Quantitative Autoradiographic Delineation of the Distribution of β-Adrenergic Receptors in Canine and Feline Left Ventricular Myocardium

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The distribution of adrenergic receptors in specific components of the heart such as vessels and myocytes cannot be determined easily with assays of membranes prepared from homogenates of whole tissue. Accordingly, we characterized the binding of the potent nonsubtype selective antagonist [125I]cyanopindolol to β-receptors in unfixed transmural slices of feline and canine left ventricle. Specific binding ratios >90% were achieved at radioligand concentrations near Kd and >80% at saturating ligand concentrations. Binding of radioligand to receptors in transmural slices was rapid, saturable, stereoselective, and displaceable by antagonists and agonists with the rank order of potency expected of β-adrenergic receptors. Analysis of binding isotherms indicated maximum binding capacities of 27.8 ± 6.6 and 40.6 ± 5.1 fmol/mg tissue protein and dissociation constants of 10.1 ± 1.8 and 21.3 ± 1.6 nM in feline and canine ventricular slices, respectively. The distribution of β-receptors in myocytes and selected vascular components of the heart was determined with quantitative film autoradiography and high resolution computer-based analysis and display of the density of binding sites, maximum binding capacity, and binding affinity measurements. The results of autoradiographic analysis revealed a uniform transmural distribution of receptors in regions composed primarily of ventricular myocytes but an inverse relation between the density of β-receptors and the diameter of coronary vessels. Large epicardial conductance arteries had half the receptor density of subjacent myocytes; small mural arteries had approximately 60% of the β-receptor density of nearby myocytes, and the coronary resistance arterioles had the highest receptor density of any vascular compartment, which was equivalent to that of myocytes. The methods developed should be of particular value in characterizing the distribution and function of receptor subtypes and mechanisms of regulation of adrenergic responsiveness in intact myocardium. (Circulation Research 1987;60:568–579)
Materials and Methods

Preparation of Tissue Slices

Hearts were excised rapidly from adult mongrel dogs anesthetized with thiopental (10 mg/kg i.v.) and cats anesthetized with α-chloralose (75 mg/kg i.v.) and rinsed briefly in saline at 4°C as previously described. Transmural blocks of left ventricular myocardium were dissected, cooled on dry ice, submerged slowly in liquid nitrogen, and stored in a sealed container at −70°C until used in experiments. Unfixed frozen sections, 12 μm in thickness, were mounted on gelatin-coated slides. Three serial sections were placed on each slide.

Radioligand binding assays

Unfixed slide-mounted transmural sections of myocardium were incubated at 22° or 37°C in buffer (NaCl 154 mM, MgCl2 10 mM, Tris-HCl 10 mM, pH 7.4) containing selected concentrations of (-)[125I]cyanopindolol (ICYP) (2.200 Ci/mmol, New England Nuclear, Inc., Boston, Mass.) and unlabelled displacer. Nonspecific binding was defined as binding of radioligand in the presence of 1 μM l-propranolol, which was kindly provided by Ayerst Laboratories, New York, N.Y. Sections were incubated with radioligand in large volumes of buffer so that the concentration of free radioligand did not change measurably during the incubations. Nonspecifically bound radioactivity was removed by incubating slides in buffer without radioligand or unlabelled displacer for selected intervals at 22° or 37°C. Following rinsing in buffer, sections were dipped briefly in distilled water to remove buffer solutes, dried under a gentle stream of air, and either scraped off slides for quantification of radioactivity with gamma scintillation spectrometry or prepared for autoradiographic analysis as described below. Radioactivity in each section was determined by scraping sections from the slides with a razor and quantifying radioactivity in each with gamma scintillation spectrometry (66% counting efficiency). Radioactivity was normalized to account for variations in section thickness or section size. Total cross-sectional area and total tissue protein were measured in groups of sections selected at regular intervals during the preparation of large numbers of serial sections for an individual experiment. Cross-sectional areas were measured by carefully tracing the outlines of the sections and digitizing the traced areas. Total tissue protein was assessed in individual sections scraped from acid-washed slides (nongelatin coated) using the Lowry assay with bovine serum albumin standards. In all experiments, data points were calculated as means of triplicate determinations.

