Biochemical Pharmacology, Vol. 24, pp. 1651 1658. Pergamon Press. 1975. Printed in Great Britain.

COMMENTARY

IDENTIFICATION OF ADENYLATE CYCLASE-COUPLED BETA-ADRENERGIC RECEPTORS WITH RADIOLABELED BETA-ADRENERGIC ANTAGONISTS

ROBERT J. LEFKOWITZ
Duke University Medical Center, Durham, N.C. 27710, U.S.A.

Rapid advances in identifying cellular receptors for cholinergic and polypeptide hormones by direct binding studies have occurred in the past few years [1–5]. Successful labeling of these receptors in membrane fractions with appropriate radioactive ligands has been followed by solubilization and even purification in certain cases.

The diversity of adrenergic effects which are mediated through beta-adrenergic receptors and adenylate cyclase [6, 7] has prompted a great deal of interest in identification and characterization of these receptors. However, attempts to achieve secure identification of beta-adrenergic receptors by direct binding techniques have, until quite recently, been frustrated. The major problems have been the lack of a highly specific radioactively labeled adrenergic ligand which could be used to label the receptors, and the presence of numerous potential competing uptake mechanisms which also bind catecholamines [7].

Several fortuitous occurrences have greatly facilitated identification of the nicotinic cholinergic receptors. First, a greatly enriched starting material was available in the electric organ of certain eels [2]. Second, it was found that the venoms of certain snakes contained peptides which specifically and irreversibly blocked the cholinergic receptors. These peptides, labeled to high specific activity with radioiodine, provided a unique, highly specific, irreversible label for the cholinergic receptors [2].

Neither markedly enriched sources of beta-adrenergic receptors nor specific irreversible beta-adrenergic agents exist to facilitate the task of beta-adrenergic receptor identification. Accordingly, a variety of reversible adrenergic ligands, generally tritium-labeled agonists, were used in early studies attempting to label the beta-adrenergic receptors. These studies met with only limited success. An earlier "Commentary" in this Journal [7] focused on these early binding studies and their interpretation.

Over the past year, rapid new developments have occurred which have now made possible unequivocal identification of beta-adrenergic receptors by direct binding techniques. This commentary will review these developments with an emphasis on the conceptual and methodological features involved in these advances.

Receptor studies: indirect vs direct

Prior to the advent of direct binding studies, receptors were defined indirectly by observation and quantitation of certain physiological or pharmacologic effects occurring subsequent to the hormone or drug–receptor interaction [8]. The observed effects, moreover, were often quite distal to the actual receptor-binding interaction, an unknown number of steps being interposed. Nonetheless, such studies have provided a great deal of information and have served to define the main patterns of drug specificity, which in turn define most receptors. Thus, on the basis of a large body of experimental data, beta-adrenergic receptors are defined as cellular structures which bind agonist catecholamines with a certain order of potency (isoproterenol > epinephrine > norepinephrine) [9]. Similarly, antagonists have definable potency series. Propranolol is among the most potent beta-adrenergic antagonists. Dichlorisoproterenol is less potent. Alpha-adrenergic antagonists, such as phentolamine or phenoxybenzamine, are generally inert.

Largely as a result of the pioneering work of Sutherland and Robison [10], it has become clear that the stimulatory effects of catecholamines on adenylate cyclase in many tissues are mediated by typical beta-adrenergic receptors. Experimentally, this conceptual advance has meant that a pharmacological effect of catecholamines could be measured (enzyme stimulation), which is considerably closer to the initial beta receptor interaction than had previously been possible. Numerous studies have confirmed the apparent identity of the beta-adrenergic receptors linked to adenylate cyclase and those coupled to physiological processes such as glycogenolysis, and cardiac contractility [11]. Nonetheless, despite the advantages of studying beta receptors coupled to adenylate cyclase, such experiments are still "indirect", i.e. the characteristics of the receptors are inferred from observed changes in enzyme activity.

Over the past 5 years, many laboratories have repeatedly demonstrated the feasibility of directly investigating hormone and drug receptors by binding radioactively labeled forms of these agents to the receptor structures in appropriate fractions derived from hormone- or drug-responsive tissues [1–5]. The earliest studies generally utilized intact tissues or pieces of tissue and were complicated by heterogeneity of the observed uptake or binding of the labeled materials. In addition to true receptor binding, drugs were also bound to other non-receptor tissue sites [12]. More recently, it has become clear that the preferred starting materials for such binding studies are simple systems such as homogeneous sus-

BCP 24/18—A

pensions of intact cells (e.g. adipocytes) or subcellular membrane fractions enriched in plasma membranes.

The use of such membrane fractions, however, does not eliminate the potential problem of binding to non-receptor sites. Accordingly, a major concern in all direct hormone binding studies is that of valid criteria for receptor identification. Put more simply, the question is: "How do we know whether or not we have labeled the true physiologically significant receptors?"

Few of the receptors recently studied by these methods have proved as elusive as the beta-adrenergic receptor. Hence, a discussion of criteria for beta-adrenergic receptor identification by direct binding studies serves to highlight these issues in a particularly useful way.

