Characterization of $[^3H](±)$Carazolol Binding to $\beta$-Adrenergic Receptors

Application to Study of $\beta$-Adrenergic Receptor Subtypes in Canine Ventricular Myocardium and Lung

ALLAN S. MANALAN, HENRY R. BESCH, JR., AND AUGUST M. WATANABE

SUMMARY $[^3H](±)$Carazolol, a newly available $\beta$-adrenergic receptor antagonist, has been used to characterize $\beta$-adrenergic receptor subtypes present in membrane vesicles derived from canine ventricular myocardium and canine lung. $[^3H](±)$Carazolol binding is saturable, of high affinity, and displaceable by $\beta$-adrenergic agents in accordance with their known pharmacological potencies. The interaction of carazolol with $\beta$-adrenergic receptors is stereospecific; the (−) stereoisomer demonstrates greater potency than the (+) stereoisomer. Kinetic analysis of $[^3H](±)$carazolol interaction with $\beta$-adrenergic receptors suggests the presence of two phases of interaction, consistent with initial rapidly reversible "low" affinity association of ligand with receptor, followed by isomerization to form a high affinity, slowly reversible complex. Through use of a $[^3H](±)$carazolol binding assay based on the high affinity complex, pharmacological specificities of $\beta$-adrenergic receptor populations of canine myocardium and lung were quantified. Analysis using computer-assisted techniques suggests a $\beta_2/\beta_1$ receptor ratio of approximately 85%/15% for canine myocardium and 5%/95% for canine lung. In the absence of added guanine nucleotides, comparison of potencies of $\beta$-adrenergic agonists in the two membrane systems suggests significant $\beta_2$ selectivity of $\alpha$-isoproterenol and $\alpha$-epinephrine, and non-selectivity of norepinephrine. In the presence of saturating levels of guanine nucleotides, comparison of agonist potencies confirms the non-selectivity of $\beta_1$-isoproterenol and $\beta_1$-epinephrine, and $\beta_2$ selectivity of norepinephrine. These results demonstrate that the state of guanine nucleotide regulation of receptors should be defined when examining agonist selectivities for $\beta$-adrenergic receptor subtypes in vitro.


IN recent years, radioligand binding techniques have facilitated major advances in the understanding of $\beta$-adrenergic receptor function. With these techniques, it has been found that $\beta$-adrenergic receptors are regulated by hormones (Williams et al., 1977; Ciaraldi and Marinetti, 1977; McDonnagh et al., 1976), nucleotides (Magne et al., 1976; Lefkowitz et al., 1976), and ions (Williams et al., 1978; Bird and Maguire, 1978). Studies in many experimental systems have demonstrated that guanine nucleotides induce a decrease in $\beta$-adrenergic receptor affinities for agonists, while leaving antagonist affinities unaltered (Maguire et al., 1976; Lefkowitz et al., 1976; Hegstrand et al., 1979). This guanine nucleotide-mediated regulation of $\beta$-adrenergic receptor affinity is thought to result from the interaction of the nucleotide with a membrane-associated guanine nucleotide binding protein which is capable of regulating the conformational state of the receptor (Limbird et al., 1980a, 1980b).

In addition to analysis of mechanisms of $\beta$-adrenergic receptor control, radioligand binding techniques have been applied to re-examination of the hypothesis that there are distinct $\beta$-adrenergic receptor subtypes (U'Prichard et al., 1978; Minneman et al., 1979a, 1979b, 1979c). Tissue differences in $\beta$-adrenergic drug potency determined by classic pharmacological techniques (Lands et al., 1967) have been correlated with corresponding differences in affinity of $\beta$-adrenergic receptors for the drugs as determined by binding assays (U'Prichard et al., 1978; Minneman et al., 1979a; Hancock et al., 1979). A wide variety of $\beta$-adrenergic agents has been evaluated through use of their ability to compete with appropriate radioligands to determine their affinity for $\beta$-adrenergic receptors. Based on comparison of data obtained with $\beta$-adrenergic receptor populations derived from several different tissues, the existence of distinct $\beta$-adrenergic receptor subtypes has been confirmed directly with binding assays (U'Prichard et al., 1978; Minneman et al., 1979a; Hancock et al., 1979). Recently, calculated
affinities of β₁- and β₂-adrenergic receptor subtypes for β-adrenergic agonists and antagonists have appeared in the literature. In some experimental systems, reported values have been derived in the presence of maximal receptor regulation by guanine nucleotides (Hegstrand et al., 1979; Minneman et al., 1978b, 1979c), whereas in other receptor systems, the level of receptor regulation by guanine nucleotides has not specifically been addressed (U'Prichard et al., 1978; Minneman et al., 1979a).

