Autoradiographic Characterization of β-Adrenergic Receptor Subtype in the Canine Conduction System

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It has been hypothesized, based on physiological evidence, that there is a greater proportion of β₂-adrenergic receptors on the myocytes of the conduction system when compared with the working myocardium. The purpose of these studies was to examine β-adrenergic receptor subtype in the conduction system of the dog by using the technique of coverslip autoradiography. Scintillation studies of [¹²⁵I]pindolol binding to ventricular sections demonstrated that binding was saturable (dissociation constant of 116 pM), had the correct order of potency for a β-receptor, and was stereoselective. Both betaxolol (β₃-selective) and ICI-118,551 (β₂-selective) competition curves fit a two-site model in nonlinear curve-fitting analyses (78% β₁-receptors). Autoradiographic studies determined that the myocytes of the sinoatrial node had approximately twice as many autoradiographic grains as the surrounding atrial myocytes. The myocytes of the atrioventricular bundle had a number of grains similar to the number in surrounding septal myocytes. Autoradiographic inhibition curves with betaxolol or ICI-118,551 demonstrated that both the sinoatrial node and the atrioventricular bundle had inhibition profiles similar to the surrounding myocytes (predominantly β₁) but unlike the inhibition profiles of arterioles (predominantly β₂). Calculations using the dissociation constants derived from the nonlinear curve-fitting analysis and the percent specific binding in the presence of 4x10⁻⁷ M betaxolol or ICI-118,551 determined that the proportion of β₁ to β₂-receptors was the same (70–80% β₁) when comparing the sinoatrial node and the surrounding atrial myocytes. The atrioventricular bundle had a higher percentage of β₂-receptors (95%) than the surrounding septal myocytes (79%) with betaxolol, but with ICI-118,551 the difference was not significant. Thus, by using techniques that can precisely quantify adrenergic receptors over myocytes of the conduction system, it was concluded that, whereas the myocytes of the sinoatrial node have approximately twice as many β-receptors as the surrounding atrial myocytes, the proportion of β₁ to β₂-receptors is the same. (Circulation Research 1992;71:51–57)

KEY WORDS • β-adrenergic receptors • subtype • heart • conduction system

Physiological evidence suggests that heart rate is more responsive than contractility to β-adrenergic receptor agonists.¹–⁴ Thus, it has been hypothesized that there may be a greater proportion of β₂-receptors in the conduction system than in the working myocardium. Autoradiographic data in the rat has found a greater percentage of β₂-receptors on the sinoatrial (SA) node than on the working myocardium.⁵ Additional autoradiographic analyses in the guinea pig have suggested that the atrioventricular (AV) node and bundle have a greater percentage of β₂-receptors than is found in the surrounding myocardium,⁶ though data in the rat has suggested that the AV node has a similar ratio of β₁ to β₂-receptors as the septal myocardium.⁷

The purpose of these studies was to examine the β-receptor subtype in two regions of the conduction system in the dog, a species commonly used for cardiovascular studies, and to compare subtype to working myocardium (predominately β₁) and arterioles (predominately β₂). We used the technique of coverslip autoradiography to precisely quantify grains over conduction system myocytes, thus excluding blood vessels, nerves, and connective tissue elements that are present in the conduction system.

Materials and Methods

Five mongrel dogs were anesthetized with pentobarbital sodium (30 mg/kg), and the hearts were removed. Serial blocks were removed at the junction of the superior vena cava with the right atrial appendage and in the area of the AV bundle. Additional blocks were removed from the anterior wall of the left ventricle adjacent to the interventricular septum. The blocks of tissue were mounted on cork and immediately frozen in freon cooled with liquid nitrogen.

Validation of Binding

Sections (10 μm) were cut from the left ventricular blocks and used for validation of [¹²⁵I]pindolol (Dupont/
New England Nuclear, Boston) binding to the sections. For binding, sections were preincubated in 50 mM Tris (pH 7.4), 10 mM MgCl2, 1 mM EDTA, and 10−4 M ascorbic acid for 10 minutes to remove endogenous ligand. Sections were then incubated at room temperature in the same buffer with [125I]pindolol. Nonspecific binding was determined by incubation in [125I]pindolol in the presence of 5 μM (±)-propranolol HCI (Sigma Chemical Co., St. Louis, Mo.). Preliminary studies determined that equilibrium was reached at 60 minutes; therefore, incubations were carried out for 70 minutes. Preliminary experiments also determined that optimal removal of nonspecific binding was achieved by two 20-minute ice-cold buffer washes. These washing conditions did not remove specific binding. After washing, the slides were quickly dipped in ice-cold distilled water and dried with a stream of cold air; the sections were scraped off the slide and analyzed by gamma counting.

