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Simultaneous Determination of *Beta*-1 and *Beta*-2-Adrenergic Receptors in Tissues Containing Both Receptor Subtypes

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SUMMARY

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A method for determining the relative concentrations and properties of β-1 and β-2-adrenergic receptors in tissues containing both receptor subtypes has been developed. Since (^{125}I)-iodohydroxybenzylpindolol has similar affinities for β-1 and β-2-adrenergic receptors, it is possible to determine the total concentration of β-adrenergic receptors in a tissue by Scatchard analysis of specific (^{125}I)-iodohydroxybenzylpindolol binding. In the presence of GTP, inhibition of specific (^{125}I)-iodohydroxybenzylpindolol binding in rat heart, lung and five regions of rat brain by agonists and antagonists that are not specific for β-1 or β-2-adrenergic receptors yields linear Hofstee plots with Hill coefficients of approximately 1.0. On the other hand, the inhibition of specific (^{125}I)-iodohydroxybenzylpindolol binding by drugs which have been shown to have different affinities for heart (β-1) and lung (β-2) receptors *in vitro* results in nonlinear Hofstee plots in each of these tissues. Two of these drugs (practolol and metoprolol) are more potent on β-1 than β-2 receptors and two of these drugs (zinterol and salmefamol) are more potent on β-2 than on β-1 receptors. The nonlinear Hofstee plots are consistent with there being two types of binding sites in each of the tissues with different affinities for the drugs. The relative number of each type of binding site and the affinity of each drug for each of the two types of site has been calculated using a computer based iterative procedure. Using this method, the relative percentages of the two receptor subtypes in rat heart, lung, cerebral cortex, caudate, cerebellum, hippocampus and diencephalon were determined. In each tissue, the use of four different drugs with different *in vitro* selectivity (two β-1 selective and two β-2 selective) resulted in approximately the same calculated β-1/β-2 ratio. This suggests that the assumption that the nonlinear Hofstee plots are composed of only two components is correct. In addition, the calculated affinity of each drug for β-1 and β-2 receptors was quantitatively similar in each of the seven tissues examined. The calculated ratios of β-1:β-2-adrenergic receptors are: heart 83:17; lung 15:85; cortex 81:19; caudate 76:24; cerebellum 15:85; hippocampus 81:19; and diencephalon 71:29. The absolute concentrations of β-1-adrenergic receptors in the brain regions examined varied by almost 20-fold. However, the absolute concentration of β-2-adrenergic receptors varied less than 3-fold. This suggests that β-2-adrenergic receptors in rat brain are associated with a more homogeneously distributed cellular element than are β-1-adrenergic receptors.

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INTRODUCTION

In the preceding paper the *in vitro* pharmacological specificity of β-adrenergic receptors in rat heart and lung has been defined to obtain an estimate of the pharmacological specificity to be expected of β-1 and β-2-adrenergic receptors in the central nervous system. A number of drugs that are cardiac- or bronchial-selective *in vivo* were also shown to have markedly different affinities for these β-adrenergic receptors *in vitro*. Four drugs showed a marked selectivity for inhibition of specific IHYP[3] binding in heart as compared to lung. Two of these drugs (practolol and metoprolol) have been previously classified as β-1-selective antagonists *in vivo* and were found to be significantly more potent in inhibiting IHYP binding in rat heart than in rat lung. The other two (zinterol and salmefamol) have been classified as β-2-selective agonists and were significantly more potent in inhibiting specific IHYP binding in rat lung than in rat heart.

Increasing evidence suggests that in some organs, including cat and dog heart and guinea pig trachea, both β-1 and β-2-adrenergic receptors may co-exist and may mediate the same physiological response (1, 2). If this is true the exact pharmacological specificity of a particular response will depend on the ratio of β-1/β-2 receptors mediating that response as well as on the relative affinities of the two types of receptor for the drug in question.

Unfortunately, as documented in the preceding paper, the selectivity of particular drugs for β-1 or β-2-adrenergic receptors is not absolute *in vitro*. Even the most selective drugs affect both receptors, although with different affinities. This means that it is not possible to devise conditions under which one can selectively measure only the β-1 receptors, or only the β-2 receptors. A method has therefore been developed to analyze the kinetics of the inhibition of specific IHYP binding in various tissues by selective β-1 or β-2 drugs to determine the relative concentrations of the two receptor subtypes.

Conditions have been established such that the inhibition of IHYP binding by nonselective drugs follows simple Michaelis-Menten (mass action) kinetics, yielding linear Hofstee plots and Hill coefficients of 1.0. Under these conditions, the inhibition of specific IHYP binding by β-1 and β-2 selective drugs yields nonlinear Hofstee plots and nonlinear Hill plots. As a working hypothesis we have assumed that the nonlinear Hofstee plots were composed of only two independent components, each of which obeys simple Michaelis-Menten kinetics. The curves were then dissected into their discrete components by a computerized iterative procedure, yielding the relative proportion of each receptor subtype and the affinity of the drug for each subtype of receptor.

In this report results obtained with four drugs with different selectivities for β-1 and β-2-adrenergic receptors are analyzed. The results suggest that each of seven different tissues, (including heart and lung) contain both β-1 and β-2 adrenergic receptors.

METHODS

Preparation of tissue homogenates. Male Sprague-Dawley rats were killed by decapitation; the hearts and lungs were removed and placed on ice. Brains were removed from the skull and the cerebellum removed and placed on ice. A cut was made through the corpus callosum and the cortex was peeled back, exposing the hippocampus and caudate which were carefully removed and placed on ice. Any remaining white matter was removed from the cortex which was then placed on ice. A cut was then made anterior to the pons to remove the "diencephalon" which included the thalamus and hypothalamus.

