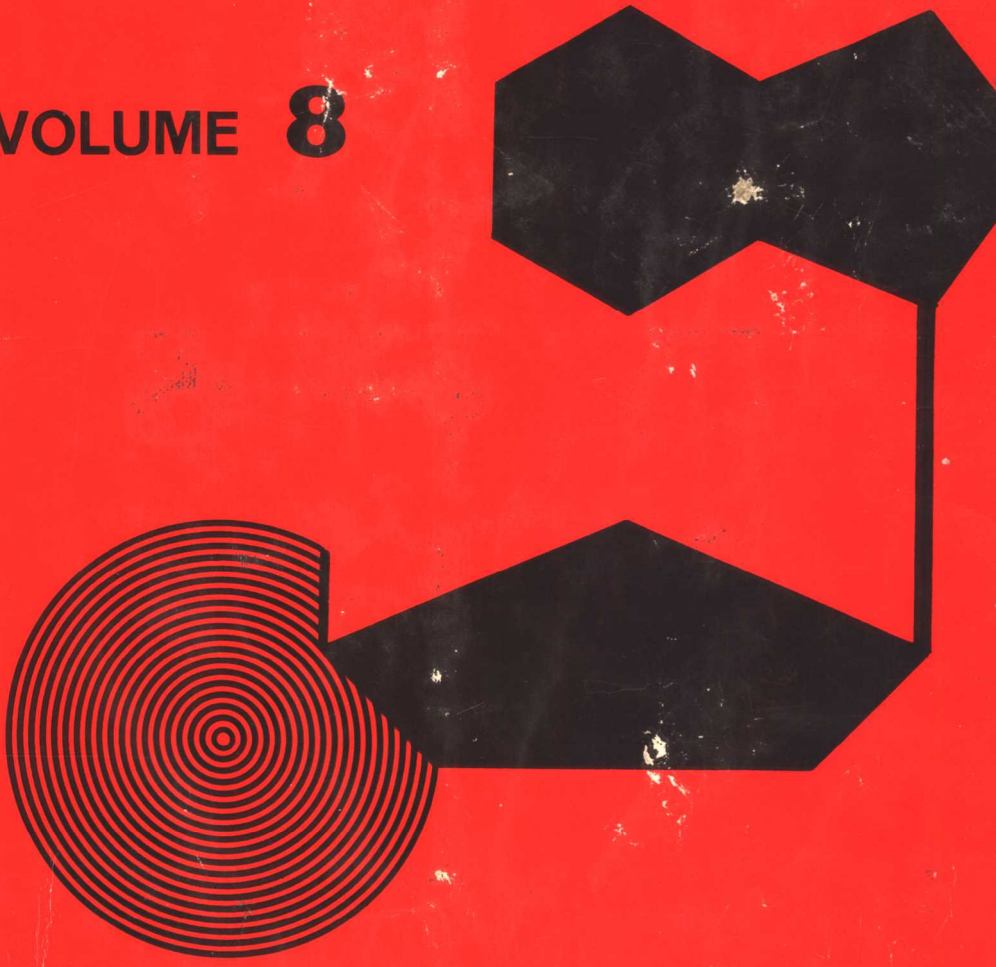
Greengard and Robison

ADVANCES IN CYCLIC NUCLEOTIDE RESEARCH

VOLUME 8



Raven Press

Advances in Cyclic Nucleotide Research

Volume 8

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Raven Press ▪ New York

Raven Press, 1140 Avenue of the Americas, New York, New York 10036

Made in the United States of America

International Standard Book Number 0–89004–169–5

Advances in cyclic nucleotide research. v. 3–
New York, Raven Press, 1973–
v. illus. 24 cm.
Earlier vols. classified separately in L. C.