Carazolol [4-(2-hydroxy-3-isopropylamino-propoxy)-carbazole], a newly available β-adrenergic receptor antagonist, has been shown to exhibit extremely high potency both in vivo (Bartsch et al., 1977) and in vitro (Morris et al., 1978). Preliminary evidence of its extraordinarily high potency suggested its potential usefulness as a radioligand for labeling β-adrenergic receptors. Recently, its suitability as a radioligand has been confirmed in a comparative study of [3H](±)carazolol and [3H](—)dihydroalprenolol binding to β-adrenergic receptors present in membranes derived from rat brain (Innis et al., 1979). In the present study, [3H](±)carazolol has been used to characterize β-adrenergic receptor subtypes present in membrane vesicles derived from canine myocardium and canine lung. Through use of this high affinity radioligand, comparison of β-adrenergic receptor subtypes was examined, with specific attention to receptor regulation by guanine nucleotides. The present findings demonstrate that correct interpretation of such comparative studies can be performed only when the level of guanine nucleotide regulation is defined.

Methods

Preparation of Membrane Vesicle Fractions

Preparation of tissue homogenates and subsequent isolation of membrane vesicle fractions were performed with the use of a slight modification of the procedure previously described for canine ventricular myocardium (Jones et al., 1979). All preparatory procedures were performed at 4°C. Canine lung parenchyma was trimmed to remove major blood vessels and cartilaginous airways. Canine ventricular myocardium was trimmed as previously described. Briefly, for each tissue, a 20% homogenate was prepared by disrupting 25–35 g of tissue three times for 30 seconds in four volumes of ice-cold 0.25 M sucrose, 30 mM histidine with a Polytron PT-20 (Brinkmann Instruments) at a setting of half maximal speed. To obtain membrane vesicle fractions from these crude homogenates, 20-minute low-speed centrifugations at 11,600 g max and 15,000 g max were employed to remove larger tissue fragments, nuclei, and mitochondria. The membrane vesicle fractions then were sedimented at 43,800 g max for 30 minutes. For purposes of comparison, recentrifugation of membrane vesicle fractions in 0.6 M KCl, a procedure designed to extract residual contractile proteins from myocardial membranes, was performed on membrane vesicle fractions derived from canine myocardium and canine lung. Crude homogenates and membrane vesicle fractions either were used fresh on the day of preparation or were stored frozen at −20°C in 0.25 M sucrose, 30 mM histidine until use. Activities remained stable for at least 1 month. In pilot studies, β-adrenergic receptor affinities for agonists and antagonists, as well as receptor regulation by guanine nucleotides, were found to be quantitatively identical in crude homogenates, membrane vesicle fractions, and membrane vesicle fractions subjected to KCl extraction. For purposes of the present comparative study, results of binding experiments in the two tissues are reported for membrane vesicle fractions of heart and lung prepared in an identical fashion, with the KCl extraction step omitted.

[3H](±)Carazolol Binding Assay

[3H](±)Carazolol, a potent β-adrenergic receptor antagonist, was used to label β-adrenergic receptors present in the membrane vesicle preparations. Membrane vesicles (50–200 μg protein) were incubated in 1 or 5 ml of medium containing 50 mM Tris buffer (pH 7.5 at 22°C), 9 mM MgCl₂, 1 mM ascorbic acid, [3H](±)carazolol, and relevant drugs. For radioligand competition curves, [3H](±)carazolol was present at a concentration approximately two times its KD (270 pm for myocardium, 100 pm for lung) to facilitate graphic comparison of experimental results obtained in the two preparations. Unless otherwise noted, incubations were conducted at 37°C for 60 minutes, a reaction time that allowed complete equilibration of [3H](±)carazolol binding. Reactions were terminated by rapid filtration through Whatman GF/C filters. Filters were rinsed with four 5-ml aliquots of ice-cold 50 mM Tris buffer containing 9 mM MgCl₂. Filters then were dried, and retained radioactivity was determined with a Beckman liquid scintillation counter (counting efficiency 56%). All determinations of total and non-specific binding were performed in triplicate. Specific binding was taken as that binding which was displacable by 20 μM (±) propranolol. Specific [3H](±)carazolol binding was linear with protein concentration over the entire range used in the present study. β-Adrenergic receptor concentration in incubations was in the range of 5–10 pm, a concentration that minimized changes in free radioligand concentration during the course of the binding reaction. Even at the low receptor concentration used, specific [3H](±)carazolol binding was greater than 70% of total binding. Protein determinations were performed by the method of Lowry et al. (1951), with the use of bovine serum albumin as standard.

