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Identification of the mRNA for the Novel α_{1D}-Adrenoceptor and Two Other α_1-Adrenoceptors in Vascular Smooth Muscle

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SUMMARY

In situ hybridization histochemistry, radioligand binding, and *in vitro* contractile studies were used to characterize the vascular distribution of the recently discovered α_{1D}-adrenoceptor. *In situ* hybridization with an antisense probe localized the mRNA for the α_{1D}-adrenoceptor to the medial layer of the rat aorta renal and mesenteric resistance arteries. If the tissues were first treated with RNase or a sense probe was used, no specific hybridization signal was detected. The extent to which this receptor was expressed as protein was assessed with radioligand binding studies. A series of ligands used to characterize α_1-adrenoceptors interacted with two sites labeled by [^{3}H]prazosin in homogenates from the aorta and mesenteric vascular bed. The high affinity site had the characteristics of an α_{1A}-adrenoceptor. However, the low affinity site had ligand binding characteristics distinct from those of the α_{1D}-adrenoceptor or any other known α_1-adrenoceptor subtype. mRNA for the α_{1B}- and α_{1C}-adrenoceptors was also detected in the aorta, renal arteries, and mesenteric resistance arteries. Chloroethylclonidine (CEC) (1 and 10 μM) had differential effects on phenylephrine-induced contractions of vascular smooth muscle. CEC completely inhibited the response in the aorta and caused a partial inhibition in the mesenteric resistance artery. The same concentrations of CEC had little effect on phenylephrine responses in the renal artery. The data suggest the following. 1) mRNA for the novel α_{1D}-adrenoceptor is localized in vascular smooth muscle. 2) Definitive identification of expression of this receptor was not possible; this may be related to coexpression of other subtypes of α_1-adrenoceptors. 3) mRNA for the α_{1C}-adrenoceptor was detected in rat peripheral vasculature. 4) The α_{1A}-adrenoceptor is also localized in the vasculature. 5) Three and possibly four α_1-adrenoceptors participate in vascular smooth muscle regulation.

α_1-Adrenoceptors associated with vascular smooth muscle play a vital role in the modulation of sympathetic nervous system activity and the regulation of arterial blood pressure (1). This receptor is a member of the superfamily of G protein-coupled receptors and shares the common structural motif of seven membrane-spanning regions and intracellular and extracellular binding domains (2). Four α_1-adrenoceptors, α_{1A} through α_{1D}, have been identified. The α_{1A}-adrenoceptor has been identified based solely on pharmacological properties (3, 4), whereas the α_{1B}- (5), α_{1C}- (6, 7), and α_{1D}-adrenoceptors (8) have been cloned. This poses the difficult question of which of these receptors plays a functional role in the regulation of the peripheral vascular system. To date the α_{1C}-adrenoceptor has not been identified in any rat peripheral tissue (6, 7). This would suggest that the potential regulatory receptors in blood vessels are the α_{1A}-, α_{1B}-, and/or α_{1D}-adrenoceptors.

There is good *in vitro* as well as *in vivo* evidence that the α_{1A}-adrenoceptor, which exhibits high affinity for catecholamines (3, 4), plays a prominent role in vascular regulation (9–15). The α_{1D}-adrenoceptor was cloned in 1991 by Perez *et al.* (8). This receptor is >60% homologous to either the α_{1B}- or α_{1C}-adrenoceptors. To date it has been studied only in model expression systems (16). Nothing is known about the vascular distribution and regulatory function of this newest member of the α_1-adrenoceptor family. This receptor has been difficult to study in mammalian systems, in part because there is no specific antagonist that clearly discriminates between the α_{1B}- and α_{1D}-adrenoceptors. For example, it has been shown (8, 17) that only a modest difference in affinity exists between these receptors for ligands such as WB 4101, 5-methylurapadil, phentolamine, and (+)-niguldipine, which are commonly used to define receptor subtypes. This would make differentiation by traditional radioligand binding techniques very difficult. Unlike the α_{1B}-adrenoceptor, Perez *et al.* showed the α_{1D}-adrenoceptor is only partially sensitive to CEC inactivation.

In this report we have taken a molecular biological approach to characterize the distribution of the α_1-adrenoceptor subtypes in rat vascular smooth muscle. *In situ* hybridization histochem-

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ABBREVIATIONS: CEC, chloroethylclonidine; SSC, saline sodium citrate.

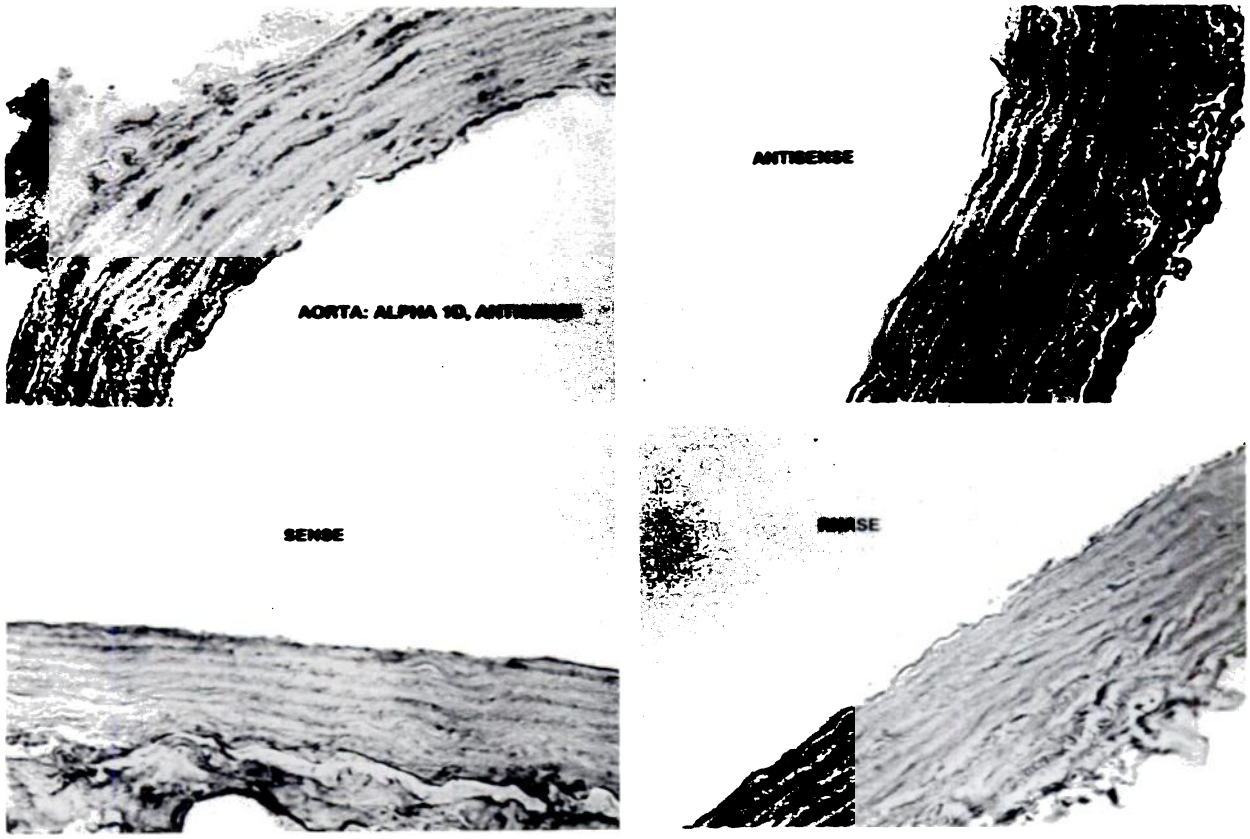

Fig. 1. Photomicrograph of the detection of the mRNA for the α_{1D}-adrenoceptor in rat aorta using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 368-base antisense probe for the mRNA for the α_{1D}-adrenoceptor was hybridized to frozen sections of the aorta. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 232-base sense probe for the α_{1D}-adrenoceptor.

