**α-Adrenoceptors in Canine Mesenteric Artery Are Predominantly 1A Subtype: Pharmacological and Immunochemical Evidence**

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Accepted for publication July 6, 1999

This paper is available online at http://www.jpet.org

**ABSTRACT**

We wanted to determine which α-adrenoceptor subtypes mediate phenylephrine (PE) contraction of dog mesenteric artery in vitro. We studied antagonisms in response to prazosin, 2-(2,6-dimethoxyphenoxymethyl)-aminomethyl-1,4-benzodioxane, 5-methylurapidil, N-[2-(2-cyclopropyl methoxy phenoxo)ethyl]5-chloro-α,α-dimethyl-1H-indole-3-ethanamine HCl (RS 17053), 8–3-[4-(2-methoxyphenyl)-1-piperazinyl]propylcarbamoyl)-3-methyl-4-oxo-22-phenyl-4-indole-3-ethanamine HCl (RS 17053), 8–3-[4-(2-methoxyphenyl)-1-piperazinyl]propylcarbamoyl)-3-methyl-4-oxo-22-phenyl-4-indole-3-ethanamine HCl (RS 17053), 7-chloro-2-bromo-3,4,5,6-tetrahydro-4-methylfurol[4,3,2-ef]-3-benzapine; WB 4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane-7,9-dione HCl; MDL 72832, and 7-chloro-2-bromo-3,4,5,6-tetrahydro-4-methylfurol[4,3,2-ef]-3-benzapine. pKᵦ values for prazosin, 5-methylurapidil, MDL 72832, and RS-17053 were consistent with action on α₁A-adrenoceptors but decreased with concentration. pKᵦ values (9.6) for Rec 15/2739 (α₁L/1A-adrenoceptor selective) were constant. Antagonism by BMY 7378, 7-chloro-2-bromo-3,4,5,6-tetrahydro-4-methylfurol[4,3,2-ef]-3-benzapine, and 8–2-[1,4-benzodioxan-2-ylmethylamino)ethyl]8-azaspirol[4,5]decane-7,9-dione HCl gave pKᵦ values between those expected for α₁A- and α₁D-adrenoceptors. Chlorothiocynoline (100 μM) shifted EC₅₀ values for PE rightward and decreased Eₘₐₓ values but left large residual responses. After 100 μM chlorothiocynoline, either BMY 7378 (100 nM) or RS-17053 (300 nM) increased EC₅₀ values for PE contractions with pKᵦ values like those of controls. At 6 nM, phenoxybenzamine increased the EC₅₀ values and reduced Eₘₐₓ values; prior Rec 15/2739, but not prior BMY 7378, protected receptors against inactivation. An antibody against the α₁B-adrenoceptors immunostained muscle of aorta but not mesenteric artery. We conclude that dog mesenteric artery contains α₁A-adrenoceptors. Discrepancies among responses expected if only these receptors are present may result from pleiotropic functional effects at this receptor and the presence of α₁L-adrenoceptors.

Shi et al. (1989a,b, 1990) showed that receptors of dog mesenteric and saphenous veins (DMVs and DSVs, respectively) had similar Kᵦ (dissociation constant in saturation ligand binding studies) values for and densities of [³H]prazosin (PR) binding sites. However, in dog mesenteric arteries (DMAs), the Kᵦ value for prazosin binding was lower at a similar receptor density. Only DMVs and DSVs, which had higher densities of [³H]rauwolscine binding sites than DMAs, responded by contraction to α₂-adrenoceptor agonists. All three vessels responded to phenylephrine (PE) with similar pD₂ values and similar efficacies. Responses to norepinephrine were effected only through α₁-adrenoceptors in DMAs, but α₂-adrenoceptor activation also contributed to the norepinephrine-induced contraction of DMVs and DSVs. Later, Shimamoto et al. (1992) showed that responses of DMAs to UK 14304, an α₂-adrenoceptor-selective agonist, were promoted by agents that produced threshold contractile stimulation by enhanced Ca²⁺ entry, but α₁-adrenoceptors as well as α₂-adrenoceptors mediated these responses.

**ABBREVIATIONS:** DMV, dog mesenteric vein; C-E, concentration-effect; CEC, chloroethylclonidine; DMA, dog mesenteric artery; DMSO, dimethyl sulfoxide; DSV, dog saphenous vein; Eₘₐₓ, maximum response to phenylephrine; Kᵦ, calculated antagonist dissociation constant in functional studies; KᵦG, dissociation constant in saturation ligand binding studies; MDL 72832, 8–3-[4-(2-methoxyphenyl)-1-piperazinyl]propylcarbamoyl)-3-methyl-4-oxo-22-phenyl-4H-1-benzopyran 2HCl; PBZ, phenoxybenzamine; Rec 15/2739, (SB216469; 8–3-[4-(2-methoxyphenyl)-1-piperazinyl]propylcarbamoyl)-3-methyl-4-oxo-22-phenyl-4H-1-benzopyran 2HCl; RS-17053, 8–3-[4-(2-methoxyphenyl)-1-piperazinyl]propylcarbamoyl)-3-methyl-4-oxo-22-phenyl-4H-1-benzopyran 2HCl; SFK& SKF 105854, 7-chloro-2-bromo-3,4,5,6-tetrahydro-4-methylfurol[4,3,2-ef]-3-benzapine; WB 4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane-7,9-dione HCl.
However, the question of which of the various subtypes of α₁-adrenoceptors mediate contraction in DMAs remains unanswered. After the subclassification of α₁-adrenoceptors into α₁A and α₁B subtypes based on greater sensitivity of the latter to inactivation by chloroethylclonidine (CEC; Han et al., 1987a,b), molecular biological studies have defined three subtypes, now known as α₁A, α₁B, and α₁D, all with high affinity for prazosin (Lomasney et al., 1991a,b). The α₁B-adrenoceptor defined pharmacologically proved to be similar to the cloned α₁B-adrenoceptor, with a lower affinity for 2-(2,6-dimethoxyphenoxethyl)-aminomethyl-1,4-benzodioxane (WB 4101) and 5-methylurapidil (5-MU) than the α₁A-adrenoceptor, which had high affinity for these antagonists, as reviewed by Ford et al. (1994).