Quantitative Autoradiography

Two complementary light microscopic autoradiographic methods were used to quantitatively localize binding sites in transmural sections of myocardium. The first was the coverslip method of Young and Kuhar. Acid-washed, gelatin-coated coverslips were dipped in melted Kodak NTB2 (Eastman Kodak Co., Rochester, N.Y.) nuclear track emulsion at 45°C and dried in a light-tight box containing desiccant at room temperature for at least 3 hours. The emulsion-coated coverslips were affixed to slides bearing radiolabelled sections with cyanoacrylate glue at one end and a binder clip at the other. Following exposure of the emulsion for 18–96 hours, the binder clips were removed, the coverslips gently lifted from the slides at one end, and the emulsions developed with Kodak D19 developer (diluted 1:1 with water) for 4 minutes and fixed with Kodak fixer for 4 minutes at 25°C. After photographic processing the tissue sections were stained with hematoxylin and eosin and the coverslips sealed permanently to the slides. The tissue and overlying grains in the emulsion layer were examined by light microscopy, photographed, and grain densities quantified by counting grains per unit area of selected regions of the section.

The second autoradiographic method employed highly sensitive film (Ultrofilm, LKB Instruments, Inc., Gaithersburg, Md.) and analysis with automated computer reconstruction of digitized film autoradiographic images. Slides bearing radiolabelled sections were placed in direct contact with Ultrofilm for 18 hours to 2 weeks, after which the film was developed with Kodak D19 developer (4 minutes at 4°C), rinsed with dilute acetic acid, fixed with rapid thiofix for 15 minutes at room temperature, and washed with distilled water. The developed images were scanned with an Eikonix 785 flatbed scanner/digitizer and digitized in units of film optical density in a 1024 × 1024 picture element (pixel) array. Background optical density, assessed in areas of film adjacent to exposed regions, was subtracted automatically from the optical density measurements obtained over the sections. Digitized image data were stored on a DEC VAX 11/730 computer and analyzed with the LONISP software package developed by the Laboratory of Neuro Imaging at Washington University. The analysis system is capable of reconstructing digitized optical density images and, using standards to convert optical density to values of specific radioactivity, can display reconstructed images showing radioligand binding density. The system can analyze binding isotherm data over a broad concentration range, perform Eadie-Hofstee analysis on a pixel-by-pixel basis and display reconstructed images showing maximum binding capacity (Bmax) and dissociation constant (Kd) in each pixel.

Tissue radioactivity standards were used to calibrate film autoradiographs and to convert optical density measurements to units of specific radioligand binding. Tissue standards were prepared by grinding canine myocardium into a smooth paste and adding known quantities of ICYP over a range of concentrations (0.2–9.6 μCi/g wet wt) equivalent to that encountered in binding isotherm experiments. Wet and dry weights, protein content, and specific radioactivity were determined in each of the labelled tissue paste standards with the Lowry protein assay and gamma scintillation spectrometry. The standards were frozen,
sections 12 µm in thickness were prepared, and a series of standard sections mounted on slides were placed on film along with experimental sections containing radioligand. A standard curve was generated for each sheet of film used to determine the distribution of myocardial β-receptors in transmural sections of myocardium.

Statistical Analysis

All data are expressed as means ± SD unless otherwise indicated. Binding isotherm data were transformed according to the methods of Scatchard21 and Hill.22 Simple linear regression was used in determining intersections and slopes in Scatchard and Hill plots.

The statistical significance of differences in grain density measurements in light microscopic autoradiographs was determined with analysis of variance using the SAS general linear models procedures.23

Results

Initial experiments were performed to determine optimal rinsing conditions to remove nonspecifically bound radioactivity without removing specifically bound radioactivity. Frozen transmural sections of canine and feline left ventricle were incubated with saturating concentrations of ICYP for prolonged intervals to promote nonspecific binding. Subsequently, the sections were incubated with buffer not containing radioligand or unlabelled displacers for selected intervals, rinsed briefly in distilled water, and then dried and scraped for quantification of radioactivity with gamma scintillation spectrometry. As shown in Figure 1A, the amount of specific binding in sections of feline myocardium remained constant during 180 minutes of incubation with buffer not containing radioligand or unlabelled displacer at room temperature (22°C). Specific binding ratios of 80–85% were routinely achieved at saturating radioligand concentrations while >90% specific binding was observed at ICYP concentrations near KD. In canine myocardium, the ratio of specific binding observed following rinsing at 22°C was lower than in the cat (Figure 1B). However, rinsing of sections at 37°C enhanced removal of nonspecifically bound radioactivity without causing removal of specifically bound radioactivity (Figure 1C). Based on these experiments it was determined that optimal removal of nonspecific radioactivity in feline myocardium was achieved after 60 minutes of incubation at room temperature, while in the dog excellent specific binding ratios were achieved following 60 minutes of incubation at 37°C. These rinsing conditions were used in all subsequent experiments.