1. Cyclic nucleotides–Collected works.
QP625.N89A36 574.8'732 71–181305
ISSN 0084–5930 MARC–S
Library of Congress 74 [2]

Preface

Progress continues to be made towards a better understanding of the formation, metabolism, and biological function of the cyclic nucleotides. Many of the reviews in this volume cover subjects previously reviewed in this series, and it is clear from reading them, especially in the light of their predecessors, that progress in some areas has been very substantial indeed. The reviews by Maguire and his colleagues and by Gill could be regarded as updating Perkins' review on adenyl cyclase, which appeared in Volume 3. Wells and Hardman have reviewed progress in the phosphodiesterase area, emphasizing material published since the review by Appleman and his colleagues, which also appeared in Volume 3. The rather enormous progress that has been made in the protein kinase area since Langan's review (also in Volume 3) is well covered not only in the reviews by Nimmo and Cohen and by Johnson but also in several of the other reviews. The last review in Volume 3 was the one by Murad on some of the more clinical aspects of cyclic nucleotide research, a subject which is brought up to date in this volume by Broadus. Evidence relating to the role of cyclic AMP in cardiac function, previously reviewed in Volume 4 by Entman, is reassessed in this volume by Tsien. Finally, the review by Strewler and Orloff on cyclic nucleotides and transport, and that by Kebabian on cyclic nucleotides in the nervous system, cover areas that were not previously reviewed comprehensively in this series. Despite the progress that has so obviously been made during the past few years, there are still a number of murky areas that remain to be elucidated, as pointed out more or less emphatically by all of the authors. Our hope is that each of these reviews will be as useful as those published previously in this series, not only in putting things into perspective but also in stimulating further productive research. As always, we are grateful to the authors and to our publishers for their help in this endeavor.

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(March 1977)

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Advances in Cyclic Nucleotide Research, Vol. 8, edited by P. Greengard and G. A. Robison. Raven Press, New York © 1977.

β-Adrenergic Receptor: Ligand Binding Properties and the Interaction with Adenylyl Cyclase

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I. INTRODUCTION

A. General Considerations

The study of chemoreceptors, particularly those for hormones and drugs, is obviously a provocative endeavor, at least as gauged by the quantity of effort devoted to the area during this century. In most cases, however, the word receptor evokes only a concept, rather than a definable entity. The chemical nature of most receptors is unknown; it follows that there is poor understanding of the more important question—the mechanism of translating the message "receptor ligand is present" into an effect.

Receptor theory has evolved by consideration of their bipartite nature. That is, a receptor must be capable of recognizing the ligand that regulates its function (affinity), and it must also be capable of influencing the rate of the reaction or sequence of reactions that results in the ultimate *effect* characteristic of the ligand–receptor interaction. The existence of antagonists and the fact that members of a series of agonists (which presumably act at the same site) can produce different maximal effects led to consideration and quantification of the concept of efficacy (1).

Historically, it was a challenge to envision mechanisms for generation of a *graded* signal from the interaction of a ligand with its receptor, and one type of rationalization is found in the concepts developed to explain allosteric regulation of enzymatic activity (2). Initially here we consider two states of a receptor, inactive (R) and active (R′), that exist in equilibrium with each other. Receptor agonists (A), by definition, have much greater affinity for R′ than for R and thus displace the equilibrium toward R′: $R + A \rightleftharpoons R' \cdot A$. Partial agonists have a greater affinity for R′ than for R, but the ratio of affinities is not as great as that for a full agonist, and a significant fraction of the receptors exist in the R form. An antagonist, by this formulation, has equal affinity for R and R′ and does not displace the equilibrium.

If the *unperturbed* equilibrium greatly favors R, an antagonist could bind preferentially to the inactive species; however, if R′ is present in a significant relative concentration, a ligand that binds preferentially to R is an inhibitor of the system rather than a pure antagonist of the effect of an agonist drug or hormone.

The functional side of the receptor has thus been incorporated into the designation R′. What aspect of the receptor has been altered when it is in this hypothetical state? The effector site of the receptor and that for recognition of ligand may be one and the same, or they may reside at separate points on the same covalently linked structure. R′ would then represent a different conformation with, for example, enhanced or inhibited enzymatic activity or with an "open" or "closed" ion channel. Alternatively, receptors may act by regulating the activity of distinct effector molecules, in which case R and R′ would differ in their ability to interact with the other components of the system. In such situations the functional sites of the receptor are those involved in molecular interactions, and, as we discuss in detail below, this type of model is generally invoked for hormone-sensitive adenylyl cyclase systems. It may be noted that this situation is immediately productive of semantic squabbles: What is the extent of "the receptor" and what should it be named? The β-adrenergic receptor is the subject of this review; at the moment its physical and functional relationships to other molecules, both type and number, remain unknown. We therefore define the receptor, for the purpose of this discussion, as a component of the system that binds appropriate adrenergic ligands. The question of the extent of this designated entity is discussed, but only partially answered, below.