Analysis of Data

Data from radioligand competition curves were subjected to mathematical transformation to obtain
linearized plots of log \( [RC/(RC_{\text{max}} - RC)] \) vs. log \( A \), where \( RC_{\text{max}} \) is the amount of \( [\text{H}]^{+}\text{carazolol} \) bound in the absence of competing drug. \( RC \) is the amount of radioligand bound in the presence of competing drug, and \( A \) is the concentration of competing drug. Linear regression analysis of these derived data was performed to obtain the slope factor ("pseudo-Hill coefficient") and X-intercept (equivalent to log \( (IC_{50}) \)). \( K \), for the competing drug was then calculated from the \( IC_{50} \) by the method of Cheng and Prusoff (1973). Guanine nucleotide-induced changes in \( K \) for competing \( \beta \)-adrenergic receptor agonists were analyzed for statistical significance by use of unpaired t-tests of \( K \)'s derived from radioligand competition curves performed in the presence and absence of added guanine nucleotides.

Kinetic data were subjected to mathematical transformation in accordance with the integrated rate equations for a simple reversible bimolecular ligand-receptor interaction, in an attempt to obtain linearized plots amenable to calculation of association and dissociation rate constants. For the binding of \( [\text{H}]^{+}\text{carazolol} \) to \( \beta \)-adrenergic receptors, such plots were distinctly nonlinear, and precluded such a straightforward quantitative analysis. Accordingly, simple alternative models to account for the observed biphasic kinetic plots have been developed and available kinetic data reanalyzed.

The possibility of co-existence of both \( \beta_{1} \) - and \( \beta_{2} \)-adrenergic receptor subtypes within the same preparation was explored using computer assisted techniques similar to those reported by Minneman et al. (1979b). Briefly, radioligand competition curves for \( \beta_{1} \)-selective antagonists were transformed mathematically to obtain Hofstee plots for these competing antagonists. The resultant nonlinear plots were then fit to a model of two noninteracting, noninterconvertible \( \beta \)-adrenergic receptor subtypes with differing affinities for those antagonists. Briefly, the computer program, patterned after that described in detail by Minneman et al. (1979b), involves an iterative sequence of linear regression analysis and graphic subtraction to obtain a pair of lines whose sum represents the best fit of the derived data. Slopes and intercepts of those lines provide an approximation of affinity and relative number of the two receptor subpopulations present in a given preparation. Internal consistency of computed parameters for all selective agents studied supports the validity of the estimates of relative contribution of \( \beta_{1} \) and \( \beta_{2} \) receptor subpopulations present in the membrane vesicle preparations.

Materials

\( [\text{H}]^{+}\text{Carazolol} \) (specific activity 23.55 Ci/mmol), S(—)carazolol, and R(+)carazolol were obtained from New England Nuclear. Drugs kindly were donated as follows: EMD 33-512, E. Merck; (±)terbutaline, (±)metoprolol, Ciba-Geigy; (±)dobutamine, Eli Lilly, (±)salbutamol, Schering; (±)practolol, (+)propranolol, and (−)propranolol, Ayerst; (±)isotolol, Mead Johnson; (±)atenolol, ICI of the Americas. (−)Isoproterenol, (−)epinephrine, and (−)norepinephrine were purchased from Sigma. 5'-Guananylimidodiphosphate was obtained from Boehringer Mannheim or Sigma. All other chemicals used were of reagent grade.

Results

Characterization of \( [\text{H}]^{+}\text{Carazolol Binding to } \beta \text{-Adrenergic Receptors} \)

\( [\text{H}]^{+}\text{Carazolol binding to } \beta \text{-adrenergic receptors present in membrane vesicle fractions is depicted in Figure 1. For membrane vesicles derived from both heart (Fig. 1A) and lung (Fig. 1B), specific } [\text{H}]^{+}\text{carazolol binding is saturable. For both membrane preparations, Scatchard plots (insets) appear linear (r = 0.99), consistent with radioligand binding to an apparently homogeneous population of receptors. Based on Scatchard analysis of saturation curves, the } K_{D} \text{ for } [\text{H}]^{+}\text{carazolol binding to } \beta \text{-adrenergic receptors is 135 pm for myocardium and 50 pm for lung. } [\text{H}]^{+}\text{Carazolol competition curves generated with unlabeled S(−) and R(+)carazolol demonstrate stereospecificity of the interaction of carazolol with } \beta \text{-adrenergic receptors in the two membrane vesicle preparations. In membrane vesicles derived from heart, S(−)carazolol is 60 times more potent than R(+)carazolol in competing with } [\text{H}]^{+}\text{carazolol for binding to } \beta \text{-adrenergic receptors (Table 1). Similarly, S(−)carazolol exhibits 22-fold greater potency than R(+)carazolol in competing for binding to } \beta \text{-adrenergic receptors in the lung membrane vesicles (Table 1). For both preparations, inhibition constants (K_{I}) for S(−)carazolol calculated from radioligand competition curves are approximately half that of dissociation constants (K_{D}) derived from } [\text{H}]^{+}\text{carazolol saturation curves, suggesting that the major fraction of binding of racemic } [\text{H}]^{+}\text{carazolol at equilibrium is the result of } \beta \text{-adrenergic receptor association with the S(−) stereoisomer (see kinetic analysis below).}