The association kinetics of ICYP binding were studied over a broad range of radioligand concentrations and at different temperatures in both dog and cat myocardial sections to define intervals required to achieve equilibrium binding. As shown in Figure 2A, a plateau in specific binding was achieved in 60 minutes when sections of feline left ventricle were incubated with a

![Figure 1. Selective removal of nonspecifically bound radioactivity during prolonged rinsing of sections with unlabelled buffer. Transmural sections were incubated with ICYP in presence or absence of 1 µM l-propranolol to determine total (●), nonspecific (○), and calculated specific binding (●). After selected intervals of rinsing, sections were dried, scraped off slides, and radioactivity quantified with gamma scintillation spectrometry. A: Sections of feline myocardium incubated with 68 pM ICYP for 1 hour at 37°C and rinsed with buffer for 0–180 minutes at 22°C. B and C: Sections of canine myocardium incubated with 52 pM ICYP for 1 hour at 37°C and rinsed for 0–180 minutes at 22°C (B) or 37°C (C). Data points are means ± SD of triplicate determinations.](http://circres.ahajournals.org/doi/abs/10.1161/01.RES.60.4.570?journalCode=circ)
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Effects of temperature and radioligand concentration on ICYP binding to feline and canine myocardium. A: Sections of feline myocardium were incubated with 50 pM ICYP at 22°C (●) or 37°C (•). After selected intervals of binding, sections were rinsed to remove nonspecifically bound radioactivity, dried, scraped from slides, and radioactivity quantified with gamma scintillation spectrometry. B: Sections of canine myocardium were incubated for selected intervals with ICYP concentrations of 93 pM (upper curve), 9.5 pM (middle curve), and 1.1 pM (lower curve). Sections were rinsed and radioactivity determined as described. Data points are means ± SD of triplicate determinations of specific binding.

saturating concentration of radioligand (50 pM) at 37°C. The same level of specific binding was not reached until 180 minutes when adjacent serial sections were incubated with 50 pM ICYP at 25°C. Accordingly, subsequent binding studies were conducted at 37°C. Incubation intervals required to achieve equilibrium binding were assessed in canine myocardium incubated with ICYP at selected concentrations (Figure 2B). At a concentration of 93 pM (approximately 5 times K_d), a plateau in specific binding was achieved after 60 minutes of incubation, whereas at 9.5 pM (approximately half K_d) a plateau in specific binding was not apparent until 120 minutes. At 1 pM (approximately 0.025 times K_d) a plateau was difficult to discern although the difference in specific binding at 120 and 240 minutes was not statistically significant. Thus, in subsequent binding isotherm experiments, sections were incubated with radioligand for 60–120 minutes depending on radioligand concentration.

Figure 3 shows the expected linear relation between section thickness and amount of specific binding. A section thickness of 12 μm was considered optimal because the variation in thickness, assessed with protein assays, was modest (< 10%), sections were easy to cut and handle, and a substantial number of sections could be prepared from a single tissue block. Therefore, sections of myocardium and tissue radioactivity standards were cut at 12 μm in all subsequent experiments.

Binding isotherms of unfixed frozen sections of feline and canine myocardium incubated with ICYP are shown in Figures 4 and 5. Scatchard analysis of 14 separate isotherms in sections prepared from 7 cat hearts revealed a K_d of 10.1 ± 1.8 pM and a B_max of 27.8 ± 6.6 fmol/mg total tissue protein (Figure 4). Scatchard plots were linear and Hill plots had slopes close to 1.0 consistent with binding of ICYP to a single class of binding sites without positive or negative cooperativity. Analysis of binding isotherms in canine myocardium (4 isotherms in sections from 4 hearts) revealed a K_d of 21.3 ± 1.6 pM and a B_max of 40.6 ± 5.1 fmol/mg total tissue protein (Figure 5).