B. Scope of This Review

This chapter discusses the binding of radioisotopically labeled ligands to β-adrenergic receptors; we attempt to evaluate the efforts of several laboratories to define and characterize the β-adrenergic receptor and to elucidate mechanisms of its interaction with adenylyl cyclase. We do not discuss the pharmacology of adrenergic receptors, and we avoid all controversy with regard to the role of cyclic AMP as the ultimate mediator of various β-adrenergic effects. There are numerous excellent reviews of these topics (3–9), as well as several recent discussions of molecular aspects of the β-adrenergic receptor (10–12).

For our purpose here, the effect of binding of agonist ligands to the β-adrenergic receptor is thus to enhance the rate of synthesis of cyclic AMP. This response is now deserving of the designation "classic" in that it was elucidated in the course of the investigations by Sutherland and Rall and their associates that resulted in the discovery of the cyclic nucleotide (13,14). This emphasis on the receptor–adenylyl cyclase relationship is certainly

not meant to imply that an alteration in the rate of cyclic AMP synthesis is the only result of occupation of β-adrenergic receptors or even that it is the first such result. This is, however, an unquestioned effect of β-adrenergic agonists and one that can be quantified in fractions containing plasma membranes from appropriate cells. It thus becomes *the* response by which the β-adrenergic receptor can be characterized and for which the mechanism of receptor function can be probed.

II. CRITERIA FOR IDENTIFICATION OF THE β-ADRENERGIC RECEPTOR BY LIGAND BINDING STUDIES

We start with indirect knowledge of certain characteristics of the receptor based on previous study of the defined response (cyclic AMP synthesis). The desire is to find a specific ligand (whose concentration can be easily monitored) that allows us to identify the receptor directly by examination of its binding activity. The most obvious experimental problems in this case are the certainty that the β-adrenergic receptor is membrane-bound in its functional state and the probability that its concentration is sufficiently low to make it worthy of consideration as a trace contaminant in the membrane. The latter fact dictates the use of sensitive techniques for detection of ligand, and radioactive compounds are thus the obvious place to begin.

Identification of the receptor by virtue of its binding of ligand then becomes a correlative argument: Are there characteristic features of ligand binding, which can be measured in either equilibrium or kinetic experiments, that are consistent with knowledge of similar characteristics of response? Based on a favorable correlation, the hypothesis is espoused that ligand binding sites are identical with functional receptor sites. However, the transmogrification of the hypothesis into a conclusion is not possible until the binding activity is purified and the response that it imparts is reconstituted.

Given the level of uncertainty generated by a need to rely on correlation, the most rigorous quantitative correspondence between binding and response should be sought; and if the effect observed is the initial and immediate result of the binding of agonist to receptor, complete quantitative correspondence between binding and response may be found. If, however, there are multiple steps between binding and effect, disparate stoichiometric relationships between receptor and effector moieties, cooperative binding interactions, and/or other complexities, quantitative relationships may become obscure indeed.

The criteria to be discussed are initially based on the simpler type of model. However, we attempt to point out what would happen to these criteria under at least certain complicating circumstances. When there is quantitative divergence between that anticipated from the simple model and that found, there are two ways to proceed—either the ligand or the model must be discarded.

A. Equilibrium Binding Experiments

Binding studies performed at true equilibrium and at a variety of ligand concentrations can be analyzed to determine the number of binding sites for the ligand and the affinity of the receptor for ligand. Thus:

$$\frac{[R \cdot L]}{[R_T]} = \frac{[L]}{[L] + K_D}$$

where bracketed entities are concentrations of ligand (L), total receptor (R_T), and the ligand–receptor complex ($R \cdot L$)—the dependent variable. Knowledge of [L] and determination of $[R \cdot L]$ allows estimation of $[R_T]$ and K_D, the dissociation constant, by a variety of graphical methods, including Scatchard and double-reciprocal plots (15). Deviations from linearity of such plots are consistent with heterogeneity of binding sites, due either to the existence of different, noninterconvertible sites or to cooperative interactions between sites. Other interpretations and several other methods of analysis of anomalous binding data are available (e.g., 16,17), but are not discussed further.