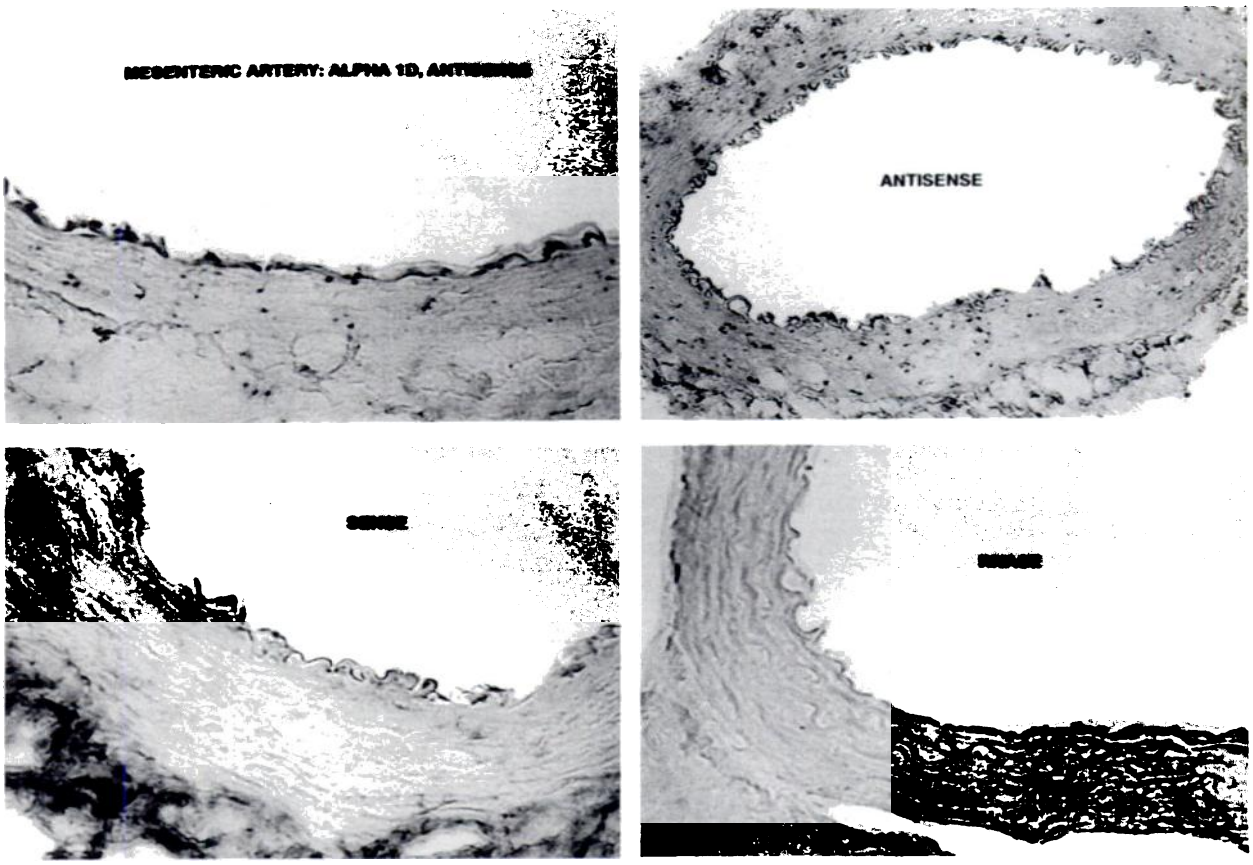

Fig. 2. Photomicrograph of the detection of the mRNA for the α_{1D}-adrenoceptor in rat mesenteric resistance artery using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 368-base antisense probe for the mRNA for the α_{1D}-adrenoceptor was hybridized to frozen sections of the artery. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 232-base sense probe for the α_{1D}-adrenoceptor.

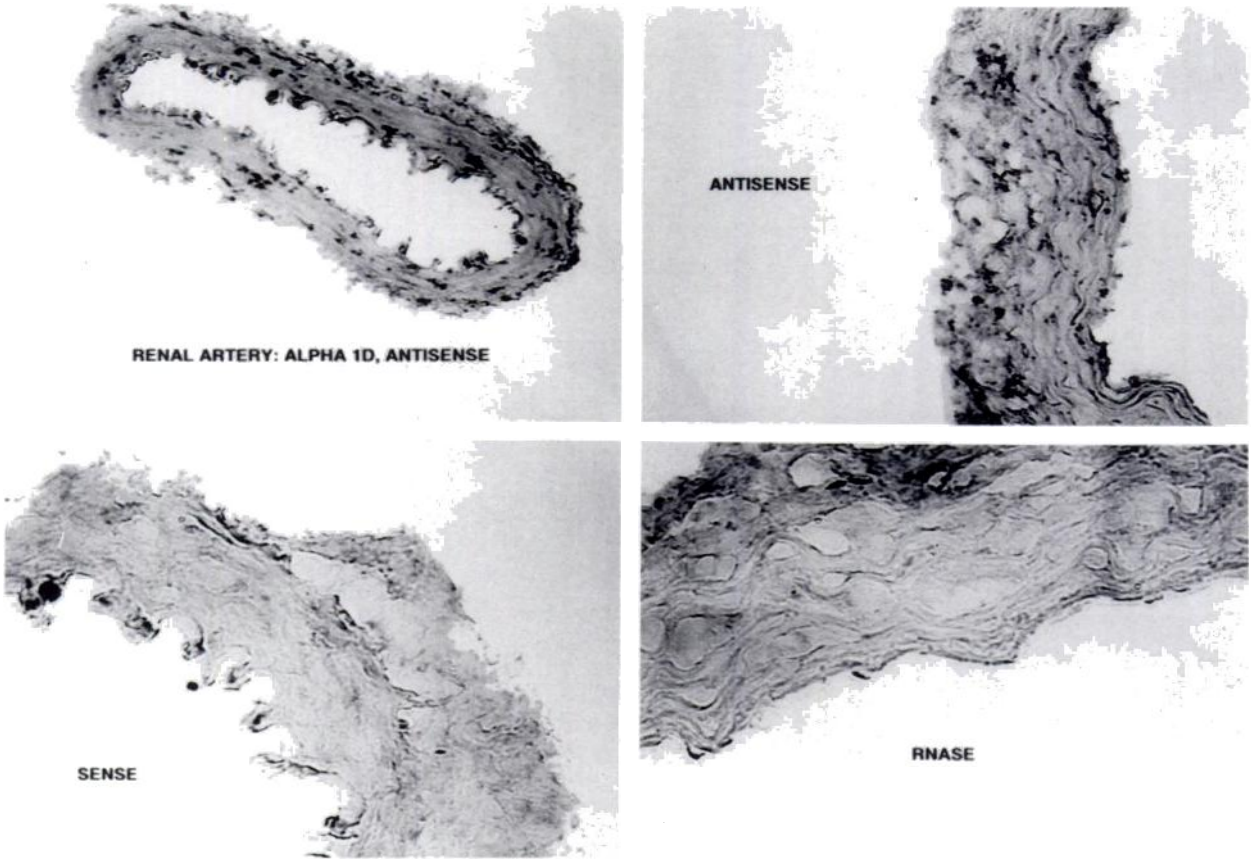

Fig. 3. Photomicrograph of the detection of the mRNA for the α_{1D}-adrenoceptor in rat renal artery using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 368-base antisense probe for the mRNA for the α_{1D}-adrenoceptor was hybridized to frozen sections of the renal artery. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 232-base sense probe for the α_{1D}-adrenoceptor.

