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Quantitative Resolution of *Beta*-Adrenergic Receptor Subtypes by Selective Ligand Binding: Application of a Computerized Model Fitting Technique

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SUMMARY

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Frog myocardium appears to possess both $beta_1$ and $beta_2$ receptors, based on the potency order of several adrenergic agonists to compete for [^{3}H]dihydroalprenolol binding. Selective *beta* blocking agents are able to distinguish two receptor subtypes in frog myocardium, but only one site in rat ventricle. Computer modeling using a PDP 11/45 indicates that all rat *beta* receptors are $beta_1$, whereas only 15%–25% of frog ventricular *beta* receptors are of the $beta_1$ subtype. Computerized curve fitting can provide a more accurate estimate of receptor parameters than currently available graphical methods of analysis.

INTRODUCTION

The use of radiolabeled ligands has facilitated the study of various properties of the *beta*-adrenergic receptors in many tissues (1). One of the characteristics of *beta* receptors investigated by this method has been the distinction between $beta_1$ and $beta_2$ receptors originally proposed from physiological observations by Lands *et al.* (2). For example, the adenylate cyclase-coupled *beta*-adrenergic receptor of the frog erythrocyte appears to possess binding properties of the $beta_2$ type (3), whereas the rat heart demonstrates binding affinities for ligands predicted for $beta_1$ receptors (4).

Until recently, it has been believed that individual tissues contain only one of the *beta* receptor subtypes. However, pharmacological studies by Carlsson *et al.* (5) demonstrated a mixture of $beta_1$ and $beta_2$ receptors in kitten, but not rat heart. Similar physiological techniques have indicated that frog myocardium might contain a small $beta_1$ component in addition to a predominant population of $beta_2$ receptors (6). Using radiolabeled ligand techniques, Barnett *et al.* (7) recently demonstrated a mixture of 25% $beta_1$ and 75% $beta_2$ receptors in rat lung, but rat heart studies demonstrated only one class of sites. A graphical method derived from the classical Scatchard data analysis ("pseudo-Scatchard") was used to estimate the relative proportions of receptor subtypes in these ligand binding experiments.

In the present study, we have applied a

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computerized non-linear least-squares curve fitting technique (8) to an investigation of the beta receptors of the frog myocardium. By the use of direct radioligand binding techniques using (−)[^{3}H]DHA[2], selective adrenergic agonists and antagonists, and a computer modeling system, we have found the frog heart to have both *beta*$_1$ and *beta*$_2$ receptors, while the rat heart contains only the *beta*$_1$ receptor type.

The purpose of the present study was not to document the properties of numerous ligands. Rather, our goals have been: 1) to demonstrate the ability of a few discriminatory ligands to interact differentially with *beta*-receptor subtypes in a model system, and 2) to introduce the use of computer modeling techniques for quantitating receptor subtypes. We believe that this computer analysis provides a much more reliable and objective estimate than previously described graphical methods (7) for determining the proportions of *beta*$_1$ to *beta*$_2$ receptors and for estimation of the relative affinity constants of ligands for different receptor types.

METHODS

Membrane preparation. Membrane vesicles were prepared by a modification of the method of Besch *et al.* (9) using ventricular tissue from southern grass frogs (*Rana pipiens*, Nasco) or male rats (C.D. strain, Charles River). Briefly, frozen ventricles stored in liquid nitrogen until use, were homogenized in 10 mM $NaHCO_3$, 5 mM Na Azide at maximum speed for 15 seconds using a Polytron. Foam was aspirated and discarded. The homogenate was centrifuged at 13,000 × *g* for 10 minutes. This step was repeated using the first supernatant. The second supernatant was centrifuged at 44,000 × *g* for 30 minutes. The resulting pellet was resuspended in 0.6 M KCl, 20 mM imidazole (pH 6.8) to dissolve contractile proteins, followed by another centrifugation at 44,000 × *g* for 30 minutes. A second resuspension in KCl-imidazole was followed by a final centrifugation at 44,000 × *g* for 30 minutes. The resultant pellet was resuspended in a small volume of 0.2 M sucrose, 30 mM D-L-histidine and stored in liquid nitrogen until use.

