Alpha-1 Adrenoceptor Subtypes in Canine Aorta

Keiko Maruyama, Noriko Ohkura, Yuko Yagi and Takafumi Nagatomo

Department of Pharmacology, Niigata College of Pharmacy, 5-13-2 Kamishineicho, Niigata 950-21, Japan

Received December 24, 1992 Accepted April 14, 1993

ABSTRACT—The present study was designed to demonstrate the existence in canine aorta of \( \alpha_1 \)-adrenoceptor subtypes, \( \alpha_{1\text{High}} \) and \( \alpha_{1\text{Low}} \), that have different binding affinities for \(^{3}\)H-prazosin and to assess the binding affinity of several drugs for each subtype by a displacement experiment. A radioligand binding assay with \(^{3}\)H-prazosin revealed the presence of two \( \alpha_1 \)-adrenoceptor subtypes in the canine aorta. One of them, which has a high affinity for prazosin, was designated as \( \alpha_{1\text{High}} \) (Kd: 12.40 pM, Bmax: 21.88 fmol/mg protein), and the other type was designated as \( \alpha_{1\text{Low}} \) (Kd: 506.03 pM, Bmax: 88.22 fmol/mg protein). The pK\(_i\) values of several drugs for each subtype were determined, and all drugs used in the present study, except for benoxathian and cloroethyliclonidine, showed significant differences between the pK\(_i\) values for \( \alpha_{1\text{High}} \) and those for \( \alpha_{1\text{Low}} \). Although it is difficult to characterize each \( \alpha_{1\text{High}} \) and \( \alpha_{1\text{Low}} \) into \( \alpha_1\text{A} \) or \( \alpha_1\text{B} \) by only the displacement potency, one structural characteristic to distinguish between \( \alpha_{1\text{High}} \) and \( \alpha_{1\text{Low}} \) could be evaluated.

Keywords: \( \alpha_1 \)-Adrenoceptor, Aorta (canine), \(^{3}\)H-Prazosin

The relationship between vascular smooth muscle contraction and the stimulation of \( \alpha_1 \)-adrenoceptors has been well established (1). In the canine aorta, the existence of an \( \alpha_1 \)-adrenoceptor was demonstrated by both radioligand binding assay and pharmacological methods (2). Recently, an increasing number of \( \alpha_1 \)-adrenoceptor subclassifications have been proposed through evidence obtained in pharmacological and/or radioligand binding studies (3–10). Our previous reports also described that \(^{3}\)H-prazosin discriminated the \( \alpha_1 \)-adrenoceptor subtypes with different affinities in bovine prostate (11) and rat brain, spleen (12) and heart (13); these subtypes were termed \( \alpha_{1\text{High}} \) and \( \alpha_{1\text{Low}} \), respectively.

The present study was performed to demonstrate the existence of these \( \alpha_1 \)-adrenoceptor subtypes (\( \alpha_{1\text{High}} \) and \( \alpha_{1\text{Low}} \)) in the canine aorta and to assess the displacement potency (pK\(_i\) value) of several drugs for each subtype by means of radioligand binding assays.

MATERIALS AND METHODS

Preparation of a crude membrane-enriched specimen from the canine aorta

This was done by essentially the same method as described previously (2). In brief, the aorta was removed, frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until used. The specimen of frozen aorta in the liquid nitrogen was crushed into a fine powder by a mortar and a pestle. After being crushed, this tissue was weighed and suspended in 10 vol. of 10 mM Tris-HCl buffer containing 250 mM sucrose, pH 7.6. The suspension was homogenized with a Poltron homogenizer about 5 times for 10 sec at setting 8 and then filtered through 1 layer of gauze. The filtrate was centrifuged for 10 min at \(1000 \times g\), and the supernatant obtained was again centrifuged for 30 min at \(40000 \times g\). The resulting pellet was rinsed once with the incubation buffer (120 mM Tris-HCl, pH 7.4) and homogenized with a glass homogenizer in 1 vol. of the same buffer. The membrane-enriched fraction was then immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until used. Protein concentration was determined by the method of Lowry et al. (14), with bovine serum albumin as the standard.