TABLE 1

Characterization of [^{3}H]prazosin sites in aorta and mesenteric artery

Experiments were carried out as described in the text. Data are equilibrium dissociation constants for the ability of ligands to displace [^{3}H]prazosin from sites on aorta or mesenteric vascular bed membranes. Values are the mean and 95% confidence limits for two to four experiments, in triplicate, on different membrane preparations.

Displacing ligand	K_d						
	Aorta		Mesenteric artery		Liver	Cloned hamster α_{1B}[a]	Cloned rat α_{1D}[a]
	K_H	K_L	K_H	K_L			
	nM						
WB 4101	0.039 (0.015–0.110)	11.8 (5.2–28.2)	0.025 (12–50)	9.5 (3.6–24.7)	4.2 (3.2–5.5)	5.9	1.9
5-Methylurapadil	0.150 (0.014–2,900)	108 (74–159)	0.192 (0.094–397)	226 (136–539)	41 (31.6–54.1)	41	15
Phentolamine	0.213 (0.054–1,517)	179 (112–278)	0.163 (0.079–679)	578 (336–1,036)	22 (20.6–25.1)	82	138
(−)-Norepinephrine	63.4 (8.9–495)	44,000 (62–214,000)	107 (44–260)	40,000 (16.4–100,000)	1,400 (1,000–1,900)	1,210	320

[a] Data taken from Ref. 8.

istry detected the mRNA for the α_{1D}-adrenoceptor in the medial layer of the aorta, renal arteries, and mesenteric resistance arteries. Radioligand binding studies detected the α_{1A}-adrenoceptor in homogenates from aorta and the mesenteric vascular bed. A low affinity site with properties similar to those of the α_{1D}-adrenoceptor was also detected in these smooth muscle homogenates. However, based on the ligand binding characteristics we cannot conclude with certainty that this represents expression of the α_{1D}-adrenoceptor. The inability to clearly identify the α_{1D}-adrenoceptor may be due to coexpression of the α_{1B}- and α_{1C}-adrenoceptors, the mRNAs for which were also detected in vascular smooth muscle. This is the first report demonstrating the localization of the α_{1C}-adrenoceptor in rat peripheral tissues. Phenylephrine-induced contractions of the aorta, renal arteries, and mesenteric resistance arteries showed different sensitivities to CEC. This spectrum of sensitivity suggests that the α_1-adrenoceptor responsible for activating muscle contraction differs in these blood vessels.

Materials and Methods

Riboprobe design. The cDNAs for the α_{1B}-, α_{1C}-, and α_{1D}-adrenoceptors were cloned into the multiple cloning site of pBS KSII. The

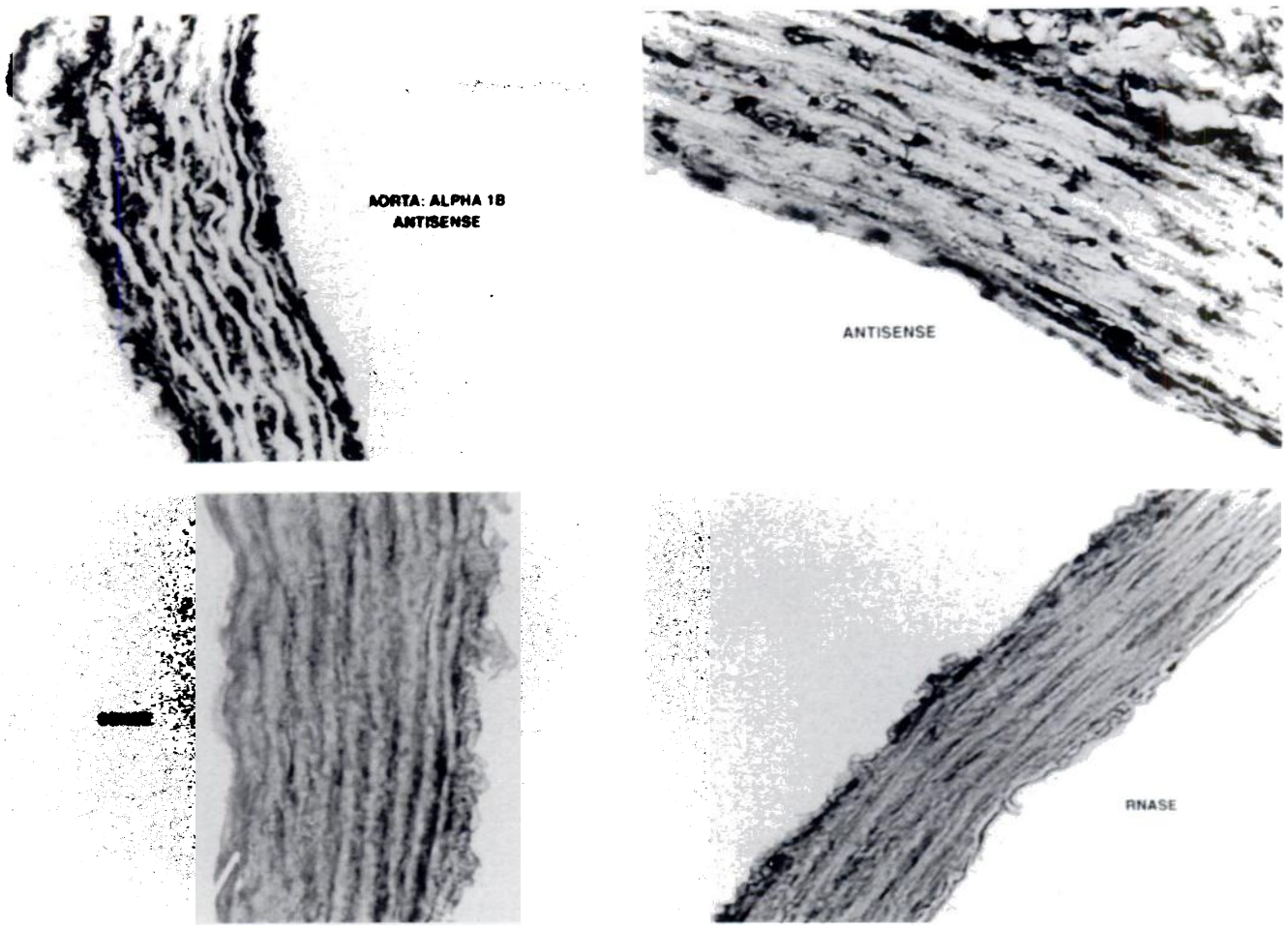

Fig. 4. Photomicrograph of the detection of the mRNA for the α_{1B}-adrenoceptor in rat aorta using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 262-base antisense probe for the mRNA for the α_{1B}-adrenoceptor was hybridized to frozen sections of the aorta. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 162-base sense probe for the α_{1B}-adrenoceptor.

rat α_{1D}-adrenoceptor was cloned in the *Eco*RI-*Not*I sites, whereas the human α_{1B}-adrenoceptor was cloned in the *Eco*RI-*Kpn*I sites. The full length rat α_{1C} cDNA was also cloned into the *Eco*RI-*Not*I sites. A sense probe for the α_{1D}-adrenoceptor mRNA was synthesized by first cutting the plasmid with *Sac*I. Transcription with T3 RNA polymerase yielded a 232-base probe. The α_{1D} antisense probe was constructed by first cutting the cDNA-containing plasmid with *Sma*I. Transcription of this linearized plasmid with T7 RNA polymerase yielded a 368-base probe. To synthesize a sense probe for the α_{1B}-adrenoceptor the plasmid containing the full length α_{1B} cDNA was cut with *Nco*I. Transcription with T7 RNA polymerase yielded a 262-base probe. To make an α_{1B} antisense probe, the plasmid was first cut with *Not*I. Transcription of this linearized DNA with T3 RNA polymerase yielded a 162-base probe. A sense probe for the α_{1C}-adrenoceptor mRNA was synthesized by first cutting the plasmid with *Xma*III. Transcription with T7 RNA polymerase yielded a 372-base sense probe. A 477-base α_{1C} antisense probe was constructed by first cutting the cDNA-containing plasmid with *Dra*I, followed by transcription with T3 RNA polymerase. All antisense probes were made to the carboxyl-terminal tail of the receptors. There is virtually no sequence homology between the three receptors in this region (8).