Kinetics of binding of \( [\text{H}]^{+}\text{carazolol to } \beta \text{-adrenergic receptors present in membranes derived from canine myocardium and canine lung are depicted in Figure 2. If the interaction of } [\text{H}]^{+}\text{carazolol with } \beta \text{-adrenergic receptors is a reversible bimolecular reaction, then the integrated rate equation}

\[
\ln \frac{RC_{\text{eq}}}{RC_{\text{eq}} - RC_{t}} = (k_{1}[C] + k_{-1}) t
\]

applies, where \( RC_{\text{eq}} \) and \( RC_{t} \) are concentrations of bound carazolol at equilibrium, and at time \( t, [C] \) is the concentration of free \( [\text{H}]^{+}\text{carazolol (constant}}

CIRCULATION RESEARCH VOL. 49, NO. 2, AUGUST 1981
under present conditions in which radioligand is present in excess, $k_1$ is the second order association rate constant, and $k_\text{-1}$ is the first order dissociation rate constant. Accordingly, plots of $\ln[R_{\text{eq}}/(R_{\text{eq}} - R_{\text{c}})]$ vs. time would be expected to be linear for a reversible bimolecular binding reaction. As shown in Figure 2, such plots of binding data are nonlinear for both heart (panel A upper) and lung (panel A lower), suggesting that binding of $[\text{3H}] (\pm)$carazolol to β-adrenergic receptors is not a simple reversible bimolecular association. Analyses of the dissociation reaction, plotted according to the integrated first order dissociation rate law

$$\ln \frac{R_{\text{eq}}}{R_{\text{c}}} = -k_1 t$$

are presented in Figure 2, panel B. Again, such plots are inconsistent with a simple first order dissociation reaction (see below).

Simple alternative mechanisms that might account for the binding data can be examined. First, possible interaction of $[\text{3H}] (\pm)$carazolol with two different receptor sites with different kinetic parameters is considered:

$$R_1 + C \xrightleftharpoons{k_1}{k_\text{-1}} R_1C$$

$$R_2 + C \xrightleftharpoons{k_2}{k_\text{-2}} R_2C$$

Examination of the data in Figure 2A reveals evidence of at least two distinct phases, an early rapid...
FIGURE 2  Kinetics of association (panel A) and dissociation (panel B) reactions between [3H](±)carazolol and β-adrenergic receptors present in membrane vesicle preparations derived from canine ventricular myocardium (panels A and B, upper) and canine lung (panels A and B, lower). To monitor the association reaction, membranes were incubated with agitation at 37°C in the presence of varying concentrations of [3H](±)carazolol (O 122 pM; □ 713 pM; △ 105 pM; ▲ 282 pM). Incubations were conducted in a total volume of 16 ml of 50 mM Tris buffer containing 9 mM MgCl₂ and 1 mM ascorbic acid. At the indicated times, 1-ml aliquots of the incubation mixture were withdrawn, added to 5 ml of ice cold 50 mM Tris buffer containing 9 mM MgCl₂ and filtered through Whatman GF/C filters. Nonspecific binding, determined in parallel incubations in the presence of 20 μM (±)propranolol, was instantaneous and constant throughout 90 minutes of incubation. Incubations were conducted in triplicate, and specific binding at time t (RC₁) calculated as the difference between mean values of total and nonspecific binding. At all concentrations of [3H](±)carazolol examined, specific binding attained a constant value by 60 minutes of incubation, and was unaltered at 90 minutes of incubation. Accordingly, equilibrium binding (RCₑ) was taken as the mean of specific binding present at 60 and 90 minutes of incubation. Data are presented transformed according to the integrated rate law for a reversible bimolecular reaction (panel A) (see text). For the dissociation reaction, membranes were incubated in triplicate under conditions identical to those for the association reaction. Parallel determinations were performed in the presence of 20 μM (±)propranolol for calculation of nonspecific binding. [3H](±)Carazolol concentrations were 210 pM (panel B upper) and 225 pM (panel B lower). At either 5 minutes (unfilled symbols) or 60 minutes (filled symbols) of association, 1-ml aliquots of the incubation mixture were withdrawn and processed as above for determination of initial specific binding (RC₁). A small volume of (±)propranolol (10 μl) was then immediately added to incubation mixtures with continuous agitation to bring the (±)propranolol concentration to 20 μM. To monitor the dissociation reaction, 1-ml aliquots of incubation mixture were subsequently removed and processed at indicated times after the propranolol addition. Specific binding at time t after propranolol addition (RCₜ) was calculated as the difference between mean values of total and nonspecific binding. Data are presented transformed according to the integrated first order dissociation rate law (panel B) (see text).

