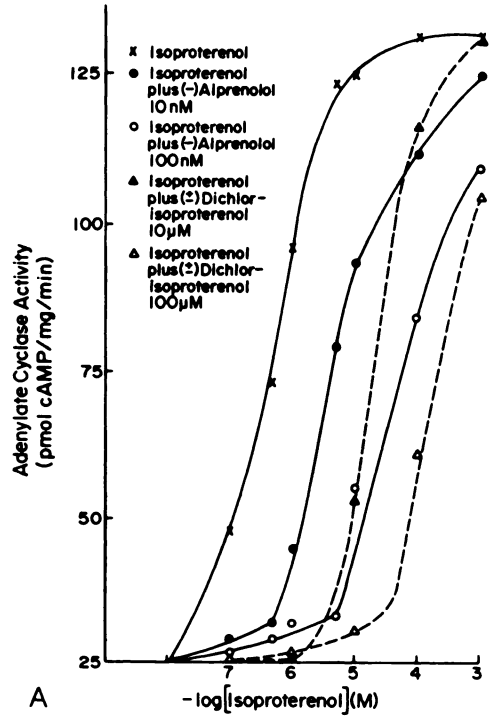


FIG. 2. *β-adrenergic antagonists*

A. Antagonism of isoproterenol stimulation of frog erythrocyte membrane adenylate cyclase by (−)-alprenolol and (±)-dichlorisoproterenol. Results are the means of two experiments determined in duplicate.

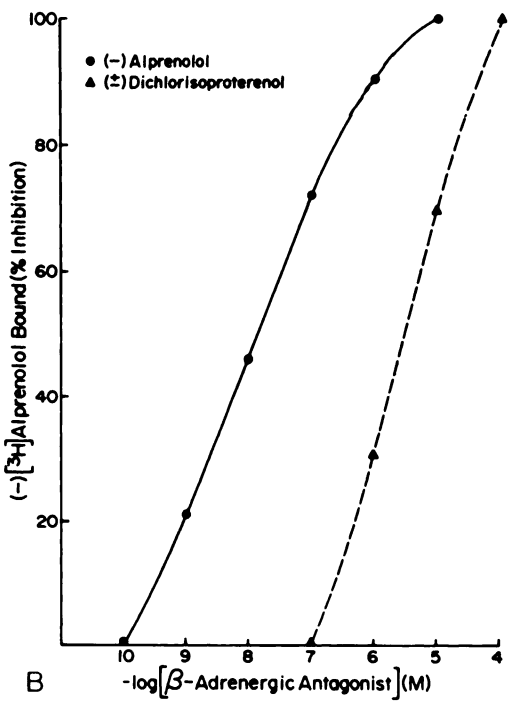


FIG. 2. *β-adrenergic antagonists*

B. Inhibition of (−)-[^{3}H]alprenolol binding to frog erythrocyte membranes by (−)-alprenolol and (±)-dichlorisoproterenol. Values shown are the means of four determinations from two experiments.

[^{3}H]alprenolol binding, the K_D is the apparent dissociation constant of the ligand. Figures 1B and 2B present typical data for several agonists and antagonists. Elsewhere we have demonstrated by saturation binding experiments that the K_D of (−)-[^{3}H]alprenolol for these *beta* adrenergic receptors is 5–8 nM (12). We also have demonstrated that binding of (−)-[^{3}H]alprenolol to frog erythrocyte membranes is rapid (equilibrium within 2–4 min at 37°) and rapidly reversible ($t_{1/2}$ about 2 min) (12, 23, 24). All K_D values listed in the tables [(−)-[^{3}H]alprenolol binding and adenylate cyclase] represent the means of values determined in two to six experiments for each agent.

Protein was determined by the method of Lowry *et al.* (26).

RESULTS

Several agents known to be devoid of *beta* adrenergic activity were inactive as agonists or antagonists on the frog erythrocyte adenylate cyclase and did not compete for the binding sites at concentrations less than 100 μM. These included phenethylamine, pyrocatechol, dihydroxymandelic acid, (±)-dihydroxyphenylalanine, the metabolite (±)-normetanephrine, and *alpha* adrenergic antagonists phentolamine and phenoxybenzamine.

