Characteristics of Adrenoceptors and \[^{3}H\]Nitrendipine Receptors of Porcine Vascular Smooth Muscle: Differences Between Coronary Artery and Aorta

Junji Nishimura, Hideo Kanaide, and Motoomi Nakamura

Characteristics of the bindings of \[^{3}H\](\(-\)dihydroalprenolol, \[^{125}I\](-)iodocyanopindolol, \[^{3}H\]prazosin, \[^{3}H\]yohimbine, and \[^{3}H\]nitrendipine to porcine coronary membranes were investigated and the results compared with studies of porcine aortic membranes. In the equilibrium binding study carried out in sarcolemma-enriched fractions, there were no major differences in the \(K_a\) values of these radioligands between coronary artery and aorta. However, the densities of \(\beta_1\), \(\alpha_1\), and \(\alpha_2\)-adrenoceptors and \[^{3}H\]nitrendipine receptors of coronary artery were 258, 12, 12, and 561 fmol/mg protein, respectively, while those of aorta were 37, 525, 1,000, and 215 fmol/mg protein. \(\beta\)-Adrenergic agonists competed with \[^{3}H\](\(-\)dihydroalprenolol binding sites in coronary artery, the order of potency being \((-\)isoproterenol > \((-\)norepinephrine > \((-\)epinephrine > \((+)\)isoproterenol. In case of aorta, the order was \((-\)isoproterenol > \((-\)epinephrine > \((-\)norepinephrine. The competition by \((\pm\)bispromol (\(\beta_1\)-selective antagonist) and ICI 118,551 (\(\beta_2\)-selective antagonist) for \[^{125}I\](-)iodocyanopindolol binding sites in coronary artery resulted in nonlinear Hofstee plots (\(\beta_1\); \(B_2 = 90\%\; 10\%\)). In case of aorta, linear Hofstee plots were obtained. From these results, we conclude that (1) coronary \(\beta\)-receptors in pigs are predominately of \(\beta_1\)-type, while those of aorta are of \(\beta_2\)-type; (2) regarding the relative population of adrenoceptors, coronary artery is \(\beta\)-dominant (\(\beta/\alpha = 11\)), while aorta is \(\alpha\)-dominant (\(\beta/\alpha = 0.02\)); (3) compared with \(\alpha\)-adrenoceptors, coronary artery has a greater number of \[^{3}H\]nitrendipine binding sites (nitrendipine/\(\alpha\)-adrenoceptor = 23) than aorta (nitrendipine/\(\alpha\)-adrenoceptor = 0.14). (Circulation Research 1987;60:6:837–844)

Numerous physiologic and pharmacologic experiments have indicated that the responses of coronary artery to various vasoactive substances are often different from those of other blood vessels. The contraction of canine coronary artery induced by stimulation of the respective sympathetic fibers is relatively weak compared with the extent of contraction of similar segments of the arterial tree. In experiments on tension development of coronary strips, the significant relaxation to norepinephrine (NE) and epinephrine (EP) has been observed in porcine or bovine coronary arteries. In contrast with the weak contraction induced by NE, a relatively strong contraction of porcine or bovine coronary artery can be induced by histamine, serotonin, acetylcholine, or excess KCl. Furthermore, it has been reported that canine coronary artery is much more sensitive to organic calcium antagonists with regard to inhibition of contraction induced by NE than is rat aorta.

Although these differences have been attributed to the corresponding variations in the kinds and numbers of respective receptors in vascular smooth muscle cells of coronary artery, direct evidence has not been documented. In the present study, using recently developed radioligand binding techniques, the affinities and densities of \[^{3}H\](\(-\)dihydroalprenolol, \[^{125}I\](-)iodocyanopindolol, \[^{3}H\]prazosin, \[^{3}H\]yohimbine, and \[^{3}H\]nitrendipine binding sites of porcine coronary membranes were investigated and ratios compared with studies of porcine aortic membranes.