Data presented in Figure 2 appear to be more consistent with a two-step reaction consisting of initial [3H](±)carazolol association with receptors, followed by isomerization to form a higher affinity, slowly dissociable ligand:receptor complex:

\[
R + C \rightleftharpoons_{k_{-1}}^{k_{+1}} RC \rightleftharpoons_{k_{-2}}^{k_{+2}} RC^*.
\]

If this model is valid, the magnitude of the dissociation rate constant \( k_{-1} \) for the rapidly reversible phase of the binding reaction can be estimated from the initial slope of the derived dissociation plots for the 5-minute preincubations (Fig. 2, panel B, open phase, followed by a slower phase of ligand:receptor interaction. Notably, the fraction of receptors involved in the slow phase of interaction (approximated by extrapolation of the slow phase regression line to the ordinate) appears to vary with radioligand concentration (Fig. 2A). In addition, the fraction of receptors involved in the slow phase of dissociation (Fig. 2B) appears to vary with time of preincubation. This apparent variation in the fraction of receptors involved in each phase of the reaction is not consistent with a model of radioligand interaction with two fixed independent populations of receptors.
symbols). By this method, estimates of $k_1$ are 0.06 min$^{-1}$ for myocardium (panel B, upper) and 0.12 min$^{-1}$ for lung (panel B, lower). Similarly, the value of $(k_1[C]+k_{-1})$ can be approximated from the initial slope of the association plots during which the rapidly reversible process appears to predominate (Fig. 2A). From association plots obtained with varying concentrations of $[3H](±)carazolol$, a series of equations of the form slope $= k_1(C) + k_{-1}$ can be obtained and solved for $k_1$. Estimates of $k_1[C]/k_{-1}$ for myocardium and 9.2 $× 10^{-11}$ M are obtained for myocardium and 9.2 $× 10^{-11}$ M for lung. These derived $K_D$ values are somewhat higher than those obtained from equilibrium plots published in Table 1. As expected, known β-adrenergic receptor antagonists compete with $[3H](±)carazolol$ for binding to β-adrenergic receptors in both myocardium (Fig. 3, panel A) and lung (Fig. 3, panel B). Specific binding is taken as that binding that was displaceable by 20 μM (±)propranolol. Specific $[3H](±)carazolol$ binding, expressed as a percentage of binding in the absence of competing drug, is plotted as a function of competing drug concentration. $S(-)carazolol$ (Δ), (−)propranolol (○), (±)metoprolol (□). Presence (filled symbols) or absence (unfilled symbols) of 10$^{-4}$ M 5′-guanylylimidodiphosphate has no effect on the position or configuration of antagonist:radioigand competition curves. To facilitate graphic comparison of experimental results obtained in the two preparations, competition curves were generated in the presence of $[3H](±)carazolol$ at concentrations of approximately two times its respective $K_D$. Slope factors and inhibition constants derived from competition curves are presented in Table 1.
Whereas the competition curve in lung displays downward deviation in the range of $10^{-7}$ to $10^{-8}$ M EMD 33-512, these systematic deviations in contour suggest the existence of a small subpopulation of $\beta_2$-adrenergic receptors in the cardiac membrane preparation, as well as a small subpopulation of the $\beta_1$-adrenergic receptors in the lung membrane preparation. Derived Hofstee plots for EMD 33-512 competition with $[\text{H}]$(±)carazolol are biphasic, consistent with the coexistence of both $\beta$-adrenergic receptor subtypes in each membrane preparation (insets, Fig. 4). Analysis of these derived plots according to the iterative least squares linear regression method of Minneman et al. (1979b) is consistent with the presence of a $\beta_1/\beta_2$ adrenergic receptor ratio of 88%/12% in the membrane fractions from canine heart, and a $\beta_1/\beta_2$ ratio of 6%/94% in membrane fractions from canine lung. Parallel analysis of competition curves for the $\beta_1$-selective antagonists metoprolol (Fig. 3) and practolol (data not shown) yield estimates of $\beta_1/\beta_2$ adrenergic receptor ratios very similar to those obtained with EMD 33-512. Based on analysis of competition curves for all three agents, $\beta_1/\beta_2$ adrenergic receptor ratios are in the range 83-88%/12-17% for heart, and 4-6%/94-96% for lung. Thus, the $\beta$-adrenergic receptor population present in each membrane preparation is not completely homogeneous with respect to subtype. As a result, the ratios of inhibition constants derived from competition curves for the two membrane preparations (Tables 1 and 2) represent only close approximations of the magnitude of receptor subselectivity of the $\beta$-adrenergic agents examined.