Criteria for beta-adrenergic receptor identification

Intensive work and some controversy in the field of hormone binding studies over the past few years have served to sharpen and focus the criteria which should be used to identify receptors. Three main criteria have come to be generally accepted: (1) specificity of binding; (2) affinity of binding; and (3) kinetics of binding.

Specificity of beta-adrenergic receptor binding. Since the well known specificity of beta-adrenergic responses is generated at the level of the receptors, binding specificity will presumably reflect all of the major structure–activity relations which characterize beta-adrenergic responses. The ability of beta-adrenergic agonists or antagonists to compete for occupancy of the receptor binding sites should be directly parallel to their biological activity as agonists or antagonists. Moreover, just as biological effects of beta-adrenergic agonists and antagonists are stereospecific, the (−) isomers being considerably more potent than the (+) isomers, so should receptor binding display marked stereospecificity.

Affinity of beta-adrenergic receptor binding. As noted, the relative pharmacologic potencies of adrenergic agents and their relative ability to compete for the binding sites should be parallel. Moreover, reasonable quantitative agreement should be found between the affinity constants of drugs for the receptors as determined by direct binding studies and indirect pharmacologic studies. It should be underscored that there is no *a priori* reason to expect that these two values will agree exactly. This is because the dissociation constants determined by classical pharmacologic techniques will depend on a number of factors other than the affinity of the drug for the receptor. Thus a variety of factors involved in coupling receptor binding to the ultimate measured physiologic response will all determine the apparent dissociation constants. By contrast, the dissociation constants determined by binding studies should represent a true estimate of the affinity of each agent for the beta-adrenergic receptor.

Kinetics of beta-adrenergic receptor binding. In membrane fractions, effects of beta-adrenergic agonists and antagonists on adenylate cyclase are quite rapid (full effects within 30–60 sec) [13, 14]. Accordingly, a reasonable expectation is that, when studied directly, binding to beta-adrenergic receptors will be rapid enough to account for this time course. Similarly, binding should be rapidly reversible [13, 14].

Early direct binding studies

(^3H)-catecholamines. The ready availability of (^{3}H)-labeled catecholamines such as (^{3}H)-isoproterenol with specific activities in the range of 1–15 Ci/m-mole led a number of groups to attempt identification of beta-adrenergic receptors with these materials. It was demonstrated that these labeled agonists bound rapidly and, in some cases, reversibly to sites in membranes from various tissues [15–22]. Although the characteristics of these binding sites resembled in certain respects those to be expected of the beta-adrenergic receptors, in other respects they diverged from the criteria outlined above [7, 23, 24]. The major discrepancies were as follows: (1) Specificity of binding—stereospecificity was not apparent; (−) and (+) isomers competed for occupancy of these sites with equal affinities. (2) Affinity of binding—potent beta-adrenergic antagonists such as propranolol, which have K_D values for the receptors in the range of 10^{-8} to 10^{-9} M as determined by pharmacological studies, have K_D values for (^{3}H)-catecholamine binding sites of 10^{-3} to 10^{-4} M. (3) Kinetics of binding—although in certain systems the forward and reverse rates of binding were quite rapid ($T_{1/2} \sim 1$ min [17, 19]), in other systems binding equilibrium was not reached for 1–2 hr and reverse rates were even slower [15, 16, 24].

As discussed previously [7], the physiological significance of (^{3}H)-catecholamine binding sites is complex and the population of sites labeled is undoubtedly heterogeneous. Several general interpretations, such as that binding represents covalent attachment to the enzyme catechol-*O*-methyl transferase [23] or irreversible oxidation [24, 25], are not consonant with much of the published data in the literature [26, 27].

(^3H), (^{14}C)-propranolol. A number of attempts to use radioactively labeled propranolol to label beta-adrenergic receptors in fractions of cardiac and neural tissue have been unsuccessful [12, 28, 29]. In each of these studies it was demonstrated that the labeled drug was bound to sites with characteristics very different from those to be expected of the true receptors. These sites were present in numbers far beyond what might be expected for the beta-adrenergic receptors [12, 28]. Binding, moreover, was non-stereospecific and did not possess beta-adrenergic specificity [28]. In one study, (^{14}C)-propranolol was shown to bind to liver mitochondrial membranes as well [30].

These results should not be interpreted as indicating that propranolol cannot be used to label beta-adrenergic receptors (see below). Rather, they indicate that, in addition to beta-adrenergic receptors, propranolol binds to a large number of other membrane sites. This large "nonspecific" binding of propranolol (possibly related to the "membrane" actions of the drug) will undoubtedly complicate attempts to use propranolol to identify beta-adrenergic receptors.