Preparation of biotinylated probes for the α_1-adrenoceptor subtypes. Biotinylated riboprobes for the α_{1B}, α_{1C}, and α_{1D} mRNAs were synthesized using a RNA-labeling system from GIBCO BRL (catalogue no. 8093SA). Reactions contained transcription buffer, 5 mM dithiothreitol, 1 mM unlabeled ribonucleotides, 2 mM Biotin-14-CTP, human placental RNase inhibitor, T3 or T7 RNA polymerase, and linearized DNA template (see above). Transcription was allowed to proceed for 2.5 hr before being stopped by the addition of EDTA. Biotinylated riboprobes were separated from unincorporated nucleotides by centrifugation through Sephadex G-50 columns and were precipitated with ethanol. The final pellet was solubilized with 2× *in situ* hybridization buffer (4× SSC (sodium chloride 600 mM, sodium citrate 60 mM), 0.2 M sodium phosphate, 2× Denhardt's solution, 0.1 mg/kg sodium azide). The final probe concentration was determined by quantitating absorbance at a wavelength of 260 nm.

***In situ* hybridization histochemistry.** Frozen 12-μm sections of aorta, renal arteries, and mesenteric resistance arteries obtained from male Sprague-Dawley rats were prepared and stored at −70° until the day of the experiment. Ten to 15 blood vessel sections were routinely mounted on each slide. On the day of experimentation, sections were fixed with 4% paraformaldehyde, rinsed with 2× SSC, and then treated with proteinase K (15 μg/ml) for 5 min at 25°. In certain experiments blood vessel sections were incubated with 100 μg/ml ribonuclease A for 1 hr at 37°, followed by two rinses with 2× SSC. The tissue was then acetylated by reaction with an 0.25% acetic anhydride/0.1 M triethanolamine, pH 8, mixture for 10 min at 25°. After acetylation, the tissue was dehydrated through increasing concentrations (50, 70, 95, and 100%) of ethanol and was finally delipidated by a 5-min incubation with chloroform. The sections were allowed to air-dry for 2 hr before hybridization. Hybridization solution contained 2× SSC, 0.1 M sodium phosphate, 1× Denhardt's solution, 10% dextran, 50% formamide, placental RNase inhibitor, and the appropriate riboprobe (80–150 ng/μl). The hybridization solution was applied directly to each slide, followed by careful placement of a coverslip. Rubber cement was placed around the coverslip to retain hydration. Hybridization was allowed to

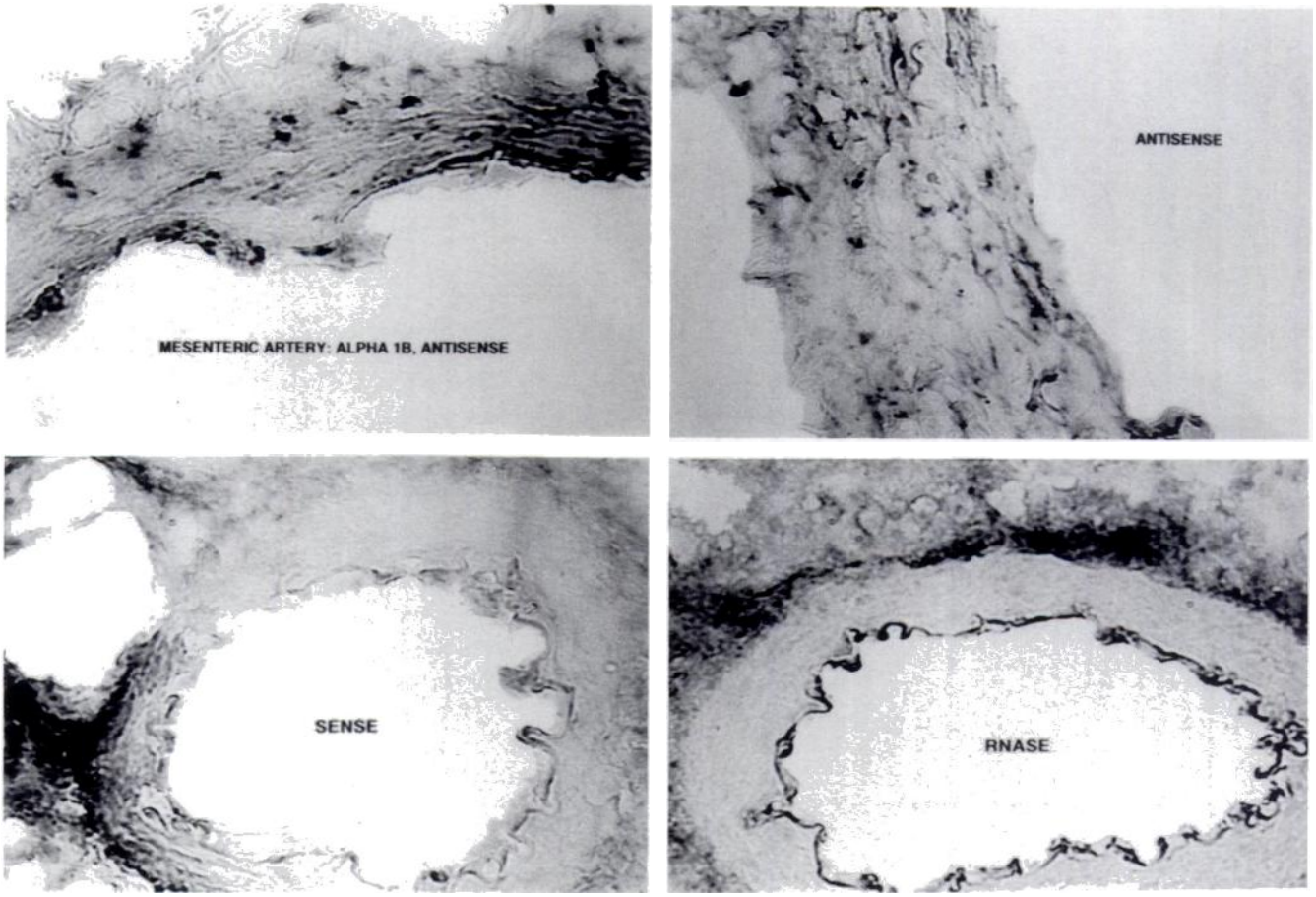

Fig. 5. Photomicrograph of the detection of the mRNA for the α_{1B}-adrenoceptor in rat mesenteric resistance artery using *in situ* hybridization histochemistry. *Top row of photomicrographs*, an 262-base antisense probe for the mRNA for the α_{1B}-adrenoceptor was hybridized to frozen sections of the artery. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 162-base sense probe for the α_{1B}-adrenoceptor.

proceed for 18–20 hr. After this time the coverslips were carefully removed and the slides were washed five times with 0.2× SSC. Detection of a cRNA-mRNA hybrid was accomplished by using a commercially available *in situ* hybridization and detection system (GIBCO/BRL catalogue no. 8250SA). This system uses alkaline phosphatase bound to strepavadin to deposit a colored, insoluble, reaction product at the site of a cRNA-mRNA hybrid. After the slides were washed and treated with a blocking solution, the blood vessel sections were exposed to the strepavidin-alkaline phosphatase conjugate for 15 min at 25°. After rinsing with Tris-buffered saline, pH 7.5, the slides were incubated with the alkaline phosphatase substrates nitroblue tetrazolium (0.3 mg/ml) and 4-bromo-5-chloro-3-indolylphosphate (0.166 mg/ml). The incubation time varied according to the tissue being probed. The reaction was stopped by rinsing with water and then dehydration with increasing concentrations of alcohol. The slides were coverslipped in preparation for microscopic examination.