The α₁A1-adrenoceptor is now recognized to be a distinct subtype (Schwinn and Lomasney, 1992; Perez et al., 1994; see reviews in Ford et al., 1994; Hieble et al., 1996a), distinguished from the α₁A2-adrenoceptor by a low affinity for 5-MU and a high affinity for recently described agents such as BMY 7378, 8-2-(1,4-benzodioxan-2-ylmethylamine)ethyl|8-azaspirol[4,5]decane-7,9-dione HCl (MDL 73005EF; Saussy et al., 1994; Goetz et al., 1995), and 7-chloro-2-bromo-3,4,5,6-tetrahydro-4-methylfurol[3,2-c]3-benzapine (SK&F 105854; Hieble et al., 1995b) in cloned and expressed rat and human receptors.

All these subclasses of receptors have high binding affinity (pKᵦ > 9) for prazosin when expressed in cell lines. Naturally occurring receptors have been found to have lower affinities (pKᵦ or pKᵦ < 9) in some tissues (Muramatsu et al., 1990; Ohmura et al., 1992) and have been classified as α₁₁-adrenoceptors in contrast to the high-affinity types, α₁₁₁-adrenoceptors. Receptors with low affinity for prazosin have not been cloned, but some antagonists [e.g., SB216469; 8-3-[4-(2-methoxyphenyl)-1-piperazinyl]propylcarbamoyl]-3-methyl-4-oxo-22-phenyl-4H-1-benzopyran 2HCl (Rec 15/2739) with high affinity for α₁₁-adrenoceptors have been reported to distinguish between them and other α₁₁ (perhaps α₁₁₁) adrenoceptor subtypes (Testa et al., 1996, 1997; Leonardi et al., 1997). Recently, Ford et al. (1997) showed that the α₁A-adrenoceptor expressed in CHO-K1 cells demonstrated binding properties [high affinity to prazosin, 5-MU, N-[2-(2-cyclopentylmethoxy)phenoxo]ethyl][5-chloro-o-acetyl-1H-indole-3-ethanamine HCl (RS-17053), Rec 15/2739, WB 4101, and (+)-niguldipine] expected of α₁₁₁-adrenoceptors. However, when a functional property, inhibition of production of inositol phosphates, was evaluated, many antagonists gave lower affinity interactions, as expected for α₁₁₁-adrenoceptors. Rec 15/2739 showed the same functional as binding affinity for the α₁₁-adrenoceptor. The authors suggested that the α₁₁-adrenoceptor was a pleiotropic expression of this receptor.

The goal of this study was to characterize the α₁₁-adrenoceptor subtypes of DMAs by using functional interactions as well as immunostaining studies. The results can be compared with those from other canine blood vessels that have different α-adrenoceptors (Daniel et al., 1996, 1997; Low et al., 1998).

### Materials and Methods

**Animal and Tissue Preparation.** Mongrel dogs of either sex, weighing 10 to 25 kg, were kept under standard conditions in our animal quarters, fasted 24 h before use, and euthanized with an i.v. overdose (100 mg/kg) of pentobarbital. These procedures were approved by the University Animal Care Committee following the guidelines of the Canadian Council on Animal Care. Segments to be used for functional studies were placed in Krebs-Ringer solution (see below for composition).

**Functional Studies.** Rings of DMAs from the first or, occasionally, the second branch (3 mm wide) were mounted individually in 10-mL organ baths filled with modified Krebs' solution composed of 115.5 mM NaCl, 4.6 mM KCl, 1.16 mM MgSO₄, 1.16 mM Na₂HPO₄, 2.5 mM CaCl₂, 21.9 mM NaHCO₃, and 11.1 mM glucose, pH 7.4; gassed with a 95% O₂/5% CO₂ gas mixture; and kept at 37°C. The endothelium was removed by rubbing with forceps, confirmed by showing that carbachol could not relax a contraction induced by PE (5 μM) or 60 mM KCl. The tissues were subjected to a 3 g preload tension, which gave the maximum contractile response, and allowed to equilibrate for 2 h. Rings were subjected to repeated exposures to 100 mM KCl, followed by washing, until contractions were regular. Cumulative dose-response curves were constructed before and 30 min after incubation with increasing concentrations of antagonists (except prazosin incubated for 45 min) were added. The concentration of agonist in the bath was increased approximately 3-fold at each step after the response to the previous dose had plateaued. Data were discarded if a 2-fold shift or more in the EC₅₀ value for PE occurred in concomitant time controls.

When CEC was used as an antagonist, it was not feasible to carry out successive exposures to different PE concentrations. In these studies, of eight arterial rings, two were time controls, and two each were exposed to different concentrations of CEC. Data are expressed as mean ± S.E.

Phenoxybenzamine (PBZ) at 6 nM for 30 min, was used as an irreversible antagonist in receptor-protection experiments. One or two untreated strips served as a time control, two strips served as antagonist controls (treated with the same concentration of antagonist as used for receptor protection), two served as PBZ controls (only PBZ added), and two served as assays for receptor protection (the antagonist added 15 min before, and during, PBZ). PE concentration-effect (C-E) curves were run initially and again with increasing concentrations of antagonists (except prazosin incubated for 45 min) were added. The concentration of agonist in the bath was increased approximately 3-fold at each step after the response to the previous dose had plateaued. Data were discarded if a 2-fold shift or more in the EC₅₀ value for PE occurred in concomitant time controls.

Some additional studies were carried out with rings of canine aortae to compare the sensitivity to PBZ of a tissue with mainly α₁B-adrenoceptors to the sensitivity of DMAs. In these studies, the handling of PBZ was similar to that above, but because of the difficulty in washing out PE contractile responses in this tissue, only one C-E curve was executed on each tissue, and the effects of PBZ were determined by comparing control C-E curves without PBZ with those after various concentrations of PBZ. Data about PBZ sensitivity from a recent study (Low et al., 1998b) of DSVs, which has α₁D-adrenoceptors, were also compared with those from DMAs.