Identification of beta-adrenergic receptors with (^3H)-(−)alprenolol and other beta-adrenergic antagonists

Despite the obviously formidable problems involved in identifying the beta-adrenergic receptors,

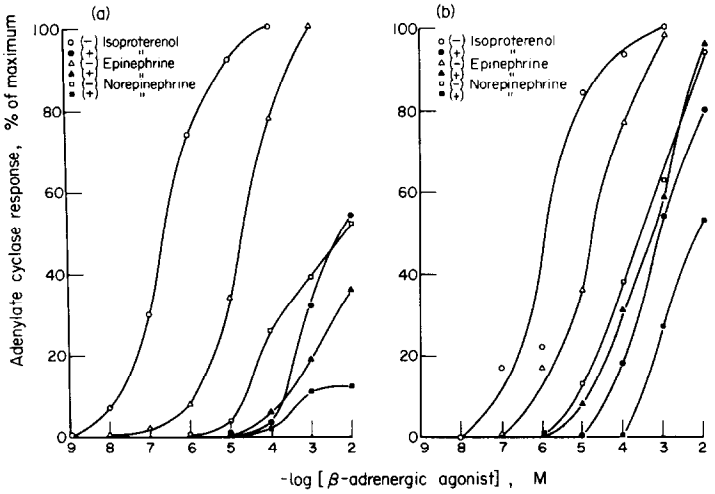


Fig. 1. (a) Stimulation of frog erythrocyte membrane adenylate cyclase by (−) and (+) isomers of isoproterenol, epinephrine and norepinephrine. (b) Inhibition of (^{3}H)(−)alprenolol binding to frog erythrocyte membranes by (−) and (+) isomers of isoproterenol, epinephrine and norepinephrine. 100% inhibition refers to complete inhibition of "specific binding", i.e. that which is displaceable by 10 μM (±)propranolol.

within the past year, several laboratories working independently have succeeded in labeling binding sites having the essential characteristics of these receptors [14, 31–38]. Although each group used different methods, certain conceptual and methodical features were common to each of the experimental approaches. Since these features were undoubtedly crucial in determining the success of recent as opposed to earlier efforts they are worth highlighting.

High affinity. The ideal agent for labeling any specific hormone or drug receptor is one with very high affinity for the receptor in question. In the case of the adenylate cyclase-coupled beta-adrenergic receptors, the most potent competitive antagonists have K_D values for the receptor (10^{-8} to 10^{-9} M) which are several orders of magnitude lower than those of potent agonists such as isoproterenol (about 10^{-6} M). Accordingly, recent approaches have utilized highly potent radio-activity labeled beta-adrenergic antagonists such as (−)alprenolol [14, 31–35], (±)hydroxybenzylpindolol [36], and (±)propranolol [37, 38].

Absence of catechol function. Previous studies with labeled agonists indicated the presence of certain (non-receptor) binding sites with a major specificity for the catechol group of beta-adrenergic agonists. Hence, the specificity of any ligand for the beta receptors would be enhanced by the absence of a catechol group. This feature characterizes all of the potent beta-adrenergic antagonists.

High specific radioactivity. Since the receptors are likely to be present in very small numbers, ability to identify them effectively with radioactive materials depends on the specific radioactivity of the agent. All of the materials used successfully for beta receptor identification have had specific activities of greater than 1 Ci/m-mole.

Methodological considerations. Since binding to beta-adrenergic receptors is rapid, incubations have generally been short, 10 min or less. Further, since the binding should be rapidly reversible, the method used to separate receptor-bound drug from free drug should be rapid. Previously used separation methods such as Millipore filtration, which involve prolonged washing of membrane fractions, are likely to "wash off" all of the receptor-bound drug [39]. Accordingly, recent studies have utilized rapid centrifugation of membranes, as with a table-top "microfuge". Alternatively, equilibrium dialysis can be used, though this is more cumbersome.

Another technical feature of some importance relates to membrane protein concentration during receptor binding incubations. Since receptor concentrations are quite low, membrane protein concentrations must be quite high to achieve receptor concentrations high enough to be effectively labeled with the radioactive materials available. In many of the earlier studies reported, membrane protein concentrations were below those necessary to have permitted successful identification of the receptors.

(^{3}H)(−)alprenolol binding sites and beta-adrenergic receptors

The characteristics of (^{3}H)(−)alprenolol* binding sites in membranes derived from frog erythrocytes and several other tissues meet the strict criteria for identification of beta-adrenergic receptor binding sites outlined above [14, 31–35].

* "(^{3}H)(−)alprenolol" has been used throughout the manuscript to denote the compound resulting from catalytic reduction of (−)alprenolol with tritium. (It can be noted from Fig. 6 that alprenolol contains an unsaturated bond in the aliphatic chain on the 2-position of the ring.) The compound therefore might appropriately be referred to as (^{3}H)(−)dihydroalprenolol. The nature of the reduction process, however, is such that tritium may also exchange with hydrogen in other portions of the molecule yielding (^{3}H)(−)alprenolol. The labeled material has biological activity and chromatographic properties identical to those of native alprenolol.