Studies with agonists. Table 1 depicts the structures of a series of *beta* adrenergic catecholamines with substituents of increasing size on the amino nitrogen. All these compounds were tested as racemic mixtures. Affinity for the binding sites progressively increased with increasing size of the substituent on the amino nitrogen. Branched alkyl-aryl substituents (Cc34, Cc25, protokylol) were associated with particularly high affinity. However, a nonaromatic, 5-membered ring (cyclopentylbutanephrine) was associated with much lower affinity. All the more potent agonists in this series had intrinsic activity equal to or greater than that of isoproterenol.

The presence of a substituent on the α-carbon had variable effects on affinity. A methyl or ethyl group on the α-carbon of norepinephrine (Cobefrin, ethylnorepinephrine) produced a slight increase in affinity. However, an ethyl substituent on the α-carbon of isoproterenol (isoetharine) produced a decrease in affinity.

The *beta* adrenergic agonists depicted in Table 2 are not catecholamines. In place of the hydroxyl group on position 3 of the aromatic ring there is methanesulfonamide substituent. In agreement with the findings of Grunfeld *et al.* (10), these agents displayed only partial intrinsic activity (Fig. 1A). Nonetheless, they had reasonably high affinity for the receptors, as shown by the binding studies. As with the catecholamine agonists, a large substituent on the amino nitrogen increased affinity. Thus MJ 9184-1, which has a very large substituent, had considerably higher affinity than soterenol, which bears an isopropyl group on the amino nitrogen. Still the intrinsic activity of MJ 9184-1 was no greater than that of soterenol, suggesting that the size of the substituent on the amino nitrogen is not an important determinant of intrinsic activity of agonists. The greater potency (affinity) of MJ 9184-1 over soterenol is quite analogous to the greater potency of Cc34 over isoproterenol (Table 1). The direct binding assays greatly facilitated the evaluation of potency of agonists with low intrinsic activity, because the ability of agents to compete for the receptor binding sites depended only on their affinity and not on intrinsic activity. A potent, high-affinity *partial agonist* such as MJ 9184-1 gives a *full* displacement curve in the binding assays (compare Fig. 1A and B).

The affinities of the agonists shown in Table 3 for the *beta* adrenergic receptor binding sites were compared in order to assess the role of the β-carbon hydroxyl in determining affinity and activity. (+)-Norepinephrine and dopamine had comparable, albeit weak, affinity for the sites, as did (+)-soterenol and β-deoxysoterenol. This suggests that the β-carbon hydroxyl is not essential for affinity and that the weak effects of the (+) isomers of agonists were likely due to the (+) isomers themselves, rather than to a contaminating (−) isomer. (+)-Norepinephrine and (+)-soterenol had weak intrinsic activity on adenylate cyclase, whereas dopamine and β-deoxysoterenol had no activity, suggesting that the β-carbon hydroxyl may play a role in

TABLE 1

Interaction of catecholamine agonists with (−)-[³H]alprenolol binding sites and adenylate cyclase

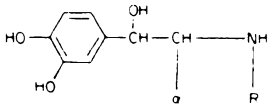
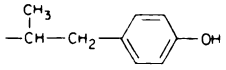
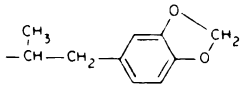
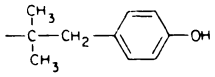
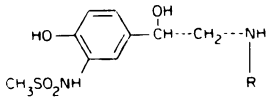
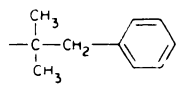
Compound		(−)-[³H]alprenolol binding, K_D	Adenylate cyclase	
			K_D	Intrinsic activity
	α	R	μM	μM	
(±)-Norepinephrine	H	H	>200	90 ± 42	0.54 ± 0.18
(±)-Cobefrin	CH_3	H	28 ± 11	41 ± 6.9	0.87 ± 0.18
(±)-Ethylnorepinephrine	C_2H_5	H	18 ± 10	38 ± 25	0.71 ± 0.10
(±)-Epinephrine	H	CH_3	8.8 ± 0.7	10 ± 7.0	0.75 ± 0.14
(±)-Isoproterenol	H		1.7 ± 0.6	1.4 ± 0.6	1.00 ± 0
(±)-Isoetharine	C_2H_5		24 ± 2.8	45 ± 20	0.72 ± 0
(±)-Cyclopentylbutanephrine	C_2H_5		39 ± 0	31 ± 2.8	0.64 ± 0.05
(±)-Cc25	H		0.68 ± 0.01	0.47 ± 0.12	1.08 ± 0.15
(±)-Protokylol	H		0.68 ± 0.01	0.42 ± 0.02	1.09 ± 0.18
(±)-Cc34	H		0.060 ± 0.003	0.08 ± 0.02	1.20 ± 0.15