To characterize the stereospecificity of binding and to determine whether binding of ICYP to β-receptors could be displaced by agonists and antagonists with the rank order of potency characteristic of β-adrenergic receptors, sections were incubated with ICYP and selected concentrations of the d- and 1-stereoisomers of the antagonist propranolol and the agonists isoproterenol and norepinephrine. In 2 separate experiments, the 1-stereoisomers were 2–3 orders of magnitude more potent than the corresponding d-isomers in displacing binding of ICYP as shown in Table 1 and Figure 6. Competitor dissociation constant values (K_c) values were determined according to the method of Cheng and Prusoff.24 The affinity of the antagonist 1-propranolol for ICYP binding sites was greater than that of the agonist 1-isoproterenol, which, in turn, displaced ICYP with greater affinity than 1-norepinephrine. Thus, binding of ICYP to unfixed transmural sections of canine and feline myocardium was stereoselective and was displaced by antagonists and agonists with the rank order of potency expected of the β-adrenergic receptor. In addition, the propranolol displacement curve was steep while those of the agonists spanned a

Figure 3. Relation of section thickness and amount of specific binding of ICYP to canine myocardium. Sections were incubated with 27 pM ICYP for 1 hour at 37°C, rinsed, and radioactivity measured with gamma scintillation spectrometry. Data points are means ± SD of triplicate determinations of specific binding.
FIGURE 4. Binding isotherm (upper panel) and Scatchard (middle panel) and Hill (bottom panel) plots of ICYP binding to transmural sections of feline left ventricle. Sections were incubated under equilibrium binding conditions at 37°C with ICYP (1–125 pM), rinsed, dried, scraped, and radioactivity quantified with gamma scintillation spectrometry. Isotherm data points are means of triplicate determinations of total (○), non-specific (●), and specific (●) binding. Standard deviation bars are indicated for the measured values of total binding. Standard deviations of means of nonspecific binding are contained within the symbols.

FIGURE 5. Binding isotherm (upper panel) and Scatchard (middle panel) and Hill (bottom panel) plots of ICYP binding to transmural sections of canine left ventricle. Sections were incubated under equilibrium binding conditions at 37°C with ICYP (0.9–120 pM), rinsed, dried, scraped, and radioactivity quantified with gamma scintillation spectrometry. Isotherm data points are means of triplicate determinations of total (○), non-specific (●), and specific (●) binding. Standard deviation bars are indicated for the measured values of total binding. Standard deviations of means of nonspecific binding are contained within the symbols.
Table 1. Stereospecificity of Binding of ICYP to Transmural Sections of Canine Left Ventricle

<table>
<thead>
<tr>
<th>Displacer</th>
<th>Experiment</th>
<th>IC50 (M)</th>
<th>Kd (M)</th>
<th>Kp (dM/d)</th>
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<tbody>
<tr>
<td>1-propranolol</td>
<td>1</td>
<td>6.3 x 10^{-6}</td>
<td>9.6 x 10^{-10}</td>
<td>135 (1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.2 x 10^{-8}</td>
<td>1.9 x 10^{-9}</td>
<td>51 (2)</td>
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<tr>
<td>d-propranolol</td>
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<td>8.0 x 10^{-7}</td>
<td>1.3 x 10^{-7}</td>
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<td></td>
<td>2</td>
<td>6.0 x 10^{-7}</td>
<td>9.6 x 10^{-8}</td>
<td>789 (2)</td>
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<tr>
<td>l-isoproterenol</td>
<td>1</td>
<td>1.0 x 10^{-8}</td>
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<td>48 (1)</td>
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<td>2</td>
<td>1.2 x 10^{-8}</td>
<td>1.9 x 10^{-9}</td>
<td>110 (2)</td>
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<td>d-isoproterenol</td>
<td>1</td>
<td>3.0 x 10^{-6}</td>
<td>4.8 x 10^{-7}</td>
<td>48 (1)</td>
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<tr>
<td></td>
<td>2</td>
<td>9.5 x 10^{-6}</td>
<td>1.5 x 10^{-6}</td>
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<tr>
<td>l-norepinephrine</td>
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<td>2</td>
<td>1.8 x 10^{-7}</td>
<td>2.9 x 10^{-8}</td>
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<td>d-norepinephrine</td>
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<td></td>
<td>2</td>
<td>2.0 x 10^{-5}</td>
<td>3.2 x 10^{-6}</td>
<td>110 (2)</td>
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*Ratios of Kd of d- and l-stereoisomers in experiments 1 and 2.

greater concentration range and appeared biphasic, consistent with the known high and low affinity states of the β-receptor for agonists.