**Data Analysis for Functional Studies.** Data were expressed in terms of the initial responses to 100 mM KCl as 100%. In all experiments, time controls were run, and corrections were made to C-E curves if needed. EC₅₀ values were estimated by fitting each concentration-response curve (logistic function) using MicroCal Origin Software (Northampton, MA). Changes in dose ratios from EC₅₀ values with antagonist concentration were evaluated using ANOVA. In some experiments after CEC pretreatment, subsequent exposure to other antagonists resulted in C-E curves that at very high PE concentrations began to show increased responses after a flex point at the expected plateau level. We used the response level that corresponded to the EC₅₀ response before the antagonist exposure to determine the new EC₅₀ value. Kᵦ (calculated antagonist dissociation constant in functional studies) values (expressed and analyzed as pKᵦ) were calculated for antagonist effects at each antagonist concentration (Furchgott, 1972). When Kᵦ values increase (pKᵦ values decrease) significantly with antagonist concentration, Schild plots have slopes of less than 1. We used pKᵦ values to emphasize the occurrence or nonoccurrence of decreases with antagonist concentration and presented mean values of dose ratios, the dependent vari-
able determining $K_B$ values. In these studies, each $n$ value refers to the mean value in a study of two or more arterial rings from a single animal. For data presentation in the tables, $pK_B$ values were expressed as mean ± S.E.

**CEC Pretreatment.** CEC pretreatment of de-endothelized arterial rings involved exposure for 30 min to several concentrations of CEC (0.3–100 μM) at 37°C, followed by washing for several exchanges of bath fluid. Then, C-E curves to PE were constructed. Finally, the shifts on C-E curves of endothelium-free arterial rings to BMY 7378 or to RS-17053 before and, in other rings, after exposure to 100 μM CEC for 30 min were determined.

**Immunocytochemical Studies.** Four healthy dogs of either sex were euthanized, and blood vessels were collected from aorta and mesenteric arteries as described above. Blood vessels were opened, rinsed free of blood, pinned out on Sylgard silicon rubber-coated dishes, and fixed with 4% paraformaldehyde with 0.1 M phosphate buffer, pH 7.4. The tissues to be used for cryostat sectioning were cut into small pieces and then stored in 15% sucrose containing PBS for cryoprotection at 4°C for 24 h and sectioned into 15-μM-thick slices in a cryostat (Leitz 1720 digital, Wetzlar, Germany). The sections were collected on the slides coated with gelatin. Cryostat sections were incubated overnight at 4°C in a 1:300 dilutions of rabbit anti-sera raised against residues 506 to 515 at the carboxyl terminus of the hamster α1A-adrenoceptor, which had been coupled to keyhole limpet hemocyanin (Fonseca et al., 1995). The antibody was visualized with CY3-labeled goat anti-rabbit goat anti-mouse antibodies (Jackson Immunoresearch, West Grove, PA). Specificity of staining was determined using preimmune serum and by saturation of the antibody with the peptide epitope during exposure of cryostat sections against which it was raised at 5 μg/ml. After washing with PBS, the sections were mounted in 80% glycerol in PBS (pH 10) and viewed on a Leitz microscope equipped with fluorescence epiluminator and I 2 filter. Kodak T-MAX 400 film was used for black-and-white photography.

**Drugs and Chemicals.** BMY 7378 was purchased from Research Biochemicals Inc. (Natick, MA). RS-17053, MDL 73005EF, and 8-[4-(1,4-benzodioxan-2-ylmethylamino)butyl]-8-azaspirol[4,5]decane-7,9-dione HCl (MDL 72832) were purchased from Torcs Cookson Chemicals (Bristol, UK). Prazosin was a gift from Pfizer Canada Inc. (Kirkland, Quebec, Canada). SK&F 105854 was a gift from Dr. J. P. Hieble (SmithKline Beecham, King of Prussia, PA). Rec 15/2739 was a gift from Dr. A. Leonardi (Recordati, S.p.a., Milan, Italy). Other chemicals, all of analytical grade, were purchased from Sigma Chemical Co. (St. Louis, MO) or Research Biochemicals Inc. except for Tris, which was purchased from Boehringer Mannheim Co. (Indianapolis, IN), and dimethyl sulfoxide (DMSO; BDH Inc., Toronto, Canada). Drug solutions were prepared in deionized water or DMSO (for prazosin, RS-17053, Rec 15/2739, and 5-MU). In all cases, the final DMSO concentration was no more than 0.1%, and time controls received the diluent solutions.

**Statistical Analysis.** Unpaired or paired (as appropriate) Student’s $t$ tests were used, and significance of difference was accepted at $P < .05$. Changes in antagonist $pK_B$ values with concentration were calculated and subjected to ANOVA with Bonferroni’s correction (Version 5; GraphPAD, San Diego, CA).

### Results

**Functional Studies**

**Effects of Selective α1-Adrenoceptor Antagonists:** Nonselective or α1D-Selective. PE was used as a α1-adrenoceptor agonist; it is poorly selective among subtypes of this receptor and is not a substrate for reuptake up by sympathetic nerves. The $EC_{50}$ values varied only slightly in different experimental series [e.g., 2.6 ± 0.6 × 10⁻⁶ M ($n = 7$) versus 1.2 ± 0.2 × 10⁻⁶ M ($n = 7$)].

Tables 1 and 2 show $pK_B$ values for 0.3 to 1000 nM α1-adrenoceptor antagonists: prazosin, WB 4101, 5-MU, RS-17053, Rec 15/2739, and SK&F 105854. Prazosin had a $pK_B$ value that decreased with concentration, whereas that for WB 4101 did not (Table 1). At 3 nM, the $pK_B$ for prazosin was 9.58, but at 30 and 300 nM, the values decreased significantly to 8.17 and 7.37, respectively. Indeed, there was no further increase in the dose ratio at prazosin concentrations higher than 3 nM; dose ratios for time controls and 3, 30, and 300 nM prazosin were 1.2 ± 0.19, 21.98 ± 11.7, 19.35 ± 9.68, and 19.05 ± 7.45, respectively. These values suggest that prazosin interacted with high-affinity receptors such as α1A-adrenoceptors but that much lower-affinity interactions occurred at higher PE or prazosin concentrations.