TABLE 2

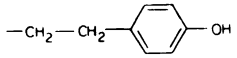
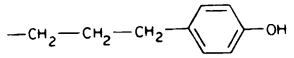
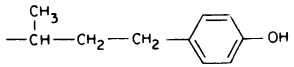
Interaction of methanesulfonamide agonists with (−)-[³H]alprenolol binding sites and adenylate cyclase

Compound		(−)-[³H]Alprenolol binding, K_D	Adenylate cyclase	
			K_D	Intrinsic activity
	R	μM	μM	
(±)-Soterenol		2.40 ± 0.46	1.0 ± 0.6	0.28 ± 0.04
(±)-MJ 8798-1		0.65 ± 0.06	3.3 ± 1.1	0.24 ± 0.09
(±)-MJ 9184-1		0.014 ± 0.001	0.08 ± 0.01	0.27 ± 0.02

determining intrinsic activity as well as affinity. The β-carbon hydroxyl is not required for intrinsic activity, however. Thus, as shown in Table 3, addition of large substituents on the amino nitrogen of dopamine produces agents such as dobutamine, which do have weak intrinsic activity even in the absence of the β-carbon

TABLE 3

Role of β-carbon hydroxyl group in determining affinity and activity of agonists

Compound	4	3	β	R	(−)-[^{3}H]Alprenolol binding, K_D	Adenylate cyclase	
						K_D	Intrinsic activity
					μM	μM	
(−)-Norepinephrine	OH	OH	OH(−)	H	49 ± 2	150 ± 10	0.50 ± 0.1
(+)-Norepinephrine	OH	OH	OH(+)	H	>200	>1000	0.10 ± 0.05
Dopamine	OH	OH	H	H	>200	>1000[a]	0
Lilly 46868	OH	OH	H		47 ± 26	21 ± 1.0	0.25 ± 0.02
Lilly 46220	OH	OH	H		24 ± 2	9 ± 2.5	0.17 ± 0.02
Dobutamine	OH	OH	H		23 ± 3	9 ± 4.0	0.25 ± 0.03
(−)-Soterenol	OH	CH_3SO_2NH	OH(−)		0.7 ± 0.1	0.30 ± 0.07	0.19 ± 0.06
(+)-Soterenol	OH	CH_3SO_2NH	OH(+)		29.8 ± 0.2	30 ± 22	0.12 ± 0.04
β-Deoxysoterenol	OH	CH_3SO_2NH	H		47 ± 5	63 ± 36[a]	0

[a] The agent had no intrinsic activity on adenylate cyclase, and its affinity was determined as an antagonist.

hydroxyl. The binding studies also clearly indicate that the addition of these substituents increases affinity for the receptors (cf. dobutamine and dopamine).

The stereoconfiguration of the β-carbon hydroxyl of *beta* adrenergic agonists and antagonists has long been recognized as a crucial structural feature in determining affinity for the *beta* adrenergic receptor (1). Table 4 summarizes inidvidual affinity values and stereoisomeric potency ratios for six pairs of *beta* agonist and antagonist stereoisomers. Data from both binding and adenylate cyclase assays are presented. In all cases the (−) isomers are considerably more potent than the (+) isomers. The potency ratios determined from binding and cyclase assays agree closely and, in turn, are in good agreement with ratios determined by others in more intact preparations (1).

