BRIEF COMMUNICATIONS

Direct Analysis of $\beta$-Adrenergic Receptor Subtypes on Intact Adult Ventricular Myocytes of the Rat

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SUMMARY. $\beta_1$- and $\beta_2$-Adrenergic receptors co-exist in the adult rat ventricle. We have employed radioligand binding and cell purification techniques to determine the cellular origin of these receptors. The $\beta$-adrenergic antagonist ligand (±)-[^125I]iodocyanopindolol binds to $2 \times 10^5$ receptors per purified adult rat cardiomyocyte, with a dissociation constant of 70 pM. The subtype-selective antagonists betaxolol ($\beta_1$), practolol ($\beta_1$), and zinterol ($\beta_2$) compete for [^125I]iodocyanopindolol-binding sites on intact myocytes in monophasic manners with dissociation constants of 46, 845, and 923 nM, respectively. [^125I]iodocyanopindolol binding to membranes prepared from nonmyocyte elements of rat ventricle occurs with a dissociation constant of 43 pM and a capacity of 88 fmol/mg membrane protein. Computer analysis of competition of [^125I]iodocyanopindolol binding by betaxolol, practolol, and zinterol in nonmyocyte membranes demonstrates biphasic curves that comprise binding to both $\beta_1$- and $\beta_2$-receptors. These data demonstrate that purified adult ventricular myocytes possess only $\beta_1$-receptors, and that the $\beta_2$-receptors found in rat ventricle are located on nonmyocyte cell types (Circ Res 56:126-132, 1985)

EVIDENCE for the co-existence of $\beta_1$- and $\beta_2$-receptors for catecholamines in the mammalian myocardium comes from both physiological and radioligand binding studies. Carlsson et al. (1972, 1977) present evidence that the inotropic and chronotropic responses of the cat heart to catecholamines are differentially antagonized by subtype-specific $\beta$-antagonists. Studies in guinea pig heart lead to the same conclusion (Dreyer and Offermeir, 1975). Radioligand binding studies, employing heterogeneous membranes prepared from homogenized hearts, reveal the presence of both $\beta$-receptor subtypes in rat (Minneman et al., 1979a, 1979b), kitten (Kaumann et al., 1983) and human heart (Stiles et al., 1983; Waelbroeck et al., 1983). Although these data demonstrate the presence of both $\beta_1$- and $\beta_2$-receptors in mammalian heart, they do not describe the distribution of $\beta$-adrenergic receptor subtypes among different cell types within the ventricle.

In the course of our own work on the hormonal regulation of cardiac function and metabolism, we have wondered whether specific hormone receptors (and, hence, distinct responses) can be assigned to particular cell types within the heart. In particular, we have considered whether $\beta_1$- and $\beta_2$-receptors co-exist on ventricular myocytes. To approach these questions experimentally, we have combined the techniques of radioligand binding and cell purification. Specifically, we have measured the distribution of $\beta$-receptor subtypes on purified cardiomyocytes from adult rat ventricle and on the nonmyocyte elements of ventricle by employing the subtype nonselective $\beta$-adrenergic antagonist (±)-[^125I]iodocyanopindolol ([^125I]ICYP), the $\beta_1$ subtype-selective antagonists, betaxolol and practolol, and the $\beta_2$-selective antagonist, zinterol.

Methods

Preparation of Ventricular Cardiomyocytes

Male Sprague-Dawley rats were given heparin (1500 U/kg, ip) and sodium pentobarbital (12 mg, ip), and were killed 10 minutes later. Hearts were rapidly removed and immediately perfused with Ca++-free basal medium Eagle (BME, 18°C) via the aorta to stop spontaneous beating and to remove blood cells. Hearts were trimmed of extraneous tissue including pericardium, and mounted via the aorta in a recirculating perfusion apparatus. Ventricular myocytes were prepared by collagenase perfusion in the absence of Ca++ by a method we have previously described in detail (Buxton and Brunton, 1983). For experiments, purified myocytes were resuspended in BME containing CaCl$_2$ (1 mM) and BSA (0.01% wt/vol). Viability of myocytes was determined by their capacity to exclude trypan blue (1 volume of cells plus 9 volumes of 0.3% trypan blue). Myocytes were routinely 80–90% rod-shaped cells excluding trypan blue. Myocytes were used in experiments 15–20 minutes after their purification.

