Immunocytochemical Localization of the Alpha-1B Adrenergic Receptor and the Contribution of This and the Other Subtypes to Vascular Smooth Muscle Contraction: Analysis with Selective Ligands and Antisense Oligonucleotides

MICHAEL T. PIASCIK, SANDRA L. HROMETZ, STEPHANIE E. EDELMANN, RICHARD D. GUARINO, ROBERT W. HADLEY and R. DALE BROWN

Department of Pharmacology and Vascular Biology Research Group, University of Kentucky College of Medicine, Lexington, Kentucky (M.T.P., S.L.H., S.E.E., R.D.G., R.W.H.), and Department of Pharmacology, University of Illinois at Chicago, Chicago, Illinois (R.D.B.)

Accepted for publication July 18, 1997

ABSTRACT

The contribution of the alpha-1B adrenergic receptor (AR) to vascular smooth muscle contraction has been assessed using a combination of immunological, molecular biological and pharmacological approaches. A subtype-selective antibody detected alpha-1B immunoreactivity in the medial layer of the aorta, caudal, femoral, iliac, mesenteric resistance, renal and superior mesenteric arteries. Receptor protection assays and antisense oligonucleotides were used to assess the contribution of the alpha-1B AR to contraction. The alpha-1B AR was implicated in mediating the phenylephrine-induced contraction of the mesenteric resistance artery. The alpha-1D AR was implicated in mediating the contraction of the aorta, femoral, iliac and superior mesenteric arteries. Similarly, the alpha-1A AR was implicated in mediating contraction of the caudal and renal arteries. In vivo application of antisense oligonucleotides targeted to the translational start site of the alpha-1B AR had no effect on the phenylephrine-induced contraction of the femoral or renal arteries. In contrast, antisense oligonucleotides directed against the alpha-1D AR significantly inhibited the phenylephrine response in the femoral artery but had no effect on the renal artery. Application of alpha-1A AR antisense oligonucleotides inhibited the contraction of the renal artery without effect on the femoral artery. These data show that (1) alpha-1B AR immunoreactivity is widely distributed in the same peripheral arteries in which previous studies detected its mRNA, and (2) despite this distribution, receptor protection and antisense oligonucleotide studies indicate that the alpha-1B AR mediates the contraction of only the mesenteric resistance artery.

The alpha-1 AR family is a member of the G protein-coupled superfamily of receptors. In analogy to bacteriorhodopsin, these receptors have the now very familiar proposed structure of seven transmembrane spanning domains connected by hydrophilic loops alternately exposed to the extracellular and intracellular environment. The structure of G protein-coupled receptors in general and alpha-1 ARs specifically have been the subject of several reviews (Bylund et al., 1995; Graham et al., 1996; Guarino et al., 1996; Minneman and Esbenshade, 1994; Strader et al., 1994). Three genes encoding unique alpha-1 AR subtypes (Cotecchia et al., 1988; Lomasney et al., 1991; Perez et al., 1991; Schwinn et al., 1990) have been discovered. Despite early controversy and confusion, a nomenclature consisting of the alpha-1A (formerly referred to as the alpha-1c), the alpha-1B and alpha-1D (formerly the alpha-1a or alpha-1/di) AR subtypes has been proposed and gained acceptance in the field (Hieble et al., 1995).

There is longstanding evidence that multiple alpha-1 AR subtypes participate in the regulation of peripheral vascular function (Bylund et al., 1995; McGrath, 1982; Minneman, 1988). However, the individual contribution of each of the alpha-1D AR subtypes has not been established. mRNA for the alpha-1A AR is expressed at very high levels in peripheral arteries, ~90% of the total alpha-1 AR message pool (Guarino et al., 1996). High-affinity antagonists have been used to implicate the alpha-1A AR in the regulation of the caudal and renal arteries (Han et al., 1990; Elhawary et al., 1992; Piascik et al., 1995). Although expressed at low levels compared with the other receptor subtypes, alpha-1D AR mRNA can also be detected throughout the peripheral vasculature (Guarino et al., 1996; Piascik et al., 1995). Studies

ABBREVIATIONS: AR, adrenergic receptor; PSS, physiological saline solution; CEC, chloroethylclonidine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PBZ, phenoxybenzamine.
Fig. 1. Immunocytochemical localization of the alpha-1B AR in the aorta, caudal and femoral arteries. The experiments were carried out as described in the text. Left, presence of an affinity-purified alpha-1B AR antibody. Right, presence of preimmune serum alone.
Fig. 2. Immunocytochemical localization of the alpha-1B AR in the iliac, mesenteric resistance, renal and superior mesenteric arteries. The experiments were carried out as described in the text. Left, presence of an affinity-purified alpha-1B AR antibody. Right, presence of preimmune serum alone.
with the high-affinity alpha-1D AR antagonist BMY 7378 have shown that the alpha-1D AR mediates the contraction of the aorta, iliac artery and skeletal muscle arterioles (Kenny et al., 1995; Leech and Faber, 1996; Piascik et al., 1995; Testa et al., 1995) and is involved in the regulation of systemic arterial blood pressure (Zhou and Varga, 1996).

