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A Quantitative Method of Analyzing the Interaction of Slightly Selective Radioligands with Multiple Receptor Subtypes

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SUMMARY

Subclasses of receptors exist for most neurotransmitters. Frequently, two subtypes of receptors coexist in the same tissue and, in some cases, they mediate the same physiological response. In tissues with two classes of binding sites for a given hormone, an estimate of the proportion of each class of binding sites is obtained by inhibiting the binding of a single concentration of a radioligand with a selective unlabeled ligand. Accurate estimates of the density of each class of receptors will only be obtained, however, if the radioligand is entirely nonselective. Selectivity of just 2- to 3-fold can markedly influence the results of subtype analysis. The conclusion that a radioligand is nonselective is usually based on the results of a saturation binding curve. If Scatchard analysis of such data results in a linear plot, then it is concluded that the radioligand is nonselective. However, Scatchard analysis cannot distinguish between a radioligand that is nonselective and one that is slightly selective. The use of a slightly selective radioligand can lead to errors of 50% or more, depending on the concentration of the radioligand relative to the K_d values of the two classes of sites. A new analytical method has been developed that can be used to quantitate 2- to 3-fold differences in the affinity of two distinct classes of binding sites for a radioligand. This new approach requires that a series of inhibition experiments with a selective unlabeled ligand be performed in the presence of increasing concentrations of the radioligand. Analysis of the resulting inhibition curves, utilizing the mathematical modeling program MLAB on the PROPHET system, yields accurate estimates of the density of each class of receptor as well as the affinity of each receptor for the labeled and unlabeled ligands. This approach was used to determine whether ^{125}I-iodopindolol shows selectivity for β_1- or β_2-adrenergic receptors. A series of inhibition curves was generated with the unlabeled ligands ICI 89,406 (β_1-selective) and ICI 118,551 (β_2-selective), using membranes prepared from C_6 glioma cells. These cells contain both β_1- and β_2-adrenergic receptors. ^{125}I-Iodopindolol was determined to be 3-fold selective for β_2-adrenergic receptors. Since the sensitivity of this approach is superior to that of Scatchard analysis, it is likely that other radioligands, previously thought to be nonselective, will be shown to be selective when analyzed by this method.

The concept that most known and putative neurotransmitters interact with multiple subtypes of receptors is now well established. Radioligand binding techniques are used widely to quantitate the properties and densities of receptor subtypes in a variety of tissues. Measurement of the binding of radioligands to receptors in disrupted cell preparations overcomes many of the limitations inherent in studies that rely only on the measurement of biological responses. Interpretation of the results of such biological studies is often complicated by factors such as diffusion barriers or neuronal and extraneuronal uptake mechanisms (1). Moreover, multiple subtypes of receptors frequently coexist in the same tissue and may even mediate the same biochemical and physiological responses. For example, β_1- and β_2-adrenergic receptors have been shown to coexist in a variety of mammalian tissues (2). Both subtypes appear to stimulate the enzyme adenylate cyclase (3), and both contribute to the electrophysiological effects of catecholamines on the heart (4, 5). Binding assays with radioligands have been used to characterize multiple receptor subtypes in a single tissue, and they represent the only means to estimate accurately the relative proportions of these subtypes (1).

Two approaches have been developed to study receptors in tissues that contain multiple subtypes of receptors. The first approach utilizes direct binding assays with a radioligand that is highly selective for one of the subtypes. If the radioligand is sufficiently selective, the density of a single subtype of receptor can be determined from a saturation experiment. The pharmacological profile for this receptor subtype can be determined from studies of the inhibition of the binding of this selective radioligand. Because selective radioligands are not available for every class of receptor, a second approach has been developed to take advantage of the availability of numerous selective ligands that either have not been prepared in a radiolabeled form or are otherwise not useful as radioligands. This second

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ABBREVIATIONS: ^{125}I-IPIN, ^{125}I-iodopindolol; HEPES, 4-(2-hydroxyethyl)-1-piperazine-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

approach involves measuring the inhibition of the binding of a single concentration of a nonselective radioligand by multiple concentrations of a highly selective unlabeled ligand. The relative proportions of the subtypes and their affinities for the competing ligand can be determined by nonlinear regression analysis of the inhibition curves (6) or by iterative analysis of curvilinear plots resulting from Hofstee transformation of the inhibition data (7). De Lean *et al.* (8) determined the limits of resolution of nonlinear regression analysis of inhibition curves under conditions typically encountered in the β-adrenergic system. Mixtures of receptor subtypes were created by combining in various proportions preparations of receptors from turkey and frog erythrocyte membranes. These receptor subtypes have markedly different affinities for norepinephrine and closely resemble mammalian β_1- and β_2-adrenergic receptors, respectively. The limits of resolution of this analytical method were also tested by using Monte Carlo simulation techniques. A 6-fold selectivity for the unlabeled ligand could be quantitated with a tissue containing a 50:50 mixture of subtypes and a 100-fold selective ligand was necessary to quantitate a 90:10 mixture of subtypes.

A nonselective radioligand is required for this analysis to ensure that the fractional occupancies of all receptor subtypes are identical. If the radioligand is selective, the fractional occupancy of the subtype with high affinity for the radioligand will be greater than the fractional occupancy of the subtype with low affinity unless the concentration of radioligand is sufficiently high to saturate both classes of receptors. Since experiments cannot generally be carried out with saturating concentrations of radioligand, erroneous estimates of the relative proportions of the subtypes and of the affinity of these subtypes for the unlabeled ligand usually result. Errors of this type will occur regardless of the selectivity of the competing ligand. Typically, the selectivity or nonselectivity of a radioligand is determined by analysis of saturation isotherms measured in a tissue containing both receptor subtypes. Iterative analysis of curvilinear Scatchard plots or nonlinear regression analysis of the untransformed saturation isotherm is used to assess the selectivity of the radioligand. A linear Scatchard plot, or a saturation isotherm for which the goodness of fit is not significantly improved by assuming the presence of two classes of binding sites, is interpreted as evidence that both subtypes have the same affinity for the radioligand.

The ability of either of these analytical methods to determine the selectivity of a radioligand is limited. Even under ideal conditions, selectivity is unlikely to be detected reliably when analyzing untransformed saturation data unless the two classes of binding sites differ in their affinity for the radioligand by at least 5- to 7-fold (9). Moreover, the ability to resolve two classes of sites by this method is affected by properties of the tissue that are not under the control of the investigator, such as the proportion of high and low affinity sites and the percentage of total binding that is specific. Iterative analysis of curvilinear Scatchard plots is even less likely to result in reliable quantitation of radioligand selectivity since linear regression analysis of the transformed data in a Scatchard plot generally yields less accurate estimates of the parameters than nonlinear regression analysis of untransformed data (9).