At 3 and 30 nM, the $pK_B$ values for WB 4101 were 9.8 and 9.4, respectively, whereas at 300 nM, the inhibitory effect of WB 4101 was such that the contraction to PE did not reach 50% of the maximum even at 2.2 mM, which is consistent with a $pK_B$ of more than 9.5. Because the C-E curve did not plateau over the concentration range of PE used, it is unclear whether 300 nM WB 4101 reduced the maximal response to PE.

Both α1D- and α1A-adrenoceptors, but not α1D-adrenoceptors, have a high affinity for WB 4101. To evaluate whether α1D-adrenoceptors mediated PE-induced contractions, BMY 7378 and SK&F 105854 were tested (Table 1). $pK_B$ values for BMY 7378 were 7.34, 7.29, and 7.06 when increasing concentrations of 10⁻⁷, 3 × 10⁻⁷, or 10⁻⁶ M were used. These values were not significantly different. Even at 10⁻⁵ M, the $pK_B$ value was similar: 6.69. Goetz et al. (1995) and Saussy et al. (1996) reported $pK_B$ values (in binding studies) for α1A of 6.1 to 6.5 and for α1D of 8.2. With SK&F 105854, our $pK_B$ values were 7.14, 6.87, 7.12, and 6.72 when concentrations of 10⁻⁷, 3 × 10⁻⁷, 10⁻⁶, or 3 × 10⁻⁶ M were used. Hieble et al. (1995)

### Table 1

$pK_B$ values for non- or α1D-selective adrenoceptor antagonists against PE-induced contractions of DMA

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>3 nM</th>
<th>30 nM</th>
<th>300 nM</th>
<th>3,000 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prazosin*</td>
<td>9.58 ± 0.29 (4)</td>
<td>8.17 ± 0.26 (4)</td>
<td>7.37 ± 0.21 (4)</td>
<td></td>
</tr>
<tr>
<td>WB4101</td>
<td>9.80 ± 0.07 (7)</td>
<td>9.38 ± 0.07 (7)</td>
<td></td>
<td>9.4*</td>
</tr>
<tr>
<td>10 nM</td>
<td>8.02 ± 0.12 (4)</td>
<td>7.00 ± 0.11 (4)</td>
<td>6.48 ± 0.16 (4)</td>
<td></td>
</tr>
<tr>
<td>100 nM</td>
<td>7.34 ± 0.05 (8)</td>
<td>7.29 ± 0.06 (8)</td>
<td>7.06 ± 0.06 (8)</td>
<td></td>
</tr>
<tr>
<td>1,000 nM</td>
<td>7.14 ± 0.17 (5)</td>
<td>6.87 ± 0.18 (4)</td>
<td>7.12 ± 0.12 (7)</td>
<td>6.72 ± 0.08 (7)</td>
</tr>
<tr>
<td>3,000 nM</td>
<td>6.9 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values significantly different from one another, $p < .05$.

**Effects of Selective α1A-Adrenoceptor Antagonists:** Nonselective or α1D-Selective. PE was used as a α1-adrenoceptor agonist; it is poorly selective among subtypes of this receptor and is not a substrate for reuptake up by sympathetic nerves. The $EC_{50}$ values varied only slightly in different experimental series [e.g., 2.6 ± 0.6 × 10⁻⁶ M ($n = 7$) versus 1.2 ± 0.2 × 10⁻⁶ M ($n = 7$)].
reported $pK_R$ values for the $\alpha_{1A}$-adrenoceptors ($\alpha_{1A}$-adrenoceptors at that time) of 6.5 and for the $\alpha_{1D}$-adrenoceptors of 8.1.

MDL 73005EF, which, like BMY 7378, is $\alpha_{1D}$ selective (Saussy et al., 1996), demonstrated $pK_R$ values that decreased significantly with increasing concentration, from 8.02 to 7.00 and 6.48 at concentrations of 10, 100, and 1000 nM. The dose ratios failed to increase significantly with increasing concentrations of MDL 73005EF, being 2.17 ± 0.24, 1.95 ± 0.36, and 4.69 ± 1.21 at 10, 100, and 1000 nM. Saussy et al. (1996) reported $pK_R$ values for the rat and human $\alpha_{1D}$-adrenoceptor subtypes as 7.31 and 8.16, respectively, for MDL 73005EF and values for the $\alpha_{1A}$ subtypes of rat and humans of 5.75 and 6.2, respectively. These data did not support the possibility that the $\alpha_{1D}$-adrenoceptor mediated PE-induced contractions of DMAs.

### $\alpha_{1A}$-Adrenoceptor Selective Antagonists

5-MU was a potent antagonist (Table 2), with a $pK_B$ value of 9.12 at 3 nM. Values of $pK_B$ decreased slightly but significantly to 8.64 at both the concentrations of 30 and 300 nM. The dose ratios increased significantly from $5.23 \pm 0.81$ at 3 nM to $14.84 \pm 2.41$ and $176.48 \pm 81.31$ at 30 and 300 nM, respectively. All of these $pK_B$ values were within the range expected for $\alpha_{1A}$-adrenoceptors (Hieble et al., 1995). Because we found no precedent in the published literature for changes in $pK_B$ values with increasing 5-MU concentrations, we repeated these studies 3 years later and obtained similar results (Table 2 and Fig. 1). The dose ratios were also similar (no significant differences from the previous study), increasing from $4.57 \pm 0.60$ at 3 nM to $13.82 \pm 2.41$ and $74.67 \pm 26.22$ at 30 and 300 nM, respectively.

RS-17053 was a potent antagonist with $pK_B$ values of 9.3 when 0.3 nM was applied (Fig. 2), but the $pK_B$ values decreased to 8.6, 8.0, and 7.6 at 3, 10, and 300 nM (Table 2). Although the dose ratios increased with concentration from $1.61 \pm 0.09$ to $2.42 \pm 0.39, 4.97 \pm 1.76$, and $18.6 \pm 8.38$ at 0.3, 3.0, and 300 nM, respectively, the highest concentration was significantly different from the other values.

MDL 72832, which is also $\alpha_{1A}$ selective (Saussy et al., 1996), had $pK_B$ values of 8.49, 8.26, and 8.15 when applied in concentrations of 10, 100, and 1000 nM (Table 2). These values were not significantly different from one another. The dose ratios increased significantly from $4.48 \pm 1.66$ to $19.92 \pm 3.25$ and $173.30 \pm 58.32$. These $pK_B$ values are similar to the $pK_B$ values (Saussy et al., 1996) for rat and human $\alpha_{1A}$-adrenoceptors (i.e., 8.58 and 8.41, respectively, and somewhat more than the $pK_B$ values for the $\alpha_{1D}$-adrenoceptor of 7.42 and 8.11, respectively).