Tissues were homogenized with a Brinkmann Polytron (setting 5–7) for 10–15 sec in 20 vol of "isosaline" (0.9% NaCl containing 20 mM Tris HCl, pH 7.5). The homogenates were centrifuged at 20,000 × *g* for 10 min, the supernatants decanted and discarded, and the pellets resuspended in 150 (cerebellum, thalamus, hippocampus), 200 (heart, cortex, caudate) or 600 (lung) volumes of isosaline.

β-Adrenergic receptor binding assay. Specific binding of IHYP was determined

[3] The abbreviation used is: IHYP, (^{125}I) iodohydroxybenzylpindolol.

as described in the preceding paper. The concentration of IHYP ranged from 50–100 pM. GTP was included in all binding assays at a final concentration of 300 μM. In binding assays of regions of rat brain it was necessary to include 100 μM phentolamine to reduce nonspecific binding. This concentration of phentolamine did not affect specific IHYP binding (3; see below). Specific binding of IHYP was defined as the amount of IHYP bound in the absence of competing ligand minus the amount bound in the presence of 10 μM *l*-isoproterenol. All binding assays were conducted such that bound ligand was less than 10% of total ligand. Average protein concentration was 65 μg/tube. Specific binding (as a percentage of total binding) routinely represented 70% in heart, 60% in lung, 75% in cortex, 70% in caudate, 60% in cerebellum, 60% in hippocampus and 50% in diencephalon.

Kinetic analysis and computerized dissection of two component Hofstee plots. All kinetic analyses were carried out on a WANG 2200-T computer system. Nonweighted linear regression analysis of Scatchard, Hill and linear components of Hofstee (modified Scatchard) plots were done by the least squares fit method.

For analysis of nonlinear Hofstee plots, it was initially assumed that the curvature was due to the presence of only two components, each of which followed simple Michaelis-Menten kinetics (the validity of this assumption is discussed below). The magnitude of the contribution of each component to the other is a function of the difference in affinity of the ligand for the two classes of receptor as well as the relative number of each class of binding site. A computer based method has been developed which iteratively corrects the kinetic values for the two classes of sites by accounting for the contribution of the other component (4).

The Hofstee plots were arbitrarily divided, and the first half of the points assigned to the first (higher affinity) component and the second half of the points assigned to the second (lower affinity) component. A line was then calculated for the first component using linear regression through the points assigned to that component. The slope and y intercept value for this line yielded apparent values for the K_d (affinity) and B_{max} (density of binding sites) for this component. Using the values calculated for the first component, the contribution of this component to the binding data obtained at each concentration of ligand was used to correct the data needed to calculate the kinetic constants of the second component. A line was then calculated for the second component by linear regression using the corrected data points. This line yielded apparent K_d and B_{max} values for the second component. Revised kinetic constants of the first component were then determined using the corrected values for the first component. A corrected line was then calculated for the first component, yielding a new value for the K_d and B_{max}. The contribution of this new first component to the original data points of the second component was then calculated and the process continuously reiterated until the B_{max} values of the two components changed less than 0.5% in ten iterations. For convenience, the program was written such that a minimum of 20 iterations were performed. In many cases this was sufficient to obtain the desired precision; however, in some cases up to 100 iterations were required. The calculated K_d and B_{max} values for each component at this point were taken to represent the relative percentage of binding sites and affinities of the drug for each binding site. As discussed below, neither the degree of specificity of a given drug for the two classes of receptors, the proportion of each class of receptors, or the data points initially assigned to each class of receptors had any effect on the ultimate values obtained. These variables did affect the number of iterations required. Final K_d values were corrected for the concentration of IHYP by dividing EC_{50} values obtained from the slopes of the Hofstee plots by $(1 + (IHYP)/K_d)$.

This analysis is based on the shape of the curve and we have shown, using theoretical data, that it makes no difference which points are assigned to each component, or even which points are included on the graph. It is obvious, however, that both of these considerations would strongly bias

visual analysis of data of this type. Analyzing the data by eye would also consistently give a significant overestimation of the proportion of the high affinity component as the contribution of the low affinity component would not be subtracted out. With theoretical data (Fig. 1) we have shown that assuming only a 20-fold specificity (i.e., K_d values of 1 and 20) and equal B_{max} values (50 in each case), the computer calculated the correct kinetic characteristics of the two components even when the lowest concentration of competing drug was 10 times the K_d value of the high affinity site (at this point over 62% of total binding and 95% of the binding to the high affinity site is already inhibited). This calculation required 160 iterations. One could not expect such accuracy from real data due to errors in measurements; however, the concentrations of drugs routinely range from well below the K_d of the high affinity site to well above that of the low affinity site.

This method can be useful for analyzing two component Hofstee plots. It must be stressed, however, that two very important assumptions are involved: 1) that there exist only two components, and 2) that each component obeys simple Michaelis-Menten (mass-action) kinetics. The validity of these assumptions will be dealt with in detail in the DISCUSSION.