The role of the alpha-1B AR in mediating vascular smooth muscle contraction is less certain. Recently, Leech and Faber (1996) showed that the alpha-1B AR mediates constriction of skeletal muscle venules. Early studies with CEC suggested this receptor was involved in the contraction of several blood vessels (Minneman, 1988). However, it is now known that the alpha-1A and alpha-1D ARs can also be inactivated by CEC to varying degrees (Lomasney et al., 1991; Perez et al., 1991; Schwinn et al., 1990). Therefore, this ligand cannot be considered a subtype-selective alkylating agent that can be used to define alpha-1B AR-mediated processes. Furthermore, the alpha-1B and alpha-1D ARs exhibit similar affinities for most ligands used for receptor characterization (Lomasney et al., 1991; Perez et al., 1991). We are not certain of the extent to which the alpha-1B AR mediates vascular smooth muscle contraction. For example, the alpha-1B AR was initially thought to regulate the contraction of the aorta (Han et al., 1990; Piascik et al., 1993). However, more recently, several groups of researchers have used BMY 7378 to demonstrate that aortic contraction is mediated by the alpha-1D AR (Kenny et al., 1995; Piascik et al., 1995; Testa et al., 1995). Recently, a selective alpha-1B AR antagonist was synthesized (Giardina et al., 1996) and may prove useful in dissecting the role of this receptor in the regulation of smooth muscle function.

mRNA for the alpha-1B AR is widely distributed in peripheral arteries (Piascik et al., 1994; Guarino et al., 1996). We

### TABLE 1

<table>
<thead>
<tr>
<th>Artery</th>
<th>Mean fluorescence</th>
<th>Postimmune staining</th>
<th>Preimmune staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relative units</td>
<td>n</td>
<td>relative units</td>
</tr>
<tr>
<td>Aorta</td>
<td>161 ± 9.9</td>
<td>3</td>
<td>131 ± 17.3</td>
</tr>
<tr>
<td>Caudal</td>
<td>50 ± 6.9</td>
<td>8</td>
<td>29 ± 3.6</td>
</tr>
<tr>
<td>Femoral</td>
<td>113 ± 7.8</td>
<td>5</td>
<td>94 ± 5.4</td>
</tr>
<tr>
<td>Iliac</td>
<td>80 ± 2.5</td>
<td>11</td>
<td>54 ± 6.4</td>
</tr>
<tr>
<td>Mesenteric resistance</td>
<td>136 ± 19.2</td>
<td>6</td>
<td>88 ± 18.3</td>
</tr>
<tr>
<td>Renal</td>
<td>137 ± 10.7</td>
<td>3</td>
<td>97 ± 12.1</td>
</tr>
<tr>
<td>Superior mesenteric</td>
<td>108 ± 11.9</td>
<td>6</td>
<td>62 ± 12.0</td>
</tr>
</tbody>
</table>

Each point represents the mean ± S.E.M. All postimmune values are statistically significantly different from preimmune staining as determined by t test for unpaired data.

![Graph A](image1.png)  
**Fig. 3.** Effect of BMY 7378 (30 nM) on the inhibitory actions of PBZ. Arteries were incubated in the presence of BMY 7378 alone, PBZ alone, both BMY 7378 and PBZ or vehicle, as detailed in the text. Each point represents the mean ± S.E.M. of eight individual experiments in the aorta, four in the femoral artery, eight in the iliac artery and two in the superior mesenteric artery. *p < .05* significant difference from the curve obtained in the presence of PBZ alone.
assessed the extent of protein expression of the \(\alpha\)-1B AR with a subtype-selective antibody. We then used pharmacological methods and antisense oligonucleotides to characterize the extent to which the \(\alpha\)-1B AR mediates smooth muscle contraction in the peripheral vasculature. A pluronic gel delivery system was used to apply the antisense oligonucleotides to arteries \textit{in vivo}, and the effect on contractility was assessed 24 hr later. Pluronic gels are liquid at 4°C and solidify at room temperature (Schmolka, 1972). The gels have been used as a system to deliver antisense oligonucleotides into vascular smooth muscle \textit{in vivo} (Bennett et al., 1994; Simons et al., 1992; Villa et al., 1995). It has been shown that antisense c-myb oligonucleotides delivered with a pluronic gel inhibit smooth muscle cell growth after vascular injury (Simons et al., 1992; Villa et al., 1995). Our results indicate that the protein expression of an \(\alpha\)-1D AR subtype on peripheral blood vessels cannot be taken as evidence that the receptor is involved in contractile regulation.