Several studies were done to determine [125I]pindolol binding characteristics. Sections were incubated in six to eight concentrations of [125I]pindolol to determine the Bmax and the equilibrium dissociation constant. Agonist order of potency and stereoselectivity were determined by incubating sections in 120 pM [125I]pindolol in the presence of several concentrations of agonists (the bitartrate salts of l-norepinephrine, l-epinephrine, l-isoproterenol, and d-norepinephrine; Sigma).

Sections were incubated in [125I]pindolol in the presence of several concentrations of the β-selective ligand betaxolol (Laboratoires d’Etudes et de Recherches Synthelabo, Paris) and the β-selective ligand ICI-118,551 (Imperial Chemical Industries PLC, Macclesfield, England). Both the saturation curves and the drug displacement studies were analyzed using the LIGAND program of Munson and Rodbard as modified by McPherson for the personal computer (BIOSOFT).

Autoradiography

Sections were incubated in 120 pM [125I]pindolol in the presence of five concentrations (3×10−7 to 2×10−6 M) of either betaxolol or ICI-118,551, 5 μM propranolol (nonspecific binding), or without drug (total binding). The sections were incubated as described above and processed for autoradiography according to previously described methods.9−11 Coverslips were dipped in NTB2 emulsion (Kodak), diluted 1:1 with distilled water (43°C), and the coverslips were allowed to dry for 3 hours in the dark. A coverslip was glued (Superglue, Loctite) to one end of each slide that had been incubated in [125I]pindolol, and the coverslips were apposed to the tissue section using a No. 20 binder clip and a plastic spacer to protect the coverslip from breakage. The slides were exposed in boxes with Drierite for 5 days.

After exposure, the binder clip was removed, the coverslip was gently lifted, and an alligator clip was placed between the coverslip and the slide. The slides were then developed in D19 (1:1 with distilled water, Kodak), fixed, stained with hematoxylin and eosin, and mounted with Permount.

The autoradiographic grains were quantified with a Nikon Magiscan according to previously described methods.11 For features that did not fill the video screen, the instrument allowed an outline of the feature to be analyzed and then determined the area of the feature, as well as the number of grains over that feature. Ten fields were counted from the following tissues: sinoatrial myocytes, atrial myocytes, atrial arterioles, myocytes of the AV bundle, septal myocytes, and septal arterioles. The fields were selected randomly using a low-power objective in which the grains could not be visualized. Because there are few arterioles in the sections, arterioles oriented in any way were used. Only vessels less than 90 μm in diameter and in which a lumen could be visualized were analyzed.

The SA node and the AV bundle were identified in the serial blocks using a histochemical stain for acetylcholinesterase.12 Serial sections were taken from both these regions, and every fifth section was stained for acetylcholinesterase. The serial sections were consecutively numbered, so that the autoradiographs could be compared with the acetylcholinesterase-labeled sections preceding and following the autoradiograph. This facilitated the positive identification of the SA node and the AV bundle. In addition, the SA node could be identified by smaller myocytes and the presence of the SA nodal artery present in the center of the node. The AV bundle was also identified by its position adjacent to the point where the membranous interventricular septum becomes the muscular septum, before it branches into the right and left bundle branches.

After quantitation, the grains were normalized to a standard area (10−2 mm2), and total and nonspecific grains were calculated. Nonspecific grain densities were subtracted from grain densities obtained from total, betaxolol, and ICI-118,551 slides; the percent inhibition of specific binding with each concentration of β-selective drug was calculated; and inhibition curves were constructed.

Data collected from one concentration (4×10−7 M) of either betaxolol or ICI-118,551 were analyzed using the equation of Neve et al13 as applied by Murphree and Saffitz14 in autoradiographic studies. These analyses are more thoroughly described in the “Results” section.

Statistics

Total and nonspecific grain densities over different regions of either the atrium or the septum were compared using a one-way analysis of variance followed by a Tukey’s multiple comparison test. Because the atrial samples and the septal samples were set up at different times, we did not compare grain densities between the atrial and septal sections.