Binding assay

The \( \alpha_1 \)-adrenoceptor subtypes in the canine aorta were examined with \(^{3}\)H-prazosin as the radioligand. All binding assays in the present study were carried out in duplicate, and the results were reported as the means obtained from 3–6 experiments. In the saturation experiment, the concentration of \(^{3}\)H-prazosin ranged from 0.01–3.5 nM; and in the displacement experiment for \( \alpha_{1\text{High}} \) and \( \alpha_{1\text{Low}} \), it was 0.01 and 0.3 nM, respectively. The binding assay of \( \alpha_{1\text{Low}} \) alone was carried out with the membrane suspension in the presence of 1 nM bunazosin as well as by a
previously described method (11). The prepared medium that contained $^3$H-prazosin, some $\alpha_1$-antagonist and membrane (0.15 mg of protein) in 0.5 ml of 60 mM Tris-HCl buffer, pH 7.4, was incubated at 23°C for 30 min and then terminated by rapid filtration under vacuum through glass fiber filters (Whatman GF/C, Whatman International Ltd., Maidstone, UK) using an Automatic Cell Harvester Labomash (LM-101, Labo Science, Tokyo). The resulting filters were added to 1 ml of toluene-triton-based scintillation fluid, and the radioactivity was counted by a liquid scintillation counter (Packard 2200 Tri-Cab Scintillation Analyzer, Packard Japan K.K., Tokyo). The specific binding of $^3$H-prazosin was defined as the difference between the total binding and the nonspecific binding obtained in the presence of 10 $\mu$M phentolamine. The values of the inhibition constants ($K_i$) were calculated by a previously described method (15) and expressed as a pKi ($-\log K_i$).

**Drugs used**

$^3$H-Prazosin (76.2 Ci/mmol) was purchased from Du Pont Company (Boston, MA, USA) and was stored at -20°C. It was then diluted to appropriate volumes with distilled water and stored at 4°C. Prazosin (Funakoshi, Tokyo), WB-4101 (Amersham, Buckinghamshire, UK), phenoxybenzamine (Nacalai Tesque, Kyoto), phenotolamine (Ciba-Geigy, Basel, Switzerland), benoxathian, 2-[[2-(2,6-dimethoxyphenoxy)ethyl] amino]methyl]-1,4-benoxathian hydrochloride hydrate, chlorethylclonidine (Research Biochemicals, Natick, MA, USA), yohimbine (Nacalai Tesque) and labetalol (ICI Pharma, Maclesfield, UK) were purchased from the manufacturers. Bunazosin (Eisai, Tokyo), HV-723 (Hokuriku Seiyaku, Katsuyama), SGB-1534, 3-[2-[4-o-methoxyphenyl]-1-piperazininyl]-ethyl]2,4-(1H,3H)-quinazolinedione monohydrochloride (Chugai, Tokyo), terazosin (Mitsubishi Kasei, Tokyo) and nifedipine (Sumitomo Chemical, Osaka) were kindly donated by each company. All compounds used in the present study, except for nifedipine, were dissolved in and diluted with distilled water. Nifedipine was dissolved in ethanol and diluted with distilled water to the appropriate concentrations. All prepared compounds were stored at 4°C.

**RESULTS**

Figure 1 (a and b) shows the results from a typical saturation experiment. The ratio of specific binding per total binding were 50–80% at the concentration of 0.01–0.6 nM $^3$H-prazosin used. An increase of the specific binding in Fig. 1a appears to stop in the range of about 0.4 to 1.3 nM $^3$H-prazosin and more than 2.2 nM. In addition, the Scatchard analysis in Fig. 1b shows the curve having a Hill coefficient value of 0.53. This Hill plot is displayed in the inset of Fig. 1b. These results suggest that there
are two binding sites of $^3$H-prazosin in the canine aorta.