[^{3}H]Dihydroalprenolol binding assay. Assays were conducted in a final volume of 900 μl, consisting of 150 μl of water or competitive ligand, 150 μl of DHA (3 nM, 58 Ci/mmole, NEN), and 600 μl of membrane preparation. Concentrated frozen membranes were diluted prior to the assay using 75 mM Tris-HCl, 25 mM $MgCl_2$ (pH 7.65) to give a final protein concentration of 0.4 to 0.6 mg/ml with frog heart and 0.7 to 1.0 mg/ml with rat heart. Assay procedures were essentially the same as described previously (10).

Specific DHA binding, determined as the difference between radioactivity bound in the presence and absence of 1 μM (±)propranolol, was approximately 90% of total binding at 3 nM ligand in frog and 75–80% in the rat. Sources for ligands have been previously documented (11).

Computer modeling. The experimental binding data, in terms of concentration of DHA bound versus concentration of competitor used, were subjected to non-linear least-squares curve fitting (12) using a generalized model for complex ligand-receptor systems as described by H. Feldman (8). The model involves m ligands binding to n classes of binding sites. The concentration of each ligand bound (B_i, $i = 1$ to m) is given by:

$$B_i = \sum_{b=1}^{n} \left\{ K_{ib} F_i R_b \Big/ \left(1 + \sum_{a=1}^{m} K_{ab} F_a \right) \right\} \quad (1)$$

where K_{ib} and K_{ab} are the affinity constants of ligand "i" and "a", respectively, binding to site "b", F_i and F_a are the free concentrations of ligands "i" and "a", respectively, and R_b is the concentration of receptor site "b". Because total, rather than free, ligand concentrations are known, the predicted value of B_i was calculated from the total ligand concentration (H_i) and the current estimates of the affinity constants (K_{ib}) and receptor concentrations (R_b) such that both equation (1) and the equation for conservation of mass of ligands (equation 2) were satisfied.

$$H_i = F_i + B_i \quad (2)$$

[2] The abbreviation used is: DHA, dihydroalprenolol.

In the present application, for each curve the number "*m*" of ligands is two, ligand 1 being the labeled ligand (DHA) and ligand 2 being the competitor. The number "*n*" of classes of binding sites is set to either 1 or 2, but could be larger. A Scatchard transformation of data (not shown) from saturation studies resulted in a straight line, indicative of high affinity binding of DHA with equal affinity for all receptors. Secondly, in the presence of a competitive ligand (e.g., propranolol) there was an apparent decrease in the affinity of DHA for the receptors without any change in the maximum amount of DHA bound, as predicted for true competitive binding. DHA appears to be non-selective for either *beta*$_1$ or *beta*$_2$ receptor subtypes and the same value was assigned to its two affinity constants, K_{11} and K_{12}, for sites 1 and 2, respectively.

The deviations of the observed points from the predicted values were weighted according to the reciprocal of the predicted variance (13).[3] The data were repeatedly fit using the model for one, two, or more classes of binding sites. The model providing the best fit was chosen on the basis of the lowest value of mean squares of residuals. The computer programs provided the best estimates (with their standard error) for the affinity constants of each ligand and the concentration of receptors in each subtype. All computations were performed using an interactive program in PL/1 using a PDP 11/45.[4]

RESULTS

Binding of DHA to membrane vesicles from both frog and rat ventricle demonstrated appropriate stereoselectivity and a high affinity for DHA. The K_D's obtained from Scatchard analysis (data not shown) were 3.6 nM and 2.6 nM in the frog and rat, respectively. The reciprocal of the K_D's were utilized as the affinity constants of DHA in subsequent computer analyses. The maximum number of binding sites for DHA was approximately 100 fmole/mg protein in frog ventricle and 35 fmole/mg protein in the rat.