**Methods**

**Animals.** Male Sprague-Dawley rats weighing between 250 and 350 g were used in all experiments. The aorta, caudal, femoral, iliac, mesenteric resistance, renal and superior mesenteric arteries were removed and processed according to the experimental protocols described below.

**Immunocytochemistry.** After removal, the blood vessels were cleaned of extraneous tissue and cryoprotected by immersion in increasing amounts of sucrose in PBS (15% for 2 days, 30% for 2 days) at 4°C. The vessels were then placed in O.C.T. mounting media and quick-frozen (−50°C in isopentane cooled with dry ice); 13-μm sections were cut on a cryostat. Sections were placed on poly-L-lysine-coated, circularly etched slides and stored at −80°C until use. Sections were thawed, fixed in cold 4% paraformaldehyde for 10 min and then washed. These sections were incubated in blocking solution (PBS containing 10% normal goat serum, 1% bovine serum albumin and 0.1% Triton X-100) for 2 hr at room temperature and then incubated with \(\alpha\)-1B AR antisera (1:100–150 dilution) for 24 hr at 4°C in the blocking solution without goat serum. Slides were then washed in PBS followed by application of an affinity-purified FITC-conjugated goat anti-rabbit IgG (1:200 dilution; Jackson Immunolabatories, West Grove, PA) secondary antibody. After 1 hr at room temperature, the secondary antibody was removed by washing with PBS, and the slides were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) mounting media and then examined by fluorescence microscopy. The anti-receptor IgG, originally described by Fonseca et al. (1995), was affinity-purified against the immunizing peptide immobilized on a solid support matrix (Sulfolink; Pierce Chemicals, Rockford, IL). Preimmune IgG was prepared by fractionating rabbit serum over the same column.

**Laser scanning confocal microscopic analysis of immunofluorescent images.** Microscopic sections were placed on the stage of an inverted microscope and imaged using a laser-scanning confocal microscope (RCM 8000; Nikon, Melville, NY). FITC fluorescence was excited using the 488-nm wavelength output of an argon laser. Emitted fluorescence was collected using a Nikon \(40\times\) water-immersion objective (N.A. 1.15). Image quality was enhanced by averaging 64 scans/image. Images were immediately stored on an optical disk recorder as eight-bit, 512 × 483 pixel TIFF files. The files were later transferred to an 80486 microcomputer for off-line analysis using the program Metamorph (Universal Imaging, West Chester, PA). Fluorescence measurements were confined to the vascular smooth muscle regions between the highly autofluorescent elastin bands. This was facilitated by setting the analysis program threshold settings to exclude the bright elastin autofluorescence. The mean fluorescence in these defined regions was calculated for both preimmune and postimmune arterial sections. Differences in these mean fluorescent values were determined by a \(t\) test for unpaired data.

**In vitro assessment of contractile function.** Isolated blood vessel segments were prepared by techniques routinely used in our laboratory (Piascik et al., 1994, 1995). Arterial segments were removed and placed in a cold PSS of the following composition (in mM): NaCl, 130; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄·7H₂O, 1.17; CaCl₂·2H₂O,
TABLE 2
Effect of BMY 7378 pretreatment on the inhibition of developed tension produced by 10 nM PBZ

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Developed tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Aorta</td>
<td>1.25 ± 0.09</td>
</tr>
<tr>
<td>Caudal</td>
<td>1.32 ± 0.17</td>
</tr>
<tr>
<td>Femoral</td>
<td>0.83 ± 0.08</td>
</tr>
<tr>
<td>Iliac</td>
<td>1.29 ± 0.17</td>
</tr>
<tr>
<td>Mesenteric resistance</td>
<td>1.15 ± 0.15</td>
</tr>
<tr>
<td>Renal</td>
<td>0.73 ± 0.14</td>
</tr>
<tr>
<td>Superior Mesenteric</td>
<td>1.03 ± 0.21</td>
</tr>
</tbody>
</table>

* Statistically greater than the response to PBZ alone.
N.D., not determined.