Preparation of Ventricular Membranes

Rat hearts were removed and handled in a manner identical to that used to prepare myocytes, except that enzyme perfusion was omitted. Ventricles were dissected away from atrial and valvular tissue and immediately frozen, using Wallenberger clamps precooled in liquid
nitrogen. Membranes were prepared from frozen tissue as described below.

Preparation of Myocyte, Nonmyocyte, and Liver Membranes

Tissue determined to be "nonmyocyte" in origin included tissue pieces remaining after filtration of enzyme-released myocytes, as well as cells contained in the first supernatant from myocyte purification. Microscopic examination of this material confirmed the presence of numerous small nonmuscle cells, damaged myocytes, and collagen fibers. Thus, while not devoid of myocyte membrane, this material was enriched for nonmyocyte, cell types present in rat ventricle. Both purified myocytes and nonmyocytes were collected by centrifugation (1000 x g for 2 minutes) and quick-frozen in liquid nitrogen. Whole rat ventricle and liver were freeze-clamped at the temperature of liquid nitrogen. For the preparation of membranes, frozen tissues and frozen cell pellets were powdered at -70°C and then homogenized (50 mm Tris, 10 mm MgCl2, 1 mm EDTA, pH 7.5, 4°C) in glass-glass homogenizing tubes (Kontes) and filtered through Nitex cloth (149 μm). Membranes were collected by centrifugation (3000 x g for 5 minutes) and suspended in homogenizing buffer at 1-2 mg protein/ml. Protein was determined by the method of Bradford (1976).

Radioligand Binding Assay

Radioiodinated cyanopindolol was the generous gift of Dr. Paul Insel, and was prepared as described previously (Engle et al., 1981). Binding assays were performed in triplicate by incubating 2-3 x 10^8 myocytes, or 30-50 μg of membrane suspension with 0.1 ml of [125I]ICYP (2200 Ci/mmol) and 0.1 ml of various β-adrenergic antagonists or diluent in a final volume of 1.0 ml in polypropylene test tubes (16 x 105 mm Sarstedt; washed). Assays were initiated by the addition of myocytes or membranes diluted in BME immediately prior to addition to the assay. All tubes containing myocytes were gassed with O2/CO2 (95%/5%) and capped. Equilibrium binding was carried out for 60-90 minutes at 34°C in a rotary water bath at 120 rpm. Assays were terminated with the addition of 10 ml of cold (4°C) hypotonic buffer (10 mm Tris, 1 mm EDTA, pH 7.5). Bound and free [125I]ICYP were separated by rapid filtration over glass fiber filters (Whatman GF/C). Filters were washed with an additional 10 ml of hypotonic buffer at 4°C. Radioactivity retained on filters was determined using a gamma counter at 86% efficiency. Replicate variation was less than 5% in all experiments. Nonspecific binding for both membranes and intact myocytes was determined by [125I]ICYP binding in the presence of 1 μM l-propranolol. The subtraction of nonspecific binding from total binding yielded specific binding which was routinely 60% for intact myocytes and 70% for membranes at 100 pm [125I]ICYP. Incubation of both intact cells and membranes with [125I]ICYP resulted in the rapid appearance of specific binding that reached equilibrium in 45 minutes and was appropriately reversed by propranolol (1 μM). Binding was linear with increasing myocyte number or amount of membrane protein. Viability of myocytes maintained under identical conditions during binding assays remained between 90 and 95% of starting figures.