To characterize accurately subtypes of receptors in mixed tissues, it is necessary to identify a radioligand that is nonselective. One possible approach utilizes a diagnostic procedure in which inhibition curves are generated at several different concentrations of radioligand (10). Systematic variation in the ratio of the IC_{50} values of the unlabeled ligand or in the relative proportions of binding sites is indicative of a difference in affinity of the two classes of binding sites for the radioligand. The sensitivity of this approach was not determined because data were presented only for radioligands with 10-fold selectivity. Inasmuch as this approach does not provide an estimate of the actual selectivity of a radioligand, it serves only to eliminate radioligands that appear to be nonselective on the basis of analysis of saturation data.

The use of radioligands and highly selective competing ligands has been particularly useful in the characterization of subtypes of β-adrenergic receptors that coexist in a variety of mammalian tissues (2). Several radioligands, presumed to be nonselective on the basis of saturation data, have been used to characterize subtypes of β-adrenergic receptors in mixed tissues. These include ^{125}I-iodohydroxybenzylpindolol (7, 11), ^{3}H-dihydroalprenolol (3, 12, 13), ^{125}I-IPIN (14, 15), and ^{125}I-iodocyanopindolol (16). As a class, these radioligands display properties considered desirable for the characterization of multiple receptor subtypes including high affinity, low levels of nonspecific binding, high specific activity, and rapid kinetics. Linear Scatchard plots are observed with all of these ligands in a variety of tissues. ^{125}I-IPIN possesses the most favorable combination of these properties and is widely used for characterization of subtypes of β-adrenergic receptors. It has been suggested (17), however, that ^{125}I-IPIN is slightly selective for β_2-adrenergic receptors. Lower K_d values have been obtained from saturation isotherms measured in the cerebellum, which contains primarily β_2-adrenergic receptors, as compared to the cortex, which contains primarily β_1-adrenergic receptors. However, differences in K_d values in different tissues may result from differences in the amount of protein in the assay or the lipid composition of the membranes (18, 19).

In the present study, theoretical data were used to demonstrate that, under appropriate conditions, 3-fold selectivity of a radioligand can be detected reliably by measuring the relative proportions of sites defined by a highly selective unlabeled ligand at several concentrations of radioligand. The error associated with the estimates of the proportions of sites when a single concentration of a slightly selective radioligand is used for the determination was also defined. A method to quantitate slight differences in the affinity of two distinct classes of binding sites for a radioligand is described. This approach requires that a series of inhibition experiments with a highly selective unlabeled ligand be performed in the presence of increasing concentrations of radioligand in a tissue containing both classes of binding sites. Simultaneous nonlinear regression analysis of these multiple inhibition curves yields accurate estimates of the density of each class of binding site as well as the affinity of each class for both the labeled and unlabeled ligands. The results of this type of analysis show that ^{125}I-IPIN is 3-fold selective for β_2-adrenergic receptors in C_6 glioma cells.

Materials and Methods

Cell culture and preparation of membranes. C_6 glioma cells of the BU1 subclone between passages 53 and 70 (2 and 19 in this laboratory) were grown in Dulbecco's modification of Eagle's medium (Flow Laboratories, Inc., McLean, VA) supplemented with 5% fetal bovine serum (Sterile Systems, Inc., Logan, UT) in a humidified

atmosphere of 10% CO_2/90% air at 37°. Cells were plated at 20,000 cells/cm² in 150-mm dishes (Lux; Miles Scientific, Naperville, IL), fed or subcultured on day 3, and harvested on day 5. The cells were lysed by replacing the growth medium with ice-cold hypotonic buffer (1 mM Na^+-HEPES, pH 7.4, 2 mM EDTA). After swelling for 10–15 min, the cells were removed with a rubber policeman and centrifuged at 24,000 × *g* for 20 min. This crude membrane preparation was resuspended with a Brinkmann Polytron homogenizer at setting 6 for 10 sec in Tris-isosaline (20 mM Tris-HCl and 0.9% NaCl) and stored at −70°. Before use, the membrane preparation was thawed, centrifuged at 24,000 × *g* for 20 min, and resuspended in Tris-isosaline.

Preparation of radioligands. (−)-Pindolol (50 nmol, dissolved in ethyl acetate to 5 mM) was added to 25 μl of 0.3 M KH_2PO_4 (pH 7.4), 25 μl of chloramine T (0.17 mg/ml), and 4–5 mCi of $Na^{125}I$ for 3 min at room temperature. The reaction was stopped by the addition of 300 μl of $Na_2S_2O_3$ (1 mg/ml) and 10 μl of 1 N NaOH. ^{125}I-IPIN was extracted into ethyl acetate containing 0.01% phenol and was separated from pindolol by chromatography for 6–8 hr in 0.5 M ammonium formate (pH 8.5) containing 10% methanol and 0.01% phenol. The radioligand (specific activity 2.2 Ci/μmol) was eluted into methanol containing 0.01% phenol and stored at −20°.

Receptor binding assay. Aliquots of a membrane preparation were added to assay tubes containing 20 mM Tris-HCl (pH 7.4), 0.9% NaCl, 1.1 mM ascorbic acid, 0.0004% bovine serum albumin, 100 μM GTP, radioligand, and appropriate drugs. (−)-Isoproterenol (100 μM) was used to define specific binding. Incubations, carried out at 37° in a final volume of 0.25 ml, were stopped by the addition of 10 ml of 0.9% NaCl in 10 mM Tris-HCl (25°, pH 7.4) to each assay. The samples were filtered through glass-fiber filters (Schleicher and Schuell no. 30) and washed with an additional 10 ml of buffer. Radioactivity retained on the filters was determined in an LKB 1274 or a Beckman 4000 gamma counter. Protein was determined according to the method of Bradford (20) using bovine serum albumin as a standard. In each experiment, inhibition of the binding of the radioligand by 15–21 concentrations of the β_1-selective ligand ICI 89,406 or the β_2-selective ligand ICI 118,551 was investigated at each of seven to nine concentrations of the radioligand.

Data analysis. Theoretical saturation data were generated with the following equation, which describes the interaction of a selective radioligand with two classes of noninteracting binding sites:

$$B = \frac{B_{max_1} \cdot L}{L + K_{d_1}} + \frac{B_{max_2} \cdot L}{L + K_{d_2}} \quad (1)$$

In this equation, B is the amount of radioligand bound, B_{max_1} and B_{max_2} are the densities of the two classes of binding sites, L is the free concentration of radioligand, and K_{d_1} and K_{d_2} are the dissociation constants of the binding sites for the radioligand.