The binding of unlabeled ligands can be measured by competition with the labeled species. Thus if both labeled ligand (L*) and unlabeled ligand (L) are present

$$\begin{array}{ccc} R + L^* & \overset{K_{L^*}}{\rightleftharpoons} & R \cdot L^* \\ + & & \\ L & & \\ \Downarrow\Uparrow K_L & & \\ R \cdot L & & \end{array}$$

the fractional degree of binding by the labeled ligand is

$$f = \frac{K_{L^*}^{-1}[L^*]}{1 + K_{L^*}^{-1}[L^*] + K_L^{-1}[L]}$$

The concentration of competing ligand to reduce binding of L* by 50% ($[L]_{1/2}$) can thus be related to its true dissociation constant, K_L, by the equation

$$K_L = [L]_{1/2}(1 - f_{L^*})$$

where f_{L^*} is the fractional degree of saturation by labeled ligand in the absence of competing ligand. Thus by appropriate use of a receptor-specific ligand, one can determine the number of receptor sites and the affinity of such sites for the radioactive ligand and the competing ligands (agonists and antagonists) of interest. The K_D values can then be compared with values

of K_{act} or K_I[1] determined by quantification of the *effects* of agonists and antagonists in functional studies.

1. PHARMACOLOGICAL SPECIFICITY

The literature concerning adrenergic agonists and antagonists is enormous. Suffice it to say that an essentially unlimited number of compounds is available that should interact with the β-adrenergic receptor, and this interaction may be assessed by evaluating a compound's effect in a preparation of adenylyl cyclase sensitive to β-adrenergic agonists. Compounds that are true agonists or antagonists must compete for appropriate radioactive ligand binding sites. However, it should be obvious that caution is necessary. If a presumed β-adrenergic agonist were to act on a different class of receptor in the preparation or if a presumed antagonist actually were an enzyme inhibitor at a more distal site in the reaction scheme, confusion could result in the absence of appropriate controls.

Numerous binding sites for "adrenergic ligands" should exist in various tissues. These sites include those of other functional adrenergic receptors and proteins involved in the synthesis, degradation, transport, and storage of catecholamines (18). Selective inhibitors of these functions are available. To the extent that they are selective, as determined by their lack of effect in the assay of catecholamine-stimulated adenylyl cyclase, they should not compete for true binding to the β-adrenergic receptor.

2. STEREOSELECTIVITY

The term stereoselectivity is meant to imply greater pharmacological activity in one optical isomer than in the other. The β-adrenergic receptor displays a high degree of selectivity for the (−)-isomer (at the position analogous to the β-carbon atom of phenylethylamine). It is not clear if this discrimination is on the order of 10- to 100-fold or if it is complete, since minor contamination with the enantiomer could explain the weaker activity of (+)-isomers. Suffice it to say that a high degree of selectivity is observed when function is examined, and similar discrimination is thus expected when binding is the parameter in question. An excellent review is available on the activity of optical isomers of adrenergic agents (19).

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[1] K_{act}, the activation constant for an agonist, is defined as the concentration of the agonist that causes 50% of the maximal response observed with that compound. K_I, the inhibition constant for an antagonist, is calculated from the equation $K_I = (I_{50} \cdot K_{act})/(A + K_{act})$, where I_{50} is the concentration of antagonist to produce 50% inhibition, K_{act} is the value for the agonist used to stimulate activity, and A is the concentration of agonist used. When the term K_D is used, it refers to the concentration of ligand necessary to occupy 50% of binding sites (in actual binding experiments).

