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COMMENTARY

MOLECULAR PHARMACOLOGY OF BETA-ADRENERGIC RECEPTORS—A STATUS REPORT

ROBERT J. LEFKOWITZ

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

ONE OF THE most exciting current areas of research in pharmacology deals with attempts to elucidate the molecular characteristics of receptors. Receptors are the specific cellular components with which drugs and hormones first interact. They perform two functions. First, they recognize or discriminate certain biologically active molecules and bind them, thus initiating the actions of these drugs. For example, adrenergic receptors bind epinephrine and structurally related catecholamines; cholinergic receptors bind acetylcholine and other cholinergic agents, etc. Second, receptors trigger subsequent events such as activation of enzymes (e.g. adenylate cyclase) or changes in ion permeability (as with acetylcholine) which lead to the pharmacological effects typical of the particular drug.

Because the biologic effects of catecholamines are so diverse, much interest has focused on adrenergic receptors. Current research in this field has two major goals: (1) to determine the molecular nature of the specific macromolecules which function as adrenergic receptors, and (2) to determine the mechanisms by which binding of catecholamines to adrenergic receptors is translated into physiological effects.

β-RECEPTORS AND ADENYLATE CYCLASE

A good starting point for any consideration of the molecular aspects of β-adrenergic receptors is the adenylate cyclase system.[1] Over the past decade, an impressive number of studies have suggested that many, if not all, "β-adrenergic" effects are mediated via stimulation of the enzyme adenylate cyclase and the subsequent generation of the second messenger "cAMP". This conclusion has been suggested by two lines of evidence. First, the order of potency of sympathomimetic amines in stimulating the enzyme in a variety of tissues is virtually identical to the classical "β-adrenergic pattern" (i.e. isoproterenol > epinephrine > norepinephrine ≫ phenylephrine), and catecholamine stimulation of adenylate cyclase is potently blocked by β-blockers such as DCI or propranolol. Second, many of the physiological "β-adrenergic" effects of catecholamines can be mimicked by cAMP or its lipid-soluble derivative, dibutyryl cAMP.[2] On the other hand, certain β-effects (for example, stimulation of myocardial contractility) have not been unequivocally shown to be a direct result of increased cAMP generation.[3]

The enzyme adenylate cyclase is found in virtually all mammalian tissues, although its sensitivity to hormonal stimulation varies strikingly from tissue to tissue. To explain this, it has been postulated that the enzyme is composed of several

subunits.[4] These include: (1) the catalytic unit which converts ATP to cAMP, (2) hormone receptors that determine which hormones can stimulate the enzyme in any given tissue, and (3) "modulators" which somehow regulate the interplay of the receptors and the catalytic units. According to this scheme, the ability of β-adrenergic catecholamines to stimulate the cyclase in any given tissue depends on the presence of appropriate regulatory subunits, i.e. β-adrenergic receptors, and appropriate modulators. In those tissues where more than one type of hormone can stimulate the cyclase, e.g. in fat, liver or cardiac tissue, distinct receptors are assumed to be present for each hormone.

APPROACH TO ISOLATION OF HORMONE RECEPTORS

The membrane-bound nature of the "β-adrenergic receptor–adenylate cyclase system" has greatly hindered attempts at purification. A further complication is that as one disrupts cell membranes and purifies their components there is no guarantee that the properties by which the receptors are to be identified will remain intact and unchanged.

Within the past few years significant progress has been made toward identifying and characterizing certain hormone receptors which regulate the functioning of the catalytic unit of adenylate cyclase. The game plan in all of these studies has been similar to work originally reported for polypeptide hormones such as ACTH.[5,6] In brief summary, the approach is as follows. An appropriate membrane fraction is prepared from tissue which contains the hormone-responsive adenylate cyclase. Using a biologically active, radioactively labeled form of the hormone, binding studies are performed by incubating the membranes and hormones together and separating the bound labeled hormone by some technique such as centrifugation or Millipore filtration. To assess whether binding is in fact occurring at physiologically significant receptor sites, competition studies are performed. The ability of a series of hormones or drugs to compete for binding sites and to activate the cyclase can be compared and is expected to be parallel. Once an appropriate binding site is identified, it may be characterized and purified using conventional techniques. Throughout such purification procedures, the receptor is followed by its ability to bind labeled hormone. In the brief span of 3 years, these techniques have been applied to receptor cyclase systems responsive to a wide variety of hormones and drugs.[7]