Studies with antagonists. Tables 5–7 summarize the structure-activity relationships for a variety of *beta* adrenergic antagonists. In addition to determining the affinity of each agent for the (−)-[^{3}H]alprenolol binding sites, we also determined an equilibrium dissociation constant of each agent for the *beta* adrenergic receptors linked to adenylated cyclase. As described under MATERIALS AND METHODS, this was determined by testing the ability of several concentrations of each antagonist to shift the isoproterenol dose-response curve to the right. All antagonists were also tested as agonists and had no intrinsic activity on the frog erythrocyte membrane adenylate cyclase. Although several laboratories have previously studied the effects of some of the agents we used on erythrocyte or other adenylate cyclase systems, in most of these studies K_B values were not determined and only relative potencies were reported (9, 10). It is apparent from the data in Tables 5–7 that the affinities of the various antagonists for the receptors determined by competition for the (−)-[^{3}H]alprenolol binding sites and by competitive inhibition of isoproterenol-stimulated adenylate cyclase are comparable.

The antagonists shown in Table 5 are all phenylethanolamine derivatives, as are the *beta* adrenergic agonists. However, in each case some modification of the basic catechol nucleus of the agonist molecule was made. This led to a complete loss of intrinsic activity, as previously noted by others (1, 10). None of the agents shown in Table 5 stimulated adenylate cyclase in concentrations tested up to 100 μM. As with the agonists, increasing the size of the substituent on the amino nitrogen in-

TABLE 4

Stereoisomeric potency ratios of beta adrenergic agonists and antagonists determined by (−)-[^{3}H]alprenolol binding and adenylate cyclase studies

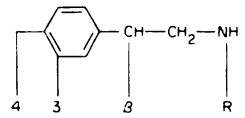
Compound	K_D		Stereoisomeric potency ratio	
	(−)-[^{3}H]Alprenolol binding	Adenylate cyclase	(−)-[^{3}H]Alprenolol binding	Adenylate cyclase
	μM			
(−)-Norepinephrine	49 ± 2	150 ± 10	4	—[a]
(+)-Norepinephrine	>200	>1000		
(−)-Epinephrine	4.6 ± 0.2	15 ± 0	30	53
(+)-Epinephrine	137 ± 4	800 ± 57		
(−)-Isoproterenol	0.400 ± 0.005	0.3 ± 0	407	2333
(+)-Isoproterenol	183.0 ± 0.6	700 ± 114		
(−)-Soterenol	0.7 ± 0.1	0.30 ± 0.07	42	100
(+)-Soterenol	29.8 ± 0.2	30 ± 22		
(−)-Alprenolol	0.0034 ± 0.0002	0.007 ± 0.002	50	30
(+)-Alprenolol	0.15 ± 0.005	0.21 ± 0.05		
(−)-Propranolol	0.0046 ± 0.0003	0.004 ± 0.001	70	45
(+)-Propranolol	0.286 ± 0.008	0.18 ± 0.07		

[a] (+)-Norepinephrine was too weak to calculate a valid K_D value (adenylate cyclase) or potency ratio.

TABLE 5

Affinity of some "phenylethanolamine" antagonists for (−)-[^{3}H]alprenolol binding sites and adenylate cyclase

K_D values were determined as described under MATERIALS AND METHODS.

Compound					(−)-[^{3}H]Alprenolol binding, K_D		Adenylate cyclase, K_B	
	4	3	β	R	μM		μM	
Tyramine	OH	H	H	H	>200		>1000	
(±)-Octopamine	OH	H	OH	H	>200		>1000	
(±)-16483-6	H	Cl	OH	H	83	± 25	310	± 140
(±)-Amidephrine	H	CH_3SO_2NH	OH	CH_3	65	± 11	220	± 106
(±)-Phenylephrine	H	OH	OH	CH_3	52	± 4	135	± 54
(±)-S-40032-7	H	OH	OH	C_2H_5	22	± 4	51	± 22
(±)-Deoxyisoproterenol	OH	H	OH		15	± 2	11	± 4.1
(±)-*p*-Chloroisoproterenol	Cl	OH	OH		4.2	± 0.8	1.3	± 0.4
(±)-Dichlorisoproterenol	Cl	Cl	OH		0.57	± 0.03	0.4	± 0.1
(±)-Sotalol	CH_3SO_2NH	H	OH		1.3	± 0.1	1.6	± 0.7

creased affinity for the binding sites. The stereoconfiguration at the β-carbon hydroxyl was also an important factor in determining affinity in antagonists (as shown in Table 4).