The percent specific binding with either betaxolol or ICI-118,551 was compared among six regions analyzed using a one-way analysis of variance. Since the percent specific binding was independent of the variability in grain density from experiment to experiment, this was a valid analysis. All data are expressed as mean±SEM. The equilibrium dissociation constants are expressed as the geometric mean±SEM.

Results

Validation studies of tissue sections determined that binding was saturable (Figure 1) with an equilibrium dissociation constant of 116 pM (−9.936±0.014 log M, n=3). In addition, binding had the order of potency characteristic of a β-adrenergic receptor and demonstrated stereoselectivity (Figure 2). The results of computer competition analyses with betaxolol using scintil-
were

dissociation constant of 120 pM and a $B_{\text{max}}$ of 35.9 fmol/mg protein were obtained.

The binding of betaxolol fit a two-site model ($p<0.0001$) with an equilibrium dissociation constant of $1.77 \times 10^{-8}$ M ($-7.753 \pm 0.072$ log M, $n=3$) for the high-affinity site ($\beta_1$) and $9.51 \times 10^{-7}$ ($-6.022 \pm 0.184$ log M) for the low-affinity site ($\beta_2$). In the ventricular sections, there were $78 \pm 3\%$ $\beta_2$-adrenergic receptors as estimated by the LIGAND program. Similar analyses were applied to the competition curves of ICI-118,551 performed on canine ventricular sections (Figure 3B) and indicated that the binding of ICI-118,551 fit a two-site model ($p<0.0001$) with an equilibrium dissociation constant of $5.08 \times 10^{-8}$ ($-8.294 \pm 0.546$ log M, $n=3$) for the high-affinity site ($\beta_1$) and $4.26 \times 10^{-7}$ ($-6.371 \pm 0.044$ log M) for the low-affinity site ($\beta_2$). In addition, simultaneous analysis of saturation curves and competition curves as described by Unnerstall$^{15}$ determined that [125I]pindolol did not show significant selectivity for $\beta_2$-receptors.

Analysis of the autoradiographs determined that the myocytes of the SA node had approximately twice as many total grains as the atrial myocytes or the atrial arterioles (Figures 4 and 5). In contrast, the myocytes of the AV bundle had a similar number of total grains as the myocytes of the interventricular septum and the septal arterioles. Nonspecific binding was low, and there were no significant differences among the three regions in either the atrium or the septum in nonspecific binding. Although there was not enough tissue in either the left or the right bundle branch present for adequate quantification, qualitatively these regions appeared to have a grain density similar to that of the AV bundle and the septal myocytes.

Examination of the inhibition curves derived from autoradiographs determined that the myocytes of the SA node and the atrial myocytes had similar profiles for either betaxolol or ICI-118,551, whereas atrial arterioles had a different profile for both drugs (Figure 6). Betaxolol was less potent for arterioles, whereas ICI-118,551 was more potent for arterioles. These profiles are characteristic of a predominance of $\beta_2$-receptors. Similar profiles were observed in the septum, with the myocytes of the AV bundle and the septal myocytes demonstrating $\beta_1$ profiles and the septal arterioles exhibiting $\beta_2$ profiles (Figure 7).

To calculate the percentage of $\beta_1$- and $\beta_2$-receptors from the autoradiographs, the results of the LIGAND analyses of betaxolol were fit to the following equation:

$$B = \frac{B_{\text{max1}} \times L}{L + K_{d1} \times (1 + 1/K_{il})} + \frac{B_{\text{max2}} \times L}{L + K_{d2} \times (1 + 1/K_{i2})}$$

(1)
where B is the amount of radioligand specifically bound, $B_{\text{max1}}$ and $B_{\text{max2}}$ are the densities of the $\beta_1$- and $\beta_2$-receptors, respectively, L is the concentration of radioligand used in the incubations (120 pM), I is the concentration of competing ligand ($4 \times 10^{-7}$ M), $K_{d1}$ and $K_{d2}$ are the equilibrium dissociation constants of the radioligand for the two sites (116 pM), and $K_{i1}$ and $K_{i2}$ are the equilibrium dissociation constants of the binding sites for the competing ligands. It should be noted that the dissociation constant of $[^{125}I]$pindolol used in these equations was the dissociation constant that was derived experimentally and not the dissociation constant from previously published calculations.