Displacement curves of agonist competition against approximately 3 nM DHA revealed a different pattern for the two species. Figure 1 illustrates the ability of four adrenergic agonists to compete for DHA binding in the frog ventricle. The pattern observed is similar to that found with *beta*$_2$ systems such as the frog erythrocyte (3). Estimates of the EC_{50}'s from dose response curves indicated a potency ratio for hydroxybenzylisoproterenol:isoproterenol:epinephrine:norepinephrine of 1200:70:7.7:1. By contrast the pattern observed in the rat ventricle was as expected for *beta*$_1$ receptor binding (Fig. 2), i.e., the relative agonist potencies of hydroxybenzylisoproterenol: isoproterenol:epinephrine:norepinephrine were 30:25:1.7:1.

The ability of three *beta*-blocking agents to compete for DHA binding to membrane vesicles was also different in the two species. Figure 3 illustrates dose-response competition curves for approximately 3 nM DHA binding in frog ventricle. The potency order of propranolol:butoxamine:practolol was 2000:6.7:1. In the rat myocardium, butoxamine and practolol were approximately equipotent, and approximately 1000 fold weaker than propranolol (Fig. 4).

In the experiments using rat heart the three antagonist dose-response curves appeared to differ only in the potency of the competitors, i.e., all three curves were parallel (Fig. 4). In the frog heart, however, the displacement of DHA by butoxamine and practolol is more complex than that obtained with propranolol. Dose response curves (Fig. 3) indicate a second component with butoxamine and practolol. In order to analyze the interaction of *beta*-receptor agents with DHA binding sites a computer modeling system was developed. Figures 1 through 4 illustrate the ability of the model to fit the experimental observations. The lines indicate the best fit from the computer model, whereas the symbols represent the actual data points. Predicted values for ligand affinities and proportions of *beta*$_1$ and *beta*$_2$ receptors obtained from agonist and antagonist competition curves are

[3] Documentation of the derivation of the variance function will be provided by the authors upon request.

[4] A listing of the computer programs employed will be provided by the authors upon request.

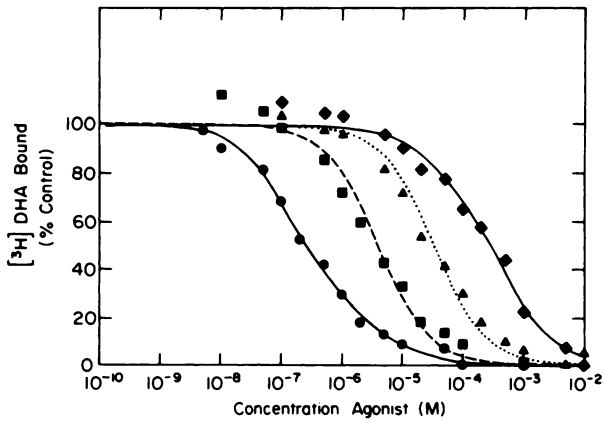

FIG. 1. *Competition curves for specific DHA binding to frog ventricular membranes by beta-adrenergic agonists*

The ordinate indicates the percent of maximal specific DHA binding, 0.0221 nM. (Specific binding is defined as the difference between binding in the absence of any competing ligand and binding in the presence of 10^{-6} M propranolol.) The abscissa is the molar concentration of various agonists. The lines are computer modeled best fits. The symbols indicate the means of actual data points for 2 (hydroxybenzylisoproterenol, ●), 11 (isoproterenol, ■), 9 (epinephrine, ▲), and 10 (norepinephrine, ◆) separate experiments with each agonist.