ATTEMPTS TO ISOLATE β-ADRENERGIC RECEPTOR BINDING SITES

Attempts to identify and characterize the regulatory subunits of the adenylate cyclase system, which discriminate and bind β-adrenergic catecholamines, have, to date, primarily utilized radioactively labeled β-agonists. Reports have described experiments utilizing cyclase-containing membranes from canine myocardium[8,9] and cultured chick embryo myocardial cells,[10] rat liver[11,12] and turkey erythrocytes.[13–15] ^{3}H-norepinephrine, ^{3}H-epinephrine and ^{3}H-isoproterenol have all been used to study the binding sites. The properties of the binding sites identified in these studies have been fairly uniform. In a number of respects, their characteristics are parallel to what could be expected of physiologic beta-adrenergic receptors. All potent β-agonists compete effectively for the binding sites, whereas α-adrenergic agonists such as phenylephrine do not. In several tissues, the ability of adrenergic drugs to stimulate the cyclase and occupy the binding sites has been parallel and for several agonists

the K_m values for occupation of the sites, activation of the cyclase and stimulation of biological processes are quite close.[8,9,13-15] In general, binding has been a rapid, reversible process, which does not alter or degrade the bound catecholamine. In the heart, the binding sites have been solubilized with detergents and purified extensively by affinity chromatography.[16] The affinity adsorbents used were prepared by covalently linking norepinephrine to agarose beads via a 30 Å side chain.[17] The properties of the purified sites are quite similar to those of the membrane-bound sites, although the affinity of binding is somewhat less.[16] These sites appear to be protein.

Despite the obvious parallels between the binding characteristics of the sites *in vitro* and the characteristics to be expected of physiologic beta-adrenergic receptors, several apparent discrepancies exist. First, whereas β-adrenergic agonists show high affinity for the binding sites, β-adrenergic antagonists such as propranolol have much lower affinities. Although in several studies β-adrenergic antagonists were more potent in competing for the sites than α-antagonists, the concentrations of drugs required for competition ($>10^{-4}$ M) were well above those generally needed for effects of these compounds *in vivo* or *in vitro*.[8,9,11-14] Second, physiological effects of catecholamines are stereospecific, i.e. the D(−) or *l* configuration is substantially more active than the L(+) or *d* form. Binding to the sites, however, is not stereospecific and the *d* and *l* forms are observed to bind equally well. Third, a number of catechol compounds which compete for the binding sites (e.g. dihydroxymandelic acid, etc.) are virtually devoid of β-adrenergic activity.

INTERPRETATION OF BINDING STUDIES

Clearly, the adrenergic binding sites possess some but not all of the characteristics to be expected of β-adrenergic receptors. Bilezekian and Aurbach[14] have suggested that the binding sites represent a portion of the β-adrenergic receptor complex. According to this scheme, the receptor complex would include sites with a predominant specificity for the catechol ring (such as those defined above) as well as other regions specific for the "ethanolamine" side chain of the catecholamine. It would be at these latter regions that classical β-adrenergic blockers would be expected to bind and at which the stereospecificity of physiological actions would be generated.[14] Their hypothesis is based on a comparison of the ability of a series of catecholamines and related compounds to interact with adenylate cyclase and to block ^{3}H-isoproterenol binding in turkey erythrocyte membranes. These studies revealed that compounds which possess an intact catechol moiety as well as the ethanolamine side chain in the D(−) configuration (e.g. isoproterenol, epinephrine, norepinephrine) were potent activators of the cyclase as well as potent inhibitors of ^{3}H-isoproterenol binding. Compounds which possessed the catechol moiety without the ethanolamine side chain (e.g. dihydroxymandelic acid, Dopa, etc.) were effective inhibitors of binding but did not activate cyclase. All of these compounds, however, were effective inhibitors of isoproterenol-stimulated cyclase. Compounds which lacked the catechol structure (e.g. phenylephrine) did not block binding, activate cyclase or inhibit isoproterenol-activated cyclase. It was found also that *d* isoproterenol at concentrations below those which activated the cyclase could inhibit activation by the *l* form of the drug. These experiments[14] clearly document that activation of adenylate cyclase by catecholamines can be blocked by compounds which specifically inhibit binding

of β-agonists at catechol specific sites as well as by conventional β-adrenergic antagonists.