Fig. 5. Effect of BMY 7378 (30 nM) on the inhibitory actions of CEC. Arteries were incubated in the presence of BMY 7378 alone, CEC alone, both BMY 7378 and CEC or vehicle, as detailed in the text. Each point represents the mean ± S.E.M. of four individual experiments in the aorta, six in the femoral artery, seven in the iliac artery, four in the mesenteric resistance artery and nine in the superior mesenteric artery. *, Statistically (P > .05) significant difference from the curve obtained in the presence of CEC alone.
1.6; NaHCO$_3$, 14.9; dextrose, 5.5 and Na$_2$EDTA, 0.03. Three-millimeter ring segments of aorta, caudal artery, femoral artery, iliac artery, renal artery, superior mesenteric artery and mesenteric resistance vessel (these were cut to 2 mm; 150–200-μm I.D.) were cut and cleaned of surrounding fat and connective tissue. The mesenteric resistance artery was isolated in the following manner: After removal of the entire mesenteric bed from its primary branching point at the abdominal aorta to the terminal arteriolar/capillary connections at the intestinal wall, a second-generation branch was isolated, cut free and cleaned. Stainless steel or platinum wires of an appropriate diameter were threaded through the lumen of each vessel. One wire was connected to a fixed base and the other to a micrometer clamp to adjust passive force on the tissues. The tissues were mounted in water-jacketed muscle baths containing PSS maintained at 37°C under constant oxygenation (95% O$_2$/5% CO$_2$, pH 7.4). Passive forces of 2 g (aorta), 0.25 g (mesenteric resistance artery) or 1 g (all other arteries) were then placed on the vessels. Previous studies have shown that these values for passive force give optimal agonist responses. Changes in force generation were recorded using Grass FT.03 force transducers connected to a Grass model 7 polygraph.

**Protection experiments.** Cumulative concentration-response curves to phenylephrine (1.0 nM to 100 μM) were generated in all vessels. The tissues were then washed repeatedly for a period of 1 hr

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Developed tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control alone</td>
</tr>
<tr>
<td>Aorta</td>
<td>1.18 ± 0.12</td>
</tr>
<tr>
<td>Femoral</td>
<td>0.73 ± 0.12</td>
</tr>
<tr>
<td>Iliac</td>
<td>1.35 ± 0.08</td>
</tr>
<tr>
<td>Mesenteric resistance</td>
<td>1.64 ± 0.13</td>
</tr>
<tr>
<td>Superior Mesenteric</td>
<td>0.85 ± 0.19</td>
</tr>
</tbody>
</table>

* Statistically greater response than CEC alone.
N.D., not determined.

---

**Fig. 6.** Effect of A-61603 (100 nM) on the inhibitory actions of PBZ. Arteries were incubated in the presence of A-61603 alone, PBZ alone, both A-61603 and PBZ or vehicle, as detailed in the text. Each point represents the mean ± S.E.M. of eight individual experiments in the aorta, 10 in the femoral artery, two in the iliac artery and two in the superior mesenteric artery.
pretreatment with protecting agents was carried out as follows: BMY 7378 (30 and 100 nM), A-61603 (100 nM) or vehicle was added and coincubated in the presence of PBZ alone. Protection from irreversible inactivation was defined as a statistically significant increase in the maximal response to phenylephrine obtained after treatment with a protecting agent compared with the maximal response obtained in the presence of PBZ or CEC alone. A-61603, the optically pure (R)-isomer, was a gift from Abbott Laboratories (Abbott Park, IL) and provided to us by Dr. Michael Meyer.

**Application of oligonucleotides.** Sense and antisense phosphorothioate oligonucleotides were synthesized by Dr. Dianne Perez (Department of Molecular Cardiology, Research Foundation, The Cleveland Clinic). The phosphorothioate backbone makes these nucleotides resistant to breakdown by nucleases. Fluorescently labeled analogs of these oligonucleotides were also made. The sequences for these oligonucleotides are sense, ACCATGGTGCTTCTC AAAATG- AATCCGGAT GAGAGCTTTTCCGA, and antisense, AGAGAGA- GCACCAT CAGATCGGGATTCAT GTCTCGGAAAGTCAT. The oligonucleotides were received as a dried pellet, resuspended in sterile water, and the concentration was determined spectrophotometrically.