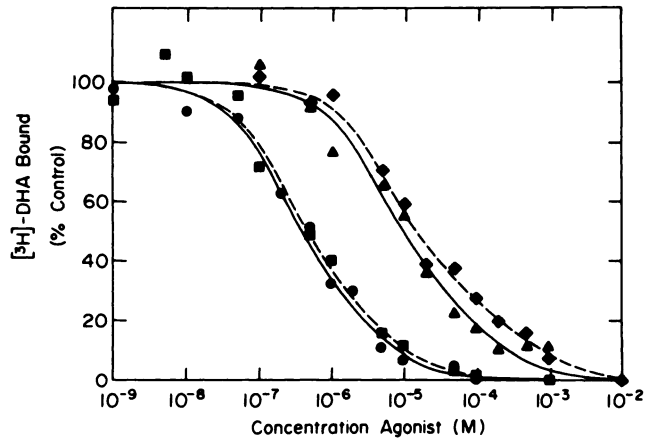

FIG. 2. *Competition curves for specific DHA binding to rat ventricular membranes by beta-adrenergic agonists*

The ordinate indicates the percent of maximal specific DHA binding, 0.0083 nM. The abscissa is the molar concentration of various agonists. The symbols indicate the means of actual data points derived from two separate experiments with each agonist (hydroxybenzylisoproterenol, ●, isoproterenol, ■, epinephrine, ▲, norepinephrine, ◆).

given in Tables 1 and 2.

The experimental data obtained in the frog ventricle were best fit with a model in which 15%–25% of the receptors are $beta_1$ and 75%–85% are $beta_2$. The relative affinities of the antagonists for these receptor types and the standard error of the mean of these estimates were also derived by the program (Table 1). Propranolol had equal affinity for both $beta_1$ and $beta_2$ receptors, whereas practolol and butoxamine had affinities for the two frog receptor subtypes that differed by nearly two orders of magnitude.

In contrast, data obtained in the rat could be fit most optimally by a model with only one binding site for both DHA and the competitor. Table 1 lists the dissociation

constants of the three antagonists for the apparently homogeneous *beta*$_1$ receptors in the rat ventricle.

Agonist competition curves (Figs. 1 and 2) for both frog and rat heart data were fit with a model involving at least two apparent classes of binding sites. Table 2 lists the relative proportions and dissociation constants of agonists for receptor subtypes. Also included in Table 2 are the agonist potency ratios for the *beta* receptor subtypes. A small fraction of frog *beta* receptors appears to possess the potency order expected of *beta*$_1$ sites, whereas in the remainder, hydroxybenzylisoproterenol, a potent *beta*$_2$-selective agonist, is 28-fold more potent than isoproterenol, and epinephrine is 15-fold more potent than norepinephrine, as expected for *beta*$_2$ receptors. Two classes of *beta* receptors were apparent in rat heart experiments, both having similar potency orders for agonists, conforming to *beta*$_1$ ex-

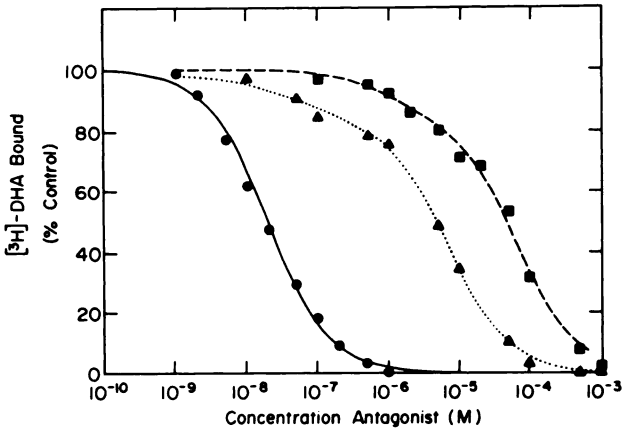

FIG. 3. *Competition curves for specific DHA binding to frog ventricular membranes by beta-adrenergic antagonists*

The ordinate indicates the percent of maximal specific DHA binding, 0.0239 nM. The abscissa is the molar concentration of various antagonists. The symbols indicate the means of actual data points for 3 (butoxamine, ▲) to 6 (propranolol, ●, practolol, ■) separate experiments with each antagonist.