Smooth muscle membrane preparation and radioligand binding studies. Membranes were prepared from the rat aorta and mesenteric vascular bed by techniques previously established in the laboratory for bovine aortic smooth muscle (18) and applied to the study of α_1-adrenoceptors in rat aorta (19). Membranes were also prepared from rat liver by centrifugation through Percoll gradients, as described by Pripic *et al.* (20). The ability of a series of ligands (see Table 1) to inhibit [^{3}H]prazosin binding to sites on smooth muscle membranes was assessed in a 500-μl volume, which contained 50 mM Tris buffer, pH 7.5, 5 mM $MgCl_2$, 0.1% ascorbic acid, 200 μl of membrane proteins, [^{3}H]prazosin, and increasing amounts of inhibiting agent. The concentration of prazosin was fixed at approximately its dissociation constant for the α_1-adrenoceptor (50–150 pM). Specific binding was determined in the presence of 10 μM phentolamine. The binding reaction was allowed to proceed for 30 min at 25° and was then terminated by rapid filtration onto Whatman GF/B filters. The filters were washed four times with 4 ml of ice-cold sodium phosphate buffer. The amount of radioactivity retained on the filters was quantitated by liquid scintillation counting.

***In vitro* assessment of contractile activity.** Anesthetized male Sprague-Dawley rats were euthanized and the thoracic aorta, mesenteric vascular bed, and renal arteries were quickly excised and placed in cold physiological saline solution of the following composition (in mM): NaCl, 130; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 \cdot 7H_2O$, 1.17; $CaCl_2 \cdot 2H_2O$, 1.6; $NaHCO_3$, 14.9; dextrose, 5.5; Na_2EDTA, 0.03. Ring segments of aorta (4 mm), renal artery (3 mm), and mesenteric resistance vessel (2 mm, ~150–200-μm i.d.) were cut and cleaned of surrounding fat and connective tissue. These segments were cut from the same area as those sections taken for *in situ* hybridization studies. Stainless steel or platinum wires of 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 physiological saline solution maintained at 37° under constant oxygenation (95% O_2/5% CO_2, pH 7.4). Passive forces of 2, 1, and 0.25 g were placed on the aorta, renal arteries, and mesenteric resistance vessels, respectively. Changes in force generation were recorded using a Grass FT.03 force transducer connected to a Grass model 7 polygraph. After an

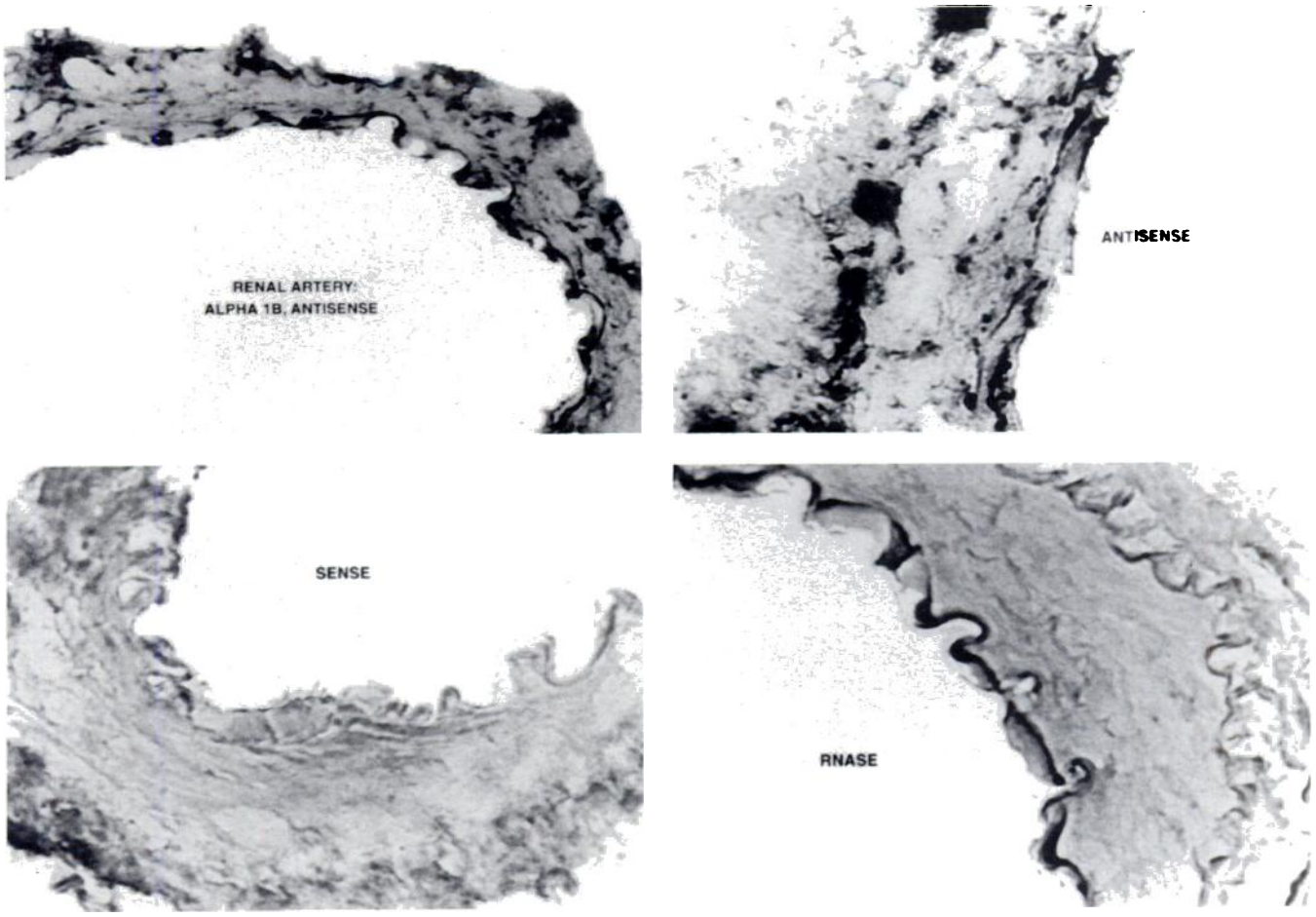

Fig. 6. Photomicrograph of the detection of the mRNA for the α_{1B}-adrenoceptor in rat renal artery using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 262-base antisense probe for the mRNA for the α_{1B}-adrenoceptor was hybridized to frozen sections of the artery. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 162-base sense probe for the α_{1B}-adrenoceptor.

equilibration period of 60–90 min, a cumulative concentration-response curve for phenylephrine (10^{-9} to 10^{-4} M) was generated in all vessels. The tissues were then washed repeatedly for a period of 30 min to allow the vessels to restabilize at their respective base-line passive forces. The tissues were then incubated with various concentrations of CEC for 30 min, after which time the antagonist was washed out repeatedly for an additional 30-min period. A concentration-response curve for phenylephrine was generated after the wash-out period.