When no competing ligand was present, the following equation (where X equals $B_{\text{max1}}$ and Y equals $B_{\text{max2}}$) was obtained based on our data:

$$B = 0.508X + 0.508Y$$  \(2\)

When $4 \times 10^{-7}$ M betaxolol was present in the incubation, the following equation was obtained:

$$B = 0.042X + 0.421Y$$  \(3\)

When $4 \times 10^{-7}$ M ICI-118,551 was present in the incubation, the following equation was obtained:

$$B = 0.348X + 0.013Y$$  \(4\)
FIGURE 6. Graphs demonstrating the inhibition of specific \[^{125}\text{I}]\text{pindolol binding by several concentrations of either betaxolol (}\beta_{1}\text{-selective)} or ICI-118,551 (}\beta_{2}\text{-selective)} over the sinoatrial (SA) node, atrial myocytes, and atrial arterioles. Betaxolol has an equal affinity for the SA node and the atrial myocytes, whereas it has a lower affinity for the arterioles. ICI-118,551 has an equal affinity for the SA node and the atrial myocytes but a greater affinity for the arterioles; \(n=5\) dogs.

The autoradiographic data (specific binding) in the absence of betaxolol or ICI-118,551 (100%) and in the presence of \(4 \times 10^{-7}\text{M betaxolol or ICI-118,551 (percent specific binding)}\) was inserted as B in the appropriate equation. The equation calculated without drug (Equation 2) was solved simultaneously with either the betaxolol equation (Equation 3) or the ICI-118,551 equation (Equation 4) using the methods of Murphree and Saffitz,\(^4\) and the percentage of \(\beta_{1}\text{- and }\beta_{2}\text{-receptor subtypes was calculated (Table 1). These data indicated that with betaxolol the myocytes of the SA node, atrial myocytes, and septal myocytes had approximately 80% }\beta_{1}\text{-receptors and that the myocytes of the AV bundle had a somewhat greater percentage of }\beta_{1}\text{-receptors (95%). Arterioles, on the other hand, had predominately }\beta_{2}\text{-receptors.}

Similar results were obtained with ICI-118,551, although in most cases the percentage of }\beta_{1}\text{-receptors was slightly lower than that obtained with betaxolol (Table 1). The exception was the percentage of }\beta_{2}\text{-receptor subtypes in the atrial arterioles, and this may be related to the fact that the variation in the specific binding to arterioles was high with betaxolol. With ICI-118,551 there was no significant difference in the percent specific binding between septal myocytes and myocytes of the AV bundle.}

**Discussion**

Pharmacological experiments carried out in vivo have led to the conclusion that }\beta_{1}\text{-adrenergic receptors are present on cardiac myocytes and, in some cases, can mediate the positive chronotropic effects of catecholamines. In one study, }\beta_{2}\text{-agonists potentiated more pronounced chronotropic than inotropic responses, whereas a }\beta_{1}\text{-selective agonist produced the same degree of inotropic and chronotropic stimulation.}^1\) These workers hypothesized that the SA node had a higher ratio of }\beta_{1}\text{- to }\beta_{2}\text{-receptors than the working myocardium. Our data suggested that in the dog, although }\beta_{1}\text{-receptor number on the SA node was twice that of the surrounding atrial myocytes, the ratio of }\beta_{1}\text{- to }\beta_{2}\text{-receptors was the same. A previous autoradiographic study in rats suggested that, in addition to increased }\beta_{1}\text{-receptor number on the SA node, the percentage of }\beta_{2}\text{-receptors was greater.}^4\) There could be species differences, but an additional explanation for the discrepancy is the fact that, in the study by Saito et al,\(^4\) the technique of film autoradiography was used. With this technique it is not possible to exclude large nerves, blood vessels, or connective tissue present in the SA node. The SA node has more connective and nervous tissue than the working myocardium. Both fibroblasts\(^1,6\) and cardiac nerves\(^5\) have predominately }\beta_{2}\text{-adrenergic receptors. By using image analysis of coverslip autoradiography, it is possible to exclude connective tissue and large nerves from the grain counting so that myocytes of the SA node can be analyzed separately. Not only were two }\beta_{1}\text{-receptor blocking agents used, but five concentrations of each blocking agent were used in the autoradiographic studies, thus obtaining competition curves that clearly indicated that the inhibition profiles of the myocytes of the SA node were similar to those of the atrial myocytes and not the atrial arterioles. Calculations of the percentage of }\beta_{1}\text{-receptors using coefficients derived from nonlinear curve-fitting analyses also supported the conclusion that myocytes of the SA node and the atrial myocytes had similar proportions of }\beta_{1}\text{-receptors.}