We employed the binding methods developed to localize β-adrenergic receptors in transmural frozen sections of the heart with light microscopic autoradiography. To facilitate quantitative analysis of film autoradiographs, film was exposed to uniformly labelled tissue paste standards containing known levels of radioactivity. As shown in Figure 7, the optical density of the exposed film was proportional to section thickness, specific radioactivity, and exposure interval. Within the range of optical densities reliably quantified by the automated microdensitometric analysis system, the relation between film optical density and tissue specific radioactivity was linear. In each experiment, a series of 12 μm sections of tissue paste radioactivity standards was exposed to Ultrasan with slides bearing radiolabelled sections to convert optical density measurements to units of specific radioactivity.

Binding isotherms were analyzed autoradiographically in 4 separate experiments with feline myocardium by exposing labelled sections to Ultrasan after incubation of tissue with a broad range of ICYP concentrations. As shown in Figure 8, the isotherm, Scatchard plot, and calculated values of Bmax and Kd in feline myocardium, determined solely from autoradiographic measurements of total section radioactivity at each ligand concentration, were in agreement with those obtained by gamma scintillation spectrometry (see Figure 4). In sections of feline left ventricle, Bmax and Kd were 19.3 ± 3.0 fmol/mg protein and 12.0 μM.

Figure 6. Stereospecificity and relative potencies of antagonist and agonist displacers of ICYP binding to sections of feline myocardium. Transmural sections were incubated for 1 hour at 37°C with 42 pM ICYP plus selected concentrations of the d- and l-enantiomers of propranolol, isoproterenol, and norepinephrine. Ascorbate (10^{-3} M) and pargyline (10^{-5} M) were included in the radioligand incubation and rinse buffers. Specific binding of ICYP at each displacer concentration was compared to maximal specific binding defined as the difference in binding in the presence and absence of 1 μM l-propranolol. Data points are means of triplicate determinations in 2 separate experiments.

Figure 7. Relation of amount of radioactivity in tissue paste standards and exposure interval to optical density in film autoradiographs. Upper panel: Sections of tissue paste standards of selected specific radioactivities cut at thicknesses of 16, 32, and 48 μm, mounted on slides, and exposed to Ultrasan for 24 hours. Data points are means of triplicate optical density measurements. Lower panel: Sections (12 μm in thickness) of tissue paste standards of selected specific radioactivities exposed to Ultrasan for 18, 48, or 120 hours. Data points are means (± SD) of 3–5 optical density measurements.
ICYP binding to transmural sections of feline myocardium. Sections were incubated with 1-64 pM ICYP, rinsed, dried, and exposed to Ultrofilm along with tissue paste standards. Specific binding at each concentration was defined as difference in optical density value was determined by computer and converted to umolol. For each autoradiographic film image, a mean optical density was determined. The density of binding sites was analyzed quantitatively in computer reconstructions of autoradiographic film images. Digitized, calibrated autoradiographic film images were compared with digitized, calibrated autoradiographic film images. The density of binding sites in each anatomic compartment and the affinity with which radioligand bound to receptors was therefore required.

Figure 10 shows computer reconstructed images selected from an isotherm in which serial transmural sections of canine myocardium were incubated with ICYP at 10 separate concentrations from 1-120 pM. Each image depicts specific binding of ICYP in a portion of epicardium that included profiles of epicardial and mural arteries. The amount of specific binding increased with increasing ICYP concentrations as indicated by the color gradations. At each ligand concentration, the density of β-receptors in epicardial and mural vessels was approximately 40 and 65%, respectively, of the density in surrounding myocytes.

Quantitative images obtained from serial sections incubated with ICYP at 1-120 pM were superimposed with the use of anatomic landmarks as fiducial marks. Eadie-Hofstee analysis was performed on a pixel-by-pixel basis over the full ligand concentration range and the computer reconstructed the resultant maximum binding capacity in the epicardial coronary artery was considerably lower than in the subjacent myocardium. Maximum binding was apparent in the connective tissue that surrounded the epicardial coronary artery, mural vessels, and surrounding ventricular myocytes. The uniformity of maximum binding capacity was apparent in specific regions of the section that corresponded to small mural coronary arteries. The sparse cellularity of the fibro fatty connective tissue resulted in a low per unit area density of binding sites in comparison with surrounding structures. However, as shown in the Kd map in Figure 11, ICYP bound to the β-receptors of the loose perivascular connective tissue, as well as those of the epicardial coronary artery, mural vessels, and surrounding ventricular myocytes with uniform affinity.