The configuration in Fig. 1b resulting from the computer analysis of the curve indicates the existence of $\alpha_{1\text{High}}$ having a high affinity and $\alpha_{1\text{Low}}$ having a low affinity for $^3$H-prazosin. Figure 1c depicts a typical Scatchard plots of the saturation experiment as examined in the presence of 1 nM bunazosin. The line that represents the $\alpha_{1\text{Low}}$ subtype seems to coincide with the line of $\alpha_{1\text{Low}}$ in Fig. 1b. This suggests the same possibility as previously described by Maruyama et al. (11) that 1 nM bunazosin could completely displace $^3$H-prazosin binding to the $\alpha_{1\text{High}}$ site. This binding assay was used in the displacement experiment shown in Fig. 2 and applied thereafter to the determination of $\alpha_{1\text{Low}}$.

Table 1 summarizes the values of the dissociation constants ($K_d$) and the maximum numbers of $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$ binding sites ($B_{\text{max}}$) for $^3$H-prazosin in the canine aorta. Data in the upper and lower rows were obtained from the Scatchard analysis of saturation experiments carried out in the absence (Fig. 1, a and b) and in the presence (Fig. 1c) of 1 nM bunazosin, respectively. The agreement with the values of the binding parameters ($K_d$ and $B_{\text{max}}$) of $\alpha_{1\text{Low}}$ in the upper and lower rows suggest that 1 nM bunazosin caused the disappearance of $\alpha_{1\text{High}}$ and enabled the analysis of $\alpha_{1\text{Low}}$ alone.

To further characterize the $\alpha_1$-adrenoceptor subtypes in the canine aorta using the displacement potencies ($pK_i$ values) of several drugs, the displacement experiment was performed for each binding site of $^3$H-prazosin. Figure 2 shows a typical displacement curve of bunazosin using different concentrations of $^3$H-prazosin. One curve is shallow with a higher concentration (0.3 nM) of $^3$H-prazosin. This suggests that bunazosin displaced two different binding sites of $^3$H-prazosin. The other curve resulting from the lower concentration (0.01 nM) of $^3$H-prazosin appears to approximate a monophasic pattern. However, this curve shows that a low concentration of bunazosin displaces $^3$H-prazosin bound to $\alpha_{1\text{High}}$ (85%) and a high concentration of bunazosin displaces $^3$H-prazosin bound to $\alpha_{1\text{Low}}$ (15%). This $^3$H-prazosin binding ratio of $\alpha_{1\text{High}}/\alpha_{1\text{Low}}$ is variable depending upon the concentration of $^3$H-prazosin used (ex. 85 : 15 using 0.01 nM $^3$H-prazosin) and can be calculated according to the method of Tsuchihashi et al. (15–17). Consequently, it was possible to obtain the $pK_i$ values of several drugs from the displacement experiments using 0.01 nM $^3$H-prazosin (Fig. 2). Furthermore, this curve suggests that a bunazosin concentration lower than 1 nM was bound only to $\alpha_{1\text{High}}$, because the displacement potency for each subtype of bunazosin is constant and independent of each other. Thus, this concentration (1 nM) was applied to the assay of $\alpha_{1\text{Low}}$ in order to mask only $\alpha_{1\text{High}}$. Figure 3 shows a typical displacement curves of prazosin. As already mentioned, the experiment using 0.01 nM $^3$H-prazosin gave us the displacement potency for $\alpha_{1\text{High}}$, whereas the experiment using 0.3 nM $^3$H-prazosin in the presence of 1 nM bunazosin gave us that for $\alpha_{1\text{Low}}$. There was no difference in the $K_i$ value obtained from the displacement curve or the $K_d$ value from the Scatchard analysis.

Table 2 summarizes the $pK_i$ values of several drugs for $\alpha_1$-adrenoceptor subtypes in the canine aorta. For most drugs, a significant difference of the potency for each subtype was found; however, no difference was shown for benoxathian or chlorethylclonidine.