All these data suggested that at low agonist concentrations, the $\alpha_{1A}$-adrenoceptors in DMA mediated contractions. RS-17053, 5-MU, and MDL 72832, all of which have higher affinity for the $\alpha_1A$ than for the $\alpha_1D$ subtype, yielded $pK_B$ values consistent with interaction at that receptor subtype under these experimental conditions. However, when higher concentrations of PE initiated contractions, another $\alpha_{1A}$-adrenoceptor interaction of lower affinity with 5-MU, RS-17053, and MDL 72832, as well as prazosin, appeared to be present. Because this receptor had as high affinity for WB 4101 as any $\alpha_{1A}$-adrenoceptor but low affinity for prazosin, it might be an $\alpha_{1L}$-adrenoceptor (Muramatsu, 1992; Leonardi et al., 1997).

### $\alpha_{1L}$-Adrenoceptor Selective Antagonist

Rec 152739, a compound highly selective for $\alpha_{1A}$-adrenoceptors and for the putative $\alpha_{1L}$-adrenoceptors (Testa et al., 1996, 1997; Leonardi et al., 1997), had $pK_B$ values of 9.63, 9.63, and 9.66 at 3, 10, and 30 nM (Fig. 3). Thus, over a 10-fold antagonist concentration range and a concentration ratio of ~200, this antagonist recognized a homogeneous group of receptors (i.e., most receptors present, including $\alpha_{1A}$-adrenoceptors, had the same functional affinity for this compound).

### Effects of CEC

The effects of increasing concentrations of CEC on responses of DMAs to PE are summarized in Fig. 4. Note that the effects of CEC are small until 10 μM was applied and that even after 10 μM, there were large residual responses to PE. The vessels were pretreated with 100 μM CEC to reduce or eliminate any contribution from $\alpha_{1D}$- or

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**Table 2**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>0.3 nM</th>
<th>3.0 nM</th>
<th>10 nM</th>
<th>30 nM</th>
<th>100 nM</th>
<th>300 nM</th>
<th>1000 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS-17053</td>
<td>9.29 ± 0.08</td>
<td>8.57 ± 0.14</td>
<td>8.04 ± 0.22</td>
<td>7.57 ± 0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rec15/2739</td>
<td>9.63 ± 0.12</td>
<td>9.63 ± 0.10</td>
<td>9.66 ± 0.17</td>
<td>8.26 ± 0.08</td>
<td>8.15 ± 0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDL 72832</td>
<td>8.49 ± 0.14</td>
<td>6.50 ± 0.14</td>
<td>8.64 ± 0.09</td>
<td>8.64 ± 0.18</td>
<td>8.60 ± 0.09</td>
<td>8.32 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>5-MU+</td>
<td>9.12 ± 0.09</td>
<td>6.49 ± 0.12</td>
<td>8.64 ± 0.09</td>
<td>8.64 ± 0.18</td>
<td>8.60 ± 0.09</td>
<td>8.32 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>5-MU+‡</td>
<td>9.06 ± 0.06</td>
<td>6.49 ± 0.12</td>
<td>8.64 ± 0.09</td>
<td>8.64 ± 0.18</td>
<td>8.60 ± 0.09</td>
<td>8.32 ± 0.15</td>
<td></td>
</tr>
</tbody>
</table>

* 5-MU, 5-methylurapidil; $pK_B$ values significantly different from one another at increasing antagonist concentrations.

† $pK_B$ value for 5-MU at 3000 nM was 8.35 (n = 2); otherwise, n = 4 except for RS-17053 at 3 nM, n = 6; at 30nM, n = 5. In no case was $B_{max}$ shown to be reduced significantly.

‡ $pK_B$ values from studies carried out 3 years after first study of 5-MU and used in Fig. 2.
α₁B-adrenoceptors, and the antagonism at residual receptors by RS-17053 (α1A selective) or BMY 7378 (α1D selective) was reexamined. If the CEC-sensitive receptors were α₁D-adrenoceptors, the pK_B values for BMY 7378 should be reduced to those expected from its interaction with α1A-adrenoceptors, whereas those for RS-17053 should be enhanced to those expected for α1A-adrenoceptors.

In fact, the pK_B values for antagonism by 1 mM BMY 7378 before and after CEC pretreatment (Table 3) were similar (7.06 versus 7.30), and the dose ratios were not significantly different (13.0 ± 1.9 versus 31.6 ± 17.4; n = 4). The pK_B values from analysis of the interaction of 100 nM BMY 7378 with the residual receptors left after CEC pretreatment also were not significantly different from those in Table 1. They had dose ratios (11.3 ± 4.7) like those at 1000 nM BMY 7378 in control arteries.

Similarly, for 300 nM RS-17053, the pK_B values before and after CEC pretreatment did not change significantly (7.48 versus 7.62), and they were not increased compared with those in Table 2. As expected, the dose ratios also were not significantly different (18.6 ± 8.4; n = 4) but not significantly different from the value after 30 nM (4.97 ± 1.76). Thus, CEC did not selectively eliminated a subpopulation of α1D-adrenoceptors, leaving only α1A-adrenoceptors.

### Table 3

<table>
<thead>
<tr>
<th>Antagonist (concentration)</th>
<th>pK_B (control-no CEC)</th>
<th>pK_B (after 30 min CEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMY 7378 (100 nM)</td>
<td>ND</td>
<td>7.88 ± 0.19</td>
</tr>
<tr>
<td>BMY 7378 (1000 nM)</td>
<td>7.06 ± 0.07</td>
<td>7.30 ± 0.22</td>
</tr>
<tr>
<td>RS 17053 (30 nM)</td>
<td>ND</td>
<td>8.55 ± 0.19</td>
</tr>
<tr>
<td>RS 17053 (300 nM)</td>
<td>7.48 ± 0.18</td>
<td>7.62 ± 0.11</td>
</tr>
</tbody>
</table>

ND, not done.