The results of quantitative localization of β-receptors with Ultrofilm autoradiography were confirmed.
FIGURE 9. Computer reconstructed image of quantitative transmural distribution of specific binding sites in section of canine myocardium incubated under equilibrium binding conditions with 30 pM ICYP. Digitized film autoradiographs of serial sections incubated with and without 1 μM l-propranolol were aligned, nonspecific values were subtracted from total, and resultant optical density values of specific binding were converted to units of fmol/mg tissue protein based on tissue paste radioactivity standards. Color-enhanced map of β-receptor distribution (top left) corresponds to photomicrograph of section used to generate film image (bottom left). High resolution reconstructions (top right) show specific binding densities in epicardial and mural coronary arteries (compare with inset photomicrograph of actual mural artery).

with conventional autoradiographic methods using emulsion-coated coverslips. Sections of canine left ventricle were incubated under equilibrium conditions with ICYP at 60 pM (saturation of specific binding sites), 20 pM ($K_d$) and 4 pM (20% of specific binding sites occupied at equilibrium) in the presence or absence of 1 μM l-propranolol, rinsed to remove nonspecifically bound radioactivity and analyzed with the emulsion-coated coverslip method of Young and Kuhar.7 Randomly selected regions of ventricular myocytes, epicardial coronary arteries, small mural arteries, and coronary arterioles (<60 μm in diameter) were photographed, grains overlying the structures of interest were counted, and the areas of the structures were digitized to determine grain density values. Specific grain density values were calculated for each structural component at each ICYP concentration by subtracting nonspecific grain densities (assessed in sections incubated with ICYP plus 1 μM l-propranolol) from total grain density measurements. At each concentration of ICYP, nonspecific binding was consistently lower in vascular than in myocyte compartments. For example, at 20 pM ICYP plus 1 μM l-propranol, the grain density of epicardial coronary arteries was only 42% of the grain density of myocytes. While not predictable a priori, it is not surprising that the extent of nonspecific binding of ICYP differs in myocytes and the coronary vasculature. Although the actual loci of nonspecific binding sites are not known, the results indicate that nonspecific bind-
FIGURE 10. Computer reconstructions of the quantitative distribution of specific binding sites in sections of canine myocardium incubated under equilibrium conditions with 4, 10, 20, and 60 pM ICYP. Each image is an average of 2 or 3 separate digitized images that were aligned, corrected for nonspecific binding, and converted to units of specific binding (fmol/mg protein).

ing of ICYP is more extensive in regions composed primarily of myocytes (higher binding affinity or capacity or both) than in the vasculature.

Table 2 compares the specific grain densities in vascular and myocyte compartments at each ICYP concentration studied. The grain densities of the vascular compartments have been expressed as proportions of the grain density of myocyte regions in each individual section to account for differences in absolute grain density measurements due to variable intervals of exposure of emulsion-coated coverslips. Over the broad concentration range of radioligand, the grain density of epicardial coronary arteries was approximately half of that of myocytes, the density of mural vessels ranged from 55–65% of that of myocytes, and the grain density of the arterioles was equivalent to the density of myocytes. These observations agree closely with the relative proportions of specific binding sites in myocytes and vascular compartments determined with film autoradiographs. The equivalent density of binding sites in coronary arterioles and adjacent myocytes demonstrated in emulsion-coated coverslip autoradiographs explained the apparent inability to resolve arterioles in the quantitative computer reconstructions of film autoradiographs.

Discussion

The purpose of this study was to quantitate and localize β-adrenergic receptors in intact transmural slices of the mammalian heart to define the distributions of β-receptors in specific myocardial and coronary vascular components. The approach developed preserves anatomic relations, thus permitting analysis of the distribution of β-receptors in specific morphologically identified components of the heart. In addition to those analyzed in the present study, such components might include the nodes and bundles of the cardiac conduction system, complex zones of ischemic injury, or regions of abnormal conduction or automaticity identified with electrophysiologic mapping. The sensitivity and resolution of the light microscopic au-
Table 2. Relative Proportions of Specific Binding Sites in Selected Vascular Compartments