The values for total and nonspecific binding of the radioligand were transformed by Scatchard analysis to estimate the K_d and B_{max} values. Each inhibition curve for ICI 89,406 or ICI 118,551 was analyzed by nonlinear regression analysis using NEWSFITSITES2 on the National Institutes of Health-supported PROPHET computer system to determine the proportion of the binding of radioligand to β_1- or β_2-adrenergic receptors at each concentration of radioligand (21). The data were fit to the following two-site equation that describes the inhibition of the binding of a nonselective radioligand by a selective competing ligand:

$$B = \frac{B_1}{1 + (I/IC_1)} + \frac{B_2}{1 + (I/IC_2)} \quad (2)$$

In this equation, B is the amount of radioligand bound, B_1 and B_2 are the total number of sites of each subtype labeled by this concentration of radioligand, I is the free concentration of the competing ligand, and IC_1 and IC_2 are the concentrations of competing ligand that inhibit 50% of the binding to each subtype.

The affinities of each class of receptors for ICI 89,406 or ICI 118,551 and for the radioligand, as well as the actual proportions of β_1- and β_2-adrenergic receptors, were determined by simultaneous nonlinear regression analysis of the inhibition curves measured at nine concentrations of radioligand. The curve fitting was performed by the mathematical modeling program MLAB (22) on the PROPHET system using the method of Marquardt and Levenberg as described in Magar (23). The data were fit to the following equation, which describes the inhibition of the binding of a selective radioligand by a selective competing ligand:

$$B = \frac{B_{max_1} \cdot L}{L + K_{d_1}(1 + I/K_{i_1})} + \frac{B_{max_2} \cdot L}{L + K_{d_2}(1 + I/K_{i_2})} \quad (3)$$

In this equation, B is the amount of radioligand bound, B_{max_1} and B_{max_2} are the densities of the two classes of binding sites, L is the free concentration of radioligand, I is the free concentration of competing ligand, K_{d_1} and K_{d_2} are the dissociation constants of the binding sites for the radioligand, and K_{i_1} and K_{i_2} are the dissociation constants of the binding sites for the competing ligand.

Simultaneous nonlinear regression analysis of the inhibition curves using Eq. 3 was performed with the additional constraint that K_{d_1} equal K_{d_2} to test the hypothesis that the radioligand was selective. Improvement of the fit was analyzed by comparing the sum of squares of the residuals for fits with and without constraints on K_d. The statistical significance of the improvement of fit was determined by performing an F test on the sum of squares of the residuals. The F value for this test was:

$$F = \frac{\frac{SS_1 - SS_2}{df_1 - df_2}}{\frac{SS_2}{df_2}}$$

where SS_1 and SS_2 are the sums of the squares of the residuals for the fits with and without constraints on K_d, respectively, and df_1 and df_2 are the degrees of freedom for the fits with and without constraints.

For the analysis of experimental data, the free concentration of radioligand in the absence of competing ligand was used. This value was determined by subtracting the total amount of radioligand bound to the tissue from the total amount of radioligand added to the sample. The concentration of receptors was sufficiently low in every experiment such that the total amount of radioligand bound to the tissue never exceeded 5% of the total amount of radioligand added to the sample. A similar correction for the free concentration of competing ligand was not feasible; therefore, the added concentration was used in the analysis. However, since the K_d values of the competing ligands were at least five times greater than those of the radioligand, the change in free concentration of competing ligand was less than 1%.

A runs test (24) was applied to the results of each Scatchard analysis to determine whether the transformed data points were randomly distributed about the regression line. Actual values exceeding the predicted values on the regression line were assigned a positive sign and actual values less than the predicted values were assigned a negative sign. The linear order corresponded to the magnitude of the amount bound. The runs test is designed to detect nonrandom clusters of signs, such as those that would result from the systematic deviation of a curvilinear set of points from a straight line. This test provides a more appropriate indicator of the curvilinearity of a set of points than does the correlation coefficient of the regression analysis.

The selectivity of the radioligand was determined by calculating the ratio of the larger to the smaller dissociation constants. The confidence limits of the selectivities were determined from the following equation:

$$`(n_1 + n_2 - 2) = \frac{X_1 - RX_2}{\frac{s_1^2}{n_1} + R^2\frac{s_2^2}{n_2}}$$

where X_1 and X_2 are the means of the larger and smaller dissociation constants, respectively, s_1 and s_2 are the respective standard deviations of the dissociation constants, n_1 and n_2 are the number of determinations of these dissociation constants, and R is the confidence limit (25).

Solution of this quadratic equation for R produces confidence limits that are not symmetric around the estimated ratio of the dissociation constants X_1 and X_2. Under these conditions, if the lower confidence limit is >1, the ratio of the means is significantly different from 1. It should be noted that the dissociation constants determined by simultaneous analysis are not independent measures. In general, however, parametric tests are sufficiently robust to tolerate some deviation from the assumption of independence, and this appears to be an appropriate test for the analysis of ratios.

Materials. C_6 cells were obtained from Dr. Mark Dibner (E. I. DuPont de Nemours and Co., Wilmington, DE). (−)-Pindolol (Sandoz, Hanover, NJ), and ICI 89,406 and ICI 118,551 (ICI Americas, Inc., Wilmington, DE) were gifts. (−)-Isoproterenol bitartrate and GTP were obtained from Sigma Chemical Co. (St. Louis, MO), and $Na^{125}I$ was purchased from New England Nuclear (Boston, MA).

Results

Theoretical saturation data were generated with Eq. 1 assuming K_d values of 1 and 3 for the receptors with high and low affinities for the radioligand. It was further assumed that 40% of the receptors had a high affinity for the radioligand. Scatchard transformation of the saturation data resulted in a linear plot with a correlation coefficient of 0.998 despite occupancy values that ranged from 5 to 95% (Fig. 1). Although there is the suggestion of curvature, the introduction of biological noise would be likely to obscure it, and a linear Scatchard plot would be interpreted as indicating that the radioligand is nonselective. A runs test applied to the results of the Scatchard analysis revealed that the transformed data points were not randomly distributed about the regression line ($p < 0.05$).

Theoretical inhibition data assuming a 3-fold selective radioligand and a 200-fold selective competing ligand, with both ligands having a high affinity for the same site, were generated for three concentrations of radioligand using Eq. 3. When these data were analyzed using a conventional two-site model, which assumes that the radioligand is nonselective (Eq. 2), the proportions of high and low affinity sites varied with the concentration of the radioligand (Fig. 2). The two-site model consistently overestimated the proportion of binding sites that had a high affinity for the radioligand. This overestimate results from greater fractional occupancy of the high affinity sites at less than saturating concentrations of radioligand. As the concentration of radioligand increased, the estimated proportion of sites from the two-site model approached the correct proportion of sites. The relative proportion of sites with a high affinity for the radioligand decreased with increasing concentrations of the radioligand.