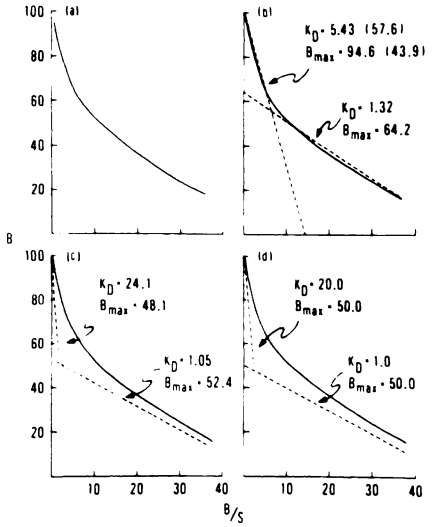

FIG. 1. *Characteristics of computerized analysis of biphasic Hofstee plots*

Part (a) shows theoretical data for a line consisting of two components. One component has a K_d of 1 and a B_{max} of 50 and the other component has a K_d of 20 and a B_{max} of 50. In part (b) the line is arbitrarily split in half and lines are drawn through each half of the curve using linear regression. The K_d and B_{max} values calculated in this way are indicated on the figure. The numbers in parentheses in part (b) indicate K_d and B_{max} values calculated for the low affinity component after the contribution of the high affinity component has been subtracted out. Part (c) shows the lines and calculated K_d and B_{max} values for each component after 5 successive iterations. Part (d) shows the lines and kinetic parameters for each component after 20 successive iterations. At this point the B_{max} has not changed by 0.5% in the last 10 iterations, and the analysis is terminated. B is the amount bound and S is the concentration of competing drug.

RESULTS

Effect of phentolamine on specific and nonspecific IHYP binding in rat brain regions. Sporn and Molinoff (3) have previously reported that phentolamine, at concentrations of up to 100 μM, selectively reduced nonspecific IHYP binding in certain regions of rat brain but had no effect on specific binding. Phentolamine appeared to inhibit specific binding in some brain regions including the hypothalamus and brain stem.

The effects of phentolamine on IHYP binding were examined in rat cortex, caudate, cerebellum, hippocampus and diencephalon. The data for the cerebellum are shown in Fig. 2. The effects of increasing concentrations of phentolamine (up to 300 μM) on total IHYP binding, IHYP binding in the presence of an arbitrary (10 μM) concentration of *d*-isoproterenol, and IHYP binding in the presence of the same concentration of *l*-isoproterenol were determined. The difference between binding in the presence of *d*-isoproterenol and binding in the presence of *l*-isoproterenol represents binding to some but not all of the β-adrenergic receptors in the preparation. This difference does not represent binding to *all* the specific binding sites since *d*-isoproterenol at this concentration also inhibits some specific binding (see preceding paper). Although total binding was markedly reduced by phentolamine the specific binding sites measured in this manner were not affected

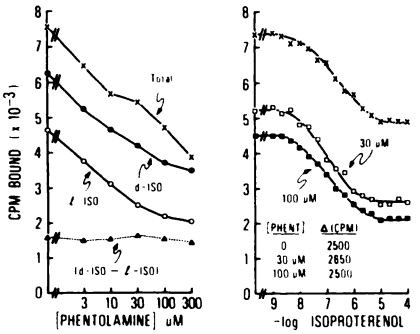

FIG. 2. *Effect of phentolamine on IHYP binding in rat cerebellum*

The left hand side of the figure shows the effect of increasing concentrations of phentolamine on total (×——×) IHYP binding, IHYP binding in the presence of 10 μM *d*-isoproterenol (●——●), IHYP binding in the presence of 10 μM *l*-isoproterenol (○——○), and the difference between binding in the presence of *d*- and *l*-isoproterenol (▲---▲). The right hand side of the figure shows the effect of increasing concentrations of *l*-isoproterenol on IHYP binding in the absence (×——×) or the presence of 30 μM (□——□) or 100 μM (■——■) phentolamine. Inset in the lower left hand corner is the difference (in cpm) between total IHYP binding in the presence of a maximal inhibitory concentration of *l*-isoproterenol (10 μM) for each phentolamine (PHENT) concentration. Conditions were as described in the text and each point is the mean of duplicate determinations from three separate animals.

by concentrations of phentolamine up to 300 μM. Similar results were obtained in each of the other brain regions examined. The effect of phentolamine has not been examined in either heart or lung. The high proportion of specific binding in these tissues makes it unnecessary to include this compound.

To define total specific binding, the experiment in the second part of Fig. 2 was performed. Dose-response curves for the inhibition of IHYP binding by *l*-isoproterenol were done in the absence and presence of 30 and 100 μM phentolamine. *l*-Isoproterenol at concentrations up to 10 μM caused a dose-dependent inhibition of IHYP binding. Increasing the concentration of *l*-isoproterenol another 10-fold caused no further decrease in binding. The difference between binding in the absence of *l*-isoproterenol and binding in the presence of 10 μM isoproterenol was identical in the presence or absence of phentolamine. However, the percentage of specific binding increased from 35% in the absence of phentolamine to 56% in the presence of 100 μM phentolamine. Binding inhibited by 10 μM isoproterenol was taken as a definition of total specific binding. Similar results were observed in the other brain regions studied.

Inhibition of nonspecific IHYP binding in rat lung and cerebellum by zinterol and salmefamol. Zinterol and salmefamol, in addition to inhibiting specific IHYP binding, also inhibited nonspecific IHYP binding in some tissues. To correct for possible errors in determining the kinetics of inhibition of specific IHYP binding by these drugs in these tissues, the effect of the drugs on IHYP binding was measured in the presence and absence of 10 μM *l*-isoproterenol. Zinterol and salmefamol inhibited nonspecific binding (i.e., binding in the presence of 10 μM *l*-isoproterenol) in the lung at concentrations where they did not completely inhibit specific IHYP binding (Fig. 3). The difference between binding in the absence and binding in the presence of 10 μM *l*-isoproterenol was taken to represent specific binding. Similar effects were observed for these two drugs in the cerebellum (data not shown). However, these drugs did not affect nonspecific binding in other tissues.