Materials and Methods
Preparation of Microsomal and Sarcolemma-Enriched Fractions

For each preparation, 20 pig hearts or 5–6 thoracic aortas were obtained from a local slaughterhouse immediately after the animals had been killed and transported to our laboratory in ice-cold buffer of 0.25 M sucrose, 10 mM morpholinopropanesulfonic acid (MOPS), and 0.05% bovine serum albumin (pH 7.4). The left and right epicardial coronary arteries were dissected from the hearts and aortas and opened longitudinally. The intima layers were scraped off, and the media layers were stripped mechanically from the adventitia. As shown in Figure 1A, the microsomal and sarcolemma-enriched fractions were prepared using the methods described by Kwan et al with minor
Binding Assays

The activity of 5'-nucleotidase was assayed according to the method of Wibo et al.13 K+Stimulated ouabain-sensitive p-nitrophenylphosphatase (K+-pNPPase) activity was measured by the method of Bers.16 Rotenone-insensitive NADH cytochrome C reductase was assayed according to Kwan et al.12 Cytochrome C oxidase was assayed according to Cooperstein and Lazarow.17 Protein was determined according to Markwell et al.18 using bovine serum albumin as the standard.

Binding Assays

[3H]Prazosin binding was used as reported.19 [3H](-)Dihydroalprenolol ([3H]DHA), [3H][(-)iodocyanopindolol ([3H]ICYP), and [3H]yohimbine binding was carried out as follows. The membrane samples were incubated with [3H]DHA, [3H]ICYP, or [3H]yohimbine at 25°C for 30 minutes in 50 mM Tris buffer (pH 7.4) containing 10 mM MgCl2 and 100 mM NaCl in the case of [3H]yohimbine binding in a total volume of 1 ml, 0.25 ml, or 0.5 ml, respectively. Binding was terminated by adding 5 ml of ice-cold buffer to the samples, which were then filtered onto Whatman GF/C glass fiber filters with 3 washes of 5 ml ice-cold buffer. Specific binding was defined as binding that was displaceable by 20 μM (—)isoproterenol (IP) for [3H]DHA, 1 μM (—)propranolol for [3H]ICYP, or 100 μM (—)norepinephrine for [3H]yohimbine binding, respectively.

In the case of [3H]nitrendipine binding, membrane preparations were incubated with [3H]nitrendipine in a total volume of 1.2 ml of 50 mM Tris buffer (pH 7.4) at 25°C for 30 minutes, with or without 1 μM unlabelled nitrendipine, to determine nonspecific or total binding, respectively. Binding was terminated by rapid vacuum filtration of 1.0 ml medium onto Whatman GF/B glass fiber filters with 4 washes of 5 ml ice-cold buffer. Labelled and unlabelled nitrendipine was protected from light during use and storage. Liquid scintillation spectrometry (Aloka LSC 900, Tokyo, Japan) and gamma counter (Aloka ARC 361, Tokyo, Japan) were used for [3H]ligands and [125I]CYP counting, respectively. All binding assays were carried out in duplicate or triplicate.

Data Analysis

The dissociation constant (Kd) and maximum binding (Bmax) of radioligand binding were determined from Scatchard analysis.20 Kinetic analysis was performed as described by Williams et al.21 Analysis of competition curves by β-adrenergic antagonists with [3H]ICYP binding sites was performed by the methods of Minneman et al.22 The inhibition constant (Ki) was determined from the formula of Cheng and Prusoff,23 K = EC50/(1 + A/Kd), where A = radioligand concentration, Kd = dissociation constant, and EC50 = concentration of competitive ligand that inhibits radioligand binding by 50%. EC50 was determined from the slopes of the Holfstee plots. Data are expressed in mean ± SEM from number of preparations. Group mean values were compared using two-tailed t test.

Chemicals

[3H](-)Dihydroalprenolol (specific activity = 96.1–105.2 Ci/mmol), [3H][(-)iodocyanopindolol (2200 Ci/mmol), [3H]prazosin (80.9 Ci/mmol),
[\text{H}]yohimbine (85.8 Ci/mmol), and [\text{H}]nitrendipine (78–79.5 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. (-)-Epinephrine, (-)-norepinephrine, (-)-isoproterenol, and (+)-isoproterenol were purchased from Sigma Chemical Co., St. Louis, Mo. The following drugs were generous gifts from the manufacturers, as indicated: (-)-propanolol, ICI 118,551 (ICI Pharma Ltd, Osaka, Japan), phen tolamine (Ciba-Geigy, Osaka, Japan), verapamil (Eisai, Tokyo, Japan), (+)-bisoproplol (EMD 33-512 in Europe and TA 4708 in Japan), (+)-cis diltiazem (Tannbe, Osaka, Japan), and nifedipine and nitrendipine (Bayer Yakuhin Ltd, Osaka, Japan). Nitrendipine and nifedipine were dissolved in 99.5% ethanol as 10 mM solutions with subsequent dilutions in 50 mM Tris buffer (pH 7.4). Ethanol, which was maximal 1% (v/v), did not affect the specific binding of [\text{H}]nitrendipine. Other drugs were prepared as 1 mM solutions in deionized distilled water just before use and then diluted in buffer. All other reagents were of the highest grade commercially available.