Data Analysis

The equilibrium dissociation constant (Kd) and maximal number of binding sites (Bmax) for [125I]ICYP, a nonspecific β-adrenergic antagonist, on ventricular myocytes and ventricular nonmyocyte and liver membranes were determined from Scatchard analysis (Scatchard, 1949) of saturation binding isotherms. Competition curves employing β-subtype-specific antagonists were analyzed by means of a computer program that performs iterative, nonlinear regression (Munson and Rodbard, 1980). This program fits the binding data to equations describing the laws of mass action for one or two classes of binding sites and determines whether the two-site model is statistically better than the one-site fit of the data.

The affinity (Kd) of the nonselective radioligand [125I]ICYP was determined separately in each competition experiment with subtype-selective antagonists and either intact myocytes or membranes, and was entered in the computer analysis as a constant. Computer-generated fits of competition data provide estimates of the affinity constant (Kd) of subtype-specific antagonists and of the concentration of each receptor subtype. Further, computer analysis determined nonspecific binding from data obtained at high competitor concentrations which was indistinguishable from simultaneous measurements of binding in the presence of 1.0 μM l-propranolol. The likelihood that more than one receptor subtype was present was decided by comparison of the residual variances obtained, assuming either one or two classes of binding sites (Munson and Rodbard, 1980). The limitation of this computer method to detect small numbers of β1-receptors on the ventricular myocyte derives from both precise measurements of binding at a given antagonist concentration (replicate variation <5%) and a large difference in the affinities (Kd) of subtype-selective agents for β1- vs. β2-receptors (Delean et al., 1981, and data of Table 1).

Results

Distribution of β-Receptors in Myocardial Membranes

As a starting point to verify our techniques, we determined the distribution of β1- and β2-receptors in a membrane fraction of an homogenate of rat heart ventricle.

The kinetics of binding of [125I]ICYP to whole ventricle membranes showed a large degree of high affinity binding (Kd = 23 pM, Bmax = 91 fmol/mg protein) that was readily competed for by β-adrenergic antagonists. Based on such competition of [125I]ICYP binding with the β-subtype-specific agents, practolol and zinterol, we calculated a distribution of β-receptors of 65-70% β1 and 30-35% β2 for whole ventricle (Fig. 1). This relatively high proportion of β2-receptors suggested to us that we might reasonably expect to detect the appearance of even a moderate fraction of either receptor subtype in more purified fractions of cardiac tissue.

Characteristics and Distribution of β-Receptors in Purified Myocytes

Binding of [125I]ICYP to the intact myocyte was saturable with increasing radioligand concentration (5-1000 pm). Scatchard analysis of saturation isotherms yielded linear plots indicating that [125I]ICYP bind to a single class of sites on the myocyte (Fig. 2). The ability of the nonselective β-adrenergic antagonist propranolol to compete for [125I]ICYP bind-
FIGURE 1. Competition of $[^{125}\text{I}]$ICYP binding sites in membranes of rat whole ventricle. Membranes were prepared from rat whole ventricle and incubated at 32°C with $[^{125}\text{I}]$ICYP (40 pM) and increasing concentrations of the $\beta_1$-receptor subtype-selective antagonists practolol ($\beta_1$) and zinterol ($\beta_5$). Data are expressed as a percentage of maximal specific binding in the absence of competitor. Curves are computer-generated best fits representing two classes of binding sites and their proportions (see Methods). Values are the mean of triplicate determinations in a single representative experiment.

ing, as well as the capacity of the $l$-isomer of the $\beta$-agonist isoproterenol to compete for $[^{125}\text{I}]$ICYP binding with a greater affinity ($K_0 = 80$ nM) than the $d$-isomer ($K_1 = 2500$ nM) confirmed that $[^{125}\text{I}]$ICYP was binding to $\beta_1$-adrenergic receptors on the myocytes. Characteristics of the cardiomyocyte $\beta$-receptor from Scatchard analyses of several experiments are listed in Table 1.