**In vivo transfection.** A 40% w/v solution of pluronic F-127 gel (Sigma Chemical, St. Louis, MO) was prepared in water at 4°C by mixing on a platform shaker overnight in a cold room. F-127 pluronic gel solutions, which are liquid at 4°C, solidify at room temperature (Schmolka, 1972). Sufficient oligonucleotide was added to the cold gel solution to give the desired concentration (see below). The addition of oligonucleotide reduces the pluronic gel concentration to 30%. Care was taken to ensure that all pipette tips and storage tubes were kept at 4°C. Animals were anesthetized with a mixture of ketamine and acepromazine. After a surgical incision, the femoral or renal artery was located and gently cleaned of adhering tissue. Forty microliters of the pluronic gel/oligonucleotide solution were applied around the artery. After the gel solidified around the artery, it was gently placed back into its original position, and the wound was closed with surgical staples. The animal was then allowed to recover until use in experimentation. To assess the degree of uptake of the oligonucleotide by the smooth muscle, an FITC-labeled oligonucleotide was applied in the gel. The artery was removed 24 hr later and prepared for fluorescence microscopy.

**Statistical analysis.** Concentration-response curves represent the mean value of a minimum of two experiments on individual blood vessel segments, of which each was from a different animal. Only two concentration-response curves were run on any segment. A two-way analysis of variance followed by Student-Newman-Kuels analysis was used to determine where statistically significant differences existed between the various treatment conditions. In all figures, data are given as mean ± S.E.M. Asterisks indicate statistically significant difference at the P < .05 level.

**Results**

mRNA for the alpha-1B AR is widely distributed in the peripheral vascular system (Guarino et al., 1996; Piascik et al., 1995). An anti-peptide antibody prepared against the alpha-1B AR (Fonseca et al., 1995) was used to determine the extent to which this mRNA is expressed as receptor protein in a series of peripheral blood vessels. Photomicrographs of the immunofluorescent staining in the aorta, caudal, femoral, iliac, mesenteric resistance, renal and superior mesen-
Fig. 8. Effect of A-61603 (100 nM) on the inhibitory actions of CEC. Arteries were incubated in the presence of A-61603 alone, CEC alone, both A-61603 and CEC or vehicle, as detailed in the text. Each point represents the mean ± S.E.M. of three individual experiments in the aorta, four in the femoral artery, three in the iliac artery, two in the superior mesenteric artery and four in the mesenteric resistance artery.

TABLE 4

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Developed tension (g)</th>
<th>A-61603 alone</th>
<th>PBZ alone</th>
<th>A-61603 + PBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>1.21 ± 0.26</td>
<td>0.86 ± 0.12</td>
<td>0.27 ± 0.08</td>
<td>0.4 ± 0.12</td>
</tr>
<tr>
<td>Caudal</td>
<td>1.37 ± 0.28</td>
<td>1.47 ± 0.14</td>
<td>0.68 ± 0.12</td>
<td>1.29 ± 0.17</td>
</tr>
<tr>
<td>Femoral</td>
<td>1.18 ± 0.11</td>
<td>1.03 ± 0.01</td>
<td>0.12 ± 0.03</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>Iliac</td>
<td>1.11 ± 0.02</td>
<td>1.25 ± 0.35</td>
<td>0.31 ± 0.08</td>
<td>0.35 ± 0.035</td>
</tr>
<tr>
<td>Mesenteric resistance</td>
<td>1.85 ± 0.17</td>
<td>1.73 ± 1.8</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Renal</td>
<td>0.95 ± 0.13</td>
<td>1.04 ± 0.1</td>
<td>0.52 ± 0.6</td>
<td>0.7 ± 0.09</td>
</tr>
<tr>
<td>Superior Mesenteric</td>
<td>0.88 ± 0.06</td>
<td>1.05 ± 0.09</td>
<td>0.13 ± 0.09</td>
<td>0.2 ± 0.11</td>
</tr>
</tbody>
</table>

* Statistically greater response than PBZ alone.
artery (fig. 6). In contrast, the ligand significantly protected the caudal and renal arteries (fig. 7). A greater level of inhibition than that observed at 30 nM. In the presence of either 30 or 100 nM BMY 7378, no protection was noted in the mesenteric resistance artery. The maximal contractile response seen in the various CEC treatment conditions is presented in table 3.

A-61603 is an agonist that has been shown to exhibit a high degree of selectivity for the alpha-1A AR (Knepper et al., 1995). At 100 nM, A-61603 had no effect on the inhibitory actions of PBZ in the caudal, mesenteric resistance or renal arteries (fig. 4). Incubation of 100 nM BMY 7378 produced a greater level of protection in the aorta, femoral, iliac and superior mesenteric arteries, whereas no protective action was noted at this higher dose in the caudal, mesenteric resistance or renal arteries. The maximal level of the contractile response (in grams) for phenylephrine in the various treatment conditions is presented in table 2. The selective protection exerted by BMY 7378 in the aorta, femoral, iliac and superior mesenteric arteries suggests that the phenylephrine response is due to activation of the alpha-1D AR.