Table 1. Binding characteristics of $^3$H-prazosin for $\alpha_1$-adrenoceptor subtypes in canine aortas

<table>
<thead>
<tr>
<th>$\alpha_{1\text{High}}$-Affinity site</th>
<th>$\alpha_{1\text{Low}}$-Affinity site</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (pM)</td>
<td>$B_{\text{max}}$ (fmol/mg protein)</td>
</tr>
<tr>
<td>12.40 ± 2.73</td>
<td>21.88 ± 3.22</td>
</tr>
<tr>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of experiments. Data express the mean ± S.E. values. Each value in the lower row was obtained from the experiments in the presence of 1 nM bunazosin.
Fig. 3. Displacement curves of prazosin using 0.3 nM $^3$H-prazosin in the presence (●) and 0.01 nM $^3$H-prazosin in the absence (○) of 1 nM bunazosin. B1 or B0 is the concentration of $^3$H-prazosin bound in the presence or absence of prazosin.

Table 2. The pK$_i$ values of various drugs for $\alpha_1$-adrenoceptor subtypes in canine aortas

<table>
<thead>
<tr>
<th>Drugs</th>
<th>$\alpha_{1\text{High}}$-Affinity site</th>
<th>$\alpha_{1\text{Low}}$-Affinity site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prazosin***</td>
<td>10.38 ± 0.23 (4)</td>
<td>8.45 ± 0.22 (8)</td>
</tr>
<tr>
<td>WB-4101***</td>
<td>9.80 ± 0.20 (4)</td>
<td>7.99 ± 0.20 (5)</td>
</tr>
<tr>
<td>Bunazosin**</td>
<td>9.73 ± 0.24 (4)</td>
<td>8.37 ± 0.19 (6)</td>
</tr>
<tr>
<td>SGB-1534***</td>
<td>9.67 ± 0.24 (4)</td>
<td>8.18 ± 0.13 (4)</td>
</tr>
<tr>
<td>HV-723**</td>
<td>9.00 ± 0.24 (3)</td>
<td>8.12 ± 0.12 (4)</td>
</tr>
<tr>
<td>Terazosin***</td>
<td>8.71 ± 0.17 (6)</td>
<td>7.30 ± 0.23 (7)</td>
</tr>
<tr>
<td>Phenoxymethylamine***</td>
<td>8.26 ± 0.24 (4)</td>
<td>6.96 ± 0.23 (4)</td>
</tr>
<tr>
<td>Benoxathian</td>
<td>8.19 ± 0.26 (6)</td>
<td>8.13 ± 0.25 (6)</td>
</tr>
<tr>
<td>Phentolamine***</td>
<td>7.89 ± 0.17 (3)</td>
<td>5.78 ± 0.06 (4)</td>
</tr>
<tr>
<td>Labetalol***</td>
<td>7.50 ± 0.20 (5)</td>
<td>6.00 ± 0.11 (5)</td>
</tr>
<tr>
<td>Yohimbine***</td>
<td>7.15 ± 0.08 (3)</td>
<td>5.86 ± 0.23 (5)</td>
</tr>
<tr>
<td>Nifedipine**</td>
<td>5.27 ± 0.18 (4)</td>
<td>4.65 ± 0.06 (4)</td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td>5.10 ± 0.19 (3)</td>
<td>5.12 ± 0.07 (4)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of experiments. Values are the mean ± S.E. values. The significant difference between the pK$_i$ values for $\alpha_{1\text{High}}$ and that for $\alpha_{1\text{Low}}$ was analyzed by Student’s t-test. Symbols indicate the following: ***$p<0.01$, **$p<0.02$.

DISCUSSION

The binding assay using $^3$H-prazosin proved the existence of $\alpha_1$-adrenoceptor subtypes ($\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$) with different affinities for $^3$H-prazosin in canine aorta. Not all the pK$_i$ values for each subtype in canine aorta (Table 2) were in agreement with those in rat brain, spleen, heart and bovine prostate in our previous reports (11–13). This different binding affinity of some drugs suggests that $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$ in the canine aorta are not the same as those in the other tissues.

