Characterization of $\beta_1$- and $\beta_2$-Adrenoceptor Subtypes in the Rat Atrioventricular Node by Quantitative Autoradiography

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We characterized $\beta$-adrenoceptor subtypes in the atrioventricular node of the rat heart by quantitative autoradiography. Consecutive 16-μm-thick sections from single rat hearts containing the atrioventricular node were incubated with increasing concentrations of $^{[125]}$Iiodocyanopindolol. After exposure to $[^{3}H]$Ultrofilm, optical densities corresponding to the atrioventricular node were determined by computerized densitometry after comparison with $[^{125}]$Istandards. The computer program LIGAND was used for analysis of receptor subtypes. Delineation of $\beta$-adrenoceptor subtypes was achieved by incubating consecutive tissue sections with 50 pM $[^{125}]$Iiodocyanopindolol in the presence of increasing concentrations of the $\beta_1$-selective antagonist atenolol or the $\beta_2$-selective antagonist ICI 118,551. The atrioventricular node contains a higher concentration of $\beta$-adrenoceptors than the adjacent interventricular septum. We estimated that the proportions of $\beta_1$- and $\beta_2$-adrenoceptors in the atrioventricular node were about 56% and 44% of the total binding capacity respectively. (Circulation Research 1988;62:173-177)

Catecholamines modulate the rate and force of cardiac contraction through $\alpha$- and $\beta$-adrenoceptor stimulation. Autonomic nerves innervate the heart where the densest innervation is found in the sinus and atrioventricular nodes, which are part of a specialized conduction system.1 Autonomic nervous system activity modulates atrioventricular conduction with stimulation of the sympathetic nerves producing effects similar to those of exogenously administered catecholamines.1

Pharmacologic evidence is consistent with the presence of $\beta$-adrenoceptors in the atrioventricular node. Sympathetic stimulation reduces the conduction time through the atrioventricular junction, an effect that is opposite to that produced by the $\beta$-adrenoceptor blockers, which are useful in reducing the ventricular responses to rapid atrial rhythms.1,2

The question has been raised of whether different $\beta$-adrenoceptor subtypes mediate inotropic and chronotropic effects in the heart. $\beta_1$- and $\beta_2$-Adrenoceptor subtypes have been identified in rat, guinea pig, cat, and human atria.3-5 Selective $\beta_2$-adrenoceptor agonists have greater effects on heart rate than on contractility.6 These differences in the effects of drugs on chronotropic and inotropic properties of the heart suggest that the cardiac conduction system may have a higher proportion of $\beta_2$-adrenoceptors than does the ventricular myocardium.

To better understand the cardiovascular effects of drugs acting at heart $\beta$-adrenoceptors, we decided to determine the possible presence and relative concentrations of $\beta$-adrenoceptor subtypes in the atrioventricular node of the rat heart and in the interventricular septum, which has contractile myocytes. The rat heart conduction system cannot be easily isolated by dissection, and it is difficult to obtain sufficient amounts of material for accurate in vitro membrane ligand studies. On the other hand, autoradiographic techniques are well-suited to such a study since they offer advantages with regard to both sensitivity and anatomical localization. We therefore employed quantitative autoradiography with a $[^{125}]$Iligand and comparison to $[^{125}]$Istandards to determine the density, affinity and subtypes of $\beta$-adrenoceptors in individual atrioventricular nodes and interventricular septa from rat heart.

Materials and Methods

Animals and Tissue Preparation

We used 6-week-old male Wistar rats from Zivic Miller, Allison Park, Penn. The animals were kept under normal laboratory conditions with access to water and rat chow ad libitum, and with lights on in the room from 0600 to 1800 hours. Animals were killed by decapitation between 0900 and 1100 hours, and the hearts, including the proximal portion of the major vessels, were immediately removed and placed while still beating in 3 mM HEPES buffer, pH 7.4, containing (in mM) NaCl 140, KCl 5, MgCl$_2$ 1, CaCl$_2$ 1.5, and glucose 11 at room temperature. After washing in