If this suggestion is correct, then we would expect similar results with CEC. Previous work has shown that neither the caudal nor renal arteries are sensitive to CEC inactivation (Han et al., 1990; Piascik et al., 1995). Therefore, these arteries were not examined in this series of studies. CEC alone produced varying degrees of inhibition in the test arteries. BMY 7378 (30 nM) protected the aorta, femoral, iliac and superior mesenteric arteries from CEC inactivation (fig. 5). Increasing the BMY 7378 concentration to 100 nM produced a greater level of inhibition than that observed at 30 nM. In the presence of either 30 or 100 nM BMY 7378, no protection was noted in the mesenteric resistance artery. The maximal contractile response seen in the various CEC treatment conditions is presented in table 3.

The Alpha-1B Receptor and Smooth Muscle
alpha-1B AR in regulating contraction of the blood vessels examined in this study. To further address this possibility, a novel approach with antisense oligonucleotides was used. We chose the femoral and renal arteries for these studies. The pluronic gel/antisense oligonucleotide mixture can be easily applied to these vessels. To demonstrate that this application technique does in fact deliver antisense oligonucleotides to smooth muscle cells, 150 μM of an FITC-labeled alpha-1D AR antisense oligonucleotide was applied to the femoral artery. Twenty-four hours later, an intense fluorescent signal was detected in the medial layer, indicating significant penetration of the oligonucleotide into the arterial smooth muscle (fig. 9). In a similar fashion, application of 250 μM FITC-labeled alpha-1A AR antisense oligonucleotide resulted in an intense fluorescence signal detected in the medial layer of the renal artery (data not shown). The application of a nonfluorescent phosphorothioate alpha-1D AR antisense oligonucleotide to the femoral artery also had no effect on the response of the artery to KCl or serotonin (fig. 10). Furthermore, an alpha-1D AR sense oligonucleotide had no effect on phenylephrine-induced contraction of the femoral artery (fig. 11B).

Fig. 9. Uptake of a fluorescent alpha-1D AR antisense oligonucleotide in the femoral artery. The oligonucleotide was applied in a F-127 pluronic gel as described in the text. Twenty four hours later, the artery was removed and prepared for microscopy.

In contrast, an antisense oligonucleotide directed against the translational start site of the alpha-1D AR significantly inhibited the femoral artery response to phenylephrine (fig. 11A). The application of 150 μM of an antisense oligonucleotide directed against the translational start site of the alpha-1B AR had no effect on the phenylephrine response in the femoral artery (fig. 11D). A similar result was observed when alpha-1A AR antisense oligonucleotides were applied to the femoral artery (fig. 11C).

When antisense oligonucleotides directed against the translational start site for the alpha-1B AR were applied to the renal artery, no inhibition of the phenylephrine response was observed (fig. 12C). The application of an antisense oligonucleotide directed against the alpha-1D AR also had no effect (fig. 12D). Similarly, sense oligonucleotides against the alpha-1A AR had no effect on the contractile actions of phenylephrine (fig. 12B). However, treatment with an antisense oligonucleotide directed against the translational start site of the alpha-1A AR significantly inhibited the response to phenylephrine in the renal artery (fig. 12A).

The inhibition of contraction produced by either antisense oligonucleotide was concentration dependent, with higher concentrations producing a greater level of inhibition than that observed at lower concentrations (fig. 13).
Discussion

Despite many significant advances in the regulatory biology of the alpha-1D AR subtypes, we have at best an incomplete understanding of the role played by these receptors in modulating physiological processes such as vascular smooth muscle contraction. Of the known alpha-1 ARs, alpha-1A and alpha-1D have most often been implicated in playing a role in regulating vascular smooth muscle tone (Guarino et al., 1996). In this report, we analyzed the contribution of the alpha-1B AR to the contraction of a series of peripheral arteries. mRNA for this receptor is widely distributed in peripheral arteries (Guarino et al., 1996). In terms of the level of mRNA expression for the entire alpha-1 AR subtype pool, the alpha-1B AR is expressed at much lower levels than the alpha-1A AR but at higher levels than the alpha-1D AR (Guarino et al., 1996; Piascik et al., 1995).

Using an antipeptide antibody, we detected specific alpha-1B AR immunoreactivity in the medial layer of all blood vessels examined. This indicates that in all arteries in which we can detect its mRNA, the alpha-1B AR is also expressed as protein. It is not known whether the expression of this receptor on vascular smooth muscle cells a priori indicates that the subtype plays a role in contraction or whether the alpha-1B AR can be expressed on blood vessels and not subserve a contractile function.