<table>
<thead>
<tr>
<th>Vascular compartment</th>
<th>ICYP Concentrations</th>
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<tr>
<td></td>
<td>4 pM (n = 3)</td>
</tr>
<tr>
<td></td>
<td>20 pM (n = 3)</td>
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<tr>
<td></td>
<td>60 pM (n = 2)</td>
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<tr>
<td>Epicardial conductance coronary arteries</td>
<td>0.49 ± 0.04 (0.40)</td>
</tr>
<tr>
<td></td>
<td>0.45 ± 0.10 (0.40)</td>
</tr>
<tr>
<td></td>
<td>0.54 ± 0.10 (0.31)</td>
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<tr>
<td>Mural coronary arteries</td>
<td>0.55 ± 0.04 (0.87)</td>
</tr>
<tr>
<td></td>
<td>0.58 ± 0.06 (0.59)</td>
</tr>
<tr>
<td></td>
<td>0.65 ± 0.05 (0.49)</td>
</tr>
<tr>
<td>Coronary arterioles (&lt;60 µm diameter)</td>
<td>0.93 ± 0.27 (1.01 ± 0.17)</td>
</tr>
<tr>
<td></td>
<td>0.97 ± 0.18 (0.97 ± 0.18)</td>
</tr>
</tbody>
</table>

Values (means ± SD) express the specific grain density of each vascular compartment as a proportion of the grain density of adjacent regions composed mainly of ventricular myocytes in emulsion-coated coverslip autoradiographs. n refers to the number of animals studied at each ICYP concentration.

One to three sections of a single transmural block of left ventricle were analyzed in each animal. In each section, grains were counted in 3–5 randomly selected photographs of each vascular compartment and adjacent myocytes. Values in parentheses are proportions of mean values of specific binding in epicardial or mural vessels and specific binding in adjacent myocytes measured in film autoradiographs at each ICYP concentration. At each concentration of ICYP the specific grain density of epicardial conductance and mural coronary arteries was significantly different (p < 0.01) than the density of grains over adjacent myocytes. The specific grain density over coronary arterioles was not significantly different than myocytes.

The high specific activity of ICYP and the high ratios of specific binding enabled us to use film autoradiography and sophisticated automated quantitative analysis and computer reconstruction of digitized autoradiographic images. This technology has been widely employed in studies of metabolism and receptor distributions in the brain but has not been applied previously to myocardium, owing in part to the low concentrations of receptors in cardiac tissues.

Our results show that a gradient in β-receptor density exists in the coronary vasculature. Large epicardial conductance arteries contained a relatively low density of receptors, mural arteries had a higher density, and coronary arterioles had the highest density, which was equivalent to that observed in regions composed mainly of ventricular myocytes. The transmural distribution of β-receptors in regions composed mainly of cardiac myocytes was uniform. Although B_max varied widely, the affinity of ICYP for β-receptors was equivalent in all tissue compartments examined.

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Because the potential for nonspecific binding of radioligand is increased by the use of whole tissue compared with partially purified membranes, we considered rigorous characterization of binding and rinsing conditions and fulfillment of basic pharmacologic criteria of receptor binding to be of critical importance before attempting to localize binding sites autoradiographically. The nonsubtype selective β-adrenergic antagonist ICYP was particularly well suited for this purpose. It has high and equal affinity for both β_1- and β_2-receptors, and its slow off-rate permits extensive washing to remove nonspecifically bound radioactivity without removal of specifically bound radioligand. Thus, specific binding ratios of 90–95% at concentrations close to K_D and 80–85% at saturating ligand concentrations were readily achieved. The high specific activity of the ligand and the ability of Ultrasoft film and nuclear track emulsions to detect the low energy secondary electron emissions of [3H]-DHA resulted in rapid formation of autoradiographic latent images even though the concentration of β-receptors in intact myocardium is low.