STRUCTURE	COMPOUND	STEREOISOMERS	EC_{50} [^{3}H](−)ALPRENOLOL BINDING	EC_{50} ADENYLATE CYCLASE ACTIVATION
			μM	μM
	Norepinephrine	− +	125–250 500–1000	150 —
	Epinephrine	− +	10–20 300–600	15 800
	Isoproterenol	− +	1–2 400–800	0.3 700
	Cc-34	±	0.15–0.3	0.10
	Cc-25	±	2–4	0.6
	Protokylol	±	2–4	0.4
	Soterenol	±	2.3–4.5	1.5
	MJ 9184-1	±	.05–.10	0.08

Fig. 2. Affinity of beta-adrenergic agonists for [^{3}H](−)alprenolol binding sites and adenylate cyclase-coupled beta-adrenergic receptors in frog erythrocyte membranes. The value listed under "binding" refers to the concentration of each agent which displaces [^{3}H](−)alprenolol (10–20 nM) 50% from the binding sites. The value listed under "cyclase" is the concentration of the agent which causes 50% of maximal adenylate cyclase stimulation.

Specificity of [^{3}H](−)alprenolol binding. The specificity of binding of [^{3}H](−)alprenolol directly parallels that of the adenylate cyclase-coupled beta-adrenergic receptors. The ability of (−) and (+) stereoisomers of the beta-adrenergic agonists, isoproterenol, epinephrine and norepinephrine, to stimulate adenylate cyclase and to compete for the [^{3}H](−)-alprenolol binding sites is shown in Fig. 1a and b [14]. Comparable data for a number of beta-adrenergic agonists are summarized in Fig. 2. For each of the agents, ability to stimulate the cyclase and to occupy the [^{3}H](−)alprenolol binding sites is directly

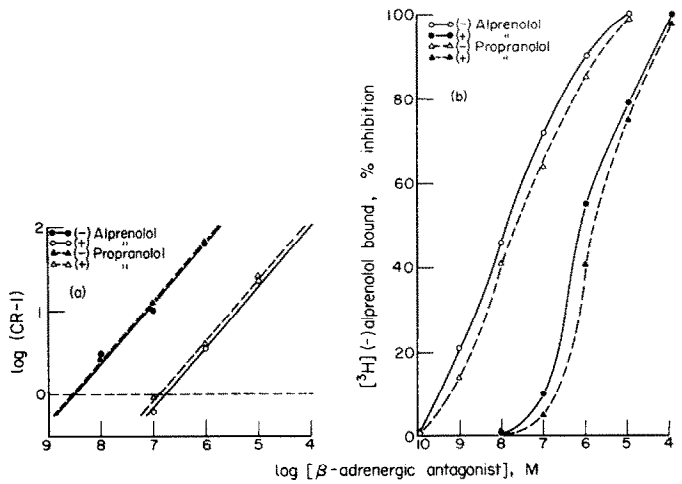


Fig. 3. (a) Schild plots [40] for competitive antagonism of isoproterenol-stimulated adenylate cyclase in frog erythrocyte membranes by (−) and (+) isomers of propranolol and alprenolol. Adenylate cyclase activity was determined in the presence of isoproterenol (10^{-7} to 10^{-4} M). The dose–response curve was then repeated in the presence of three fixed concentrations of each antagonist (indicated on the abscissa). CR refers to the concentration ratio of equiactive concentrations of isoproterenol in the presence and absence of the antagonist. (b) Inhibition of [^{3}H](−)alprenolol binding to frog erythrocyte membranes by (−) and (+) isomers of propranolol and alprenolol.

parallel. Stereospecificity is quite marked, (−) isomers being about two orders of magnitude more potent than the (+) isomers in both cyclase and binding assays.

A comparable situation exists with regard to adrenergic antagonists. Fig. 3a presents Schild plots [40] for antagonism of isoproterenol-stimulated frog erythrocyte membrane adenylate cyclase by stereoisomers of alprenolol and propranolol [14]. The abscissa intercepts are equivalent to the dissociation constants of the antagonists for the adenylate cyclase-coupled beta-adrenergic receptors. Marked stereospecificity is again apparent, as it is for ability of these agents to compete for the (^{3}H)(−)alprenolol binding sites (Fig. 3b). Data for a number of antagonists are summarized in Fig. 4.

These findings indicate the virtual identity of the structure–activity relationships which determine affinity for the adenylate cyclase-coupled beta-adrenergic receptors and the (^{3}H)(−)alprenolol binding sites [14].

Affinity of (^{3}H)(−)alprenolol binding. The data in Figs. 1–4 indicate that, in all cases tested, the dissociation constants of drugs determined by direct binding studies are in reasonable agreement with those determined by adenylate cyclase assays. It is worth noting, however, that the two sets of numbers are not exactly identical. This is because the EC_{50} or concentration of competing ligand which 50% inhibits the binding of a radioligand is equivalent to the K_D only under certain limited conditions. The EC_{50} would be expected to equal the K_D only if the radioligand were present in the binding assays at "true tracer" concentrations and cooperative interactions and spare receptors were not present. With tritiated beta-adrenergic ligands of specific activity ~20 Ci/m-mole radioligands will generally be present in binding assays at concentrations several fold higher than their respective K_D's for the β-receptors. Under such circumstances the observed EC_{50}'s will be several fold higher than the true K_D's as noted here.