Statistical analysis. Binding data for the ability of a series of compounds to inhibit [^{3}H]prazosin binding to sites on smooth muscle homogenates were analyzed with the iterative curve-fitting program LIGAND. Data were fit first to a one-site model and then to a two-site model. A two-site model was accepted only if it was a statistically better fit. The equilibrium dissociation constants for the ligands obtained from this curve fitting are presented in Table 1. *In vitro* contractile data are expressed as the mean and standard error and were analyzed by one-way analysis of variance.

Results

A biotinylated probe was used to assess the distribution of the mRNA for the novel α_{1D}-adrenoceptor in blood vessels. mRNA for the α_{1D}-adrenoceptor was detected in the medial layer of the aorta, renal arteries, and mesenteric resistance arteries. Examples of the cRNA-mRNA hybrids are presented in Figs. 1-3. Evidence that the alkaline phosphatase reaction products shown correspond to specific hybridization of the biotinylated probe to α_{1D} mRNA is also found in Figs. 1–3. In these control experiments, no signal was observed when the RNA within the blood vessel rings was destroyed by digestion with ribonuclease A before hybridization. Probing with a sense probe for α_{1D} mRNA also did not give a hybridization signal.

Radioligand binding studies were then performed to determine the extent to which this mRNA is expressed as protein. Studies were carried out in the aorta and mesenteric vascular bed. Binding studies were not performed in renal artery because of the quantities of tissue required to carry out adequate receptor characterization. Analysis of the data for a series of ligands used to define α_1-receptor subtypes revealed that all ligands interacted with two sites labeled by [^{3}H]prazosin (see Table 1). One site exhibited subnanomolar affinity for the antagonist ligands and nanomolar affinity for (−)-norepinephrine. A table listing equilibrium dissociation constants for the interaction of all test compounds used in this study with the four α_1-adrenoceptor subtypes was provided by Perez *et al.* (Table 3 in Ref. 8). The calculated dissociation constants for interactions at the high affinity site are consistent with those for interactions at the α_{1A}-adrenoceptor subtype and indicate that this subtype is located in vascular tissue. The identity of the low affinity site detected in the aorta and mesenteric vascular bed is problematic. For this site to be definitively identified as α_{1D}, it should have ligand binding characteristics consistent with those of this

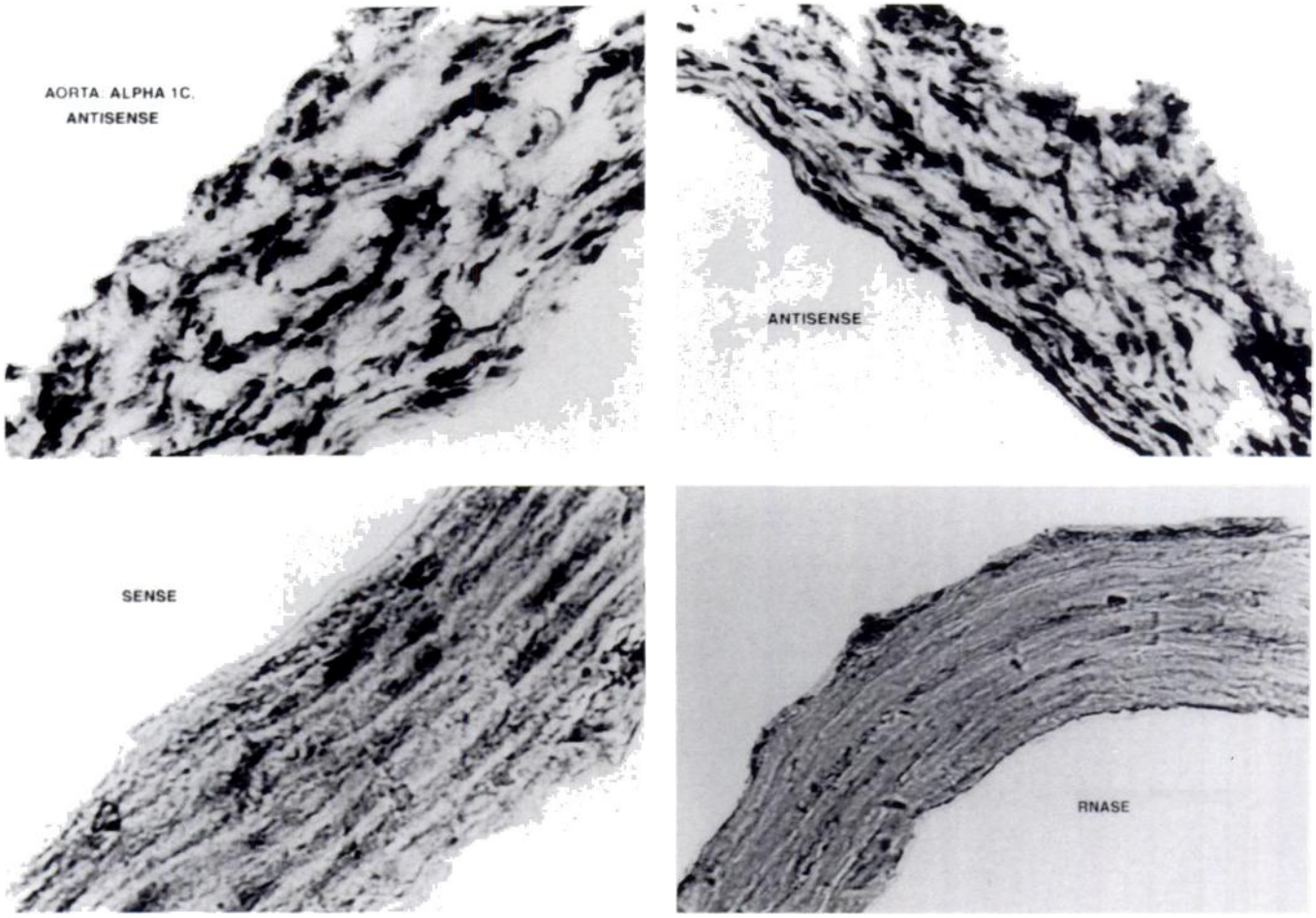

Fig. 7. Photomicrograph of the detection of the mRNA for the α_{1C}-adrenoceptor in rat aorta using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 477-base antisense probe for the mRNA for the α_{1C}-adrenoceptor was hybridized to frozen sections of the aorta. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 372-base sense probe for the α_{1C}-adrenoceptor.

receptor. An example of the ligand binding characteristics of the cloned α_{1D}-adrenoceptor expressed in COS-1 cells is presented in Table 1. Whereas the calculated dissociation constants for the low affinity site in aorta and mesenteric vascular bed are similar to those obtained with the cloned α_{1D}-adrenoceptor, differences do exist. For example, the affinities for phentolamine and 5-methylurapadil in the cloned α_{1D} system are 15 and 138 nM, respectively. Values for these same ligands are 5–10-fold higher in smooth muscle homogenates. The other α_1-adrenoceptor subtype that exhibits low affinity for these ligands is the α_{1B}-adrenoceptor. However, the properties of the low affinity site of the vascular homogenates do not correspond to results with the cloned α_{1B}-adrenoceptor (see Table 1). In contrast, our binding data from rat liver clearly indicate that this site has properties similar to those of the cloned α_{1B}-adrenoceptor.