The nature of the substituents on the phenyl ring had variable effects on affinity for the receptor binding sites. Two chlorine atoms in positions 3 and 4 [(±)-dichlorisoproterenol] were associated with reasonably high affinity, in the micromolar range. This value is quite close to that for (±)-isoproterenol, which is a strong agonist. Thus substitution of the two ring hydroxyls by chlorine atoms does not affect affinity for the receptors, but converts an agonist to a competitive antagonist. One chlorine and one hydroxyl in positions 4 and 3 on the ring (*p*-chloroisoproterenol), or one hydroxyl and one hydrogen ("deoxyisoproterenol") were associated with somewhat lower affinity. Sotalol was considerably more potent than deoxyisoproterenol, indicating that methanesulfonamide is a more effective substituent on position 4 of the ring than a hydroxyl. However, amidephrine and phenylephrine had comparable affinities, suggesting that methanesulfonamide is no more effective than a hydroxyl group when this is the only substituent on the ring and present in position 3.

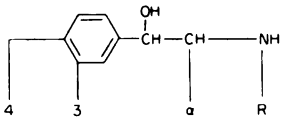
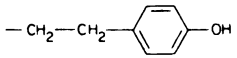
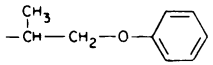
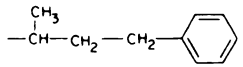
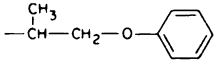
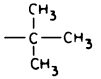
The antagonists depicted in Table 6 are all derivatives of phenylpropanololamine. As with all the other *beta* adrenergic agents studied, increasing the size of the substituent on the amino nitrogen increased affinity for the *beta* adrenergic binding sites.

In general, the presence of a methyl group on the α-carbon in this series of compounds did not strikingly affect affinity as compared with agents of comparable structure without this group shown in Table 5. A larger substituent on the α-carbon, however, such as an ethyl group, did decrease affinity (compare MJ 7434-1 in Table 6 with sotalol in Table 5).

As with the phenylethanolamine derivatives, the nature of the substituents on the phenyl ring affected affinity. Thus MJ 7963-1 was more potent than isoxsuprine. This is presumably attributable to the pres-

TABLE 6

Affinity of some "phenylpropranolamine" antagonists for (−)-[^{3}H]alprenolol binding sites and adenylate cyclase

Compound					(−)-[^{3}H]Alprenolol binding, K_D	Adenylate cyclase, K_B
	4	*3*	*α*	*R*	*μM*	*μM*
(±)-S-37260-9	H	H	C_2H_5OH	H	62 ± 14	140 ± 41
(±)-Metaraminol	H	OH	CH_3	H	35 ± 11	30 ± 3.2
(±)-Ephedrine	H	H	CH_3	CH_3	9 ± 1	85 ± 8.6
(±)-MJ 7434-1	$CH_2SO_2NH_2$	H	C_2H_5		14 ± 1	4.5 ± 1.6
(±)-Ritodrine	OH	H	CH_3		8.8 ± 1.1	1.8 ± 0.5
(±)-Isoxsuprine	OH	H	CH_3		1.4 ± 0.4	0.24 ± 0.12
(±)-Nylidrin	OH	H	CH_3		0.18 ± 0.02	0.13 ± 0.05
(±)-MJ 7963-1	OH	$CH_3SO_2N(CH_3)_2$	CH_3		0.075 ± 0.01	0.06 ± 0.02
(±)-Butoxamine	2-OCH_3	5-OCH_3	CH_3		7.8 ± 0.2	2.3 ± 1.1

ence of the $CH_3SO_2N(CH_3)$ group in position 3 of MJ 7963-1 rather than the hydrogen in isoxsuprine.