The basic question is whether these adrenergic binding sites are part of the β-adrenergic receptor which mediates stimulation of adenylate cyclase and of biological processes. Clearly their properties differ from all other known binding mechanisms for catecholamines, such as metabolizing enzymes, storage vesicles, uptake 1 or 2 etc., as has been reviewed elsewhere.[18] As noted above, the binding specificity and affinity of the sites resemble in some respects what might be expected of the intact receptors on the basis of pharmacological data. However, there is no guarantee that such characteristics, as determined by direct binding studies, will necessarily agree with the same parameters determined by conventional pharmacological techniques applied to more intact tissues. As an example, one may consider recent studies on the cholinergic receptor binding protein. There are significant discrepancies between affinity constants for drug interaction determined using the purified protein as compared with intact tissues.[19] Discrepancies appear to be greater for antagonists.[20]

There is no *a priori* reason, even for classical competitive antagonists, to assume that the antagonist and agonist drug occupy the receptor in an entirely equivalent fashion. Consider the case of an agonist which must make a perfect three-point contact with its receptor to produce a biological response. A competitive antagonist might be capable of occupying the receptor via contact with two of the three contact points. As assessed by measures of biological effect, this would produce "blockade". Nonetheless, the agonist might remain physically attached to the receptor via the third contact point. Thus, it would be "bound" though not at that moment producing biological effects. This discussion is meant to underscore the fact that there are certain inherent limitations in relying solely on the criteria of specificity and affinity of binding for identification of receptors.

At present, the data available on binding of labeled β-adrenergic agonists *in vitro* to adenylate cyclase-containing membranes from a variety of tissues are not, in themselves, sufficient to prove or disprove the hypothesis that these sites represent the β-adrenergic receptor binding sites or some fraction of these sites.

OTHER APPROACHES TO β-ADRENERGIC RECEPTOR IDENTIFICATION

Recently, derivatives of catecholamines covalently linked to agarose or glass beads have been used in studies designed to probe the molecular characteristics of β-adrenergic receptors. One group has demonstrated that leukocytes bind to agarose-norepinephrine and that this binding is inhibited by propranolol and isoproterenol.[21] The implication was that the binding observed was occurring via interaction with adrenergic receptor binding sites located at the cell surface. However, there were significant discrepancies between the specificity and affinity of this binding process and what would be expected of the β-receptors in these cells based, for example, on potency of drugs in stimulating cyclase in the cells.[22]

The major difference in this approach is that binding of intact cells to an immobilized hormone is being studied, rather than the binding of free labeled hormone to cells or membranes.

Other investigators have reported that derivatives of catecholamines and agarose[23] or glass beads[24] are biologically active on intact cells or even papillary muscles. The

implication of such studies is that the receptors are in fact located on the cell membrane. However, results of such studies must be interpreted with caution in view of recent findings that the derivatives may be unstable and release free catecholamines (which may account for the observed biological effects) during incubations with tissues.[25]

HORMONE BINDING SITES VS HORMONE RECEPTORS

As noted earlier, receptors play two important roles: one related to recognition or discrimination (and binding), the other to triggering or activation of biological processes. It is only by focusing attention on the second of these functions that conclusive identification of macromolecules as receptors is likely to be achieved. This point was recently underscored in a paper by Birnbaumer and Pohl[26] relating to "glucagon" receptors in liver plasma membranes. They have identified ^{125}I-glucagon binding sites in these membranes with specificity and affinity virtually identical to the process by which glucagon activates adenylate cyclase. Nonetheless, their experiments suggest that at best only ~ 10 per cent of these sites are likely to be related to cyclase, the other 90 per cent serving some other, as yet unknown, function.

How then can the receptors be definitively identified? There would seem to be several approaches, which are based on the "activation" function of receptors. The first might be termed the "reconstitution" approach. Here, the goal is to demonstrate that a specific macromolecule (putative receptor) possesses the "activation" properties to be expected of the true receptor. Such a demonstration requires that the binding site be reconstituted with the membrane components necessary for generating a measurable response, e.g. with the adenylate cyclase, with an ion pump, etc. One means of doing this may be to incorporate the protein into an artificial lipid membrane. Recently, Ochoa *et al.*[27] have reported just such a reconstitution with an adrenergic binding protein isolated by chloroform–methanol extraction of splenic capsule tissue. Lipid membranes containing the protein reacted with changes in conductance when exposed to adrenergic α-agonists and these responses were blocked by phentolamine but not propranolol. The results were complicated by nonspecificity of the responses, which could be mimicked by certain lipids in the absence of any binding protein. Further, virtually none of the binding characteristics of the crude protein fractions used were reported in these studies. Nonetheless, they clearly indicate one approach to the problem of reconstitution.