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HEPES buffer for about 10 seconds, the area containing the atrioventricular node was dissected as follows: a horizontal cut was made to separate the atria and the proximal end of both ventricles from the rest of the heart. The appendage of the right atrium was removed, and the wall of the right ventricle was cut open to expose the area containing the coronary sinus, the area of the tricuspid valve, and a portion of the interauricular and upper interventricular septa. The area containing the atrioventricular node, located between the coronary sinus and the aorta, was removed under a dissecting microscope, frozen by immersion in isopentane at -30°C, and stored not longer than 1 week at -70°C. Frozen, 16-μm-thick sections were cut in a cryostat at -14°C. Sections containing the atrioventricular node were identified by staining alternate sections with Toluidine blue and examined under a dissecting microscope. Alternate, unstained sections containing the atrioventricular node were thaw-mounted onto gelatin-coated glass slides placed under vacuum at 4°C and stored no longer than 24 hours until incubation.

**Section Staining**

To detect acetylcholinesterase activity, alternate sections containing the atrioventricular node were stained with 65 mM sodium hydrogen maleate buffer containing 2 mM acetylthiocholine iodide, 5 mM sodium citrate, 3 mM copper sulfate, and 0.5 mM potassium ferricyanide.

**In Vitro Autoradiography**

Alternate unstained sections on glass slides were preincubated for 15 minutes at room temperature in 170 mM Tris HCl buffer, pH 7.6, containing 10 mM phenylmethylsulfonyl fluoride, 0.5 mM MgCl₂, and 0.01% ascorbate. β-Adrenoceptors were labeled in vitro by incubation for 150 minutes at room temperature in fresh buffer containing [¹²⁵I]iodocyanopindolol (specific activity 2,050 Ci/mmol, Amersham Corporation, Arlington Heights, Ill.).

Characterization of β-adrenoceptors was performed by incubation of consecutive sections from single hearts with [¹²⁵I]iodocyanopindolol in concentrations ranging from 12 to 560 pM. Nonspecific binding was determined by incubation of alternate sections under the same conditions with addition of 1 μM (—)-propranolol (ICI, Macclesfield, England). β-Adrenoceptor subtypes were delineated by incubating consecutive tissue sections with 50 pM [¹²⁵I]iodocyanopindolol in the presence of increasing concentrations of the β₁-specific antagonist atenolol and the β₂-specific antagonist ICI 118,551 (ICI, Macclesfield, England).

Following incubation, the sections were rinsed in 170 mM Tris HCl buffer, pH 7.6, followed by two washes of 15 minutes each in the same buffer and a 30-second rinse in cold distilled water. The sections were then dried under a cold stream of air, placed in x-ray cassettes together with [³H]standards, and exposed to [³H]Ultratfilm (LKB Industries, Rockville, Md.) for 1 or 2 days. The films were developed with undiluted D19 developer (Eastman Kodak Co., Rochester, N.Y.).

**Data Analysis**

Optical densities were calculated by computerized microdensitometry. Binding data were calculated by comparison with [¹²⁵I]standards. Scatchard plots of [¹²⁵I]iodocyanopindolol binding at 7 concentrations (12 to 560 pM) were analyzed with the LIGAND computer program to determine the density of binding sites and the affinity of the receptor for the radioligand. The proportion of β₁- and β₂-adrenoceptors was estimated from experiments in which the ability of various concentrations of atenolol (a β₁-selective antagonist) and ICI 118,551 (a β₂-selective antagonist) to inhibit the binding of [¹²⁵I]iodocyanopindolol were compared. The competition curves were analyzed and pseudo Hill numbers (mean ± SEM) were obtained with the program ALLFIT. The dissociation constants (Kᵦ) of the ligand for the receptor subtypes and the relative proportions of the capacities were analyzed by nonlinear regression analysis using models of varying complexity. Data were pooled from multiple experiments and analyzed simultaneously using the LIGAND program.