Classic and contemporary pharmacological approaches were used to determine the contribution of the alpha-1B AR to the contraction of the blood vessels under study. In the first series of experiments, we assessed the ability of subtype-selective ligands (BMY 7378 and A-61603) to protect a specific alpha-1D AR from inactivation by PBZ and CEC. The dissociation constant of BMY 7378 estimated in binding studies with a recombinant form of the alpha-1D AR or in functional studies is 0.9 to 4 nM (Goetz et al., 1995; Piascik et al., 1995). The dissociation constant for either the alpha-1A or the alpha-1B AR is 500 nM (Goetz et al., 1995; Piascik et al., 1995). Therefore, at 30 nM, BMY 7378 would occupy >90% of the alpha-1D population while occupying <6% of either the alpha-1A or alpha-1B ARs. BMY 7378 protected the aorta, femoral, iliac and superior mesenteric arteries from inactivation by either PBZ or CEC, supporting our contention that the main alpha-1 AR subtype involved in the contractile regulation of these vessels is the alpha-1D AR.

The lack of complete protection seen with BMY 7378 could be taken as evidence that in addition to the alpha-1D, other alpha-1 AR subtypes are involved in the contractile regula-

Fig. 11. Effect of a variety of oligonucleotides on the contractile action of phenylephrine in the femoral artery. The femoral artery was transfected with the indicated oligonucleotide and studied 24 hours later as described in the text. Each point represents the mean ± S.E.M. of 15, 30, 14 or 10 individual rings for the alpha-1D sense, alpha-1D antisense, alpha-1B antisense and alpha-1A antisense, respectively. * Statistically significant difference from the control dose-response curve.
tion of the aorta, femoral, iliac and superior mesenteric arteries. However, there are alternative explanations for the lack of complete protection. There is a dynamic interaction between the ligands and the receptors in these protection experiments. BMY 7378 is added first in concentrations sufficient to nearly saturate the \(\alpha\)-1D AR but have little occupancy at either the \(\alpha\)-1A or \(\alpha\)-1B ARs. The ligand freely dissociates and reassociates with the receptor. When PBZ or CEC is added, it will inactivate the unprotected \(\alpha\)-1A or \(\alpha\)-1B ARs as well as compete with BMY 7378 for the \(\alpha\)-1D AR. Once the alkylating agent acquires a receptor and covalent modification occurs, no dissociation from the receptor will take place. Therefore, the presence of BMY 7378 should be expected to retard, not prohibit receptor inactivation. Indeed, if the incubation period is sufficiently long, either alkylating agent would be expected to completely wipe out the \(\alpha\)-1D AR population. The way to enhance the degree of protection is to use very high concentrations of the protecting agent. This is not practical in this instance because as the concentration of BMY 7378 increases, the chances of \(\alpha\)-1A or \(\alpha\)-1B AR occupation also increase. Therefore, in these experiments, complete receptor protection was difficult to achieve. It should be noted that when the BMY 7378 concentration was increased to 100 nM, there was a greater level of protection in the aorta, femoral, iliac and superior mesenteric arteries without any protection noted in the other arteries (tables 2 and 4). Finally, the fact that two different alkylating agents gave the same results further strengthens the conclusions made with these studies.

In logic similar to that discussed above, we used the \(\alpha\)-1A AR-selective agonist A-61603 in protection studies with PBZ. It has an estimated dissociation constant at the cloned \(\alpha\)-1A AR of 30 nM. Therefore, at 100 nM A-61603, 80% of the \(\alpha\)-1A ARs would be occupied, whereas only 9% and 7% of the \(\alpha\)-1B and \(\alpha\)-1D AR populations, respectively, would be occupied by the agonist. A-61603 showed protective activity in the caudal and renal arteries. This indicates that the \(\alpha\)-1A AR is the major regulatory receptor in these arteries and agrees with previous work using other approaches (Han et al., 1990).

The protective action of A-61603 in the femoral artery was surprising and not consistent with other results discussed above and below. The modest degree of protection could indicate that a component of the response in the femoral artery is mediated by the \(\alpha\)-1A AR. In contrast, protection was not seen when A-61603 was used against inactivation by CEC. This would argue against a role for the \(\alpha\)-1A AR in

---

Fig. 12. Effect of a variety of oligonucleotides on the contractile action of phenylephrine in the renal artery. The renal artery was transfected with the indicated oligonucleotide and studied 24 hours later as described in the text. Each point represents the mean ± S.E.M. of eight, 16, six and eight individual rings for the \(\alpha\)-1A sense, \(\alpha\)-1A antisense, \(\alpha\)-1B antisense and \(\alpha\)-1D antisense, respectively. *, Statistically significant difference from the control dose-response curve.
regulating the femoral artery contraction. Furthermore, application of antisense oligonucleotides against the alpha-1A AR to the femoral artery also had no effect on phenylephrine-induced femoral artery contraction.