3. QUANTIFICATION: K_D VERSUS K_{act} OR K_I; NUMBER OF RECEPTORS

The criteria above have been stated in a rather qualitative fashion: Compounds that appear to influence function at the receptor site should also affect binding. In the simplest model, $R + L \rightleftharpoons RL \rightarrow$ effect, where the magnitude of effect is proportional to the fractional degree of binding, there should, by definition, be a complete correspondence between binding and effect. This holds over the entire range of concentration of labeled ligand and with a variety of competing agonists and antagonists. When the midpoints of binding and effect curves are considered, K_I or K_{act} equals K_D. Some studies (discussed below) have been consistent (at least under certain circumstances) with this simple interpretation. However, if the mechanism of initiation of effect following binding is complex or if the stoichiometry between receptor and effector is not simple, strict proportionality between binding and response can be lost. To consider simple examples: If receptors are in excess of adenylyl cyclase catalytic moieties (C) by a factor of 10, and the reaction mechanism is $L + R + C \rightleftharpoons LR + C \rightleftharpoons LRC$, a concentration of ligand equal to approximately 10% of the K_D produces a maximal effect if the affinity of LR for C is high. If multiple receptors are necessary to activate a single catalytic unit, a ligand concentration in excess of the K_D may be required to produce a half-maximal effect. Disparities of this type can be far more complex and may be exquisitely dependent on incubation conditions or on the identity of the individual competing ligand used. The latter fact could be particularly confusing when different competitors are compared. The former leads to our promulgation of an edict, which unfortunately has been ignored frequently by many, including ourselves. If quantitative comparison of binding and response are to be made, *experiments must be performed under identical conditions* if at all possible. Ample documentation of this need is provided below, as is further interpretation of discrepancies between concentrations of ligands required to bind to a receptor and to produce an effect.

Another aspect of the quantitative analysis of ligand binding that must be considered is the number of receptor sites revealed by the ligand. Typical enriched plasma membrane preparations from various sources yield maximal (NaF-stimulated) specific activities of adenylyl cyclase that rarely exceed 1 nmole/min/mg protein. This corresponds to 6×10^{14} molecules/min/mg. The turnover number for the homogeneous adenylyl cyclase of *Brevibacterium liquefaciens* is 1,400 molecules/min (20), and it is not unreasonable to propose that this number is relatively conserved in evolution (21). If there are equal numbers of β-adrenergic receptors and catalytic units of adenylyl cyclase, this would yield approximately 4×10^{11} molecules of receptor per milligram of protein or 700 fmoles of receptor per milligram of protein as a

first approximation of the concentrations to be expected. Using the same assumptions for intact cells, where initial rates of cyclic AMP accumulation of 500 pmoles/min/mg protein are frequently observed, one would expect to find no more than 10^4 to 10^5 receptors per cell; this approximation exceeds the number of receptors for peptide hormones usually observed by others (22).

4. CELLULAR SPECIFICITY

It is clear that a response to a hormone cannot occur unless the cell involved is equipped with an appropriate receptor. The converse cannot be stated in absolute terms, although it seems logical that most normal cells would "choose" not to respond to a given chemical by failure to synthesize the relevant receptor. However, the response could be successfully regulated at points other than the receptor. As far as criteria for the specificity of a given ligand are concerned, responsive cells should bind ligand, and the abolition of specific ligand binding sites (by physical, chemical, developmental, or genetic manipulations) should abolish the response. If receptor is found to exist in the absence of response, important information may be available on the relationship between the sites involved in the response and the binding of ligand.

B. Kinetic Experiments

In the simple and ideal situation, the fractional degree of binding of a labeled agonist could be correlated with the fractional response as a function of time. However, the quantitative correlation between the kinetics of binding and the time course of effect may be even more subject to the difficulties of interpretation that were mentioned above. Thus any slow step in the reaction sequence between the formation of the ligand–receptor complex and the realization of the effect manifests as a discrepancy between fractional binding and the fraction of maximal response observed. Such phenomena are superimposed on any anomalies that result from other deviations from the simplest mechanism.

There are, in addition, several experimental difficulties that hamper careful kinetic experimentation on the β-adrenergic receptor–adenylyl cyclase system. The most useful radiolabeled ligands are antagonists. Association of ligand with receptor can thus be correlated only with *loss* of response to an agonist; this does not foster precision. If labeled agonists were available, this situation would be little better. Few adrenergic agonists have apparent dissociation constants lower than 10 nM. The concentration of ligand required to observe an effect would ensure that the approach to equilibrium of binding and probably response would be too fast to quantify meaning-

fully by techniques currently available (for examples of rates see section V-A).