**Results**

The atrioventricular node was localized to the border between the interatrial and the interventricular septa and stained heavily for acetylcholinesterase (Figure 1A). We detected a high concentration of [¹²⁵I]iodocyanopindolol binding sites throughout the node (Figure 1B) with lower concentrations in the interauricular and interventricular septa. The nonspecific binding accounted for less than 20% of the total binding (Figure 1C).

Our results demonstrated a single population of saturable, high affinity binding sites (Figure 2, upper panel) with average maximum binding capacities (B_max) for three separate experiments of 113 ± 22 and 79 ± 16 fmol/mg protein for the atrioventricular node and the interventricular septum, respectively. Average dissociation constants (Kᵦ) for three separate experiments were 37 ± 4 and 62 ± 8 pM for the atrioventricular node and the interventricular septum, respectively.

Competition curves with atenolol and ICI 118,551 (Figure 2, lower panel) gave pseudo Hill numbers lower than 1 (Table 1), suggesting the presence of more than one class of β-adrenoceptors. A nonlinear regression analysis of the competition curves and the [¹²⁵I]iodocyanopindolol saturation curve showed that a two-site model fit the data significantly better than a one-site model (p<0.001) for both the atrioventricular node and the interventricular septum. In the atrioventricular node, atenolol bound to a high affinity site with a Kᵦ of 528 nM (β₁-adrenoceptors) and a low affinity site with a Kᵦ of 11,400 nM (β₂-adrenoceptors). On the other hand, ICI 118,551 identified a high affinity site with a Kᵦ of 5.2 nM (β₁-adrenoceptors) and a low affinity site with a Kᵦ of 204 nM (β₂-adrenoceptors) (Table 1). The number of β₁- and β₂-adrenoceptor...
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FIGURE 1. Autoradiographic localization of β-adrenoceptors in the rat atrioventricular node. a) Acetylcholinesterase staining of the atrioventricular node and interseptal area of the rat heart. b) Total [125I]iodocyanopindolol binding. The section was incubated in the presence of 300 pM [125I]iodocyanopindolol as described in “Materials and Methods.” c) Nonspecific binding. The section was incubated as in b) with the addition of 1 μM (-)-propranolol. IAS, interatrial septum; IVS, interventricular septum; M, mitral valve leaflet; T, tricuspid valve leaflet; and A-V, atrioventricular node.

FIGURE 2. Specific [125I]iodocyanopindolol binding to atrioventricular node sections from the rat heart. Upper panel, left side: Saturation curves; right side: Scatchard analysis. Sections were incubated with increasing concentrations of [125I]iodocyanopindolol with and without 10 μM (-)-propranolol, as described in “Materials and Methods.” The figure represents a typical example, which was replicated three times. In this example, Bmax was 156 and 106 fmol/mg protein and Kd was 37 and 80 pM for A-V node and interventricular septum, respectively. o o, A-V node; O O, interventricular septum. Lower panel: Inhibition of [125I]iodocyanopindolol binding by selective β1- and β2-adrenoceptor antagonists. Left panel: Atrioventricular node; right panel: interventricular septum. Sections were incubated in the presence of 50 pM [125I]iodocyanopindolol and increasing concentrations of the antagonists. Each point is the mean of 3 experiments. • •, Atenolol; O O, ICI 118,551.

binding sites was about 56% and 44%, respectively, of the total binding capacity in both the atrioventricular node and the interventricular septum (Table 1).

Discussion
The coexistence of β1- and β2-adrenoceptors in mammalian heart has been demonstrated with radioligand binding studies using partially purified cardiac membranes.4,5,12-14 In man, both β1- and β2-adrenoceptors are coupled to adenylate cyclase,12 indicating a possible functional role for both subtypes. The highly selective β1-antagonist ICI 118,551 is more potent in inhibiting isoprenaline-induced tachycardia than the β2-selective antagonist atenolol,13 thus suggesting the possibility that β2-adrenoceptors could play a more important role in the regulation of atrioventricular conduction than β1-receptors in man.