**Results**

**Membrane Preparations**

Media layers from coronary artery and aorta were exclusively composed of smooth muscle cells surrounded by amorphous materials and were essentially free of adventitial components. Thus, the cellular components of the starting material used for the isolation were presumably all smooth muscle cells.

The enzyme profiles of the 4 subfractions obtained from coronary artery by discontinuous sucrose density gradient centrifugation are shown in Figure 1B. The activities of sarcolemmal marker enzymes (5'-nucleotidase and K+-pNPPase) peaked at F2 fraction and K+-pNPPase. As shown in Figure 3, adrenergic agonists exhibited competition of [\text{H}]DHA binding sites of coronary membranes that had been incubated with 4 nM [\text{H}]DHA or 0.8 nM [\text{H}]nitrendipine for 30 minutes at 25° C. Each point was determined in triplicate. Association rate constant (K_a) for [\text{H}]DHA binding was 6.5 × 10^5 M⁻¹min⁻¹, and dissociation rate constant (K_d) was 0.14/min. K_d(K_a/K_c) calculated from these data was 2.2 nM. In [\text{H}]nitrendipine binding, K_a was 0.16 × 10^4 M⁻¹min⁻¹, and K_d was 0.062/min. K_d was 0.38 nM. K_d values obtained in this kinetic study were consistent with values obtained in equilibrium binding study (Table 1).

**[\text{H}]DHA and [\text{I}^2\text{H}]CYP Binding**

Specific binding of [\text{H}]DHA to coronary microsomes at 25° C was rapid and reversible, as shown in Figure 2A. An incubation time of 30 minutes was chosen to represent equilibrium binding in subsequent experiments. Specific binding of [\text{H}]DHA to coronary microsomal and F2 fractions and to aortic F2 fraction was saturable and of high affinity. Scatchard analysis of the saturation data yielded a straight line, and the K_d and Bmax values measured in coronary F2 fraction and aortic F2 fraction are shown in Table 1. The K_d and Bmax of coronary F2 fraction were significantly higher than that of aortic F2 fractions (p < 0.01). There was no significant difference in K_d values between coronary microsomal and F2 fractions (for coronary microsomal fraction, K_d = 1.44 ± 0.18 nM, Bmax = 182 ± 5 fmol/mg, n = 3). The distribution of specific [\text{H}]DHA binding activities at the concentration of 8 nM in the 4 subcellular fractions obtained from sucrose density gradient centrifugation of coronary microsomes showed a peak at the F2 fraction, as in the case of sarcolemmal marker enzymes 5'-nucleotidase and K+-pNPPase. As shown in Figure 3, adrenergic agonists exhibited competition of [\text{H}]DHA binding sites of coronary membranes.
Table 1. Binding Parameters of [3H]DHA, [3H]Prazosin, [3H]Yohimbine, and [3H]Nitrendipine to Porcine Coronary and Aortic F2 Fractions

<table>
<thead>
<tr>
<th>Radioligands</th>
<th>Coronary</th>
<th>Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (nM)</td>
<td>Bmax (fmol/mg protein)</td>
</tr>
<tr>
<td>[3H]DHA</td>
<td>1.55 ± 0.06*</td>
<td>258 ± 21*</td>
</tr>
<tr>
<td>[3H]Prazosin</td>
<td>0.081 ± 0.006</td>
<td>12 ± 2*</td>
</tr>
<tr>
<td>[3H]Yohimbine</td>
<td>2.48 ± 0.38</td>
<td>12 ± 0.5*</td>
</tr>
<tr>
<td>[3H]Nitrendipine</td>
<td>0.16 ± 0.02</td>
<td>561 ± 47*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM from $n$ preparations of membranes. *Significantly different from values in aorta ($p < 0.01$).

coronary artery in the following order of decreasing potency: (−)IP ($K_i = 29 ± 6$ nM) > (−)NE ($K_i = 170 ± 10$ nM) > (−)EP ($K_i = 500 ± 70$ nM) > (+)IP ($K_i = 1700 ± 400$ nM) ((−)IP vs. (−)NE, (−)NE vs. (−)EP, (−)EP vs. (+)IP; $p < 0.01$). In case of aorta, the order was (−)IP ($K_i = 99 ± 44$ nM) > (−)EP ($K_i = 760 ± 240$ nM) > (−)NE ($K_i = 19,000 ± 4,000$ nM) ((−)IP vs. (−)NE, (−)NE vs. (−)EP; $p < 0.01$) (Figure 4).