$\beta$-Adrenergic receptor agonists also compete with $[\text{H}]$(±)carazolol for binding to $\alpha$-adrenergic receptors in myocardium (Fig. 5A) and lung (Fig. 5B). In
the absence of added guanine nucleotides, slope factors derived from agonist:radioigand competition curves are distinctly less than 1 (Fig. 5, Table 2). Interestingly, slope factors derived from control competition curves in heart are uniformly higher than those in lung. Based upon agonist potency series, β-adrenergic receptors present in heart membranes exhibit pharmacological specificity typical of β1-adrenergic receptors, whereas β2-adrenergic receptors present in lung membranes display specificity typical of β2-adrenergic receptors (Fig. 5, Table 2). However, comparison of radioligand competition curves for l-isoproterenol in the absence of added guanine nucleotides reveals an apparently greater potency of isoproterenol in interacting with the predominantly β1-adrenergic receptor population of lung (Ki 0.026 μM) than in the predominantly β2-adrenergic receptor population of heart (Ki 0.17 μM). These data surprisingly suggest that isoproterenol is a β2-selective agonist. Similarly, in the absence of added guanine nucleotides, l-epinephrine demonstrates greater potency in competing with \(^{3}H\)(±)carazolol for binding to β-adrenergic receptors present in lung membranes than in heart membranes, suggesting β2-selectivity of l-epinephrine as well. l-Norepinephrine appears to be a nonselective agonist, based on very similar potencies in the two preparations.

Effects of added guanine nucleotides on agonist:radioigand competition curves are presented in Figure 5, panels C and D. For β-adrenergic receptor populations in both canine myocardium and lung, addition of guanine nucleotides induces a rightward shift in the agonist:radioigand competition curves, consistent with a decrease in receptor affinity for the agonists. Notably, based on comparison of agonist Ki’s, the magnitude of the guanine nucleotide-induced decrease in receptor affinity is greater in lung membranes than in heart (Fig. 5, Table 2). For both preparations, guanine nucleotide addition is also accompanied by increases in slope factors derived from agonist:radioigand competition curves (Table 2). Consequences of the presence of saturating concentrations of guanine nucleotides are presented graphically in Figure 5, panels C and D. The agonist potency series are again consistent with a predominantly β1-adrenergic receptor population in canine myocardium, and a predominantly β2-adrenergic receptor population in canine lung (Fig. 5, Table 2). Comparison of competition curves in the presence of guanine nucleotides suggests that l-isoproterenol is a nonselective β-adrenergic agent, based on similar Ki’s in the two β-adrenergic receptor containing preparations. In the presence of guanine nucleotide regulation, l-epinephrine also displays similar β-adrenergic receptor affinities in the two membrane fractions. Only l-norepinephrine exhibits significant β1 selectivity, demonstrating approximately 5-fold greater potency in the predominantly β1 system than in the β2 system.