The antagonists listed in Table 7 are aryloxyethanolamine derivatives. As previously determined in a variety of intact and subcellular systems (1, 6), the presence of the ether function between the ethanolamine side chain and the aromatic ring is associated with very high affinity for the (−)-[^{3}H]alprenolol binding sites. Two agents in this series had only modest affinity for the receptors despite the presence of the ether function. Both of these, practolol and H64/52 ("*p*-alprenolol"), contain a single substituent on the phenyl ring in the *para* position. This configuration has been demonstrated to be associated with a reduction of affinity for the adenylate cyclase-coupled *beta* adrenergic receptors (10). (±)-Hydroxybenzylpindolol, which has a large substituent on the amino nitrogen as well as an ether function, was the most potent agent of all.

Figure 3 is a plot of K_D values determined by (−)-[^{3}H]alprenolol binding vs. those determined in the adenylate cyclase assays. The plot includes values for all the agonists and antagonists studied except those which were too weak to determine accurately (K_D values listed in tables as more than 1000 or more than 200). The agents studied displayed affinities for the receptors which varied over six orders of magnitude. The correlation coefficient of 0.95 indicates the good agreement between the two sets of data.

DISCUSSION

The data presented here indicate the feasibility of studying the detailed structure-activity relationships of *beta* adrenergic receptors by direct binding studies. The results are in agreement with previously reported data determined by studies of adenylate cyclase activation (5–10) as well

TABLE 7

Affinity of some "aryloxyethaolamine" antagonists for (−)-[^{3}H]alprenolol binding sites and adenylate cyclase

Compound	(−)-[^{3}H]Alprenolol binding, K_D	Adenylate cyclase, K_B
	μM	*μM*
(−)-Propranolol	0.0046 ± 0.0003	0.004 ± 0.001
(+)-Propranolol	0.286 ± 0.008	0.18 ± 0.07
(−)-Alprenolol	0.0034 ± 0.0002	0.007 ± 0.002
(+)-Alprenolol	0.150 ± 0.005	0.21 ± 0.05
(−)-Oxprenolol	0.0078 ± 0.0006	0.009 ± 0.006
(±)-H64/52 (*p*-alprenolol)	2.94 ± 0.55	6.6 ± 3.8
(±)-Practolol	20.9 ± 0.9	7.8 ± 3.2
(±)-Hydroxybenzylpindolol	0.0011 ± 0.0002	0.0014 ± 0.0003

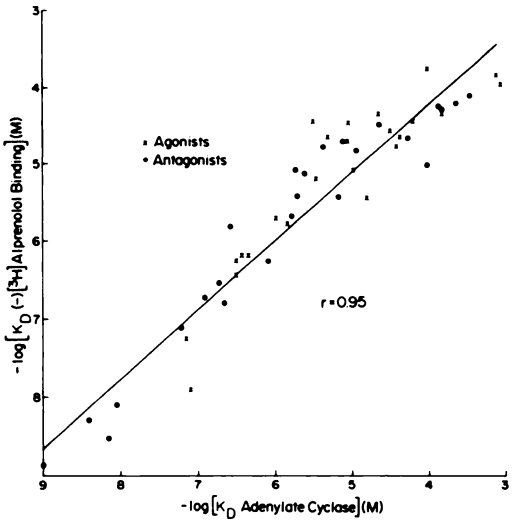


FIG. 3. *K_D values of beta adrenergic agents determined by (−)-[^{3}H]alprenolol binding and adenylate cyclase assays*

Individual values used are those listed in Tables 1–7. The line was drawn by regression analysis.

as data obtained in a variety of intact tissue preparations (1).

Previously the most direct means of assessing the molecular properties of *beta* adrenergic receptors was by studies of adenylate cyclase activation. However, binding studies such as those reported here represent the most direct way of studying these receptors (11–16, 27–29). Validation of the hypothesis that the sites labeled with (−)-[^{3}H]alprenolol are in fact equivalent to the adenylate cyclase-coupled *beta* adrenergic receptors in the membranes rests on the close agreement obtained in the binding and enzyme studies. Grunfeld *et al.* (10) underscored the fact that "characterization of purified *beta* adrenergic receptors must ultimately include correlation of binding by the receptor complex with the complete structure-activity relationships documented for adenylate cyclase."