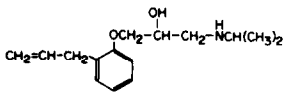
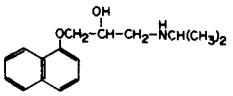
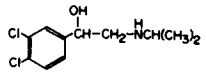
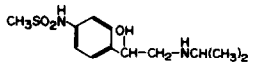
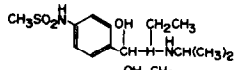
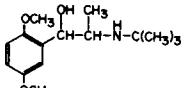
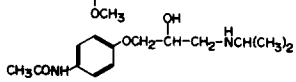
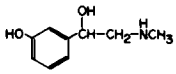
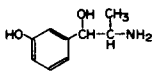
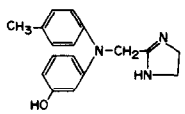
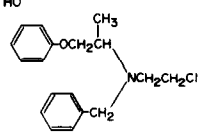
STRUCTURE	COMPOUND	STEREOISOMERS	EC_{50} [^{3}H](−)ALPRENOLOL BINDING	EC_{50} ADENYLATE CYCLASE ACTIVATION
			μM	μM
	Alprenolol	− +	0.008 – 0.016 0.40 – 0.8	0.007 0.21
	Propranolol	− +	0.012 – 0.024 0.75 – 1.5	0.004 0.18
	Dichlorisoproterenol	±	1.75 – 3.5	0.23
	Sotalol	±	7.5 – 15	1.6
	MJ 7434-1	±	50 – 100	4.2
	Butoxamine	±	20 – 40	2.2
	Practolol	±	55 – 110	8
	Phenylephrine	±	135 – 270	23
	Metaraminol	±	90 – 180	20
	Phentolamine		–	–
	Phenoxybenzamine		–	–

Fig. 4. Affinity of beta-adrenergic antagonists for (^{3}H)(−)alprenolol binding sites and adenylate cyclase-coupled beta-adrenergic receptors in frog erythrocyte membranes. The value listed under "binding" refers to the concentration of each agent which displaces (^{3}H)(−)alprenolol (10–20 nM) 50% from the binding sites. The value listed under "cyclase" is the K_D for isoproterenol-stimulated cyclase determined from Schild plots such as those shown in Fig. 3a.

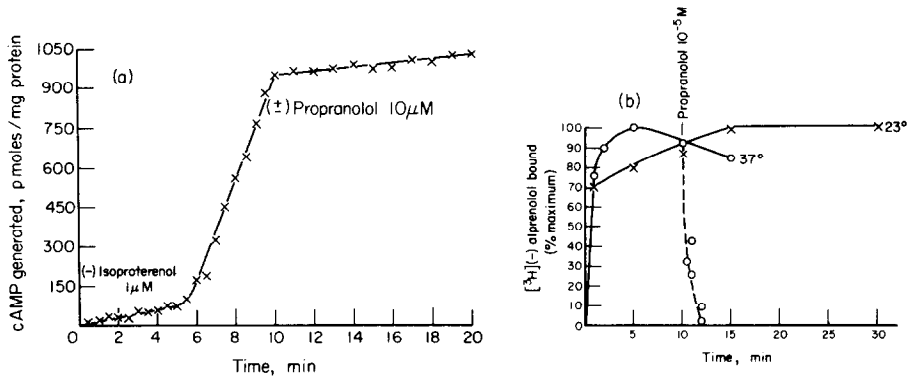


Fig. 5. (a) Time course of (−)isoproterenol stimulation and (±)propranolol inhibition of frog erythrocyte membrane adenylate cyclase. (b) Time course of (^{3}H)(−)alprenolol binding and dissociation from sites in frog erythocyte membranes.

Kinetics of (^{3}H)(−)alprenolol binding. The kinetics of beta-adrenergic agonist and antagonist interaction with adenylate cyclase-coupled beta-adrenergic receptors are demonstrated in Fig 5a. Addition of (−)isoproterenol to membrane preparations is followed by a prompt increase in the rate of cAMP generation [14]. The effect is maximal within seconds. Subsequent addition of the beta-adrenergic antagonist propranolol leads to a rapid return to the basal enzyme rate. As noted above, data such as these imply that interaction of beta-adrenergic agents with their receptors is quite rapid. This rapid interaction is reflected in the time course of (^{3}H)(−)alprenolol binding as shown in Fig. 5b. Equilibrium binding of (^{3}H)(−)-alprenolol is established within several min, and 80 per cent of the equilibrium binding value is established by the earliest point tested (30 sec). Binding is also rapidly reversible. When an excess of unlabeled (±)propranolol is added to the membranes after equilibrium binding is established, (^{3}H)(−)alprenolol dissociates with a $T_{1/2}$ of 15–30 sec [14].