Our results offer the first direct demonstration and characterization of β-adrenoceptor subtypes specifically located in discrete areas of the mammalian conduction system. These results indicate that the rat atrioventricular node contains high concentrations of β-adrenoceptors, coincident with very high acetylcho-
linesterase staining, and that the relative concentrations of both subtypes are approximately equal, with only a slight predominance of the \( \beta_1 \)-type. The affinity constants calculated by autoradiography are similar to that of \( \beta \)-adrenoceptors in human right atria calculated by membrane binding techniques and to those of \( \beta \)-adrenoceptors in rat kidney and dog splenic artery, calculated by autoradiographic techniques.

The present results do not allow the determination of the specific cellular localization of all the \( \beta \)-adrenoceptors. These sites could be predominantly localized postsynaptically on the specialized cells belonging to the conduction system. The possibility remains, however, that at least part of the \( \beta \)-adrenoceptors could be present presynaptically in the sympathetic nerve terminals innervating the atrioventricular node. In addition, some \( \beta \)-adrenoceptor sites could be located in blood vessels or connective tissue within the area.

We have reported \( \beta \)-adrenoceptor subtype characterization in the atrioventricular node of the rat; available tissue from the normal pacemaker, the sinoatral node, was insufficient for a complete characterization. Preliminary data, however, indicate that the rat sinoatral node contains a large concentration of \( \beta \)-adrenoceptors (data not shown).

Our results, together with prior physiologic data indicate that the conduction system of the heart could be under both \( \beta_1 \) and \( \beta_2 \)-adrenoceptor control. \( \beta_1 \)-Adrenoceptors have been proposed to preferentially mediate responses to neuronally released norepinephrine, whereas \( \beta_2 \)-adrenoceptors have been proposed to mediate responses to circulating epinephrine, a hormone released from the adrenal medulla into the blood. Thus, the activity of the conduction system of the heart could be selectively modified by imbalances in the autonomic nervous system, resulting in alterations of peripheral sympathetic discharge, or by humoral responses represented by changes in blood catecholamines in conditions such as stress.

The use of quantitative autoradiographic techniques will allow experiments to be conducted in convenient laboratory animal models such as the rat, permitting further elucidation of the physiologic role of \( \beta \)-adrenoceptor subtypes and their participation in atrioventricular block, heart failure, and other pathophysiologic conditions.

### Table 1. Competition for (−)-\([^{125}I]\)Iodocyanopindolol Binding by Subtype-Selective \( \beta \)-Adrenoceptor Antagonists

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Displacer</th>
<th>( K_{\text{dl}} ) nM</th>
<th>( K_{\text{eq}} ) nM</th>
<th>( B_{\text{max}} 1 )</th>
<th>( B_{\text{max}} 2 )</th>
<th>( % ) Total</th>
<th>( \text{slope pseudo } n\text{H}^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-V node</td>
<td>ICI 118,551</td>
<td>5.2</td>
<td>204</td>
<td>45</td>
<td>55</td>
<td>0.56 ± 0.03</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Atenolol</td>
<td>528</td>
<td>11,400</td>
<td>57</td>
<td>43</td>
<td>0.61 ± 0.07</td>
<td>0.63 ± 0.07</td>
</tr>
<tr>
<td>IVS</td>
<td>ICI 118,551</td>
<td>8</td>
<td>322</td>
<td>45</td>
<td>55</td>
<td>0.53 ± 0.02</td>
<td>0.66 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>Atenolol</td>
<td>245</td>
<td>18,200</td>
<td>56</td>
<td>44</td>
<td></td>
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</tr>
</tbody>
</table>

Values are means of groups of three rats, assayed individually.

\( K_{\text{dl}} \): High affinity constant; \( K_{\text{eq}} \): low affinity constant; \( B_{\text{max}} 1 \): binding capacity for the high affinity sites; \( B_{\text{max}} 2 \): binding capacity for the low affinity sites; A-V node, atrioventricular node; and IVS, interventricular septum.

*Pseudo \( n\text{H} \): pseudo Hill number (means ± SEM).

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