**Discussion**

In the present study, \(^{3}H\)(±)carazolol binding to β1- and β2-adrenergic receptor subtypes in canine
heart and lung is characterized. Consistent with the previous report by Innis et al. (1979), $[^3H]$(±)carazolol binding is saturable and displaceable by β-adrenergic agents in accordance with their known pharmacological potencies. Comparison of modified Scatchard analyses of $[^3H]$(±)carazolol saturation curves obtained from membranes from canine myocardium and lung suggests only minimal (approximately 2.5-fold) $\beta_2$ selectivity of $[^3H]$(±)carazolol in vitro. Thus, the presence of coexisting populations of β-adrenergic receptor subtypes in the same membrane preparation is not convincingly identified by modified Scatchard analysis of $[^3H]$(±)carazolol binding. In contrast to the previous report of $[^3H]$(±)carazolol binding in rat brain (Innis et al., 1979), kinetic analysis of $[^3H]$(±)carazolol binding to β-adrenergic receptors in canine myocardium and lung is not consistent with a simple reversible bimolecular reaction. The kinetic plots reveal the presence of two distinct phases of interaction of $[^3H]$(±)carazolol with both $\beta_1$- and $\beta_2$-adrenergic receptors. Analysis of available data suggests that the observed biphasic kinetics can be accounted for by initial rapidly reversible "low" affinity association of ligand with receptor, followed by isomerization to form a high affinity, slowly reversible complex. Such a sequential association/isomerization reaction between radioligand and receptor has been proposed to account for the observed kinetics of $[125I]$(±)iodohydroxybenzylpindolol binding to β-adrenergic receptors (Ross et al., 1977), as well as that of the binding of $[^3H]$(±)quinuclidinyl benzilate to muscarinic cholinergic receptors (Galper et al., 1977; Jarv et al., 1979). However, in the present kinetic analysis, potential contribution of binding of both radiolabeled stereoisomers of carazolol to β-adrenergic receptors cannot be neglected. This point is supported by the recent preliminary report of Burgisser et al. (1980). Through use of computer modeling techniques, these investigators have examined saturation curves for the racemic tracers $[^3H]$(±)carazolol and $[125I]$(±)iodohydroxybenzylpindolol in the β-adrenergic receptor system of frog erythrocytes. When high receptor concentrations are present, saturation curves for both agents are best fit to a model of two stereoisomers with different affinity constants. It should be noted that in the present study low receptor concentrations were used to minimize changes in free radioligand concentration during the binding reaction. Thus, contribution of the (+) stereoisomer to equilibrium binding is not apparent at the low receptor concentrations used. As radiolabeled preparations of pure stereoisomers of carazolol become generally available, the possible contribution of each stereoisomer to the observed kinetics of radioligand binding can be examined directly.

By analysis of a series of $[^3H]$(±)carazolol competition curves for β-adrenergic agonists and antagonists, pharmacological specificities of β-adrenergic receptor populations present in membrane vesicles derived from canine ventricular myocardium and lung have been quantified. As expected, $\beta_1$-adrenergic receptors predominate in heart, whereas $\beta_2$-adrenergic receptors predominate in lung. Consistent with previous reports, computer-facilitated analysis of antagonist:radioligand competition curves for highly selective $\beta_1$-adrenergic antagonists suggests the coexistence of small subpopulations of $\beta_2$-adrenergic receptors in myocardial tissue, and $\beta_1$-adrenergic receptors in lung tissue (Hancock et al., 1979; Minneman et al. 1979b). These small $\beta_1$-adrenergic receptor subpopulations may reflect the cellular inhomogeneity of the tissue sources from which membrane vesicles have been derived. Indeed, by using tissue culture techniques, Lau et al. (1980) have identified $\beta_1$-adrenergic receptor sites on fibroblasts present in cultures of neonatal rat myocardium.

Comparison of potencies of $\beta_1$-adrenergic antagonists in the predominantly $\beta_1$ and predominantly $\beta_2$ receptor systems confirms previously reported $\beta_2$ selectivity of metoprolol, practolol, and atenolol (U'Prichard et al., 1978; Minneman et al., 1979a, 1979c). The recently synthesized agent, EMD 33,452, has been found to be an even more highly selective $\beta_1$-adrenergic antagonist, displaying 23-fold greater potency in interacting with $\beta_1$ receptors than with $\beta_2$ receptors. In canine tissue, S(-)carazolol and sotalol behave as nonselective $\beta_1$-adrenergic antagonists, binding to $\beta_1$ and $\beta_2$ receptors with similar affinities. Interestingly, the modest $\beta_2$ selectivity of l-propranolol (3- to 5-fold) previously reported in rat tissues (U'Prichard et al., 1978; Minneman et al., 1979a) is also demonstrated in the present study of canine myocardium and lung.

Naive comparison of potencies of $\beta_1$-adrenergic agonists in the two membrane systems suggests significant $\beta_2$ selectivity of l-isoproterenol and l-epinephrine, and nonselectivity of l-norepinephrine. These surprising conclusions have been shown to arise from failure to define specifically the level of receptor regulation by guanine nucleotides in these in vitro systems. Without added guanine nucleotides, agonist competition curves in membrane vesicles derived from canine myocardium are shifted rightward (lower affinity) and demonstrate higher slope factors than those in lung. As expected, addition of saturating levels of guanine nucleotides induces a rightward shift and increased slope factor for agonist: radioligand competition curves obtained in both membrane preparations. However, alterations in affinity and slope factor resulting from addition of guanine nucleotides are of substantially greater magnitude in the $\beta_1$-adrenergic receptor preparation derived from lung than from heart. Consequently, in the presence of saturating concentrations of guanine nucleotides, l-isoproterenol and l-epinephrine behave as nonselective $\beta_1$-adrenergic
agents, whereas l-norepinephrine displays its expected β₁ selectivity. Similarly, comparison of potencies of dobutamine in the two membrane systems in the absence of added guanine nucleotides suggest substantial β₂ selectivity of this agent. In the presence of saturating levels of guanine nucleotides, dobutamine behaves as a nonselective β-adrenergic agent. In general, failure to define the level of guanine nucleotide regulation of β-adrenergic receptors in comparative studies of canine myocardium and lung leads to a substantial overestimation of the β₂ selectivity of β-adrenergic agonists (e.g., terbutaline, salbutamol).