* Significantly different (p < .05) from values for RS 17053 at 300 nM. Values are mean ± S.E.
Effects of PBZ Inactivation of \( \alpha \)-Adrenoceptors on Responses to Selective Antagonists

The effects of various concentrations of PBZ on responses to PE are shown in Fig. 5. Note that 6 nM PBZ for 30 min reduced \( E_{\text{max}} \) (maximum response to PE) values by more than 60% and shifted the response curve rightward by \(-2\) log units. \( \alpha \)-Adrenoceptors on DMAs were much more sensitive to PBZ inactivation than were those of DSV (Low et al., 1998b) or dog aorta. In DSVs, 100 nM PBZ was required to comparably shift the PE concentration-response curve and reduce \( E_{\text{max}} \). In five experiments in dog aorta, 100, 1000, and 10,000 nM PBZ reduced the \( E_{\text{max}} \) value to 78.9 \( \pm \) 6.9, 49.9 \( \pm \) 8.9, and 20.5 \( \pm \) 7.1%, respectively, of control \( E_{\text{max}} \) and shifted \( E_{50} \) values from 4.1 \( \pm \) 0.5 to 10.1 \( \pm \) 2.6, 13.7 \( \pm \) 4.1, and 69.2 \( \pm \) 15.3 \( \mu \text{M} \), respectively. Lower concentrations had no significant effects.

As shown in Fig. 6, BMY 7378 (300 nM), and Rec 15/2739 (10 nM) were tested as to the effects of each alone and with PBZ (receptor protection). BMY 7378 had no significant antagonistic effect by itself after washout and failed to affect the location of the concentration-response curve after PBZ. It did restore the \( E_{\text{max}} \) level from \(-40\) to \(-60\)% of control. In contrast, Rec 15/2739 alone after washout shifted the concentration-response curve \(-6\)-fold (apparent \( pK_B \) = 8.7). Moreover, it restored the concentration-response curve after PBZ to the value with Rec 15/2739 alone. In control experiments, we found that Rec 15/2739 alone did not wash out over the experimental time period. We interpret these experiments to suggest that BMY 7378 protects only a small subset of \( \alpha \)-adrenoceptors, responding to high concentrations of PE, from PBZ inactivation, whereas Rec 15/2739 protects nearly all receptors.

Immunochemistry for \( \alpha_{1B} \)-Adrenoceptors in DMAs and Aorta

Figure 7 shows the staining of aorta as a positive control for recognition of \( \alpha_{1B} \)-adrenoceptors. Canine aorta appears to contain predominantly \( \alpha_{1B} \)-adrenoceptors based on functional and ligand binding studies (Hoo et al., 1994; Leonardi et al., 1997; Low et al., 1998). Aortic cells were stained in particulate fashion (Fig. 6A), and the preimmune serum failed to stain these cells (Fig. 6B). Saturation of the antibody with the peptide epitope also abolished staining (Fig. 6C). In contrast, DMAs did not stain with the antibody to \( \alpha_{1B} \)-adrenoceptors (Fig. 6D).

Discussion

In this study, we used several approaches to identify the \( \alpha \)-adrenoceptors that mediate PE-induced contractions of DMAs in vitro. We quantified antagonisms by various selective and nonselective antagonists: prazosin was expected to have high and nonselective affinity at all subtypes of cloned receptors, WB 4101 had a higher affinity for \( \alpha_{1A} \) and \( \alpha_{1D} \).
adrenoceptors; 5-MU, MDL 72832, RS-17053, and Rec 15/2739 had a higher affinity for $\alpha_{1A}$-adrenoceptors, but Rec 15/2739 possibly had a special high affinity for $\alpha_{1L}$-adrenoceptors, presently defined only by their low affinity for prazosin (Hieble et al., 1995a; Testa et al., 1996, 1997; Leonardi et al., 1997). BMY 7378, MDL 73005EF, and SK&F 105854 have higher affinity for $\alpha_{1D}$-adrenoceptors (Hieble et al., 1995a; Saussy et al., 1996).

We used CEC, expecting it to inactivate $\alpha_{1B}$-adrenoceptors nearly completely and $\alpha_{1D}$-adrenoceptors mostly, whereas sparing most $\alpha_{1A}$-adrenoceptors. Thus, we expected pretreatment with CEC to eliminate or reduce any effects of $\alpha_{1B}$- or $\alpha_{1D}$-adrenoceptors. However, it might inhibit agonist action at $\alpha_{1A}$-adrenoceptors without receptor inactivation (Michel et al., 1993). In the human prostate, which may contain $\alpha_{1L}$- or $\alpha_{1A}$-adrenoceptors, 50 $\mu$M CEC for 30 min had little effect compared with vehicle control (Testa et al., 1996).

We also used receptor protection by selective antagonists of PBZ inactivation of $\alpha_{1A}$-adrenoceptors because we have found that both $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptors are relatively resistant to this agent (Low et al., 1998, 1999; current study).

Our initial main findings were that at low agonist concentrations, all antagonists behaved as if they interacted with $\alpha_{1A}$-adrenoceptors, but some of them also appeared to interact at higher antagonist concentrations with another receptor, likely unidentifiable because of interference from the most sensitive pathway.

This last receptor subtype was identified by Muramatsu et al. (1990) after finding that both $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptors are members of the $\alpha_{1H}$-adrenoceptor class. Bevan et al. (1989) reported that func-

Fig. 7. Immunoreactivities of canine aorta and mesenteric artery to antibody against $\alpha_{1B}$-adrenoceptors. A, cryostat section of canine aorta exposed to antibody against $\alpha_{1B}$-adrenoceptors. Note particulate nature of staining where cells cut tangentially. B, similar section exposed to preimmune serum form rabbit used to make antibody in A. C, similar section as a except that antibody was preabsorbed with 5 $\mu$g/ml concentration of the peptide epitope used to raise the antibody. D, cryostat section of mesenteric artery exposed to antibody under same conditions as in a. Length bars, 12.5 $\mu$m in A to C and 25 $\mu$m in D.
tional α₁-adrenoceptors have varied affinities for prazosin in different blood vessels. In our study, receptors with both high and low affinity for prazosin seem to function in DMAs. However, high-affinity receptors are not α₁B-adrenoceptors.