These data indicate that, using high specific activity radioactively labeled beta-adrenergic antagonists such as (^{3}H)(−)alprenolol, it is possible to label sites in membrane fractions having essentially all the characteristics to be expected of the adenylate cyclase-coupled beta-adrenergic receptors. Moreover, this approach answers all of the major criticisms previously directed at the several ways in which the properties of (^{3}H)-catecholamine binding sites diverged from those expected of beta-adrenergic receptors [7, 23].

Comparison of (^{3}H)(−)alprenolol binding with other methods

To date, studies utilizing radioactively labeled beta-adrenergic antagonists to label beta-adrenergic receptors have been reported with (^{3}H)(−)alprenolol [14, 31–35], (^{3}H)(±)propranolol [37, 38] and (^{125}I) (±)hydroxybenzylpindolol [36]. The structures and several other characteristics of these labeled materials are compared in Fig. 6. As noted, all of these agents are highly potent competitive beta-adrenergic antagonists, with K_D values for the adenylate cyclase-coupled beta-adrenergic receptors which are in the nanomolar range. Of the three agents, (^{3}H)(±)propranolol is probably the least desirable for several reasons. First, several groups of investigators have clearly demonstated that (^{3}H)-propranolol binds to a variety of non-beta receptor sites in membrane preparations [12, 28–30]. Second, even in purified relatively homogeneous preparations of turkey erythrocyte membranes, 60–75 per cent of all (^{3}H)-propranolol binding observed is nonspecific binding [37, 38]. Third, of the agents available, it has the lowest specific radioactivity. Moreover, because of its structure and the nature of the general labeling procedures that must be used to tritiate the compound, it is unlikely that significantly higher activities can be easily obtained.

(^{125}I) (±)hydroxybenzylpindolol has very high affinity for the beta receptors and, because of the iodine label, much higher specific activity [36]. This very high specific activity, however, is not necessarily a marked advantage. It is apparent from the calculations of Atlas *et al.* [38] that binding studies are best

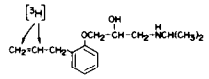
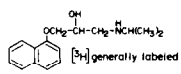
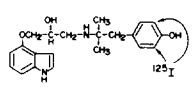
Compound	Structure and Sites of Labelling	Isotope	Specific Activity	Membranes Used For Binding Studies
(-)Alprenolol		[^{3}H]	~ 17 Ci/mmole	Frog erythrocyte, canine myocardium
(±)Propranolol		[^{3}H]	~ 4 Ci/mmole	Turkey erythrocytes
(±)Hydroxybenzylpindolol		[^{125}I]	~ 200 Ci/mmole	Turkey erythrocytes

Fig. 6. Radiolabeled beta-adrenergic antagonists used for beta-adrenergic receptor identification.

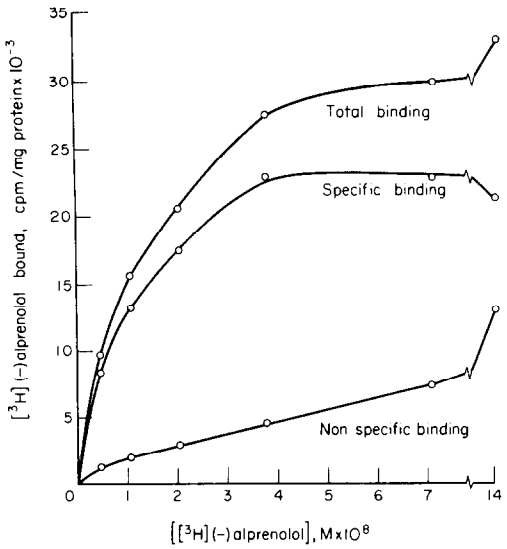


Fig. 7. "Specific" and "nonspecific" binding of (^{3}H)(−)-alprenolol to frog erythrocyte membranes. "Nonspecific" binding refers to that which is not displaced by 10 μM (±)propranolol.

performed with the radioactive ligand present at concentrations in the range of the K_D for the receptor in question. Under these circumstances, the concentrations of nonradioactive ligands causing 50 per cent displacement of radioactive ligand are approximately equal to the respective K_D values for the receptor. With iodinated ligands of extremely high specific activity, nanomolar concentrations may be equivalent to inconveniently high levels of radioactivity. The half-life of iodinated compounds is much shorter than that of tritiated compounds as well. From the data published thus far, it is not clear to what extent nonspecific binding is a problem with this ligand.

(^{3}H)(−)alprenolol has several advantageous features as a ligand for identifying the beta-adrenergic receptors. First, it is a pure (−) stereoisomer. Second, the presence of an unsaturated bond permits insertion of two atoms of tritium by catalytic reduction. The (^{3}H)(−)alprenolol we have used has a specific activity of 17 Ci/m-mole. Third, (^{3}H)(−)alprenolol has much less tendency to bind nonspecifically to membranes than does (^{3}H)(±)propranolol. In frog erythrocyte membranes, only about 15 per cent of binding is "nonspecific" (Fig. 7) [41]. As noted above, this is in marked contrast to the situation with (^{3}H)-(±)propranolol.