Specific bindings of $[125I]$CYP to coronary and aortic microsomes at 25°C were rapid, reversible, saturable, and of high affinity. The $K_i$ and Bmax of $[125I]$CYP binding for coronary microsome were 140 ± 6 pM and 121 ± 7 fmol/mg protein and for aortic microsome were 234 ± 2 pM and 26 ± 1 fmol/mg protein, respectively ($n = 3$). Figures 5 and 6 show competition curves for $[125I]$CYP binding to coronary and aortic microsomes by β-adrenergic antagonists. After transformation of these data on coronary artery into Hofstee plots, nonlinear plots were obtained for (+)bisoprolol (β₁-selective antagonist) and ICI 118,551 (β₂-selective antagonist), and linear plots were obtained for (−)propranolol (β₁, β₂-nonselective antagonist). In case of aorta, linear plots were obtained for all three compounds. An analysis of these plots according to the iterative least-square-linear-regression method of Minneman et al is consistent with the presence of β receptors in a $β_1$:β₂ ratio of about 9:10 in coronary artery and of 0:100 in aorta (Table 2).

[3H]Prazosin and [3H]Yohimbine Binding

Specific binding of [3H]prazosin to coronary membranes was rapid, reversible, saturable, and of high affinity. Appropriate specificity, kinetics, and preferential localization of binding sites in the sarcolemma were observed in porcine aortic membranes. In the case of [3H]yohimbine, these prerequisite conditions were also observed (data not shown). The $K_i$ and Bmax values measured in coronary and aortic F2 fractions are shown in Table 1. There were no significant differences in $K_i$ values between coronary artery and aorta. However, the Bmax of coronary artery was significantly lower than that of aorta ($p < 0.01$).

[3H]Nitrendipine Binding

The specific binding of [3H]nitrendipine to coronary F2 fraction was rapid and reversible (Figure 2B). The $K_i$ and Bmax values for coronary F2 and aortic F2 fractions are shown in Table 1. There was no significant difference in $K_i$ values between coronary artery and aorta. However, the Bmax value of coronary artery was significantly higher than that of aorta ($p <
Table 2. Binding Parameters of β-Adrenergic Antagonists Determined by Competition for [125I]CYP

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Coronary</th>
<th>Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β1 (nM)</td>
<td>β2 (nM)</td>
</tr>
<tr>
<td>(±)Bisoprolol</td>
<td>32 ± 3</td>
<td>2200 ± 600</td>
</tr>
<tr>
<td>ICI 118,551</td>
<td>165 ± 4</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>(-)Propranolol</td>
<td>1.4 ± 0.2</td>
<td>...</td>
</tr>
</tbody>
</table>

Values are means ± SEM from 3 preparations of membranes (microsome).

Discussion

Using radioligand binding methods, the number and affinity of β1, β2, and α1-adrenoceptors and [3H]nitrendipine receptors were measured in membrane preparations of porcine coronary artery, and the ratios were compared with those of porcine aorta. Despite a high enrichment of sarcolemmal preparations of coronary artery and aorta in the present study, a comparison of Bmax values obtained in binding assays of these two arteries cannot be discussed in terms of absolute values (mol/mg protein) because there is no evidence that the sarcolemma of F2 fractions from these two anatomically different arteries are equal with respect to purity. Thus, a comparison of only the ratios of populations of these receptors in coronary artery with those in aorta is valid.

The radioligands utilized in the present study, [3H]DHA, [125I]CYP, [3H]prazosin, [3H]yohimbine, and [3H]nitrendipine, have been widely used to identify β1-, β2-, α1-, and α2-adrenoceptors and [3H]nitrendipine receptors, respectively, in various tissues from various species. In the present study, we also observed appropriate specificity, kinetics, affinity, saturability, and preferential localization of the binding sites in the sarcolemma for each radioligand.

The interactions of nifedipine, verapamil, and diltiazem with [3H]nitrendipine binding sites of both coronary artery and aorta were similar and consistent with data obtained by other investigators who used various tissues. These data substantiate the proposals that more than one type of binding site is associated with the calcium channel and with probably allosteric interactions between the sites.