We appreciate that these types of experiments lead to indirect conclusions regarding alpha-1 AR regulation. However, certain hypotheses can be made and tested with antisense oligonucleotides. In control experiments, we demonstrated that a fluorescently labeled antisense oligonucleotide can be taken up and retained by either the femoral or renal arteries 24 hr after application in a pluron gel (fig. 9). We are in the process of quantifying the extent of oligonucleotide uptake using laser scanning confocal microscopy. Our preliminary results indicate that oligonucleotide uptake is very inefficient. The application of 150 μM to the outside of the vessel in pluron gel yields an estimated 4 μM intracellularly. The surgical procedure and application of antisense directed against the alpha-1D AR did not affect the contractile actions of KCl or serotonin. This indicates that neither our application technique nor the mere presence of a 15-residue oligonucleotide accounts for the inhibitory actions observed. If the predictions from the protection studies are correct, then application of antisense constructs directed against the alpha-1A or alpha-1B AR should have no effect on the response of the femoral artery to phenylephrine. This is exactly what was observed. In contrast, treatment with alpha-1D AR antisense oligonucleotides significantly decreased the femoral artery response to phenylephrine. These data support the conclusion that the alpha-1D but not the alpha-1A or alpha-1B ARs are involved in mediating the contraction of the femoral artery.

In the renal artery, application of antisense oligonucleotides directed against the start site for translation of the alpha-1B AR also had no effect on phenylephrine-induced smooth muscle contraction. A similar effect was noted for alpha-1D AR antisense oligonucleotides. The application of an alpha-1A AR sense oligonucleotide also had no effect on phenylephrine responsiveness. In contrast, the application of an alpha-1A AR antisense oligonucleotide significantly inhibited the phenylephrine response. Therefore, the inhibition of contractile function in the renal artery appears to be specific to the alpha-1A AR. These data provide independent evidence that this receptor is the dominant alpha-1 AR in the renal artery. Antisense oligonucleotide application did not result in a complete inhibition of contractile function. There are several possible reasons for this. The phosphorothioate oligonucleotides, while more stable than those containing phosphodiester linkages, still could be broken down by cellular nucleases, thus reducing the effective concentration. Higher concentrations of oligonucleotides may be necessary to achieve complete inhibition. In this regard, we did note a concentration-dependent inhibition (fig. 13) with either alpha-1A or alpha-1D AR antisense oligonucleotides.

This is the first demonstration of the use of antisense oligonucleotides delivered in vivo to inhibit alpha-1 AR responses. It is reasonable to suggest that we merely observed nonspecific effects due to surgery or introduction of foreign nucleotides into the smooth muscle and not specific blockade of the translation of alpha-1 AR protein. However, these studies with two different arteries demonstrated inhibition by a specific antisense construct that is predicted from pharmacological studies. A further argument for specificity is the fact that we implanted three antisense constructs each in the femoral and renal arteries. Only one of these, the alpha-1D in the femoral and the alpha-1A in the renal, inhibited phenylephrine contractility. If the inhibition was simply due to surgical manipulation or introduction of oligonucleotides, then we would expect every antisense construct to inhibit function. Despite a wide distribution of this receptor in the peripheral vasculature, the only artery in which we obtained evidence for an alpha-1B AR-mediated contraction was the mesenteric resistance artery. This is interesting because the main mesenteric artery is protected by BMY 7378. Therefore, it appears that the alpha-1A subtype regulating contraction differs along the mesenteric vascular bed. It may be that the alpha-1A and alpha-1D AR regulate the larger vessels, whereas the alpha-1B AR controls the smaller resistance vessels. In this regard, Leech and Faber (1996) demonstrated that alpha-1B AR mediates regulation of skeletal muscle venules. Alternatively, the alpha-1B AR may serve other regulatory functions. In recent work, Siwik and Brown (1996) showed that the alpha-1B AR regulates protein synthesis in cultured aortic smooth muscle cells. Finally, we have shown the feasibility of the in vivo application of antisense oligonucleotides to the femoral and renal arteries and that this
application results in a specific inhibition of alpha-1 AR subtype-mediated contractile responses.

References


Send reprint requests to: Michael T. Piascik, Ph.D., Associate Professor, Director, Vascular Biology Research Group, Department of Pharmacology, The University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY 40536. E-mail: mtp@pop.uky.edu