The characteristics of beta-adrenergic receptors delineated with the various radioactive antagonists appear, thus far, to be reasonably consistent. One discrepancy is worth underscoring. Levitzki *et al.* [37, 38] contend that binding to beta-adrenergic receptors displays "absolute" stereospecificity. Thus, these investigators state: that (+) isomers of beta-adrenergic agonists do not activate adenylate cyclase; that (+) isomers of beta-adrenergic antagonists do not inhibit catecholamine-stimulated cyclase; and that (+)-isomers do not bind to beta-adrenergic receptors. As demonstrated above, our own data indicate that (−) isomers of beta-adrenergic agents are considerably more potent than (+) isomers, as assessed by both binding and cyclase studies. We do not find "absolute stereospecificity", however. Parallel displacement curves are obtained in binding assays with (+) and (−) isomers, with the (+) isomers shifted about two orders of magnitude to the right. Similarly, (+) isomers of agonists and antagonists stimulate or competitively inhibit stimulation of adenylate cyclase with about 1/50 to 1/100 the potency of the (−) isomers (Figs. 1–4). Comparable results are obtained with cardiac [32] or erythrocyte membranes [14, 31]. We find that (+)alprenolol and (+)propranolol have affinities for the beta receptor which are equivalent to that of (±)dichlorisoproterenol and significantly greater than those of a wide variety of other beta-adrenergic antagonists tested (Fig. 4). Our findings in this regard are in excellent agreement with those previously published for interaction of (+) and (−) isomers of beta-adrenergic agents with more intact preparations [42] (Table 1). The failure of Levitzki *et al.* [37, 38] to detect the interaction of (+) isomers with beta-adrenergic receptors is most likely explained by the rather limited range of concentrations tested by these investigators.

Physiological regulation and development of beta-adrenergic receptors

The development of these new methods provides a powerful new approach for studying directly the molecular basis of physiological control and development of beta-adrenergic receptors. Several examples illustrate this point. Tolerance or tachyphylaxis to chronically administered drugs is a well documented phenomenon. We have reproduced this phenomenon in a simple model system, by injecting frogs with beta-adrenergic catecholamines. When membranes are pre-

Table 1. Potency ratios of (−) and (+) isomers of adrenergic agents

	Potency ratio (−) isomer/(+) isomer		
	(^{3}H) (−)alprenolol binding	Adenylate cyclase	Physiological responses (Intact tissue)
Agent	Frog erythrocytes [14, 31]	Frog erythrocytes [14, 31]	Guinea pig atrium Calf tracheal muscle [42]
Norepinephrine	4	*	20
Epinephrine	30	53	40–50
Isoproterenol	400	700–2300	300–500
Propranolol	62	45	40–50† (42)

* The (+) isomer is too weak to calculate a valid ratio.
† Value for propranolol refers to antagonism of isoproterenol-induced tachycardia in the cat.

pared from erythrocytes from such treated frogs, the adenylate cyclase is found to be markedly subsensitive to isoproterenol stimulation *in vitro*, although fluoride stimulates the enzyme normally [41]. The catecholamine subsensitivity is associated with a striking decline in the number of beta-adrenergic receptors in the cells, as assessed by (^{3}H)(−)alprenolol binding. These data suggest that catecholamines are capable of regulating the number of beta-adrenergic receptors and thus of providing a safety mechanism in the face of chronically elevated catecholamine levels.

Developmental changes in beta-adrenergic receptors and catecholamine sensitivity also occur. Rosen and Rosen [43] found that the adenylate cyclase of tadpole erythrocytes was unresponsive to catecholamines, although it was sensitive to fluoride stimulation. During metamorphosis, the enzyme acquired sensitivity to stimulation by beta-adrenergic catecholamines. We find that metamorphosis is associated with a highly significant ($P < 0.005$) 3-fold increase in the number of beta-adrenergic receptors present in the erythrocyte membranes as measured by direct binding studies [33].

These methods should be equally applicable to the study of a variety of models of altered beta-adrenergic responsiveness. Gilman and Minna [44] have described a series of somatic hybrid lines of cultured glioma cells. Sensitivity of adenylate cyclase to catecholamine stimulation varies markedly among the strains and can be studied in a series of hybrids. Whether or not this sensitivity is mediated by the presence or absence of the beta-adrenergic receptors in these cells is not known at present. The methods described here should permit a direct approach to such problems.

Acknowledgements—This study was supported by H. E. W. Grant no. HL16037 and by a Grant-in-aid from the American Heart Association with funds contributed in part by the North Carolina Heart Association. Dr. Lefkowitz is an Established Investigator of the American Heart Association.