Inhibition of specific IHYP binding by nonselective agonists and antagonists. Previous work has shown that although the interaction of antagonists with the β-adrenergic receptor follows simple Michaelis-Menten kinetics (i.e., has a Hill coefficient of 1.0) the effects of agonists on inhibiting IHYP binding often show shallow displacement curves consistent with apparent negative cooperativity (i.e., Hill coefficients of 0.6–0.7) (3, 4). This apparently agonist-specific negatively cooperative inhibition of binding disappears in the presence of GTP. Inclusion of GTP in the assay leads to a four- to ten-fold reduction in the potency of agonists. However, the kinetics of the inhibition of binding appear to follow simple Michaelis-Menten kinetics (with Hill coefficients of 1.0) (5, 6). To avoid added complexity and permit a simple kinetic analysis, all binding assays routinely included GTP at a final concentration of 300 μM (see METHODS).

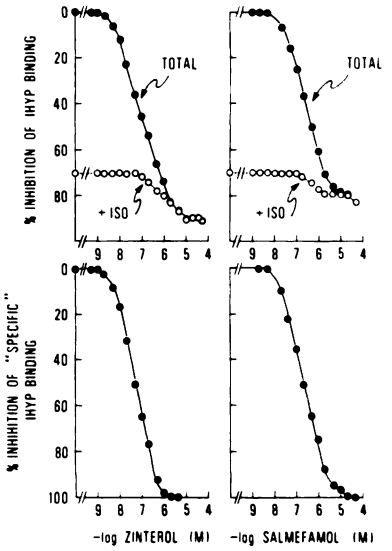

FIG. 3. *Effect of zinterol and salmefamol on total and nonspecific IHYP binding in rat lung*

Effect of increasing concentrations of zinterol (left) and salmefamol (right) on total IHYP binding (top ●——●) and IHYP binding in the presence of 10 μM *l*-isoproterenol (top O- - -O) in rat lung. Each point is the mean of duplicate determinations from 6 animals. On the bottom is plotted the percent inhibition of "specific" IHYP binding, where "specific" binding is calculated as the difference between IHYP binding in the absence and presence of 10 μM *l*-isoproterenol.

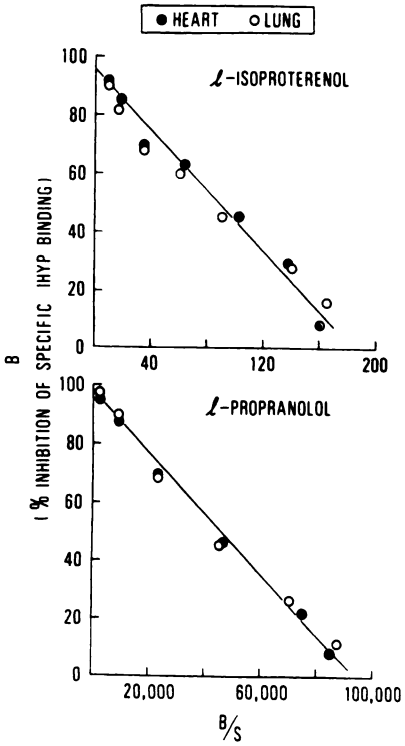

FIG. 4. *Hofstee plots for the inhibition of specific IHYP binding by l-isoproterenol and l-propranolol in rat heart and lung*

The effect of *l*-isoproterenol (top) and *l*-propranolol (bottom) on inhibiting specific IHYP binding in rat heart (●——●) and lung (O——O) is shown. Conditions were as described in the text and each point is the mean of duplicate determinations on 4 separate animals. Plotted on the ordinate is the percent inhibition of specific IHYP binding, and on the abscissa is this value divided by the concentration of competing drug.

Under these conditions (Fig. 4) inhibition of IHYP binding by nonselective agonists and antagonists such as *l*-isoproterenol and *l*-propranolol followed simple Michaelis-Menten kinetics in both heart and lung, and yielded linear Hofstee plots and Hill plots with coefficients of approximately 1.0. The K_d value for isoproterenol was 0.27 μM, for epinephrine 4 μM and for propranolol was .0005 μM in both heart and lung. Similar results were obtained in the 5 regions of rat brain investigated and with other nonselective agonists and antagonists, including epinephrine, sotalol, pindolol and dihydroalprenolol which showed similarly simple kinetics in these tissues (data not shown).

Inhibition of specific IHYP binding in rat heart and lung by β-1 and β-2-selective drugs. The β-1 antagonists practolol and metoprolol were more potent in inhibiting IHYP binding in rat heart than in rat lung, and the β-2 agonists zinterol and salmefamol were more potent in inhibiting IHYP binding in rat lung than in rat heart (see preceding paper). These data were interpreted to suggest that these drugs, which have previously been shown to have different affinities for β-1 and β-2 receptors *in vivo* and *in vitro* with cellularly intact isolated organs and tissues also have different affinities for β-1 and β-2-adrenergic receptors as studied *in vitro* with direct binding assays. Hofstee plots for the inhibition of specific IHYP binding by these β-1 and β-2 selective drugs exhibited a marked curvature in both heart and lung (Fig. 5). This is in contrast to the linear Hofstee plots obtained with nonselective drugs (Fig. 4). If

it is assumed that these drugs are binding with different affinities to two classes of receptors, it can be seen that for the β-2-selective drugs zinterol and salmefamol, the high affinity component (which would be the β-2 component) is the small component in the heart and the large component in the lung (Fig. 5). However, with β-1 selective drugs such as practolol and metoprolol, the reverse is true and the high affinity component (which in this case is the β-1 component) is the large component in the heart and the small component in the lung (Fig. 5).