To determine the relative proportion of $\beta_1$- and $\beta_2$-receptor subtypes on intact ventricular cardiomyocytes, we employed the $\beta_1$-selective antagonists betaxolol and practolol and the $\beta_2$-selective agent zinterol to compete for $[^{125}\text{I}]$ICYP binding to these intact adult muscle cells. Competition for $[^{125}\text{I}]$ICYP binding by each antagonist resulted in monophasic curves, demonstrating a single class of $\beta$-adrenergic binding sites (Fig. 3). The $K_i$ value for both betaxolol (46 nM) and practolol (800 nM) is consistent with competition of these drugs for binding to a $\beta_1$-receptor, whereas the $K_i$ value calculated for zinterol binding (1.2 $\mu$M) to the same receptor demonstrates a much lower affinity than would be predicted for the binding of zinterol to a $\beta_2$-receptor [20 nM (Minneman et al., 1979a, 1979b, and Table 1)].

Similar results were obtained in membranes prepared from freshly purified myocytes. Myocyte membranes bound $[^{125}\text{I}]$ICYP with high affinity ($K_0 = 52$ pM) to a large number of sites, 188 fmol of receptor/mg membrane protein. This density agrees well with our estimate of $2.1 \times 10^9$ receptors/cell, given that each cell has a measured membrane protein content of 2.2 ng. Practolol competed for this binding in a monophasic manner with an affinity ($K_0$) of 780 nM. This affinity is in excellent agreement with data for competition of $[^{125}\text{I}]$ICYP binding to intact myocytes, shown above. Thus, the results with myocyte membranes confirm our finding a single subtype of $\beta$-receptor ($\beta_1$) on intact myocytes.

Distribution of $\beta$-Receptor Subtypes in Nonmyocyte Membranes

Because we detected only $\beta_1$-receptors on ventricular myocytes, we anticipated that membranes prepared from the nonmyocyte components of the ventricle would reveal the presence of $\beta_2$-adrenergic receptors. Saturation isotherms produced under conditions identical to those used to assay $[^{125}\text{I}]$ICYP binding to purified myocytes gave linear Scatchard curves (Fig. 4), indicating that the nonselective radiolabeled antagonist recognizes a single class of binding sites in nonmyocyte membranes (kinetic parameters summarized in Table 2). However, competition of $[^{125}\text{I}]$ICYP binding to nonmyocyte membranes by betaxolol, practolol and zinterol occurred over a broader concentration range than expected for a single component interaction, indicating the presence of both $\beta_1$- and $\beta_2$-receptors (Fig. 5). Data of competition for $[^{125}\text{I}]$ICYP binding by betaxolol and practolol were best satisfied by a two-site fit.
TABLE 1

<table>
<thead>
<tr>
<th>Characteristics of [125I]ICYP binding</th>
<th>Distribution of β-adrenergic receptor subtypes</th>
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<tbody>
<tr>
<td></td>
<td>Receptor prevalence (%)</td>
</tr>
<tr>
<td>Receptor subtype</td>
<td>Practolol (β₁)</td>
</tr>
<tr>
<td>Nonmyocyte membranes</td>
<td>51.5 ± 8.5</td>
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<tr>
<td></td>
<td>0.66 ± 0.14</td>
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<tr>
<td>Purified myocytes</td>
<td>100</td>
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<tr>
<td></td>
<td>0.85 ± 0.06</td>
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<td></td>
<td>None</td>
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Radiolabeled antagonist affinity (K₀) and receptor density were determined by incubating tissue with increasing concentrations of [125I]ICYP (5–1000 pM) in the presence and absence of 1 μM (-)-propranolol. Data obtained with [125I]ICYP and the subtype-selective agents practolol (β₁) and zinterol (β₂) were analyzed by computer as described in Methods. Values are the mean ± SE of four to six experiments. Measurements of protein refer to membrane protein.