Several investigators (3–10) have also reported that $\alpha_1$-adrenoceptor subtypes could be distinguished by the binding affinity of an antagonist or pharmacological specificity. Among them, Morrow and Creese (7) reported that the $\alpha_1$-adrenoceptor could be subclassified into $\alpha_{1A}$ and $\alpha_{1B}$ by the radioligand binding assay method using $^3$H-prazosin and $^3$H-WB4101. Namely, $\alpha_1$-adrenoceptor subtypes were characterized as $\alpha_{1A}$ when the ratio of the potency ($K_i$ or $K_d$) of phentolamine versus prazosin was about 4 and the $\alpha_{1B}$ was about 80. In the present study, this ratio was 309.0 at $\alpha_{1\text{High}}$ and 467.7 at $\alpha_{1\text{Low}}$. These values suggested that both $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$ in the canine aorta may be characterized as $\alpha_{1B}$. Furthermore, Han et al. showed that the involvement of extracellular Ca$^{2+}$ in contraction is different between the $\alpha_{1A}$ and $\alpha_{1B}$ subtypes (4), and they described the characteristics of each subtype as follows: The $\alpha_{1A}$ has a high affinity for WB-4101, phentolamine, 5-methylurapidil and (+)niguldipine and is not sensitive to chlorhexylnoludine (CEC). The $\alpha_{1B}$-subtype has a 20- to 1500-fold lower affinity for the antagonists mentioned and is sensitive to CEC (5). The binding affinity of WB-4101 and phentolamine to $\alpha_{1\text{High}}$ was 65- and 129-fold higher than that to $\alpha_{1\text{Low}}$, respectively. On the other hand, the binding affinity of CEC to $\alpha_{1\text{High}}$ was very low and showed no significant difference from that to $\alpha_{1\text{Low}}$. These observations suggested that data on only the binding affinity of WB-4101, phentolamine and CEC could not clearly characterize each $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$ into $\alpha_{1A}$ or $\alpha_{1B}$. Further investigation for the optimum experimental method or conditions (e.g., the incubation time, temperature or CEC-treated condition) may solve this problem of characterizing each $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$ into $\alpha_{1A}$ of $\alpha_{1B}$. Thus, the $\alpha_1$-adrenoceptor subtypes in the present study should still be called $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$.

One structural characteristic that may distinguish between $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$ was suggested by the comparison of the chemical structures of benoxathian and WB-4101 or CEC and phenoxybenzamine (PBZ). As shown in Table 2, benoxathian and CEC could not distinguish between $\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$. On the other hand, WB-4101 and PBZ, although having partially the same structures as benoxathian and CEC, respectively, could be distinguished by each subtype. First, WB-4101 contains an oxygen atom in the benzo-1,4-dioxan group that is replaced with a sulfur atom in benoxathian. This oxygen atom in WB-4101 may result in the higher affinity for $\alpha_{1\text{High}}$ than that for $\alpha_{1\text{Low}}$. Secondly, both PBZ and CEC contain the β-chlorethylbenzylamine skeleton. It is well known that this skeleton changes to the benzylaziridium ion and irreversibly binds to the $\alpha_1$-adrenoceptor. Thus, the recognition of each $\alpha_1$-adrenoceptor subtype may be due to the side-chain binding to the nitrogen atom.

In conclusion, using the radioligand binding assay method, we have proved the existence of $\alpha_1$-adrenoceptor subtypes ($\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$) in canine aorta and demonstrat-
ed the binding characteristics of each site. There are some difficulties in correlating the subclassification in the present study ($\alpha_{1\text{High}}$ and $\alpha_{1\text{Low}}$) and that in the previous reports ($\alpha_{1A}$ and $\alpha_{1B}$). To clarify the $\alpha_{1}$-adrenoceptor subclassification, not only the binding affinity of drugs for each subtype, but also their roles in contraction should be used as criteria.

Acknowledgment
We would like to thank Dr. H. Tsuchihashi for his valuable advice.

REFERENCES

4 Han, C., Abel, P.W. and Minneman, K.P.: $\alpha_{1}$-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular $\text{Ca}^{2+}$ in smooth muscle. Nature 329, 333–335 (1987)
17 Tsuchihashi, H., Nagatomo, T. and Imai, S.: Three binding sites of $^{125}$I-Iodocyanopindolol, i.e. $\beta_{1}$, $\beta_{2}$-adrenergic and 5-HT$_{1A}$-serotonergic receptors in rat brain determined by the displacement and Scatchard analysis. J. Pharmacobiodyn. 12, 509–516 (1989)