Evidence Excluding Participation of α₁B-Adrenoceptors in Contraction of DMAs. Piascik et al. (1997) reported that the α₁B-adrenoceptor and its mRNA were present and that the receptors contributed to the contractile responses of rat mesenteric arteries. Zhu et al. (1999) reported that contractile responses of rat mesenteric arteries were mediated primarily by α₁A-adrenoceptors, suggesting that the situation is similar to that in DMAs. Multiple evidence rules out α₁B-adrenoceptors playing a major functional role in canine DMAs. First, the high potency of WB 4101 to inhibit PE responses with subnanomolar Kᵦ values over the concentration range of 3 to 300 nM is inconsistent with interactions with α₁B-adrenoceptors (Schwinn and Lomasney, 1992). Second, immunocytochemical studies with an antibody to α₁B-adrenoceptors stained cells of canine aorta but not DMA. Aorta appeared to contain predominantly α₁B-adrenoceptors but not α₁A-adrenoceptors (Hoo et al., 1994; Leonardi et al., 1997; Low et al., 1998) and immunostained strongly with the antibody to that receptor, whereas the DMA did not contain antigens that recognized this antibody. Third, in DMAs, CEC was less potent than canine aorta, in which 10 μM CEC reduced the maximum response to PE to 11% of the control value and 100 μM abolished all responses (Low et al., 1998). At 10 μM, CEC reduced the maximum response of DMA rings to 73% of control, and in 100 μΜ, the maximal response to PE was 53% of control and had not reached a plateau. Fourth, PBZ was much more potent to inactivate receptors in DMAs compared with canine aorta. We conclude that DMA has few or no functional α₁B-adrenoceptors and that the persistent responses after CEC likely reflect effects on α₁A-adrenoceptors (see below). In the absence of a tool to selectively and irreversibly eliminate α₁A-adrenoceptors, we could not characterize residual adrenoceptors after CEC in DMAs.

Identification of α₁-Adrenoceptor Subtypes in DMAs. Most of the prazosin interaction sites in DMAs had pKᵦ or pKi values for prazosin and other antagonists near the values expected from binding studies of cloned human and rat α₁A-adrenoceptors (Hieble et al., 1995). DMA had a Kᵦ value of 0.8 nM for prazosin receptors in earlier studies (Shi et al., 1998a). To evaluate functional receptors, we discounted Kᵦ values derived from concentration ratios of less than 2-fold more than for time controls as subject to large errors (e.g., values for 3 and 30 nM MDL 73005EF and 0.3 or 3 nM RS-17053). The remaining values for functional receptors also corresponded to α₁A-adrenoceptors in their high-affinity interactions with prazosin, WB 4101, MLD 72832, RS-17053, Rec 15/2739, and 5-MU and their resistance to inactivation by CEC. However, at higher agonist concentrations, lower-affinity interactions occurred with prazosin and possibly with 5-MU but not with WB 4101 or Rec 15/2739.

Leonardi et al. (1997) and Testa et al. (1996, 1997) reported that the urinary tracts of humans, dogs, and rabbits contained α₁-adrenoceptors that had many pharmacological characteristics of the α₁A-adrenoceptor subtype but had functional interactions that correlated best overall with the putative α₁A-adrenoceptor subtype, based on results from studies of a series of quinazolyl-amino derivative such as Rec 15/2739.

Ford et al. (1997) found that recombinant α₁A-adrenocep-

 tors expressed in CHO-K1 cells without native receptors demonstrated antagonist binding interactions (pKi values) typical of 1A subtype (prazosin, 9.9; RS-17053, 9.3; WB 4101, 9.8; 5-MU, 9.2; and Rec 15/2739, 9.6). However, when the pKi values for agonists at these receptors were determined for inhibition of [³H]inositol phosphates accumulation, the values differed (prazosin, 8.7; RS-17053, 8.3; WB 4101, 8.8; 5-MU, 8.1; and Rec 15/2739, 9.4) and resembled those at the α₁A-adrenoceptor determined in human prostate. Only Rec 15/2739 had a functional affinity comparable to its binding affinity. In several respects, these data resemble ours in DMAs.

However, in the study by Ford et al. (1997), there were no cases among the above antagonists in which the p Ki values for functional antagonism were significantly different from 1. Our data yielded several cases in which Schild plots would have yielded slopes less than 1, due to significant decreases in pKi values with increasing antagonist concentrations. Our decreasing pKi values might result from interaction of antagonists with different receptors or with one receptor and different G proteins at increasing agonist concentrations. In view of this uncertainty, we carried out additional experiments to rule out participation of α₁B-adrenoceptors in the responses of DMAs to PE.

Possible Contributions of α₁D- or α₁L-Adrenoceptors to DMA Contractions. If α₁D-adrenoceptors are present in DMAs along with α₁A-adrenoceptors, removing or minimizing their contribution of non-α₁A-adrenoceptor with CEC should leave classic α₁A-adrenoceptors. As shown in Table 3, elimination of CEC-sensitive receptors did not reduce the pKi value of BMY 7378 to levels expected for α₁A-adrenoceptors, nor was there a major effect to increase the pKi of higher concentrations of RS-17053. Thus, the CEC-resistant and CEC-sensitive receptors behaved similarly to antagonists that were selective for both the 1D and 1A subtypes. The effects of CEC to shift C-E curves to PE for DMAs might result from noncompetitive antagonism of the α₁A-adrenoceptors rather than inactivation of α₁-adrenoceptors. These results are inconsistent with a model in which there are two functional receptors: the α₁A-adrenoceptor and the α₁D-adrenoceptor. A model in which all receptors recognize the same receptor population before and after CEC was suggested. Thus, either CEC has no selectivity for α₁D-adrenoceptors over α₁A-adrenoceptors in DMA, or the receptors are all the same and the differences in pKi values at higher concentrations of some antagonists result in nonclassic, possibly pleiotropic behavior of the receptor. However, the presence of another receptor, the α₁L-adrenoceptor subtype, has not been excluded by these data.