The inability to clearly identify the α_{1D}-adrenoceptor in vascular homogenates could be due to coexpression of other α_1-adrenoceptors. Previous work has shown that the mRNA for the α_{1B}-adrenoceptor can be detected in rat aorta (21, 17). mRNAs for both α_{1B}- and α_{1C}-adrenoceptors were also detected in the aorta, renal arteries, and mesenteric resistance arteries (see Figs. 4-9). Specificity of the hybridization signal is supported by the fact that hybridization with a sense probe or prior treatment with RNase produced no hybridization signal.

Another test for the functional expression of the α_1-adrenoceptor subtypes is to examine the contraction of isolated vascular smooth muscle segments. These contractile studies were performed on areas similar to those used for *in situ* hybridization. Phenylephrine induced contractions in the isolated aorta, renal arteries, and mesenteric resistance arteries (Fig. 10). CEC (10 μM) completely blocked the aortic response to phenylephrine. At the same concentrations of CEC the renal artery was minimally affected, whereas the phenylephrine response in the mesenteric resistance artery was partially sensitive to this alkylating agent.

Discussion

Pharmacological and molecular cloning studies have expanded our understanding of the nature and number of adrenergic receptors well beyond the original subdivision of α and β proposed by Ahlquist (22). Indeed, it now appears that there are at least three β-adrenoceptors and eight α-adrenoceptors (four α_1 and four α_2). The α_1-adrenoceptors are used by the sympathetic nervous system to regulate peripheral vascular function and systemic arterial blood pressure. Current research indicates that all known α_1 subtypes can potentially utilize the same intracellular signaling pathways (15). A vital question is the physiological significance of having four similarly struc-

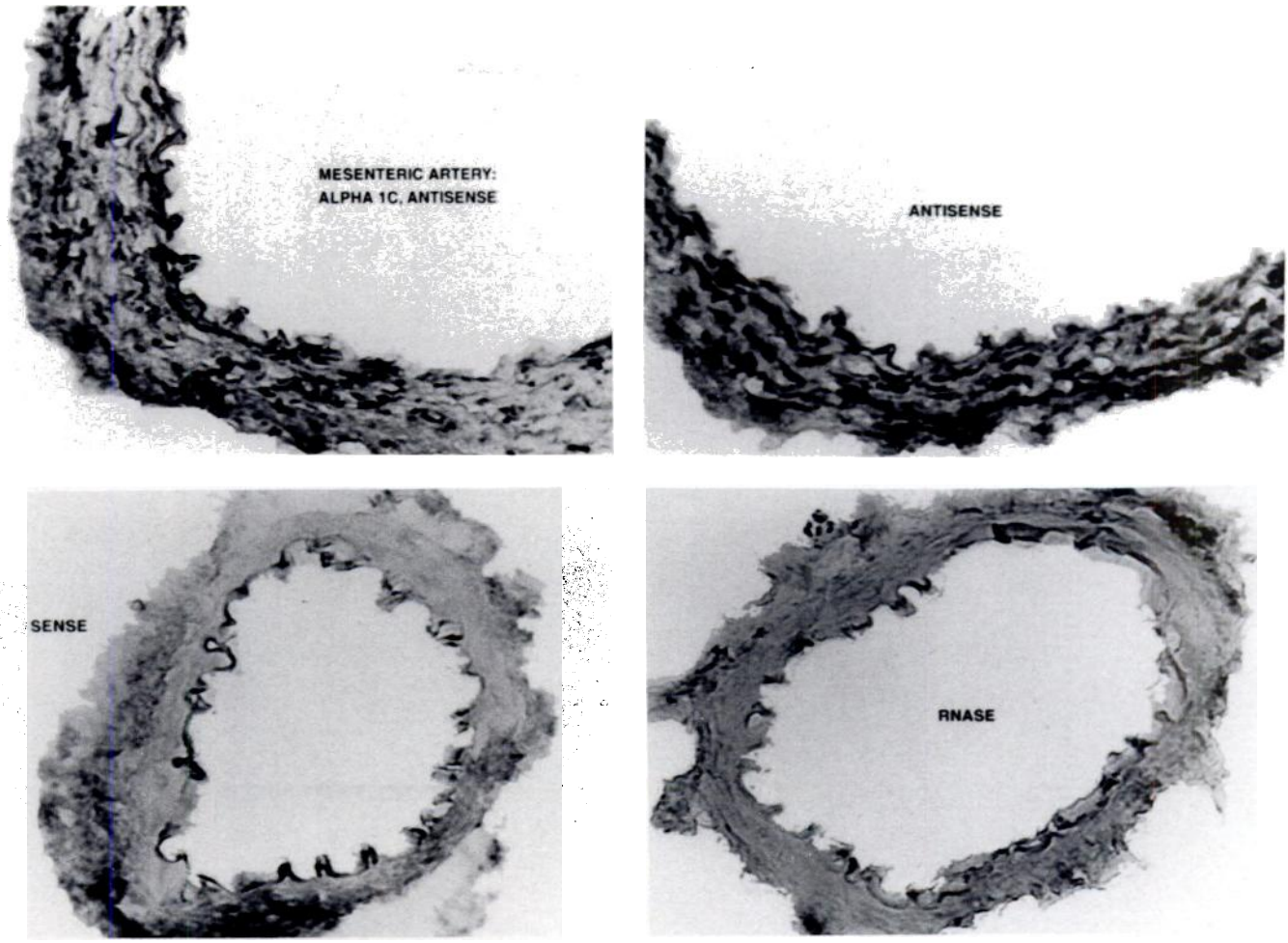

Fig. 8. Photomicrograph of the detection of the mRNA for the α_{1C}-adrenoceptor in rat mesenteric resistance artery using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 477-base antisense probe for the mRNA for the α_{1C}-adrenoceptor was hybridized to frozen sections of the artery. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 372-base sense probe for the α_{1C}-adrenoceptor.

tured receptors that utilize the same intracellular signal. An equally important issue is whether all α_1-adrenoceptors are distributed in vascular smooth muscle and the extent to which each subtype participates in blood pressure regulation. *In vitro* studies from several laboratories have clearly shown that multiple α_1-adrenoceptors can regulate the contraction of vascular smooth muscle (9, 11–14, 23). Data from conscious unrestrained rats indicate that more than one α_1-adrenoceptor participates in the regulation of blood flow in the gut, hindlimb, and kidney, as well as systemic arterial blood pressure (10, 12, 24, 25). These *in vitro* and *in vivo* studies clearly suggest that one of the α_1-adrenoceptors that plays a prominent role in peripheral vascular regulation is the α_{1A}. The identity of the other α_1-adrenoceptor(s) involved in vascular regulation is less certain. Studies have shown that CEC can inhibit the contraction of vascular smooth muscle, which has led to the suggestion that the α_{1B}-adrenoceptor is involved in vascular regulation. However, this conclusion was made before the discovery of the α_{1D}-adrenoceptor and its partial sensitivity to CEC (8) and our present observations that the α_{1C} mRNA can be localized to peripheral blood vessels. Therefore, the contribution of CEC-sensitive α_1-adrenoceptors to the overall regulation of the cardiovascular system by the α_1-adrenoceptor family is not well understood.

Using *in situ* hybridization histochemistry, we have shown that the mRNA for the most recently discovered α_1-adrenoceptor, the α_{1D}-adrenoceptor, can be detected in the aorta, renal arteries, and mesenteric resistance arteries. Detection of mRNA for the α_{1D}-adrenoceptor in renal and mesenteric resistance arteries, blood vessels that play a prominent role in regulating systemic vascular resistance and blood pressure, suggests, but by no means proves, that this subtype is involved in peripheral vascular regulation.