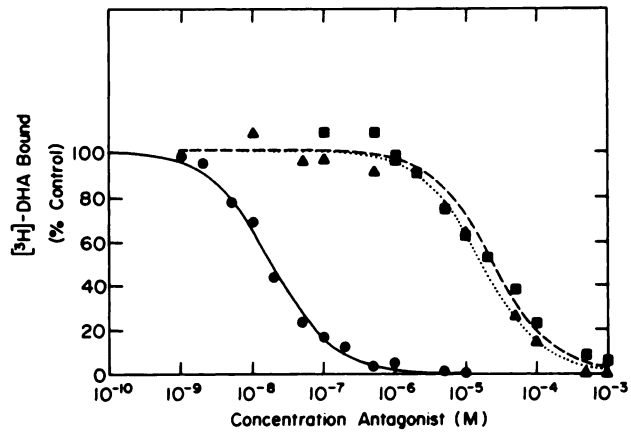

FIG. 4. *Competition curves for specific DHA binding to rat ventricular membranes by beta-adrenergic antagonists*

The ordinate indicates the percent of maximal specific DHA binding, 0.0186 nM. The abscissa is the molar concentration of various antagonists. The symbols indicate the means of actual data points for two separate experiments with each antagonist (propranolol, ●, butoxamine, ▲, and practolol, ■).

TABLE 1

Comparison of relative proportions of beta$_1$ and beta $_2$ receptors in frog and rat heart and dissociation constant estimates from computer analysis of antagonist competition curves

Species	Receptor subtype	*Beta* receptor distribution	Ligand dissociation constants (M)		
			Propranolol	Practolol	Butoxamine
Frog	*Beta*$_1$	8.4 ± 1.8 pM (15%)	$1.04 \pm .07 \times 10^{-8}$	$4.1 \pm 3.4 \times 10^{-7}$	$2.1 \pm 1.9 \times 10^{-8}$
Frog	*Beta*$_2$	48.0 ± 1.6 pM (85%)	$1.04 \pm .07 \times 10^{-8}$	$3.5 \pm .33 \times 10^{-5}$	$3.8 \pm .36 \times 10^{-6}$
Rat	*Beta*$_1$	35.6 ± .06 pM (100%)	$8.6 \pm .82 \times 10^{-9}$	$1.1 \pm .11 \times 10^{-5}$	$7.6 \pm .84 \times 10^{-6}$

Values for receptor concentration are shown in picomolar DHA bound ± S.E.M. The relative percent of each subtype is indicated in parentheses. Ligand dissociation constant values (M) are indicated ± S.E.M. These values are derived by computer from antagonist curves shown in Figs. 3 and 4.

TABLE 2

Comparison of relative proportions of beta$_1$ *and* beta$_2$ *receptors in frog and rat heart, dissociation constants and potency ratios from computer analysis of agonist competition curves*

Species	Receptor subtype	Beta receptor distribution	Ligand dissociation constants (M) and potency ratios			
			Hydroxybenzylisoproterenol	Isoproterenol	Epinephrine	Norepinephrine
Frog	*Beta*$_1$	11.4±1.9 pM (26%)	$2.1 \pm .21 \times 10^{-6}$ 9:	$2.1 \pm .21 \times 10^{-6}$ 9:	$1.8 \pm .18 \times 10^{-5}$ 1:	$1.8 \pm .18 \times 10^{-5}$ 1
Frog	*Beta*$_2$	32.7±1.8 pM (74%)	$7.2 \pm 1.7 \times 10^{-8}$ 3800:	$2.1 \pm .21 \times 10^{-6}$ 130:	$1.8 \pm .18 \times 10^{-5}$ 15:	$2.7 \pm .45 \times 10^{-4}$ 1
Rat	*Beta*$_1$-High affinity state	10.0±0.08 pM (71%)	$8.8 \pm 2.2 \times 10^{-8}$ 32:	$1.0 \pm .28 \times 10^{-7}$ 28:	$1.9 \pm .54 \times 10^{-6}$ 1.4:	$2.8 \pm .84 \times 10^{-6}$ 1
Rat	*Beta*$_1$-Low affinity state	4.1±0.09 pM (29%)	$1.5 \pm .73 \times 10^{-6}$ 113:	$2.1 \pm 1.1 \times 10^{-6}$ 80:	$5.4 \pm 2.7 \times 10^{-5}$ 3.1:	$1.7 \pm 1.0 \times 10^{-4}$ 1