PBZ as an irreversible antagonist was much more potent to inactivate receptors of DMA compared with DSV (α₁D-adrenoceptors; see Daniel et al., 1996; Low et al., 1999) and dog aorta (Hoo et al., 1994; current study), suggesting that the receptors inactivated by PBZ were neither α1D nor α1B subtype. Thus, the concentration of PBZ used in DMAs should have inactivated α₁A-adrenoceptors but left α₁L- and α₁B-adrenoceptors intact or enriched because they are resistant to PBZ at that concentration. Consistent with the fact that PBZ-sensitive receptors were not α₁D subtype was the limited ability of BMY 7378 to protect them against inactivation. In contrast, Rec 15/2739 provided strong protection. Thus, these studies and those with CEC speak against the presence of BMY 7378-sensitive α₁D-adrenoceptors in DMA. The ability of Rec 15/2739 to shift C-E curves to PE for DMAs might result from noncompetitive antagonism of the α₁A-adrenoceptors rather than inactivation of α₁-adrenoceptors. These results are inconsistent with a model in which there are two functional receptors: the α₁A-adrenoceptor and the α₁D-adrenoceptor. A model in which all receptors recognize the same receptor population before and after CEC was suggested. Thus, either CEC has no selectivity for α₁D-adrenoceptors over α₁A-adrenoceptors in DMA, or the receptors are all the same and the differences in pKi values at higher concentrations of some antagonists result in nonclassic, possibly pleiotropic behavior of the receptor. However, the presence of another receptor, the α₁L-adrenoceptor subtype, has not been excluded by these data.

FBZ as an irreversible antagonist was much more potent to inactivate receptors of DMA compared with DSV (α₁D-adrenoceptors; see Daniel et al., 1996; Low et al., 1999) and dog aorta (Hoo et al., 1994; current study), suggesting that the receptors inactivated by PBZ were neither α1D nor α1B subtype. Thus, the concentration of PBZ used in DMAs should have inactivated α₁A-adrenoceptors but left α₁L- and α₁B-adrenoceptors intact or enriched because they are resistant to PBZ at that concentration. Consistent with the fact that PBZ-sensitive receptors were not α₁D subtype was the limited ability of BMY 7378 to protect them against inactivation. In contrast, Rec 15/2739 provided strong protection. Thus, these studies and those with CEC speak against the presence of BMY 7378-sensitive α₁D-adrenoceptors in DMA. The ability of Rec 15/2739 to shift C-E curves to PE for DMAs might result from noncompetitive antagonism of the α₁A-adrenoceptors rather than inactivation of α₁-adrenoceptors. These results are inconsistent with a model in which there are two functional receptors: the α₁A-adrenoceptor and the α₁D-adrenoceptor. A model in which all receptors recognize the same receptor population before and after CEC was suggested. Thus, either CEC has no selectivity for α₁D-adrenoceptors over α₁A-adrenoceptors in DMA, or the receptors are all the same and the differences in pKi values at higher concentrations of some antagonists result in nonclassic, possibly pleiotropic behavior of the receptor. However, the presence of another receptor, the α₁L-adrenoceptor subtype, has not been excluded by these data.

PBZ as an irreversible antagonist was much more potent to inactivate receptors of DMA compared with DSV (α₁D-adrenoceptors; see Daniel et al., 1996; Low et al., 1999) and dog aorta (Hoo et al., 1994; current study), suggesting that the receptors inactivated by PBZ were neither α1D nor α1B subtype. Thus, the concentration of PBZ used in DMAs should have inactivated α₁A-adrenoceptors but left α₁L- and α₁B-adrenoceptors intact or enriched because they are resistant to PBZ at that concentration. Consistent with the fact that PBZ-sensitive receptors were not α₁D subtype was the limited ability of BMY 7378 to protect them against inactivation. In contrast, Rec 15/2739 provided strong protection. Thus, these studies and those with CEC speak against the presence of BMY 7378-sensitive α₁D-adrenoceptors in DMA. The ability of Rec 15/2739 to shift C-E curves to PE for DMAs might result from noncompetitive antagonism of the α₁A-adrenoceptors rather than inactivation of α₁-adrenoceptors. These results are inconsistent with a model in which there are two functional receptors: the α₁A-adrenoceptor and the α₁D-adrenoceptor. A model in which all receptors recognize the same receptor population before and after CEC was suggested. Thus, either CEC has no selectivity for α₁D-adrenoceptors over α₁A-adrenoceptors in DMA, or the receptors are all the same and the differences in pKi values at higher concentrations of some antagonists result in nonclassic, possibly pleiotropic behavior of the receptor. However, the presence of another receptor, the α₁L-adrenoceptor subtype, has not been excluded by these data.
to protect against PBZ inactivation was consistent with its functional antagonism at DMA receptors, antagonism that could involve α1A- or α1D-adrenoceptors.

Both α1A- and α1D-Adrenoceptors in DMAs? The occurrence of blood vessels with relatively low affinity for prazosin at high concentrations of agonist but many pharmacological characteristics of the α1A subtype is not confined to canine blood vessels. Recently, van der Graaf et al. (1996) reported a similar result for rat mesenteric arteries. They suggested that the pharmacologically defined α1L subtype operated in that resistance vessel. Testa et al. (1996) suggested that human mesenteric artery contained either exclusively α1A- or α1L-adrenoceptors. Lachnit et al. (1997) suggested that rat caudal artery contained at least two subtypes of α1-adrenoceptors: mostly α1A-adrenoceptors and another of lower affinity to RS-17053. If the findings of Ford et al. (1997) with recombinant α1A-adrenoceptors apply to resistance blood vessels, it is possible that those in DMA have α1A-adrenoceptors in an environment in which their functional behavior at high agonist levels corresponds to the α1L-adrenoceptors.

If, in contrast, the correct explanation is that more than one α1-adrenoceptor subtype is present, we were unable to unmask or characterize it, as noted above.

Conclusions and Future Perspectives. The results of this study suggest that the main functional adrenoceptor subtype in DMA are α1A-adrenoceptors and that DMA lacks functional α1B- or α1D-adrenoceptors. Low-affinity interactions of these receptors may reflect the functional behavior of the α1A-adrenoceptor at high agonist concentrations.

Acknowledgments

We thank Angela Demeter and Tony Kwan for technical assistance in data analysis and graphic presentation.

References


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