Analyzing these data by the computer based iterative method described above yielded consistent percentages of β-1 and β-2 receptors in heart and lung, irrespective of whether the drug used for analysis was β-1-selective or β-2-selective (Table 1). In addition, the K_d value calculated for each drug for each component was similar in heart and lung (Table 2). Varying the receptor concentration by a factor of 3 caused no change in the affinity or specificity of any of the drugs examined. The K_d value for the small population of sites showed more variation than that for the large population of sites probably due to the smaller set of data points which contribute to this component. The results are consistent with the idea that two classes of receptor exist in both tissues, and that although they have similar pharmacological specificities, they exist in very different proportions in the two tissues.

Inhibition of specific IHYP binding in rat brain regions by β-1 and β-2-selective drugs. Hofstee plots for the inhibition of specific IHYP binding by zinterol, salmefamol, practolol and metoprolol in various regions of rat brain were consistently nonlinear. Hofstee plots for these four drugs in caudate, cortex and cerebellum are shown in Fig. 6. Similar data were obtained in hippocampus and diencephalon (data not shown). As in heart and lung, calculation of the percentage of β-1 and β-2 receptors in each brain region by the computer-based iterative procedure described above gave consistent percentages of β-1 and β-2-adrenergic receptors in each brain region regardless of which drug was used for analysis (Table 3). It is clear that the caudate, cortex, hippocampus and diencephalon contain mainly β-1 receptors, while the cerebellum contains mainly β-2 receptors (Table 3). The affinities of each drug for β-1

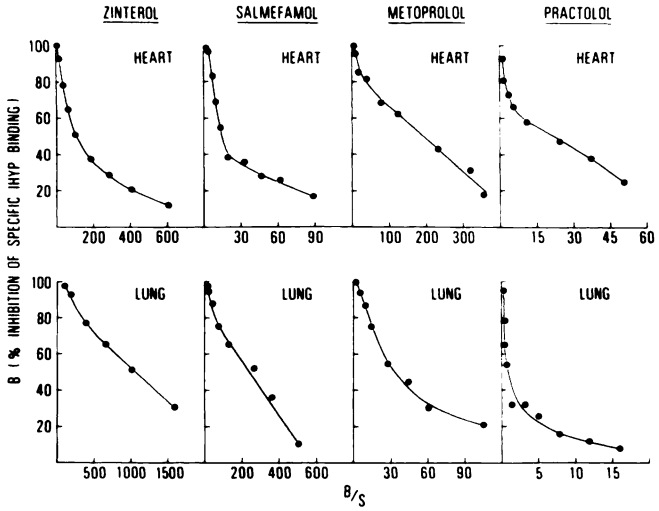

FIG. 5. *Hofstee plots for the inhibition of specific IHYP binding by β-1 and β-2 selective drugs in rat heart and lung*

Effect of zinterol, salmefamol, metoprolol and practolol on specific IHYP binding in rat heart (top) and lung (bottom). Each point is the mean of duplicate determinations on 6 animals. Plotted on the ordinate is the percent inhibition of specific IHYP binding, and on the abscissa is this value divided by the concentration of drug.

TABLE 1

Relative concentrations of β-1 and β-2-adrenergic receptors in rat heart and lung

The kinetics of inhibition of specific IHYP binding were determined for each drug in each tissue at fourteen different concentrations as described in METHODS. The resulting Hofstee plot was then analyzed by the computerized iterative procedure described in the text resulting in two components. For the β-2 selective drugs zinterol and salmefamol, the high affinity component was taken to represent the β-2 component and the low affinity component was the β-1 component. For the β-1 selective drugs practolol and metoprolol the high affinity component was taken to represent the β-1 component and the low affinity component was the β-2 component. Each value is the mean ± SEM of 6 separate experiments.

Drug used to calculate percentage	Heart		Lung	
	% β-1	% β-2	% β-1	% β-2
Zinterol	83.4 ± 4.6	16.6 ± 4.6	15.7 ± 4.2	84.3 ± 4.2
Salmefamol	86.2 ± 7.6	13.8 ± 7.6	19.8 ± 1.9	80.2 ± 1.9
Practolol	75.8 ± 5.2	24.2 ± 5.2	17.1 ± 2.7	82.9 ± 2.7
Metoprolol	86.3 ± 6.2	13.7 ± 6.2	9.0 ± 5.0	91.0 ± 5.0
Mean	82.9 ± 2.47	17.1 ± 2.47	15.4 ± 2.30	84.6 ± 2.30

TABLE 2

Apparent affinities of β-1 and β-2 adrenergic receptors for β-1 and β-2 selective drugs in rat heart and lung

The inhibition of specific IHYP binding was determined for each drug in each tissue as described in Fig. 1 and Table 1. Each value shown is the mean ± SEM of values determined in 6 separate experiments.

	Zinterol K_d (μM)		Salmefamol K_d (μM)		Practolol K_d (μM)		Metoprolol K_d (μM)	
	β-1	β-2	β-1	β-2	β-1	β-2	β-1	β-2
Heart	1.0 ± .23	0.02 ± .004	4.2 ± .82	.25 ± .11	2.1 ± .53	34 ± 5.6	.15 ± .03	2.8 ± .66
Lung	1.0 ± .15	0.04 ± .005	3.2 ± .65	.22 ± .07	0.5 ± .18	20 ± 4.8	.05 ± .03	1.5 ± .20

and β-2-adrenergic receptors in the various regions of rat brain (Table 4) were similar to each other and to those values calculated in experiments with heart and lung (Table 2).