Values for receptor concentration are shown in picomolar DHA bound ± S.E.M. The relative percent of each subtype is indicated in parentheses. Ligand dissociation constant values (M) are indicated ± S.E.M. These values are derived by computer from agonist curves shown in Figs. 1 and 2. Potency ratios (read across the table) for agonists are given below the K_D values. For example, in the frog *beta*$_1$ receptor population, the potency ratio for hydroxybenzylisoproterenol:isoproterenol:epinephrine:norepinephrine is 9:9:1:1.

pectations. All the dissociation constants for agonists are lower (higher affinity) for the first apparent class of sites, than for the second subtype. These two apparent classes of *beta*$_1$ sites in the rat are likely analogous to the high and low affinity states for agonists previously reported for frog erythrocyte *beta* adrenergic receptors (14).

DISCUSSION

The results of this investigation document the different binding characteristics of *beta*-adrenergic receptors in frog and rat myocardium. Computer estimates indicate a predominance of *beta*$_2$ receptors in the frog myocardium in agreement with previously reported physiological data (6, 15). The power of the computer technique is revealed by its ability to detect a small (15%–25%) population of *beta*$_1$ receptors in frog myocardium, as well. In contrast, rat ventricular tissue appears to be similar to other mammalian cardiac muscle typical of a *beta*$_1$ system (2, 7, 10, 16–19).

Recently, a mixture of *beta*$_1$ and *beta*$_2$ receptors has been described in rat lung (7). In order to quantitate the properties of each receptor subtype, these authors employed a "pseudo-Scatchard" analysis to estimate the receptor proportions and affinities for a competitive ligand. We have attempted to use this method in our systems and believe it to provide less accurate predictions than our computer model. Using the "pseudo-Scatchard" method in the frog heart provides a two-fold higher estimate

of the proportion of *beta*$_1$ receptors and an underestimate of the discriminating power of the competitive ligand for the two receptor types. When parameter estimates derived from "pseudo-Scatchard" plots are used in the computer model, the data from both frog and rat antagonist competition curves can not be satisfactorily fit and predicted dose-response curves are shifted as far as three-fold to the right of the actual data points. A more quantitative measure of the error of these fits is found in the sum of squared deviations of the points from the fitted lines. Using the parameter estimates from the "pseudo-Scatchard" plots produces as much as an eight-fold greater sum of squares than our computer model estimates.

There are several reasons that this discrepancy in results might be expected to occur. One of the potential errors of a graphical approach is the application of a method for studying direct ligand binding (the Scatchard plot) to indirect ligand binding (i.e., competition curves). A second problem lies in the assumption that percent inhibition of labeled ligand binding by the competitor is synonymous with the percent occupancy of receptors by the competitor ligand. Although not a major source of error with tritiated ligands, this factor is particularly relevant when very high affinity and high specific activity iodinated ligands (in which a much higher proportion of total ligand is bound) are considered. From a statistical point of view, the "pseudo-Scatchard" plot is subject to several possible sources of error in regression analysis (20). Also, the utilization of asymptotes to the lines, as used in this method (7), relies too heavily on the least accurate points of the curve and underestimates the influence of one class of sites on ligand binding to the other class of sites (21). In general, curve fitting methods of the "pseudo-Scatchard" type become inaccurate because of the nature of the data transformation techniques, in which numbers subject to experimental error are divided by the low concentrations of competitor to produce the graphical coordinates. These transformed values are exceedingly sensitive to small errors in the data obtained and can produce large fluctuations in the shape of the fitted curve.