The results of the initial phase of this study indicate that binding of ICYP to unfixed transmural sections of canine and feline left ventricle was rapid, saturable, stereoselective, and displaceable by antagonist and agonists with the rank order of potency characteristic of the β-adrenergic receptor. Furthermore, agonist dis-
effects of subtype-selective drugs on coronary vascular resistance and perfusion patterns suggest that $\beta_1$-receptors are concentrated in the microvasculature. $^4,5,7,10,32$

Furthermore, [H]-DHA appears to bind with greater affinity to membranes of tissues rich in $\beta_1$-receptors than tissues in which $\beta_2$-receptors are predominant. For example, reported values of $K_d$ for [H]-DHA in rat myocardial membranes $^25,33$ or isolated rat cardiac myocytes $^34$ (predominantly $\beta_2$-subtype) range from 4–8 nM, whereas reported values for binding to rat lung $^35,36$ or erythrocytes $^36$ (predominantly $\beta_2$-subtype) are 0.25–0.5 nM. ICYP, however, binds with equal affinity to $\beta_1$- and $\beta_2$-receptors. $^37$ Thus, the greater affinity of [H]-DHA for binding sites in arterioles observed by Muntz et al $^31$ may be due to a greater proportion of $\beta_2$-receptors in the microvasculature as opposed to myocytes.

The light microscopic methods used to quantitate and localize $\beta$-adrenergic receptors cannot resolve radioligand binding sites in individual cells or closely apposed structures. Thus, autoradiographic measurements expressed as grains per unit section area necessarily encompass structurally heterogeneous regions. This limitation has important implications. First, measurements of the density of binding sites in the compartment referred to as ventricular myocytes in the present study actually included the contributions of cardiac myocytes, capillary endothelium, and other interstitial cells. Although cardiac myocytes comprise approximately 85% of the volume of canine left ventricular myocardium, $^38$ they have modest membrane surface per unit volume (0.25–0.30 $\mu$m$^2$ sarcolemmal surface/$\mu$m$^3$ myocyte vol). $^38,39$ Interstitial cells (mainly capillary endothelium) constitute only 4% of canine myocardial tissue volume $^38$ (the remaining 12% is interstitial and intravascular space) but have a much greater surface per unit volume due to the flattened configuration of endothelium. Based on the morphometric data of Gerdes and Kasten, $^38$ we estimate that the endothelial surface to volume ratio is approximately 25 times that of myocytes. Thus, more than half of total cell surface membrane in myocardium may be derived from nonmyocytic cells, a conclusion that obviously applies to binding assays in membrane preparations as well as to autoradiography in tissue sections. The relative densities of $\beta$-receptors in cell membranes of cardiac myocytes and capillary endothelial cells are not known. Ultrastructural resolution of the distribution of receptors is required to resolve this question.

A second consequence of the limited resolution of light microscopic autoradiography concerns the relative amounts of cell surface membrane per unit area of different classes of coronary vessels. Our observations that large epicardial coronary arteries contained a lower density of $\beta$-receptors than smaller intramural arteries could be due in part to a lower proportion of cells and a greater proportion of noncellular structures (e.g., collagen and elastin fibers) per unit section area of large vs. small arteries. As shown in Table 2, however, the greatest difference in receptor density was observed between small mural arteries (60–300 $\mu$m in diameter) and arterioles (< 60 $\mu$m in diameter). It seems unlikely that disparities in cellularity or the relative proportions of cell surface membrane per unit area could account for the significant grain density difference observed. Furthermore, pharmacologic observations that coronary vessels of different caliber vary in their responses to catecholamines $^3,10$ support our findings, which suggest that the density of $\beta$-receptors on a per cell basis increases as the caliber of coronary arteries decreases. Ultrastructural morphometric analysis of the volume proportion of medial smooth muscle cells and cell surface-to-volume relations will be required, however, to resolve this issue.

Despite the limitations of resolution, quantitative light microscopic autoradiographic localization of adrenergic receptors does offer insight that cannot be obtained with other approaches. It holds considerable promise in analyses of receptor distribution and function in normal and pathologic myocardial tissue.

Acknowledgments

We wish to acknowledge the technical assistance of James R. Baker. We are grateful to Drs. Burton E. Sobel and Peter B. Corr for their advice. We thank Dr. Kenneth Schectman for statistical analysis, Emily Santori and Mariam Samae of the Laboratory of Neuro Imaging for assistance in analysis of film autoradiographs, Louise Schoelch and Margaret McHugh for photographic assistance, David Rees for technical assistance, and Susan Johnson for preparation of the typescript.

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KEY WORDS • β-adrenergic receptors • quantitative autoradiography • myocardium • [125I]iodo-cyanopindolol
Quantitative autoradiographic delineation of the distribution of beta-adrenergic receptors in canine and feline left ventricular myocardium.

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Circ Res. 1987;60:568-579
doi: 10.1161/01.RES.60.4.568

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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