Furthermore, nonspecific binding of labeled ligands is, by definition, poor affinity binding. (This subject is discussed in more detail below.) Since $K_D = k_{-1}/k_1$, where k_1 and k_{-1} are the forward and reverse rate constants for the reaction of ligand with binding site, higher values of K_D imply higher values of k_{-1}. (The variation of k_{-1} is usually *much* greater than that of k_1 when a range of values of K_D are compared.) The amount of ligand bound at time = $t(f_t)$, as a function of the amount bound at equilibrium (f_{eq}), is given by the following expression:

$$f_t = f_{eq}[1 - e^{(-k_1[L]+k_{-1})t}]$$

Thus if k_{-1} is large, the exponential term becomes insignificant at short times and equilibrium is achieved rapidly ($f_t = f_{eq}$). In general, therefore, nonspecific binding equilibrates very rapidly compared with binding to the receptor. If the *association* reaction is studied, nonspecific binding may predominate at early times, even though it is a relatively insignificant fraction at equilibrium.

Greater success should be possible with the reverse reaction—particularly if a high-affinity ligand with a small value of k_{-1} is available. In this case reversal of binding of an antagonist ligand should be slow enough to measure and may be correlated with the appearance of the ability to respond to an agonist (section *V*-A, Fig. 4). Anomalies in this correlation are obviously again apparent if either the response is not proportional to the availability of binding sites (for a variety of reasons) or if the labeled ligand has not bound to functional receptor sites.

The final point of this section relates to a common misstatement about the relationship between the kinetics of binding of experimental ligands and the time course of the effect of natural agonists: Since the physiological effect of the agonist is rapid in onset and offset, the binding of any labeled ligand should be similarly quick. The appearance of bound ligand results from a bimolecular reaction governed by the equation

$$\frac{d[LR]}{dt} = k_1[L][R]$$

When high-affinity, high-specific activity ligands are utilized, they are by necessity and desire present in low concentrations ([L]). The rate of binding is of course correspondingly slow. Very high affinity implies small values of k_{-1}, since k_1 cannot exceed constraints imposed by the speed of diffusion. High-affinity ligands then obviously dissociate slowly. By contrast, physiological agonists must *not* have great affinities for their receptors if the system is to respond quickly (unless the receptor is a catabolic enzyme for the ligand). Were acetylcholine to have a very high affinity for its receptor at the neuromuscular junction, animals would be vegetables.

III. ATTEMPTS TO IDENTIFY THE β-ADRENERGIC RECEPTOR

A variety of ligands have been employed with varying degrees of success in attempts to identify binding sites with the properties expected of the β-adrenergic receptor. The merits of these ligands are described in this section; the structures of the compounds discussed are shown in Fig. 1, and certain of their general properties are summarized in Table 1.

A. [^{3}H]Catecholamines

It is clear that the majority of experiments that relied on [^{3}H]catecholamines as ligands failed to identify the β-adrenergic receptor. Haber and Wrenn (11) and Lefkowitz et al. (10) reviewed the inadequacy of these data, and the pertinent references are cited in those reviews. There are, however, at least two reasons to provide a few further comments. First, the early binding studies with [^{3}H]catecholamines met certain of the criteria discussed above, and it is instructive to note the manner in which other criteria were not met and the explanations for at least certain of the responsible artifacts. Second, at least two groups appear to have demonstrated a binding site for [^{3}H]isoproterenol with properties expected of the β-adrenergic receptor (23,24), and [^{3}H]dopamine has been employed to identify receptor sites that interact preferentially with this catecholamine (26,27). Thus in certain situations and with appropriate caution based on experience, it may be possible to employ these compounds. The ligands that are currently generally available and that yield the most useful data are both antagonists: [^{3}H]dihydroalprenolol ([^{3}H]DHA) and [^{125}I]iodohydroxybenzylpindolol ([^{125}I]IHYP). It becomes apparent below that it would be beneficial to have a radioisotopically labeled agonist of high affinity and specificity to compliment experiments with [^{3}H]DHA and [^{125}I]IHYP.

1. CRITERIA APPLIED TO [^{3}H]CATECHOLAMINE BINDING STUDIES

We can summarize the difficulties with [^{3}H]catecholamines under the headings used above to describe criteria for appropriate binding (see refs. 28–30 for documentation). The two recent reports about [^{3}H]isoproterenol (23,24) are exceptions to the following discussion.

a. Pharmacological specificity

The compounds that were usually shown to compete effectively for [^{3}H]catecholamine binding sites were the three commonly used agonists: isoproterenol, epinephrine, and norepinephrine. Major differences in the