with 40% β₂-receptors (K₀: betaxolol 0.3 μM, practolol 12.2 μM). The balance (60%) of receptors interacted with both betaxolol and practolol with the high affinity (0.004 and 0.5 μM, respectively) expected for binding to a β₁-receptor. Competition [125I]ICYP binding to the nonmyocyte membranes by the β₂-selective antagonist zinterol revealed two components of binding in the proportion β₂:β₁, 45:55. The dissociation constants for the interactions of these receptors with zinterol were 40 nM (β₂) and 800 nM (β₁). The ability of zinterol to compete with high affinity for a large fraction (45%) of [125I]ICYP binding to nonmyocyte membranes is not the result of the absence of added guanine nucleotide (GTP). When zinterol competition of [125I]ICYP binding to these membranes was compared with and without 100 μM added GTP, the guanine nucleotide caused only a small rightward shift in the high and low affinity components of binding. This slight shift caused no change in the estimation of proportions of β₁- and β₂-receptors present (Fig. 6).

The resolution of β₁- and β₂-receptors by betaxolol, practolol, and zinterol, the appropriateness of their affinities, and the excellent agreement on the relative densities of the receptor subtypes determined by these agents, demonstrate convincingly that the nonmyocyte fraction contains β₂- as well as β₁-receptors.

Confirmation of the ability of methods applied here to detect β₂-adrenergic receptors was provided by identical studies performed on a tissue known to be a homogeneous source of β₂-adrenergic receptors (Minneman et al., 1979c). Binding of [125I]ICYP to rat liver membranes showed a single class of high affinity sites (K₀ = 25 pm; Bmax = 69 fmol/mg membrane protein). Competition of [125I]ICYP binding to liver membranes by subtype-specific antagonists gave monophasic curves with affinities for zinterol and practolol appropriate for a β₂-receptor (15 and 760 nM, respectively).

Discussion

We have employed radioligand-binding techniques to investigate the distribution of β-adrenergic receptors in rat myocardium, a tissue known to contain both β₁- and β₂-receptors (Minneman, 1979a, 1979b, and Table 1). Our work differs from previous studies in two principal ways. First, we have purified ventricular myocytes from rat heart and are thus able to compare receptor binding data from whole ventricle, purified cardiomyocytes, myocyte membranes, and the nonmyocyte fraction of ventricle. Second, we provide data on β₂-adrenergic receptors present on fully differentiated, intact car-
Employing antagonists selective for $\alpha_1$- or $\alpha_2$-receptors and the $\beta$-specific radioligand $[^{125}I]ICYP$, we find a single class of $\beta_1$-receptors on purified ventricular myocytes. In the nonmyocyte fraction, however, approximately 50% of $\beta$-receptors are of the $\beta_2$-subtype, data consistent with reports of as many as 24% $\beta_2$-receptors in rat heart ventricle (Table 1, and Minneman et al., 1979a, 1979b), since our preparation represents a purification of both myocyte (all $\beta_1$) and nonmyocyte elements (contributing $\beta_2$-receptors). Indeed, it seems likely to us that many of the $\beta_1$-receptors identified in the nonmyocyte fraction of ventricle are contributed by damaged myocytes that are excluded during myocyte purification.

It is, of course, possible that cardiomyocytes have a small fraction of $\beta_2$-receptors that we failed to detect. Applying the analysis described by DeLean et al. (1981), we can estimate the limits of our ability to discern a small population of $\beta_2$-receptors. In using a subtype-specific antagonist with a nonspecific labeled ligand in a competitive binding assay, one needs an antagonist that exhibits a selectivity between $\beta_1$- and $\beta_2$-receptors of ~50 to detect as few as 10% $\beta_2$-receptors. In our experiments, the selectivities of the compounds were ~20 for practolol and zinterol and ~60 for betaxolol. Thus, within these limits (10% $\beta_2$-receptors), which are the likely limits imposed by experimental error, we do not detect $\beta_2$-receptors on the adult cardiomyocyte.