Nonlinear curve-fitting analyses of canine ventricular sections yielded a dissociation constant for \[^{125}\text{I}]\text{pindolol of 116 pM. This is very close to that obtained with }^{[125]}\text{I]pindolol in canine atrium (120 pM). The rationale for using }^{[125]}\text{I]pindolol instead of }^{[2]}\text{I]cyanopindolol was that }^{[125]}\text{I]pindolol had a lower nonspecific binding (-5% at the dissociation constant) than }^{[125]}\text{I]cyanopindolol (20%) in canine heart sections (author's unpublished observations).}

In doing simultaneous nonlinear ligand analyses of Scatchard plots with competition curves of either betaxolol or ICI-118,551, I did not find that \[^{125}\text{I}]\text{pindolol was selective for }\beta_{1}\text{-receptors. Neve et al,}^1\text{ in more extensive studies, have reported that }^{[125]}\text{I]pindolol was more selective for }\beta_{2}\text{-receptors. If this is the case, I would be underestimating the number of }\beta_{1}\text{-receptors at the concentrations of ligand used in our studies. However, the present data are in line with other studies using}
radioligand binding to membranes and tissue sections in which the atrial2,5,17,18 and septal5 myocytes have approximately 70–80% β2-receptors. Regardless, a slight selectivity of [125I]pindolol for β2-receptors would in no way alter the conclusion that the relative proportions of β1 to β2-receptors are the same in the myocytes of the SA node and the atrial myocytes. Because [125I]pindolol was not selective for β2-receptors in my system, I elected to use the dissociation constant derived with my methods (116 pM for both β1- and β2-receptors) rather than try to estimate constants from the data of Neve et al13 in another system.

It has been reported that the AV bundle and the AV node in the guinea pig have a higher proportion of β2-receptors than the myocardiun of the interventricular septum, whereas the rat AV node has proportions of β2-receptors similar to those found in interventricular septal myocytes. In our study, the AV bundle of the dog did not have a greater proportion of β2-receptors when compared with the interventricular septum. Indeed, there was evidence for an increased proportion of β2-receptors in the AV bundle, at least with betaxolol. Variability was greater with ICI-118,551 in the AV bundle and septal myocytes, which may explain why a significant difference was not detected with this ligand.

Since a higher proportion of β2-receptors was not found in the SA node when compared with the atrial myocytes, how does one explain the increased sensitivity of heart rate to β2-agonists when compared with contractility? It has been shown in canine atrium with radioligand methods that approximately 25% of the β2-receptors are of the β2-receptor subtype. However, 50% of the shortening of the action potential duration is due to β2-receptors, suggesting that β2-receptors may be more tightly coupled to physiological responsiveness. These authors ruled out a role for presynaptic β2-receptors by depleting catecholamines in some animals. Presynaptic β2-receptors are thought to facilitate cate-

### Table 1. Percent specific [125I]Pindolol Binding and Percentage of β1- and β2-Receptors in Several Regions of Canine Heart

<table>
<thead>
<tr>
<th></th>
<th>Specific [125I]pindolol binding (%)</th>
<th>X (%)</th>
<th>Y (%)</th>
<th>β1 (%)</th>
<th>β2 (%)</th>
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<td>Betaxolol</td>
<td></td>
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<td>22.46±4.56</td>
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<td>37</td>
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<td>87.78</td>
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<td>SA node myocytes</td>
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<td>39</td>
<td>80.03</td>
<td>19.97</td>
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<tr>
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<td>156</td>
<td>40</td>
<td>79.49</td>
<td>20.51</td>
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<tr>
<td>Septal arterioles</td>
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<td>49</td>
<td>148</td>
<td>24.78</td>
<td>75.22</td>
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<td>AV myocytes</td>
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<td>10</td>
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<tr>
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<td>157</td>
<td>40</td>
<td>79.91</td>
<td>20.09</td>
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SA, sinoatrial; AV, atrioventricular. Values are mean±SEM for specific [125I]pindolol binding; n=5 dogs. X, Y, and percentage of β1- and β2-receptors were calculated using methods described in the text.

* p<0.001 vs. myocytes.
† p<0.05 vs. other myocytes and arterioles.

### References


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