Density of β-1 and β-2-adrenergic receptors in rat brain regions. Since IHYP nonselectively labels both receptor subtypes (see preceding paper) Scatchard analysis of IHYP binding provides an estimate of the total number of β-adrenergic receptors in a given tissue. The percentage of β-1 and β-2-adrenergic receptors in each brain region was then determined by averaging the percentages obtained with the four drugs (Table 3). These calculations yield the actual density of β-1 and β-2-adrenergic receptors in each brain region (Table 5). It is interesting to note that although the density of β-1-receptors varies almost 20-fold in the regions examined, the density of β-2-receptors varies by less than 3-fold. Thus the β-2-adrenergic receptors in rat brain are associated with a more homogeneously distributed tissue constituent than are β-1-adrenergic receptors.

DISCUSSION

A number of drugs which show selectivity for either heart (β-1) or lung (β-2) receptors *in vivo* also show selectivity for these receptors *in vitro* (see preceding paper). This selectivity is seen with both β-1 selective (metoprolol and practolol) and β-2-selective (zinterol and salmefamol) drugs. These observations provide direct evidence to support the hypothesis that there exist more than one type of β-adrenergic receptor with differing pharmacological specificities (7).

To examine this phenomenon in a more precise manner, it was necessary to devise a method to measure the relative concentrations of β-1 and β-2-adrenergic receptors in tissues containing both receptor subtypes and to determine their affinities for a variety of drugs. This objective was made more difficult by the fact that no drug so far

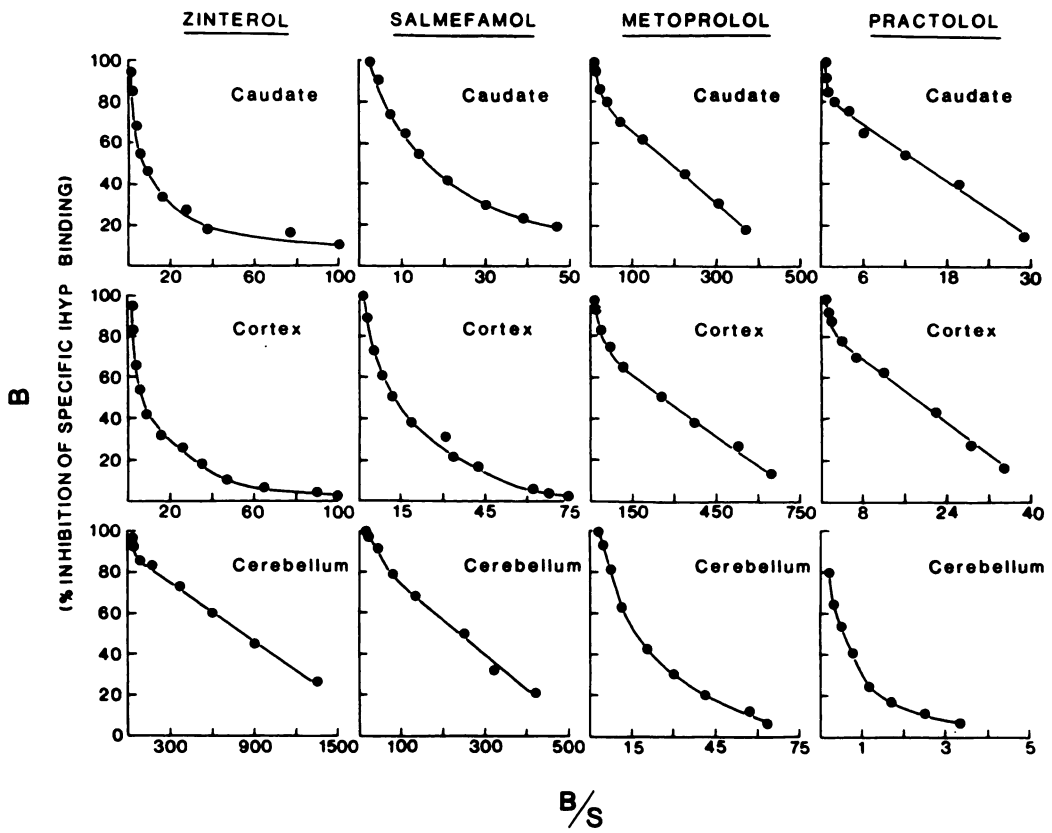

FIG. 6. *Hofstee plots for the inhibition of specific IHYP binding by β-1 and β-2 selective drugs in rat caudate, cortex and cerebellum*

Effect of zinterol, salmefamol, metoprolol and practolol on inhibiting specific IHYP binding in rat caudate (top), cortex (middle) and cerebellum (bottom) is shown. Each point is the mean of duplicate determinations on 6 animals. Plotted on the ordinate is percent inhibition of specific IHYP binding, and on the abscissa is this value divided by the concentration of competing drug (μM).

examined shows an absolute selectivity for β-1 or β-2-adrenergic receptors.