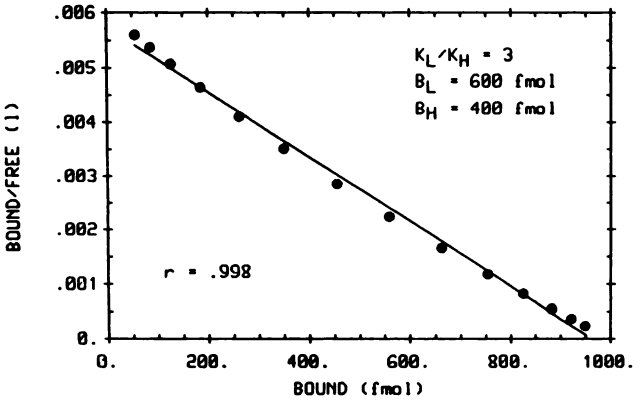

Fig. 1. Scatchard transformation for a 3-fold selective radioligand. Theoretical saturation data were generated using Eq. 1, which describes the interaction of a selective ligand with two classes of binding sites. A 3-fold selectivity for the radioligand (K_L/K_H) and a 40% population of high affinity sites (B_H) were assumed. The saturation data were transformed by the method of Scatchard and the line of best fit was determined by linear regression analysis.

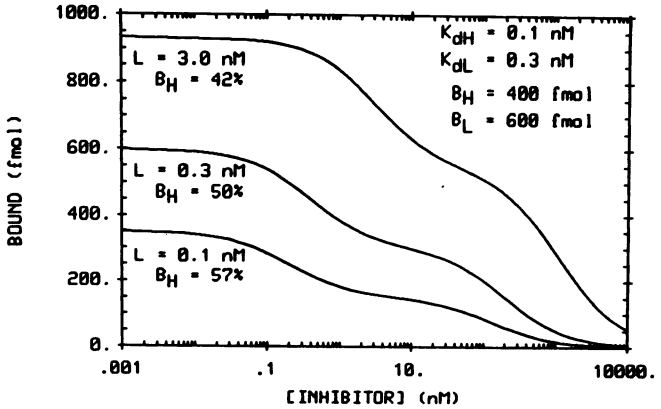

Fig. 2. Inhibition curves produced by varying the concentration of a 3-fold selective radioligand. Theoretical inhibition data assuming a 3-fold selectivity for the radioligand (K_{dL}/K_{dH}) and a 200-fold selectivity for the inhibitor were generated for three concentrations of radioligand (L) using Eq. 3 which describes the interaction of a selective radioligand and a selective inhibitor with two classes of binding sites. The proportion of high affinity to low affinity sites (B_H:B_L) was 40:60 for this analysis. The data were analyzed using Eq. 2, which describes the interaction of a nonselective radioligand and a selective inhibitor with two binding sites.

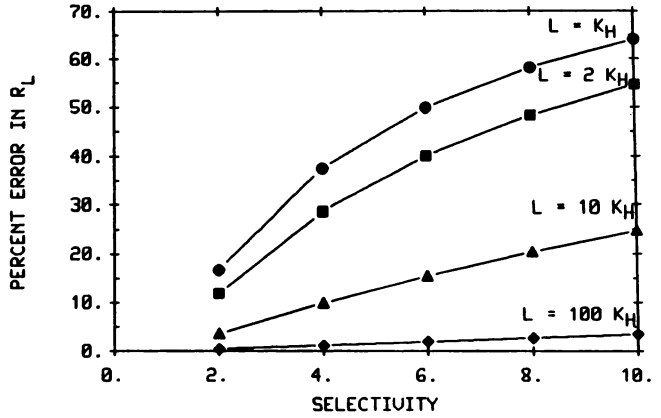

Fig. 3. Effect of the selectivity of a radioligand on the estimate of the proportions of binding sites. Theoretical inhibition data assuming 2- to 10-fold selectivity for the radioligand (*abscissa*) were generated using Eq. 3 and the resulting curves were analyzed with Eq. 2, which assumes that the radioligand is nonselective. A 200-fold selectivity for the inhibitor and a 60% population of low affinity sites (B_L) were assumed. The error in the estimate of the proportion of low affinity sites that resulted from the incorrect assumption that the radioligand was nonselective was calculated from the following equation: % error = 100 × (60 − estimated proportion)/60. L is the free concentration of radioligand and K_H is the dissociation constant of the radioligand at the high affinity sites.

The error in the estimated proportion of low affinity sites that results from the incorrect assumption that a selective radioligand is nonselective was determined for varying conditions assuming that one class of sites had a 2–10 times higher affinity for the radioligand than the other class. Theoretical inhibition curves were generated using Eq. 3 and analyzed with the two-site model (Eq. 2). The error in the estimate of the proportions of subtypes increased as the selectivity of the radioligand increased. This error decreased as the concentration of the radioligand increased (Fig. 3).

Inhibition of the binding of ^{125}I-IPIN by the β_2-selective ligand ICI 118,551 was measured in C_6 glioma cells. Each experiment consisted of inhibition curves measured at 15–21 concentrations of ICI 118,551 and 7–9 concentrations of ^{125}I-IPIN (20–720 pM). Total and nonspecific binding were measured at each concentration of radioligand. Scatchard transformation of specific binding measured in the absence of ICI 118,551 resulted in a linear plot (Fig. 4) with a correlation coefficient greater than 0.98. Moreover, no suggestion of curvature was observed, and the transformed data points were randomly distributed about the regression line as determined by the runs test. These results are consistent with the hypothesis that ^{125}I-IPIN is nonselective for the subtypes of β-adrenergic receptors.

When the inhibition curves were analyzed using the conventional two-site model, however, the estimated proportion of β_1- and β_2-receptors changed in a systematic fashion. In a typical experiment (Fig. 5A), the estimated proportion of β_2-receptors ranged from 69% at the lowest concentration of ^{125}I-IPIN to 50% at the highest concentration. This systematic change indicated that, at low concentrations of ^{125}I-IPIN, the proportion of β_2-adrenergic receptors was overestimated, suggesting that ^{125}I-IPIN is β_2-selective. To determine the affinity of each receptor subtype for ^{125}I-IPIN, the data used to generate the eight inhibition curves were simultaneously fit to Eq. 3 by nonlinear regression analysis. Results from the analysis of one individual experiment are illustrated in Fig. 5B. In this experiment, ^{125}I-IPIN was estimated to be 4.4-fold selective for β_2-receptors, ICI 118,551 was estimated to be 170-fold selective for β_2-receptors, and 46% of the receptors had a high affinity for ICI 118,551 and are presumed to be β_2-adrenergic receptors. The K_d value of 70 pM determined by Scatchard analysis of the specific binding data in the absence of ICI 118,551 (Fig. 4) was intermediate between the K_d values of 40 pM at β_2-receptors and 220 pM at β_1-receptors determined by simultaneous analysis. The inhibition data were also fit to Eq. 3 with the K_d values constrained to be equal to test the contribution of the selectivity of the radioligand to the goodness of fit of the simultaneous analysis. The improvement of fit that resulted from permitting the K_d values to differ was significant at $p < 0.0001$. The results of the simultaneous analysis for four experiments in which the binding of ^{125}I-IPIN was inhibited by ICI 118,551 are summarized in Table 1. ^{125}I-IPIN was found to be 3-fold selective for β_2-receptors. This 3-fold selectivity was statistically significant at the 95% confidence level. ICI 118,551 was 110-fold selective for β_2-receptors, and C_6 glioma cells contain 59% β_1-receptors and 41% β_2-receptors.