Utilizing affinity columns of agarose-norepinephrine,[16] it is possible to adsorb the adrenergic binding sites while permitting the catalytic units of the adenylate cyclase to pass through the columns.* Combinations of binding sites and cyclase might be made to see if sensitivity of the cyclase to adrenergic stimulation is crucially dependent on the binding sites in question (as it should be if these represent true receptors).

Alternatively, the binding sites could be combined with cyclase preparations from tissues normally unresponsive to catecholamines (e.g. adrenal cortex) in an attempt to render these responsive to adrenergic stimulation. Such recombinations are likely to be technically difficult and possible only within the milieu of lipid-containing membranes, artificial or otherwise.

* R. J. Lefkowitz, unpublished data

The second approach to testing the activation function of a hormone binding site is to determine if specific inhibition or shielding of the site is accompanied by the expected loss of hormonal sensitivity. One procedure would be to raise antibodies to the "receptor" molecule and see if exposure of tissue to the antibody blocked hormone response. Just such an approach has very recently been successfully applied in the case of the cholinergic receptor protein.[19]

For the most part, experiments of the type described above lie in the future. The common thread of each of these is to document the "activation" properties of hormone binding (recognition) sites. Only when such documentation is achieved should binding sites be truly considered to have been proved to be physiologic receptors.

BETA-ADRENERGIC RECEPTOR–ADENYLATE CYCLASE COUPLING

Earlier it was stated that there are two major goals of current research dealing with molecular mechanisms of β-adrenergic receptors. The status of the first, attempts to determine the molecular characteristics of the receptors, has been the subject of much of this communication.

I would like now to describe briefly what is known about the second, the mechanisms by which β-receptor binding is coupled to activation of adenylate cyclase. To date; at least two cellular components have been shown to modulate the activation of adenylate cyclase by the β-receptor; these are lipids and guanyl nucleotides.

Lipid effects have been demonstrated in several ways. When membrane lipids are disrupted by solubilization with detergents[28] or digestion with phospholipases,[29] fluoride-stimulated adenylate cyclase remains relatively intact while catecholamine sensitivity of the enzyme is virtually abolished. In several studies, readdition of certain lipids restored catecholamine sensitivity. However, the specifics of the lipid requirement have varied greatly. Thus, Levey[28] found that in solubilized myocardial membranes only monophosphatidylinositol restored catecholamine sensitivity, whereas phosphatidylserine restored glucagon[30] and histamine responsiveness.[31] In liver membranes digested with phospholipase A, Rethy *et al.*[29] found that phosphatidylserine restored both catecholamine and glucagon stimulation. In my own studies with phospholipase A-treated myocardial membranes, only a total lipid extract of heart was capable of restoring responsiveness of the cyclase to catecholamines.[32] Further, we have been entirely unable to restore hormone responsiveness to solubilized myocardial cyclase with any lipid.* Thus, although it seems documented that lipids are crucially involved in the chain of events by which β-adrenergic receptor stimulation of cyclase is mediated, the details remain to be clarified. Most likely, lipids are involved in orienting the receptor and catalytic units in certain configurations favoring their interaction. However, it is also possible that lipids function as an integral part of the receptor itself.

Recently guanyl nucleotides such as GTP and GDP have been found to augment markedly the stimulation of adenylate cyclase caused by β-adrenergic catecholamines and other hormones.[32,33] The K_m for enzyme stimulation by catecholamines does not appear to be altered by the nucleotides, suggesting that the observed effects are not due to increased affinity of binding to the β-adrenergic receptor.[32] Rather, they are

* L. Limbird and R. J. Lefkowitz, unpublished data.

presumably due to effects exerted either directly on the catalytic unit of the cyclase or on the so-called "modulators". It is not yet known whether these regulatory actions of guanyl nucleotides on adenylate cyclase *in vitro* have a counterpart *in vivo*.

Although the mechanisms by which β-receptor binding is coupled to activation of adenylate cyclase are not entirely clear, an intact membrane structure may be required for this interaction, hence the involvement of lipid. It should be borne in mind that the coupling need not necessarily involve a direct physical interaction or binding of the drug–receptor complex to the cyclase. It is also possible that interaction of the receptor with the catecholamine might lead to a dissociation of the receptor from the cyclase, producing an unmasking effect with consequent increase in enzyme activity, in analogy with the mechanism by which cAMP activates protein kinases.

The emphasis of this report on the β-receptor adenylate cyclase link is not meant to imply that all beta receptors must of necessity be coupled to this enzyme. Nonetheless, the close relationship of beta receptors and the cyclase in many tissues seems to make this the best model for biochemical study currently available.

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