To be able to carry out a straightforward kinetic analysis, it is necessary that the interaction of drugs with β-adrenergic receptors follow simple Michaelis-Menten (mass action) kinetics. This has previously been shown to be true for the inhibition of IHYP and dihydroalprenolol binding by nonselective antagonists such as propranolol and alprenolol in a number of different tissues (5, 6). The interaction of these drugs as well as the nonselective antagonists dihydroalprenolol, pindolol and sotalol with IHYP binding sites in rat heart and lung followed simple Michaelis-Menten kinetics yielding linear Hofstee plots and Hill plots with coefficients of 1.0. On the other hand, the inhibition of specific IHYP binding by a variety of nonselective β-adrenergic *agonists* showed an apparent negative cooperativity with Hill coefficients ranging from 0.6 to 0.7 (5, 6). In the presence of GTP the affinity of agonists for β-adrenergic receptors is reduced and negative cooperativity is no longer apparent. GTP causes the inhibition of specific IHYP binding by nonselective β-adrenergic agonists to follow simple Michaelis-Menten kinetics yielding linear Hofstee plots and Hill coefficients of 1.0. Therefore, under the conditions used (i.e., in the presence of GTP) the inhibition of specific IHYP binding by nonselective agonists and nonselective antagonists follows simple Michaelis-Menten kinetics and shows no evidence of cooperativity in either

TABLE 3

Relative concentrations of β-1 and β-2-adrenergic receptors in discrete regions of rat brain

The kinetics of inhibition of IHYP binding was determined for each drug in each tissue as described in Fig. 1. Computer analysis of the resulting Hofstee plots resulted in the relative percentages of each component in each tissue. Each value is the mean ± SEM of 6 separate experiments.

	Caudate		Cortex		Cerebellum		Hippocampus		Diencephalon	
	% β-1	% β-2	% β-1	% β-2	% β-1	% β-2	% β-1	% β-2	% β-1	% β-2
Zinterol	74.7 ± 3.1	25.3 ± 3.1	80.5 ± 2.9	19.5 ± 2.9	15.0 ± 1.9	85.0 ± 1.9	80.6 ± 4.7	19.4 ± 4.7	74.0 ± 8.0	26.0 ± 8.0
Salmefamol	76.5 ± 5.1	23.5 ± 5.1	83.6 ± 1.7	16.4 ± 1.7	17.4 ± 4.3	82.6 ± 4.3	78.3 ± 3.7	21.7 ± 3.7	67.1 ± 4.3	32.9 ± 4.3
Practolol	76.0 ± 1.0	24.0 ± 1.0	81.5 ± 4.4	18.5 ± 4.4	17.6 ± 8.9	82.4 ± 8.9	84.0 ± 8.8	16.0 ± 8.8	69.1 ± 5.2	30.9 ± 5.2
Metoprolol	78.9 ± 7.5	21.1 ± 7.5	80.2 ± 6.5	19.8 ± 6.5	11.7 ± 3.2	88.3 ± 3.2	82.2 ± 3.6	17.8 ± 3.6	73.3 ± 8.9	26.7 ± 8.9
Mean	76.5 ± 0.88	23.5 ± 0.88	81.5 ± 0.77	18.5 ± 0.77	15.4 ± 1.38	84.6 ± 1.38	81.3 ± 1.21	18.7 ± 1.21	70.9 ± 1.66	29.1 ± 1.66

TABLE 4

Apparent affinities of β-1 and β-2-selective drugs for β-1 and β-2-adrenergic receptors in discrete regions of rat brain

The inhibition of specific IHYP binding was determined for each drug in each tissue as described in Fig. 1. Computer analysis of the resulting Hofstee plot yielded the apparent K_d values of each drug for each component in each tissue. Each value is the mean ± SEM of 6 separate experiments.

	Zinterol K_d (μM)		Salmefamol K_d (μM)		Practolol K_d (μM)		Metoprolol K_d (μM)	
	β-1	β-2	β-1	β-2	β-1	β-2	β-1	β-2
Caudate	1.9 ± 0.32	.034 ± .010	6.9 ± 1.8	.33 ± .12	2.3 ± .51	63 ± 34.8	.18 ± .03	3.2 ± 1.15
Cortex	1.5 ± .30	.026 ± .009	2.9 ± .42	.25 ± .10	5.0 ± 1.18	91 ± 24.4	.17 ± .04	3.5 ± .64
Cerebellum	1.7 ± .42	.030 ± .006	3.3 ± .13	.17 ± .05	2.1 ± .48	71 ± 28.3	.13 ± .02	4.2 ± 1.29
Hippocampus	3.0 ± 1.02	.034 ± .012	3.1 ± .39	.18 ± .06	1.9 ± .55	47 ± 16.5	.13 ± .02	3.8 ± 1.21
Diencephalon	1.8 ± .30	.038 ± .009	4.4 ± .30	.17 ± .05	1.7 ± .33	30 ± 15.4	.20 ± .06	2.9 ± .23

TABLE 5

Densities of β-1 and β-2-adrenergic receptors in discrete areas of rat brain

The total number of β-adrenergic receptors in each brain area was determined by Scatchard analysis of specific IHYP binding as described in text. Each value is the mean ± SEM of 3 animals. The number of β-1 and β-2-adrenergic receptors in each brain region was determined by calculating the percentage of each receptor subtype as measured with four different drugs in Table 3 and multiplying by the total number of β-adrenergic receptors in that region. The age of the rats was 4–5 weeks.

	Total β-adrenergic receptors	β-1-adrenergic receptors	β-2-adrenergic receptors
	(fmole IHYP bound/mg protein)	*(fmole IHYP bound/mg protein)*	*(fmole IHYP bound/mg protein)*
Cerebellum	21.7 ± 2.24	3.3 ± 0.46	18.4 ± 1.92
Diencephalon	27.4 ± 1.69	19.4 ± 1.28	8.0 ± 0.67
Hippocampus	38.1 ± 5.11	31.0 ± 4.18	7.1 ± 0.95
Caudate	54.0 ± 3.41	41.3 ± 2.65	12.7 ± 0.93
Cortex	71.3 ± 8.38	58.1 ± 6.84	13.2 ± 1.55

heart or lung.