The frog erythrocyte membranes used for these experiments provide a simple, convenient model system for study of *beta* adrenergic receptors linked to adenylate cyclase (5, 9, 10, 18, 30). Rosen *et al.* (9) and Grunfeld *et al.* (10) studied the structure-activity relationships of agents which stimulate or inhibit the frog erythrocyte membrane adenylate cyclase. Nonetheless, we chose to determine K_D values for both interaction with adenylate cyclase and the (−)-[^{3}H]alprenolol binding sites for all the agents we studied, for several reasons. First, although there is some overlap between the agents we studied and those studied by other groups, many of the agents we used have not previously been tested in this system. Second, even where agents had been previously tested, actual K_D values were often not reported (9, 10), precluding quantitative comparison of potencies determined by enzyme and binding studies.

The data presented here indicate that the detailed specificity apparent in *beta* adrenergic responses can be attributed solely to the specificity of binding of *beta* adrenergic agents to the *beta* adrenergic receptor binding sites. The data also suggest that agonists and antagonists compete for the same set of binding sites. In no case was the potency of an agonist or antagonist as determined by adenylate cyclase studies discordant with the potency determined simply on the basis of ability to compete for occupancy of the (−)-[^{3}H]alprenolol binding sites.

The K_D values of the various agents determined by binding and adenylate cyclase studies were quite comparable. It should be borne in mind, however, that a variety of factors may influence the relationship between the binding EC_{50} and K_D, such as the concentration of receptors present, cooperative interactions between receptors, or presence of spare receptors (31).

By performing binding and enzyme studies in parallel on all agents, we were able to distinguish those structural features of ligands which are major determinants of receptor affinity and those which determine intrinsic activity. Different features of the *beta* adrenergic ligand molecule determined these two parameters of drug action. The binding data clearly indicate that the major determinants of receptor affinity were the stereoconfiguration of the β-carbon hydroxyl and the size of the substituent on the amino nitrogen. Substituents on the α-carbon had relatively little effect on affinity. The nature and number of substituents on the catechol ring had

only a small effect on receptor affinity. By contrast, the nature of the substituents on the catechol ring was crucial in determining intrinsic activity. Only the 3,4-dihydroxyl configuration was compatible with full intrinsic activity. CH_3SO_2NH in position 3 of the catechol ring produces agents, such as soterenol or MJ 9184-1, which have reduced intrinsic activity. Deletion of one or both hydroxyls, or their substitution by chlorine atoms, produces agents with no intrinsic activity (antagonists), but only minimally altered affinity for the receptor binding sites.

The β-carbon hydroxyl appeared to be important in determining both affinity and intrinsic activity. Thus, as noted above, a (−) configuration of this substituent conferred approximately 100 times more affinity than the (+) configuration. Compounds with no hydroxyl group on the β-carbon generally had affinity equivalent to that of similar agents having a (+)-hydroxyl group at this position. However, the agents with no β-carbon hydroxyl had reduced intrinsic activity in this system. Thus agents such as dobutamine had only partial intrinsic activity, indicating a role for the β-carbon hydroxyl in determining activity at the *beta* receptors.

The binding data and the considerations outlined above highlight the independence of receptor affinity and intrinsic activity. High-affinity partial agonists such as soterenol or MJ 9184-1 gave binding displacement curves indistinguishable from those obtained with full agonists such as isoproterenol or Cc34. Thus binding assays provide information only about receptor affinity and not about intrinsic activity. The latter can be assessed only by an assay which measures some parameter of biological activity, such as activation of an enzyme or change in a physiological variable. The most complete understanding of structure-activity relationships can be obtained by studying receptor binding and biological activity in parallel.

ACKNOWLEDGMENT

We gratefully acknowledge the expert assistance of the analytical chemistry department of New England Nuclear Corporation in developing chromatographic procedures for (−)-alprenolol.

Note added in proof. It has recently been determined by mass spectroscopy that the structure of the ligand used in these studies is, as anticipated, that of (−)-[^{3}H]dihydroalprenolol.