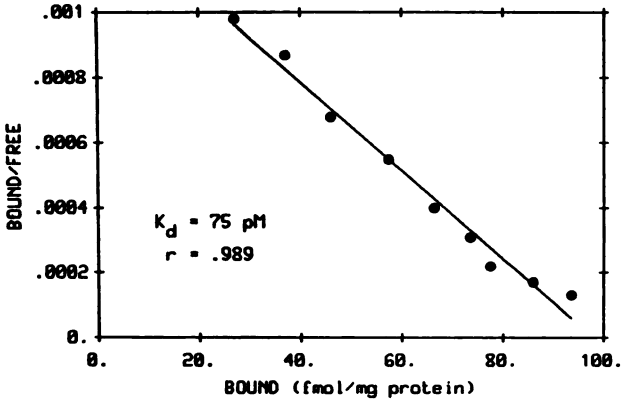

Fig. 4. Scatchard plot for the binding of ^{125}I-IPIN. Saturation isotherms for ^{125}I-IPIN were measured as described in Materials and Methods and the data were transformed by the method of Scatchard. The dissociation constant and correlation coefficient for this representative analysis are shown. Each *point* is the mean of triplicate determinations. Results are representative of seven similar experiments. Free radioligand is in units of fM.

Inhibition of the binding of ^{125}I-IPIN by the β_1-selective ligand ICI 89,406 was also investigated using the same experimental design as described for ICI 118,551. Analysis of the inhibition curves using the conventional two-site model (Eq. 2) revealed that the estimated proportions of β_1- and β_2-receptors changed in a systematic fashion. Results from a typical experiment are presented in Fig. 6. In this experiment, the estimated proportion of β_1-receptors ranged from 31% at the lowest concentration of ^{125}I-IPIN to 50% at the highest concentration. Just as with ICI 118,551, a decrease in the estimated proportion of β_2-receptors was observed as the concentration of ^{125}I-IPIN increased, supporting the conclusion that ^{125}I-IPIN is selective for β_2-receptors. The inhibition curves were simultaneously fit to Eq. 3 by nonlinear regression as described in Materials and Methods. The results of the simultaneous analysis are summarized in Table 1 and are in good agreement with those obtained with ICI 118,551. ^{125}I-IPIN was found to be 3-fold selective for β_2-receptors. The percentage of β_1-receptors was 55%, and ICI 89,406 was found to be 120-fold selective for β_1-receptors. The average selectivity of ^{125}I-IPIN was 3.1 with a 95% confidence interval of 1.9–5.2

Discussion

Accurate estimates of the relative proportions of binding sites and the affinity of each site for a selective competing ligand in a mixed tissue cannot be obtained when the inhibition of the binding of a single concentration of a slightly selective radioligand is analyzed with a conventional two-site model. This occurs because the fractional occupancies of each site differ at all but saturating concentrations of radioligand. In practice, indirect binding experiments are rarely performed with saturating concentrations of radioligand due to the higher level of nonspecific binding observed and the expense of the radioligand. Although some methods of analysis correct for the selectivity of a radioligand (8), the selectivity must be predetermined in a separate experiment requiring tissues that have only a single class of binding sites. Methods currently available to measure the affinities of multiple classes of binding sites for a radioligand lack the resolution to reliably detect slight (<6-fold) selectivity. Analysis of untransformed saturation data measured even under ideal conditions cannot detect selectivities of less than 5- to 7-fold (9). Alternatively, iterative analysis of curvilinear Scatchard plots has serious theoretical and technical limitations. Both variables in a Scatchard plot (Bound and Bound/Free) are dependent variables that are subject to error, and these errors are interdependent. The ratio Bound/Free also demonstrates nonuniformity of variance, thus violating a basic assumption of regression analysis (26). The present results confirm that 3-fold selectivity of a radioligand cannot be detected by Scatchard transformation of saturation data. Using theoretically generated saturation data, a 3-fold selective radioligand produced a Scatchard plot with a correlation coefficient of 0.998. A runs test suggested that the transformed theoretical data were not randomly distributed about the regression line in accordance with curvilinearity, but in no case