Lands et al introduced the classification of β-adrenoceptors into β1- and β2-type receptors and defined these two subtypes by their affinities for EP and NE; specifically, β2-adrenoceptors display approximately equal affinities for EP and NE whereas β2-adrenoceptors have a considerably greater affinity for EP than for NE. Recently, not only agonist potencies but also subtype-selective antagonist potencies have been used to define β-adrenoceptor subtypes. The coexistence of β1- and β2-adrenoceptors within the same tissue has been identified by analyzing the competition curves of subtype-selective antagonists with nonselective radioligands. We have shown that β-receptors of porcine coronary artery are predominantly β1-type (β1:β2 = 90%:10%) and that those of aorta are β2-.
type by using the competition curves of β₁-selective antagonist (±)bisoprolol and β₂-selective antagonist (ICI 118,551). These data are in fairly good agreement with the results obtained by Schwartz and Velly, who found that the ratio of β₁ to β₂-adrenoceptors of porcine coronary arteries was 65:35 from the inhibition of [125I]iodocyanopindolol binding by betaxolol, zinterol, and ICI 118,551. Note that the affinity of NE for coronary β₁-receptors was significantly higher than that of EP (Table 1). The predominance of β₁-adrenoceptors and their relatively high affinity for NE (because they are β₁-type) may account for the weak contraction, or relaxation, of coronary artery induced by sympathetic nerve stimulation or exogenously applied NE or EP.

We reported that Bmax of [H]mepyramine binding to histamine H₁-receptors of sarcolemma was 310 and 681 fmol/mg protein in porcine coronary artery and aorta, respectively. Thus, the ratio of histamine H₁-receptors to α-adrenoceptors of coronary artery is 13 (H₁/(α₁ + α₂) = 310 fmol/mg/(12 + 12 fmol/mg) = 13) while that of aorta is 0.4 (H₁/(α₁ + α₂) = 681/(525 + 1,000) = 0.4). In contrast to the weak contraction induced by NE, the strong contraction or spasm of porcine coronary artery induced by histamine may be accounted for by the predominance of histamine H₁-receptors compared with α-adrenoceptors.

Regarding the relative population of adrenoceptors, the present study indicates that coronary artery is β₁-dominant (β₁/(α₁ + α₂) = 258 fmol/mg/(12 + 12 fmol/mg) = 11) while aorta is α-dominant (β₁/(α₁ + α₂) = 37/(525 + 1,000) = 0.02). The sympathetic nervous control and effects of exogenously applied catecholamines on coronary arteries of various species have been investigated both in vivo and in vitro. The contraction of the conduit coronary segment induced by stimulation of the sympathetic fibers is relatively weak compared with the extent of contraction of similar-sized segments of the arterial tree. Although there are species and regional differences, the poor contraction, or relaxation, induced by exogenously applied NE or EP has been noted in experiments dealing with the responses of isolated coronary rings or strips. Since the density of innervation of the conduit coronary artery is comparable to that of other similar-sized arteries and since the conduit coronary segment is in a relatively favorable condition for diffusion across the vessel wall, the poor contraction, or relaxation, of coronary artery seems to be attributable to peculiarities of the receptor apparatus. Ito et al. reported that porcine coronary artery possessed only β₁-adrenoceptors, determined by investigating the membrane and mechanical properties of the smooth muscle cells. Shepherd and Vanhoutte hypothesized in their review article that both β₁- and α₁-adrenoceptors were present on canine coronary arteries and that, under normal conditions, these arteries relax because of the predominance of β₁-adrenoceptors. In the present study, the ratio of β₁- to α₁-adrenoceptors of porcine coronary artery was 11 while that of aorta was 0.02 (Table 1). The predominance of β₁-adrenoceptors and their relatively high affinity for NE (because they are β₁-type) may account for the weak contraction, or relaxation, of coronary artery induced by sympathetic nerve stimulation or exogenously applied NE or EP.

In summary, we obtained direct evidence that β₁-adrenoceptors and [H]nitrendipine receptors predominate over α₁-adrenoceptors in porcine coronary artery. These results are in good agreement with reported physiologic observations and postulated mechanisms for the response of coronary artery to sympathetic stimulation and exogenously applied catecholamines. Thus, it appears that the populations of receptors on the sarcolemma play a key role in the regulation of responsiveness of vascular smooth muscle cells to various vasoactive substances.

Acknowledgment

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