In contrast to the above problems, the computer modeling process possesses several strengths. Of primary importance is the fact that the model is derived directly from mass action law for multiple classes of binding sites. It may be applied to simple or complex cases in which the number of sites is greater than two. The data are used as originally obtained (i.e., labeled bound ligand versus total unlabeled competitor) in an untransformed coordinate system. In terms of statistical considerations the model derives increased accuracy from the ability of the computer to analyze several curves simultaneously, improving the precision of the estimated parameters common to all curves. Lastly, the data analysis corrects for the non-homogeneity of the variance of data at extremes of the curves.

A further problem exists in the "pseudo-Scatchard" analysis when this method is applied to agonists. When used with the data obtained in this investigation, this graphical technique produced curves with a more complex pattern suggesting more than two classes of binding sites in both rat and frog studies. Resolution of these binding components from such curves would be extremely inaccurate because so few points fall on a single component of the line, in addition to the other potential sources of error mentioned above. The complex curves were not unexpected in view of the nature of agonist binding to receptors (14).

Agonists, because they induce subsequent changes in cellular function, behave with different properties than antagonists. In the frog erythrocyte, it has been proposed that agonists form a slowly reversible complex with the receptor (14), i.e., a high affinity state that follows the initial interaction of hormone and receptor in a low affinity combination. Antagonists are believed to form only one, the low affinity, complex (14). In the rat heart, the failure to fit agonist data to only one class of sites (Fig. 3), in contrast to antagonist data (Fig. 4), may be the result of agonist interactions with both high and low affinity states of the same *beta*$_1$ receptor subtype. In the frog ventricle it is possible that agonists form both high and low affinity states with both

beta$_1$ and *beta*$_2$ receptors. It is unlikely that any data analysis could distinguish four sites with closely overlapping affinities without modifications of the present experimental protocol. In fact, modeling rat or frog agonist data to more than two sites did not significantly improve the fit.

It should be pointed out that, in the present studies, butoxamine, a proposed *beta*$_2$-selective antagonist (4, 22, 23), acted similarly to practolol, a *beta*$_1$-selective blocking agent (4, 22, 24). Most previous studies (22–25) of selectivity of butoxamine have relied on measurements of physiological or biochemical parameters. It is therefore possible that factors other than direct interaction of receptor with ligand may account for apparent differences in selectivity with this drug. In agreement with our data, it has been observed by others (4) that butoxamine was slightly more potent than practolol in inhibiting DHA binding to rat cardiac membranes. The EC_{50} values obtained in that study (4) are virtually identical to those obtained for practolol and butoxamine in the current experiments.

One observation from the present investigation that is not completely understood is the difference in dissociation constants observed in frog *beta*$_1$ and rat *beta*$_1$ receptors for practolol and butoxamine. It is possible that *beta*$_1$ receptors in different species have unequal discriminatory powers for various ligands. Within the same species (rat) the inhibition constants of practolol for DHA binding to *beta*$_1$ receptors in lung and heart have been reported to be approximately equal (7).

In summary, ligand binding techniques indicate that frog ventricular myocardium possesses a small number of *beta*$_1$ receptors in addition to a predominant *beta*$_2$ population. Computer-fitted curves coincide remarkably closely to experimental observations and provide accurate estimates of the proportions of receptor subtypes and their affinities for selective ligands. Graphical estimates of numbers and affinities of receptor subtypes appear less accurate and should not be used without an awareness of the potential inaccuracies inherent in such methods.

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