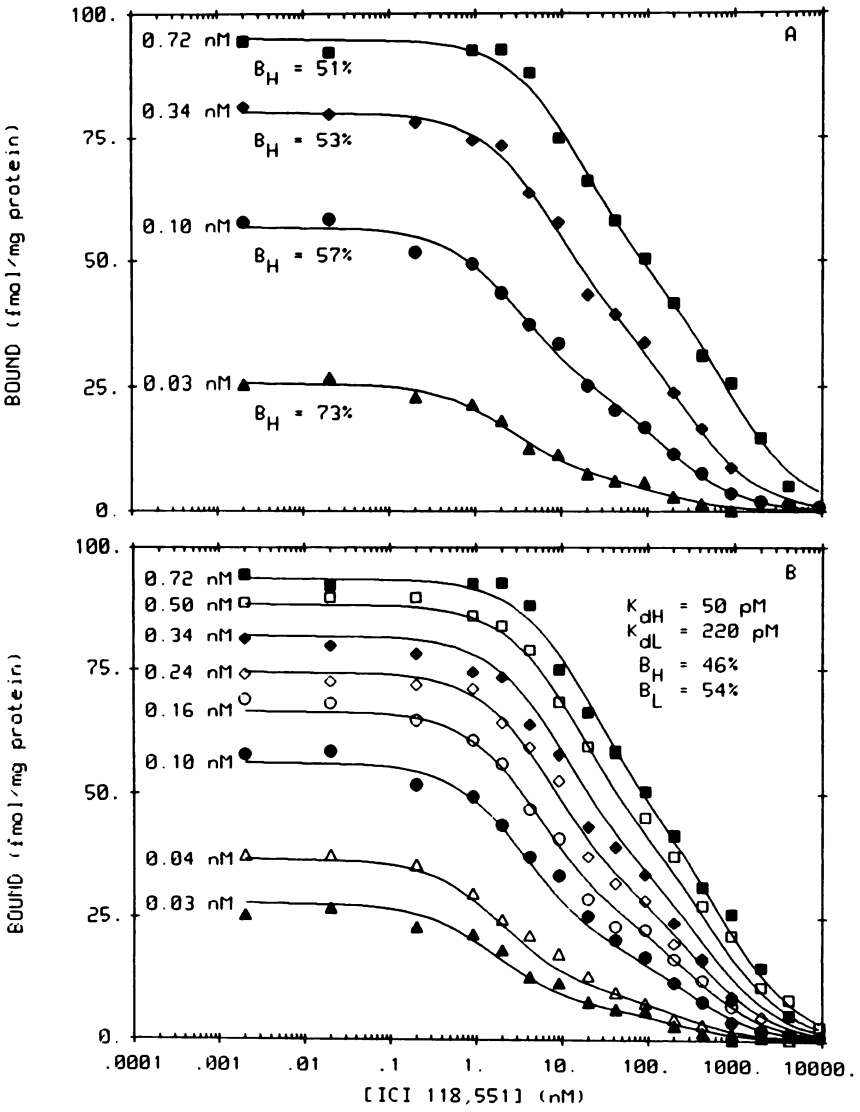

Fig. 5. Inhibition of the binding of ^{125}I-IPIN by the β_2-selective antagonist ICI 118,551. The inhibition of the binding of eight concentrations of ^{125}I-IPIN by the β_2-selective antagonist ICI 118,551 was measured as described in Materials and Methods. A. The inhibition data were fit to Eq. 2, which assumes that the radioligand is nonselective. The results of this analysis are shown for representative concentrations of ^{125}I-IPIN. B. All of the inhibition curves were fit simultaneously using Eq. 3, which can account for the selectivity of a radioligand. The curves are the best fit of Eq. 3 to the data. The concentration of ^{125}I-IPIN at which each curve was measured is given. The proportions of β_2 (B_H)- and β_1 (B_L)-adrenergic receptors and the dissociation constants of ^{125}I-IPIN for each site are shown. The selectivity of ^{125}I-IPIN for β_2-adrenergic receptors was approximately 4-fold in this experiment. Each *point* represents the mean of triplicate determinations. Results are representative of four similar experiments.

TABLE 1

Results of simultaneous analysis with ^{125}I-IPIN

The inhibition of the binding of 8–9 concentrations of ^{125}I-IPIN by 15–21 concentrations of ICI 118,551 (n = 3) or ICI 89,406 (n = 4) was measured as described in Materials and Methods. The data from each experiment were analyzed using Eq. 3, which accounts for selectivity for both the radioligand and the inhibitor. The data represent the mean ± standard error of the parameter estimates from each experiment.

	ICI 89,406	ICI 118,551
K_i (β_1) (nM)	1.1 ± 0.1	155 ± 9
K_i (β_2) (nM)	130 ± 27	1.4 ± 0.2
K_d (β_1) (pM)	211 ± 65	245 ± 28
K_d (β_2) (pM)	67 ± 13	74 ± 13
β_{max} (β_1) (%)	55 ± 2	59 ± 3
β_{max} (β_2) (%)	45 ± 2	41 ± 3
n	4	3

did the runs test on experimentally derived data suggest the presence of curvature. Thus, 3-fold selectivity of a radioligand could not be detected, much less quantitated, using Scatchard analysis.

Another approach is to determine the selectivity of a radioligand by comparing its binding characteristics in two tissues, each containing only one class of binding site. This method has been applied successfully to detect 3-fold selectivity (17); however, the tissues used did not contain a pure population of a single class of receptors. In general, such tissues are only infrequently available. Moreover, the small differences in the affinities of receptors for the radioligand are difficult to detect due to experimental variability, and it must be assumed that the properties of the receptor are entirely conserved from tissue to tissue. It is also possible that the affinity of receptors for a radioligand will differ because of differences in lipid composition of the membranes or the amount of protein (18, 19).

Slight differences in the affinities of binding sites for a radioligand cannot be measured with traditional methods of analysis. Such differences can, however, drastically affect the estimates of the relative proportions of sites and the estimate of the affinity of each class of binding site for the competing ligand when a single concentration of a slightly selective radioligand is used. The error in the estimated proportion of low affinity sites was determined to be 50% when theoretically generated data assuming 6-fold selectivity and a radioligand concentration equal to the dissociation constant at the high affinity site were analyzed. In some cases, this error can be so great that a tissue judged to contain predominantly one subtype

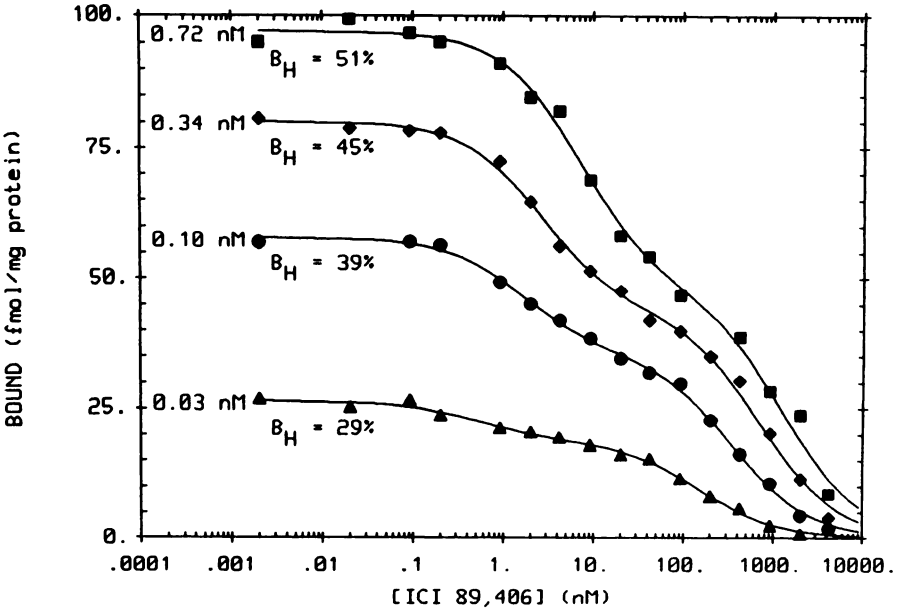

Fig. 6. Inhibition of the binding of ^{125}I-IPIN by the β_1-selective antagonist ICI 89,406. The inhibition of the binding of nine concentrations of ^{125}I-IPIN by the β_1-selective antagonist ICI 89,406 was measured as described in Materials and Methods and the resulting inhibition data were fit to Eq. 2. The best fit curves are shown for a low, an intermediate, and a high concentration of ^{125}I-IPIN. The proportion of β_1-adrenergic receptors (B_H) estimated with Eq. 2 increased as the radioligand concentration increased. When all nine inhibition curves were fit simultaneously using Eq. 3 (not shown), the selectivity of ^{125}I-IPIN was determined to be 3-fold. Each *point* represents the mean of triplicate determinations. The results are representative of three similar experiments.

of receptor could actually contain predominantly the other subtype. From analysis of theoretical data it is clear that the error associated with the estimate of the relative proportions of sites can be minimized if a high concentration of radioligand is used. The drawback of this approach is that the error in the estimate of the K_i values of the binding sites for the unlabeled competing ligand is maximized under these conditions. When a simple two-site model is applied to an inhibition curve measured at a single concentration of radioligand, the IC_{50} values are converted to K_i values according to the method of Cheng and Prusoff (27). This correction is more pronounced at concentrations of radioligand that exceed the K_d value and it is inaccurate if the K_d value of the binding sites for the radioligand are under- or overestimated. The K_d value determined from analysis of saturation data for a slightly selective radioligand will be intermediate between the high and low affinity values, thus overestimating the K_d value at the high affinity site and underestimating the K_d value at the low affinity site.