Agonist-specific regulation of β-adrenergic receptors by guanine nucleotides is a well-documented phenomenon (Maguire et al., 1976; Lefkowitz et al., 1976; Hegstrand et al., 1979). Although certain mechanistic details remain to be established, it is currently believed that guanine nucleotides regulate receptor function as a result of interaction with a membrane-associated nucleotide binding protein (Limbird et al., 1980a, 1980b). As a result of guanine nucleotide binding to this protein, a conformational change in the β₂-adrenergic receptor is thought to be induced, with resultant reduction of the receptor affinity for its agonists (Williams and Lefkowitz, 1977; Catt et al., 1979; Kent et al., 1980).

Accordingly, at least two conformational states of β-adrenergic receptors can be envisioned: (1) a conformation with high agonist affinity; and (2) a conformation with lowered agonist affinity. Indeed, application of computer-modeling techniques suggests that shallow agonist:radioisotopic curves found in the absence of added guanine nucleotides can be quantitatively accounted for by postulating the existence of two and only two β-adrenergic receptor conformations (Kent et al., 1980; Stadel et al., 1980). Based on these analyses, saturating levels of guanine nucleotides convert all β-adrenergic receptors to the low affinity conformation, and this accounts for the rightward shift and increased steepness of the agonist:radioisotopic competition curve.

In light of this model, the greater magnitude of β-adrenergic receptor regulation by guanine nucleotides seen in membranes derived from canine lung than from canine myocardium warrants comment. Although the present data are consistent with the possibility that β₁-adrenergic receptors themselves are intrinsically less susceptible to guanine nucleotide regulation than β₂-adrenergic receptors, studies in other β-adrenergic systems document equivalent levels of regulation of both receptor subtypes (Hegstrand et al., 1979). Alternatively, persistence of endogenous guanine nucleotides bound to regulatory sites in myocardial membranes could account for the rightward shift and greater steepness of control agonist:radioisotopic competition curves seen in myocardial membranes than in lung membranes. Subsequent addition of exogenous guanine nucleotides to myocardial membranes already partially regulated would result in changes of lesser magnitude than in membranes devoid of endogenous guanine nucleotides in the control state. In the present study, membrane vesicle fractions from both myocardium and lung were isolated with the use of identical preparatory methods. Nevertheless, the possibility of persistence of endogenous guanine nucleotides bound to regulatory sites on membranes derived from myocardium (but not lung) cannot be excluded. Intrinsic tissue-specific variations in interaction of the guanine nucleotide binding regulatory protein with β-adrenergic receptors remain a third possibility to account for the present findings. Whatever the source of these differences in the magnitude of β-adrenergic receptor regulation by guanine nucleotides, the results of the present study demonstrate that the state of guanine nucleotide regulation must be defined when examining agonist selectivities for β-adrenergic receptor subtypes in vitro.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Gail Wiseman and the skillful secretarial assistance of Terri Butcher and Carleen Mueller.

References


Cheng Y-C, Prusoff WH (1973) Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 percent inhibition (I₅₀) of an enzymatic reaction. Biochem Pharmacol 22: 3099-3108

Ciaraldi T, Mannetti GV (1977) Thyroxine and propylthiouracil effects in vivo on alpha and beta adrenergic receptors in rat heart. Biochem Biophys Res Commun 74: 584-591


Kent RS, DeLean A, Lefkowitz RJ (1980) A quantitative analysis...
of beta adrenergic receptor interactions: Resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. Mol Pharmacol 17: 14-23


Limbird LE, Gill DM, Lefkowitz RJ (1980a) Agonist-promoted coupling of the beta-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system. Proc Natl Acad Sci USA 77: 775-779


Minneman KP, Hegstrand LR, Molinoff PB (1979b) Simultaneous determination of beta-1 and beta-2 adrenergic receptors in tissues containing both receptor subtypes. Mol Pharmacol 18: 34-46


Characterization of [3H](+/-)-carazolol binding to beta-adrenergic receptors. Application to study of beta-adrenergic receptor subtypes in canine ventricular myocardium and lung.

A S Manalan, H R Besch, Jr and A M Watanabe

doi: 10.1161/01.RES.49.2.326

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1981 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/49/2/326