The number of $\beta_1$-receptors found on each adult myocyte, ~2 x 10^5, seems, at first glance, large. This high density of receptor on the intact myocyte manifests itself as an appropriately high content of receptors in isolated membranes, ~162 fmol/mg pro-

![Figure 4: Binding of $[^{125}I]ICYP$ to the non-myocyte fraction of rat ventricle. Panel A: membranes prepared from the nonmyocyte fraction of rat ventricle were incubated with increasing concentrations of $[^{125}I]ICYP$ (5-580 pM) in the presence (nonspecific binding, $\Delta$) and absence (total binding, $\bullet$) of propranolol ($10^{-6}$ M). Specific binding ($\bigcirc$) was determined as the difference of nonspecific and total binding. The Hill slope for specific binding was 0.97. Panel B: Scatchard plot of specific binding.

![Figure 5: Competition of $[^{125}I]ICYP$ binding sites in nonmyocyte membranes by betaxolol, practolol, and zinterol. Membranes prepared from the nonmyocyte fraction of rat ventricle were incubated at 32°C with $[^{125}I]ICYP$ (45 pM) and increasing concentrations of betaxolol, practolol, or zinterol. Data are expressed as a percentage of maximal specific $[^{125}I]ICYP$ binding in the absence of competitor. Values are the mean of triplicate determinations in a single representative experiment. Curves are computer-generated best fits of two classes of binding sites (see Methods).

![Figure 6: Effect of GTP on zinterol competition of $[^{125}I]ICYP$ binding to nonmyocyte membranes. Nonmyocyte membranes were incubated with 50 pM $[^{125}I]ICYP$ and increasing concentrations of zinterol in the presence (○) or absence of (□) of 100 μM GTP. Data, determined in triplicate, are expressed as a percentage of maximal specific binding in the absence of competitor. Curves are computer generated best-fit of the data modeled to two binding sites (see Methods).
tein. Our estimate compares favorably with that of Moustafa et al. (1978), who found 360,000 binding sites for [3H]dihydroalprenolol per rat cardiomyocyte. The report of Lau et al. (1980) of 7500 β-receptors per myocyte in primary cultures of fetal heart is probably not a relevant comparison, since those cells were immature, far smaller than the adult cells that we isolate, and possibly contaminated by other cell types. Densities of $10^3$ to $10^4$ receptors per cell are routine in many prototypic cell systems (for β-receptors: S49 cells, ~1200 cell; avian red cells, ~600/cell; BC3H-1 cells, 9000/cell; C6 glioma cells, ~9400/cell). When expressed as a function of cell size, the density of β-receptors on myocytes falls in this range. Myocytes are large cells; ours average 20 μm × 90 μm. Assuming that the myocyte is a cylinder of those dimensions, the surface density is 33 β1-receptors per μm², compared to ~3 receptors/μm² for S49 lymphoma cells, ~1 receptor/μm² for pigeon red cells, 42 receptors/μm² for C6 glioma cells (Dibner et al., 1981), and 20 receptors/μm² for the BC3H-1 smooth muscle cell (calculated from data of Hughes et al. (1982) and Brown et al. (1984). Thus, the prevalence of β-adrenergic receptors per unit area of myocyte membrane is not unusually high. Furthermore, if surface receptor density is a way in which cells regulate their adrenergic responsiveness (Shear et al., 1976), then this number of β-receptors in a highly responsive tissue such as heart seems reasonable.

The presence of β1- but not β2-receptors on rat myocytes implies that the metabolic and contractile response of rat ventricular muscle to β-agonists results exclusively from occupation of β1-receptors. This conclusion varies from that reached by Robberecht et al. (1983) and Waelbroeck et al. (1983). These workers have recently offered evidence that human cardiac tissue has equal contents of β1- and β2-receptors, and that only the β2-adrenergic receptors are coupled to adenylyl cyclase. These studies must be accepted with caution, however, since the tissues employed were largely atrial and were from patients who had been receiving β-blockade therapy. In addition, these human tissues, removed during surgical repair of coronary insufficiency or valvular disease, very likely represent diseased rather than normal tissue and were certainly a population of mixed cell types. The sort of study that we have performed emphasizes the utility of a purified cell population and provides a direct test of the proposal of Waelbroeck and colleagues (1983).

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