To avoid erroneous estimates of the relative densities of sites and of K_i values, a radioligand should be tested for selectivity. The selectivity of a radioligand can be detected with a single diagnostic experiment. The inhibition of the binding of the radioligand by a highly selective competing ligand at low, intermediate, and high concentrations is measured in a tissue that contains both of the putative classes of receptors. For this experiment, the low concentration should be less than the K_d value resulting from analysis of saturation data and the high concentration should exceed the K_d value. Furthermore, the range of concentrations should be as great as is technically feasible. If the relative proportions of sites estimated with a conventional two-site model change in a systematic fashion, the radioligand must be assumed to be selective. Furthermore, the radioligand is selective for the class of binding sites whose proportion decreases with increasing concentrations of the radioligand. In the case of ^{125}I-IPIN, the proportion of β_2-receptors in C_6 cells estimated with either ICI 118,551 or ICI 89,406 decreased as the concentration of radioligand increased, indicating that ^{125}I-IPIN is selective for β_2-receptors. This diagnostic test serves to uncover slight selectivity but does not provide quantitative estimates of the affinities of the binding sites for the radioligand.

This diagnostic method for detecting selectivity was first described by Richardson and Howlett (10). It has not been used to detect selectivities of radioligands of less than 10-fold. When this method was used to analyze the inhibition of the binding of ^{3}H-dihydroalprenolol by the selective antagonist practolol in rat brain tissue, it led to the erroneous conclusion that this radioligand is nonselective. Previous reports (8) and application of the approach described in this report (28) have established that ^{3}H-dihydroalprenolol is selective for β_2-adrenergic receptors.

In this report we have described a new method to quantitate selectivities of radioligands that are less than 6-fold selective. This method also provides accurate estimates of the density of each class of binding site and the affinity of each binding site for the unlabeled ligand. This approach requires that a series of inhibition curves with a highly selective competing ligand be generated in the presence of increasing concentrations of radioligand. Simultaneous nonlinear regression analysis of these multiple inhibition curves using Eq. 3 provides estimates of the parameters. The accuracy of these estimates will be affected by several factors including the range of concentrations of the radioligand, the number of concentrations of radioligand used, the number of concentrations of inhibitor, the selectivity of the inhibitor, and the relative densities of binding sites in the tissue. Regression analysis of multiple curves is sensitive to the variability in the raw data. A high degree of variability may make it impossible for the program to fit the data. The range of concentrations of the radioligand should be as wide as is technically feasible and should include concentrations at which the fractional occupancies of the two sites are significantly different. The K_d and K_i values of the radioligand and competing ligand should be much greater than the concentration of receptors. If this is not the case, significant changes in the free concentration of a ligand may occur during the course of the assay. The effect of a 5% change in radioligand concentration was tested by comparing the results of simultaneous analysis of the data in Fig. 5B using 100% and 95% of the total added

concentration. None of the parameter estimates differed by more than 5%, which was less than the standard error associated with each parameter listed in Table 1. On the basis of the results obtained with ^{125}I-IPIN, this method is sufficiently sensitive to detect 3-fold selectivity in a tissue containing approximately equal proportions of two classes of binding sites. Moreover, when only four of the eight inhibition curves in Fig. 5B were used in the simultaneous analysis, each parameter estimate was less than 15% different from the estimate obtained using all of the inhibition curves. No significant difference in the estimates of the parameters from the simultaneous analysis was observed when the data points were weighted according to the reciprocal of the square of their magnitude. The theoretical superiority of simultaneous analysis of multiple inhibition curves has been described by Rothman (29). The accuracy of the method has been demonstrated for a 20-fold selective radioligand by analysis of simulated binding data (30).

The validity of this approach was demonstrated by its application to the interaction of ^{125}I-IPIN with β_1- and β_2-adrenergic receptors. The diagnostic experiment with both ICI 89,406 and ICI 118,551 revealed that ^{125}I-IPIN was selective, and the decrease in the estimated proportion of β_2-receptors with increasing concentrations of ^{125}I-IPIN indicated that the radioligand was selective for β_2-receptors. Consistent with the diagnostic experiment, simultaneous analysis of the inhibition curves revealed that ^{125}I-IPIN is 3-fold selective for β_2-adrenergic receptors. The estimates of the selectivity of ^{125}I-IPIN and the densities of β_1- and β_2-receptors from the simultaneous analysis were the same regardless of the competing drug used for the determination.

Estimates of the relative proportions of sites and the K_i values for the competing ligand obtained from a conventional two-site model can be corrected to account for radioligand selectivity. The K_d values for the interaction of a radioligand with each class of receptors are determined by simultaneous analysis of inhibition curves measured in the tissue of interest. The correct K_i values for the interaction of each class of sites with the competing ligand are determined by converting the measured IC_{50} values according to the method of Cheng and Prusoff (27) using the K_d values from the simultaneous analysis. The relative proportions of high and low affinity sites measured at a single concentration of radioligand can be corrected using the following equations:

$$B_H = \frac{B_H^n/f_H}{B_H^n/f_H + B_L^n/f_L} \text{ or } B_L = \frac{B_L^n/f_L}{B_H^n/f_H + B_L^n/f_L}$$

where B_H and B_L are the correct proportions of high and low affinity sites, respectively, B_H^n and B_L^n are the relative proportions of high and low affinity binding sites, respectively, determined by conventional two-site analysis, and f_H and f_L are the fractional occupancies of the high and low affinity sites, respectively. The fractional occupancies of the high and low affinity sites are:

$$f_H = \frac{L}{L + K_{dH}} \text{ and } f_L = \frac{L}{L + K_{dL}}$$

where L is the free concentration of radioligand and K_{dH} and K_{dL} are the dissociation constants of the radioligand for the high and low affinity sites, respectively. These corrections rely heavily on the accuracy of the estimates of the two dissociation constants. Therefore, the simultaneous analysis should be carried out in the same tissue under the same conditions to attain the most reliable results.