REFERENCES

1. Ariëns, E. J. (1967) *Ann. N. Y. Acad. Sci.* **139**, 606–631.
2. Ahlquist, R. P. (1948) *Am. J. Physiol.* **135**, 586–600.
3. Powell, C. E. & Slater, I. H. (1958) *J. Pharmacol. Exp. Ther.*, **122**, 480–488.
4. Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP*, pp. 150–151, Academic Press, New York.
5. Lefkowitz, R. J. (1975) *Biochem. Pharmacol.*, **24**, 583–590.
6. Kaumann, A. J. & Birnbaumer, L. (1974) *J. Biol. Chem.*, **249**, 7874–7885.
7. Mayer, S. E. (1972) *J. Pharmacol. Exp. Ther.*, **181**, 116–125.
8. Murad, F., Chi, Y. M., Rall, T. W. & Sutherland, E. W. (1962) *J. Biol. Chem.* **237**, 1233–1238.
9. Rosen, O. M., Erlichman, J. & Rosen, S. M. (1970) *Mol. Pharmacol.*, **6**, 524–531.
10. Grunfeld, C., Grollman, A. P. & Rosen, O. M. (1974) *Mol. Pharmacol.*, **10**, 605–614.
11. Lefkowitz, R. J., Mukherjee, C., Coverstone, M. & Caron, M. G. (1974) *Biochem. Biophys. Res. Commun.*, **60**, 703–709.
12. Mukherjee, C., Caron, M. G., Coverstone, M. & Lefkowitz, R. J. (1975) *J. Biol. Chem.*, **250**, 4869–4876.
13. Mukherjee, C., Caron, M. G. & Lefkowitz, R. J. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 1945–1949.
14. Lefkowitz, R. J. (1975) in *Methods in Receptor Research* (Blecher, M., ed.), Marcel Dekker, New York, in press.
15. Limbird, L. & Lefkowitz, R. J. (1975) *Fed. Proc.*, **34**, 333.
16. Alexander, R. W., Williams, L. T. & Lefkowitz, R. J. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 1564–1568.
17. Stahl, E. (1969) *Thin Layer Chromatography*, Ed. 2, pp. 396–400, Springer, New York.
18. Lefkowitz, R. J. (1974) *J. Biol. Chem.*, **249**, 6119–6124.
19. Lefkowitz, R. J. (1975) *J. Biol. Chem.* **250**, 1006–1011.
20. Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.*, **58**, 541–548.
21. Ariëns, E. J., Simonis, A. M. & Van Rossum, J. M. (1964) in *Molecular Pharmacology* (Ariëns, E. J., ed.), pp. 148–153, Academic Press, New York.
22. Furchgott, R. F. (1967) *Ann. N. Y. Acad. Sci.*, **139**, 553–570.

23. Lefkowitz, R. J., Mukherjee, C., Limbird, L. E., Caron, M. G., Williams, L. T., Alexander, R. W., Mickey, J. V. & Tate, R. (1975) *Rec. Prog. Horm. Res.*, in press.
24. Limbird, L. W., De Meyts, P. & Lefkowitz, R. J. (1975) *Biochem. Biophys. Res. Commun.*, **64**, 1160–1168.
25. Cheng, Y. & Prusoff, W. H. (1973) *Biochem. Pharmacol.*, **22**, 3099–3108.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
27. Levitzki, A., Atlas, D. & Steer, M. L. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 2773–2776.
28. Atlas, D., Steer, M. L. & Levitzki, A. (1974) *Proc. Nat. Acad. Sci. U. S. A.*, **71**, 4246–4248.
29. Aurbach, G. D., Fedak, S. A., Woodard, C. J. Palmer, J. S., Hauser, P. & Troxler, F. (1974) *Science*, **186**, 1223–1224.
30. Caron, M. G. & Lefkowitz, R. J. (1974) *Nature*, **249**, 258–260.
31. Rodbard, D. (1973) in *Receptors for Reproductive Hormones* (O'Malley, B. W., ed.), pp. 289–326, Plenum Press, New York.