The observation that drugs which show differential affinities for β-adrenergic receptors in rat heart and lung *in vitro* yield nonlinear Hofstee plots for the inhibition of specific IHYP binding in a number of different tissues even in the presence of GTP suggests that both receptor subtypes exist in these tissues. It seems likely that the interaction of a drug with each receptor subtype follows simple kinetics since, as discussed above, the interaction of nonselective agonists and antagonists with each receptor subtype follows simple kinetics in the presence of GTP. The only drugs that yielded nonlinear Hofstee plots for the inhibition of specific IHYP binding were the drugs that showed selectivity between heart and lung receptors *in vitro*, and all drugs that showed such selectivity yielded nonlinear Hofstee plots. In this case the resulting Hofstee plots are curves obtained by the mathematical addition of two straight lines with different slopes. These curves can be dissected into their two original components by the computer-based iterative procedure described in the METHODS section (see Fig. 1).

The analysis described above suggests that both β-1 and β-2-adrenergic receptors exist in both heart and lung. For those drugs (practolol and metoprolol) that have been shown to be more potent in inhibiting IHYP binding in rat heart than in rat lung and that have been shown to be cardioselective *in vivo*, the high affinity component is termed the β-1 component and the low affinity component is termed the β-2 component. For those drugs (zinterol and salmefamol) that have been shown to be more potent in inhibiting IHYP binding in rat lung than in rat heart and that have been shown to be bronchial-selective *in vivo*, the high affinity component is termed the β-2 component and the low affinity component is termed the β-1 component. Using these definitions, the relative percentage of β-1 and β-2-adrenergic receptors in each tissue was calculated by analyzing the kinetics of inhibition of specific IHYP binding by a variety of selective drugs. In rat heart the use of both β-1-selective and β-2-selective drugs indicates that 76–86% of the β-adrenergic receptors are of the β-1 subtype, while in the lung only 9–20% are of this subtype. The existence of β-1 and β-2-adrenergic receptors in both heart and lung is consistent with previous physiological evidence which suggests that both receptor subtypes can coexist in a single organ and may in fact subserve the same physiological response (1, 2). To date there is no evidence as to whether these receptors coexist on the same cell, or whether any given cell has a homogeneous population of either β-1 or β-2-adrenergic receptors. The existence of both receptor subtypes in a single organ may reflect the existence of different cell types in that organ.

The results obtained with β-1 and β-2-selective drugs in various regions of rat brain are similar to the results obtained in

heart and lung. In each of five brain regions examined the inhibition of specific IHYP binding by practolol, metoprolol, zinterol and salmefamol resulted in nonlinear Hofstee plots. The use of four selective drugs to determine the relative proportions of β-1 and β-2-adrenergic receptors gave very similar percentages in each case. In the cerebellum, only 12–18% of the β-adrenergic receptors were of the β-1 subtype, while in the other brain regions examined this subtype predominated. In any case, the relative percentages of β-1 and β-2-adrenergic receptors varied markedly in the different brain regions. The density of β-1-adrenergic receptors varied almost 20-fold between different brain regions, being lowest in the cerebellum and highest in the cerebral cortex. The density of β-2-adrenergic receptors, on the other hand, varied less than 3-fold, being lowest in hippocampus and highest in cerebellum. The striking difference between the regional distributions of β-1 and β-2-adrenergic receptors suggests that they may be involved in different functions. The relatively small variation in the density of β-2-adrenergic receptors suggests that these receptors are located on a relatively heterogeneously distributed tissue component. It is interesting to note that β-2-adrenergic receptors have a high affinity for epinephrine and a very low affinity for norepinephrine. It therefore seems likely that epinephrine may be the natural agonist for β-2-adrenergic receptors. In view of the small amount of epinephrine in rat brain (8) it is reasonable to postulate that β-2-adrenergic receptors in the brain are associated with cerebral blood vessels which are outside of the blood brain barrier and accessible to epinephrine released from the adrenal medulla. It is interesting to note that the β-adrenergic receptors controlling vasodilation are frequently of the β-2 subtype (9,10).

It is important to emphasize that interpretation of these experiments rests on two basic assumptions: 1) that the interaction of each drug with each component follows simple kinetics; and 2) that there are only two components. The first assumption seems relatively safe; as discussed above, nonselective drugs yield simple kinetics with both receptors, only selective drugs yield complex kinetics, and *all* selective drugs yield complex kinetics. The second assumption, that there are only two components, is much less certain. Our working hypothesis has been that there are only two receptor subtypes. Consistent with this assumption is the fact that the use of four different drugs with different affinities and selectivities yielded essentially constant percentages of β-1 and β-2-adrenergic receptors in seven different tissues. In addition, the calculated affinity of each drug for the β-1 and β-2-adrenergic receptors in each of seven different tissues was essentially the same. The results generated given these assumptions are therefore internally consistent, yielding constant percentages of the two receptor subtypes in a given tissue, and constant affinity of a particular drug for each receptor subtype in a variety of tissues. This evidence therefore suggests that there are only two subtypes of β-adrenergic receptor in the rat and that the pharmacological specificity of each receptor subtype is constant in a variety of tissues. We are currently subjecting this hypothesis to more rigorous testing, using additional drugs that show selectivity for β-1 and β-2-adrenergic receptors *in vitro* and a range of tissues from different animal species.

Note added in proof. While this manuscript was in preparation a letter appeared in *Nature* which showed that the inhibition of (^{3}H)-dihydroalprenolol binding in rat lung by the β-1-selective antagonist practolol resulted in a nonlinear Hofstee plot (11). This paper reached the same general conclusion, i.e., that there are two types of β-adrenergic receptors in the lung, but since the data were analyzed by eye, the relative proportion of the high affinity (β-1) component was significantly overestimated (see RESULTS).

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