REFERENCES

1. J. Roth, Metabolism **22**, 1059 (1973).
2. Z. W. Hall, *A. Rev. Biochem.* **41**, 925 (1972).
3. P. Cuatrecasas, *A. Rev. Biochem.* **43**, 169 (1974).
4. R. J. Lefkowitz, L. Limbird, M. Caron, C. Mukherjee and L. T. Williams, in *Membrane Bound Enzymes* (Ed. A. Martinosi), in press. Plenum Press, New York.
5. C. R. Kahn, in *Methods in Membrane Biology* (Ed. E. D. Korn), Vol. 3, in press. Plenum Press, New York.
6. G. A. Robison, R. W. Butcher and E. W. Sutherland, *Cyclic AMP* Academic Press, New York (1971).
7. R. J. Lefkowitz, *Biochem. Pharmac.* **23**, 2069 (1974).
8. D. R. Waud, *Pharmac. Rev.* **20**, 49 (1968).
9. R. P. Ahlquist, *Am. J. Physiol.* **135**, 586 (1948).
10. E. W. Sutherland and G. A. Robison, *Pharmac. Rev.* **18**, 145 (1966).
11. R. J. Lefkowitz, *Biochem. Pharmac.* **24**, 583 (1975).
12. L. Potter, *J. Pharmacol. exp. Ther.* **155**, 91 (1967).
13. H. P. Bar, *Molec. Pharmac.* **10**, 597 (1974).
14. C. Mukherjee, M. Caron, M. Coverstone and R. J. Lefkowitz, *J. biol. Chem.*, in press.
15. R. J. Lefkowitz and E. Haber, *Proc. natn. Acad. Sci. U.S.A.* **68**, 1773 (1971).
16. R. J. Lefkowitz, G. Sharp and E. Haber, *J. biol. Chem.* **248**, 342 (1973).
17. J. Bilezekian and G. Aurbach, *J. biol. Chem.* **248**, 5577 (1973).
18. J. Bilezekian and G. Aurbach, *J. biol. Chem.* **248**, 5584 (1973).
19. M. Schramm, H. Feinstein, E. Naim, M. Long and M. Lasser, *Proc. natn. Acad. Sci. U.S.A.* **69**, 523 (1972).
20. S. Fiszer de Plazas and E. DeRobertis, *Biochim. biophys. Acta* **266**, 246 (1972).
21. G. V. Marinetti, T. K. Ray and V. Tomasi, *Biochem. biophys. Res. Commun.* **36**, 185 (1969).
22. V. Tomasi, S. Koretz, T. K. Ray, J. Dunnick and G. V. Marinetti, *Biochim. biophys. Acta.* **211**, 31 (1970).
23. P. Cuatrecasas, G. P. E. Tell, V. Sica, I. Parikh and K. J. Chang, *Nature, Lond.* **247**, 92 (1974).
24. M. E. Maguire, P. H. Goldman and A. G. Gilman, *Molec. Pharmac.* **10**, 563 (1974).
25. B. B. Wolfe, J. A. Zirrolli and P. B. Molinoff, *Molec. Pharmac.* **10**, 582 (1974).
26. R. J. Lefkowitz, *Biochem. biophys. Res. Commun.* **58**, 1110 (1974).
27. S. H. Koretz and G. V. Marinetti, *Biochem. biophys. Res. Commun.* **61**, 22 (1974).
28. D. Vatner and R. J. Lefkowitz, *Molec. Pharmac.* **10**, 450 (1974).
29. E. DeRobertis and S. Fiszer de Plazas, *Life Sci.* **8**, 1247 (1969).
30. A. Huunan-Seppala, *Acta chem. scand.* **26**, 2713 (1972).
31. R. J. Lefkowitz, C. Mukherjee, M. Coverstone and M. G. Caron, *Biochem. biophys. Res. Commun.* **60**, 703 (1974).
32. R. W. Alexander, L. T. Williams and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1564 (1975).
33. R. J. Lefkowitz and L. Limbird, *Biochem. biophys. Acta*, in press.
34. R. J. Lefkowitz, in *Methods in Receptor Research* (Ed. M. Blecher), in press. Marcel Dekker, New York.
35. L. Limbird and R. J. Lefkowitz, *Fedn Proc.*, in press.
36. G. D. Aurbach, S. A. Fedak, C. J. Woodward, J. S. Palmer, D. Hauser and F. Troxler, *Science, N.Y.* **186**, 1223 (1974).
37. A. Levitzki, D. Atlas and M. L. Steer, *Proc. natn. Acad. Sci. U.S.A.* **71**, 2773 (1974).
38. D. Atlas, M. L. Steer and A. Levitzki, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4346 (1974).
39. P. B. Molinoff, in *Frontiers in Catecholamine Research* (Eds. S. Snyder and E. Usdin), pp. 357–360. Pergamon Press, New York (1973).
40. H. O. Schild, *Br. J. Pharmac. Chemother.* **4**, 277 (1949).
41. C. Mukherjee, M. Caron and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1945 (1975).
42. E. J. Ariens, *Ann. N.Y. Acad. Sci.* **139**, 606 (1967).
43. O. M. Rosen and S. M. Rosen, *Biochem. biophys. Res. Commun.* **31**, 82 (1968).
44. A. Gilman and J. D. Minna, *J. biol. Chem.* **248**, 6610 (1973).