Because the sensitivity of this method is superior to that of traditional methods of analysis, it is likely that other radioligands, previously thought to be nonselective, will be shown to be selective when analyzed by this method. Indeed, this method has been used to demonstrate that ^{125}I-iodocyanopindolol, ^{125}I-iodohydroxybenzylpindolol, and ^{3}H-dihydroalprenolol are all slightly selective for β_2-adrenergic receptors (28). Furthermore, this method is not limited to characterization of the interaction of a radioligand with the subtypes of a single receptor. It can also be used to quantitate the interaction of a radioligand with different receptors or binding sites. For example, this approach has been used to determine the affinities of dopamine and serotonin receptors that coexist in striatal tissue for ^{3}H-spiroperidol.[1] Given the widespread use of indirect binding assays to quantitate multiple receptor subtypes in mixed tissues, this method should be routinely used to quantitate the selectivity of the radioligands used in these assays.

References

1. Molinoff, P. B., B. B. Wolfe, and G. A. Weiland. Quantitative analysis of drug-receptor interactions. II. Determination of the properties of receptor subtypes. *Life Sci.* **29**:427–443 (1981).
2. Minneman, K. P., A. Hedberg, and P. B. Molinoff. Comparison of *beta* adrenergic receptor subtypes in mammalian tissues. *J. Pharmacol. Exp. Ther.* **211**:502–508 (1979).
3. Homburger, V., M. Lucas, E. Rosenbaum, G. Vassent, and J. Bockaert. Presence of both beta$_1$- and beta$_2$-adrenergic receptors in a single cell type. *Mol. Pharmacol.* **20**:463–469 (1981).
4. Carlsson, E., B. Ablad, A. Brandstrom, and B. Carlsson. Differential blockade of the chronotropic effects of various adrenergic stimuli in the cat heart. *Life Sci.* **11**:953–958 (1972).
5. Liang, B. T., L. H. Frame, and P. B. Molinoff. β_3-Adrenergic receptors contribute to catecholamine-induced shortening of action potential duration in dog atrial muscle. *Proc. Natl. Acad. Sci. USA* **82**:4521–4525 (1985).
6. Hancock, A. A., A. L. DeLean, and R. J. Lefkowitz. Quantitative resolution of beta-adrenergic receptor subtypes by selective ligand binding: application of a computerized model fitting technique. *Mol. Pharmacol.* **16**:1–9 (1979).
7. Minneman, K. P., L. R. Hegstrand, and P. B. Molinoff. Simultaneous determination of *beta*-1 and *beta*-2-adrenergic receptors in tissues containing both receptor subtypes. *Mol. Pharmacol.* **16**:34–46 (1979).
8. De Lean, A. L., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).
9. Burgisser, E. Model testing in radioligand/receptor interaction by Monte Carlo simulation. *J. Recept. Res.* **3**:261–281 (1983).
10. Richardson, A., and D. R. Howlett. A method for dissecting two-site receptor interactions in ligand binding studies. *J. Recept. Res.* **3**:541–560 (1983).
11. Minneman, K. P., L. R. Hegstrand, and P. B. Molinoff. The pharmacological specificity of *beta*-1 and *beta*-2 adrenergic receptors in rat heart and lung *in vitro*. *Mol. Pharmacol.* **16**:21–33 (1979).
12. Rugg, E. L., D. B. Barnett, and S. R. Nahorski. Coexistence of *beta*$_1$ and *beta*$_2$ adrenoceptors in mammalian lung: evidence from direct binding studies. *Mol. Pharmacol.* **14**:996–1005 (1978).
13. U'Prichard, D. C., D. B. Bylund, and S. H. Snyder. (±)-(^{3}H)-Epinephrine and (−)-(^{3}H)-dihydroalprenolol binding to β_1- and β_2-noradrenergic receptors in brain, heart and lung membranes. *J. Biol. Chem.* **253**:5090–5102 (1978).
14. Rainbow, T. C., B. Parsons, and B. B. Wolfe. Quantitative autoradiography of β_1- and β_2-adrenergic receptors in rat brain. *Proc. Natl. Acad. Sci. USA* **81**:1585–1589 (1984).
15. Hedberg, A., F. Kempf, Jr., M. E. Josephson, and P. B. Molinoff. Coexistence of *beta*-1 and *beta*-2 adrenergic receptors in the human heart: effects of treatment with receptor antagonists or calcium entry blockers. *J. Pharmacol. Exp. Ther.* **234**:561–568 (1985).
16. Engel, G., D. Hoyer, R. Berthold, and H. Wagner. (±)(^{125}Iodo)cyanopindolol, a new ligand for β-adrenoceptors: identification and quantitation of subclasses of β-adrenoceptors in guinea pig. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **317**:277–285 (1981).
17. Tondo, L., P. G. Conway, and D. J. Brunswick. Labeling *in vivo* of *beta*-adrenergic receptors in the central nervous system of the rat after administration of (^{125}I)iodopindolol. *J. Pharmacol. Exp. Ther.* **235**:1–9 (1985).
18. Heron, D. S., M. Shinitzky, M. Hershkowitz, and D. Samuel. Lipid fluidity

[1] Unpublished observations.

markedly modulates the binding of serotonin to mouse brain membranes. *Proc. Natl. Acad. Sci. USA* **77**:7463–7467 (1980).
19. Seeman, P., C. Ulpian, K. A. Wreggett, and J. W. Wells. Dopamine receptor parameters detected by (^{3}H)-spiperone depend on tissue concentrations: analysis and examples. *J. Neurochem.* **43**:221–235 (1984).
20. Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
21. McGonigle, P., R. M. Huff, and P. B. Molinoff. A comprehensive method for the quantitative determination of dopamine receptor subtypes. *Ann. N. Y. Acad. Sci.* **430**:77–90 (1984).
22. Knott, G. D. MLAB—a mathematical modeling tool. *Comput. Programs Biomed.* **10**:271–280 (1979).
23. Magar, M. E. *Data Analysis in Biochemistry and Biophysics.* Academic Press, New York, 93–166 (1972).
24. Zar, J. H. *Biostatistical Analysis.* Prentice Hall, Inc., Englewood Cliffs, NJ, 406-421 (1984).
25. Tallarida, R. J., and R. B. Murray. *Manual of Pharmacological Calculations.* Springer-Verlag, New York, in press.
26. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
27. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
28. Neve, K. A., P. McGonigle, and P. B. Molinoff. Quantitative analysis of the selectivity of radioligands for subtypes of *beta* adrenergic receptors. *J. Pharmacol. Exp. Ther.* **238**:46–53 (1986).
29. Rothman, R. B. Analysis of binding surfaces: a methodology appropriate for the investigation of complex receptor mechanisms and multiple neurotransmitter receptors. *Neuropeptides* **4**:41–44 (1983).
30. Rothman, R. B. Binding surface analysis: an intuitive yet quantitative method for the design and analysis of ligand binding studies. *Alcohol Drug Res.* **6**:309–325 (1986).

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