Although detection of this mRNA species is important, it is also critical to determine the extent to which the message is expressed as protein. To this end, radioligand binding studies were performed with a series of ligands with known affinities for each of the α_1-adrenoceptor subtypes. All ligands interacted with two sites labeled by [^{3}H]prazosin in membrane homogenates prepared from the aorta and mesenteric vascular bed. The calculated affinities for WB 4101, 5-methylurapadil, phentolamine, and (−)-norepinephrine at the high affinity site are consistent with those for interactions at an α_{1A}-adrenoceptor (see Table 3 in Ref. 8). Detection of an α_{1A}-adrenoceptor in vascular tissue supports the suggestion that this receptor plays an important role in vascular regulation. An additional argument for the role of this receptor is the fact that it exhibits

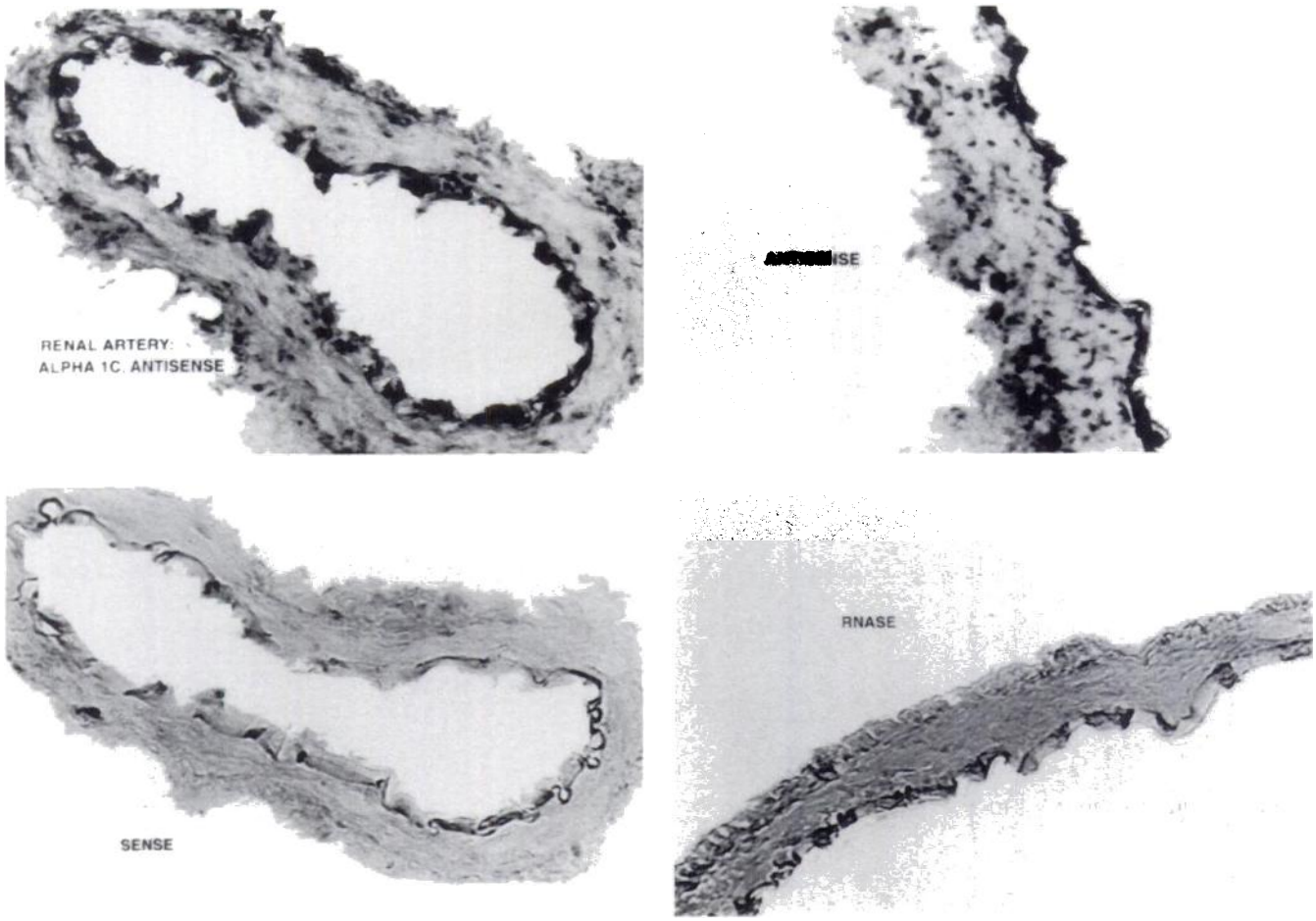

Fig. 9. Photomicrograph of the detection of the mRNA for the α_{1C}-adrenoceptor in rat renal artery using *in situ* hybridization histochemistry. *Top row of photomicrographs*, a 477-base antisense probe for the mRNA for the α_{1C}-adrenoceptor was hybridized to frozen sections of the artery. Biotinylated cRNA-mRNA hybrids were detected as described in Materials and Methods. *Bottom row of photomicrographs*, control experiments in which the mRNA was destroyed with RNase treatment or the hybridization experiment was carried out using a 372-base sense probe for the α_{1C}-adrenoceptor.

high affinity (63.4 nM) for norepinephrine, the endogenous neurotransmitter for the sympathetic nervous system.

It is tempting to conclude that the low affinity site is the α_{1D}-adrenoceptor. Indeed, it does have properties related to the α_{1D}-adrenoceptor expressed in COS-1 cells. For example, it exhibits low affinity for the inhibiting ligands. Furthermore, the rank order of potency for inhibition in smooth muscle homogenates agrees with the order obtained in the cloned receptor system. However, differences do exist. For the unambiguous conclusion that the low affinity site in blood vessel homogenates is the α_{1D}-adrenoceptor (or, for that matter, any receptor), it should have binding characteristics that make its identification clear. Examination of the data presented in Table 1 unfortunately show that this is not the case. There is at least a 5–10-fold difference in affinity for the low affinity site in homogenates, compared with the cloned α_{1D}-adrenoceptor. Therefore, we cannot conclude with certainty that the low affinity site detected in the aorta and mesenteric vascular bed represents expression of the α_{1D} mRNA. The other receptor that exhibits low affinity for the ligands is the α_{1B}. The low affinity smooth muscle site also has binding properties distinct from those of the α_{1B}-adrenoceptor expressed in COS-1 cells or the liver α_1-adrenoceptor (which is thought to be mainly an α_{1B} system). We do not understand why a clear identification of this low affinity site cannot be made. It does not appear to be due to experimental error. This is because in our hands affinity estimates for the rat liver α_{1B}-adrenoceptor agree with data from the cloned receptor system and a summary of values taken from the literature (see Table 1 herein and Table 3 in Ref. 8). Therefore, although we have detected a low affinity α_1-adrenoceptor in vascular smooth muscle, it does not have properties of other known α_1-adrenoceptors.

A potential explanation for the inability to identify the low affinity site could be the coexpression of additional α_1-adrenoceptors. Previous work using Northern blots has shown that the mRNA for the α_{1B}-adrenoceptor can be detected in the aorta (21, 17). In the present study we show that we can detect the α_{1B}- as well as the α_{1C}-adrenoceptor mRNA in all test blood vessels. Expression of these receptors could account for our inability to clearly identify the low affinity site in smooth muscle. We do know that the α_{1A}-adrenoceptor is expressed in smooth muscle. Expression of the α_{1B}-, α_{1C}-, and α_{1D}-adrenoceptors could result in four receptors clustered on the cell surface in such a way that the properties of these receptors are different from those observed if the receptors exist as single entities in the membranes or in model expression systems. An alternative explanation is that the low affinity site represents another α_1-adrenoceptor subtype specific to vascular smooth muscle.

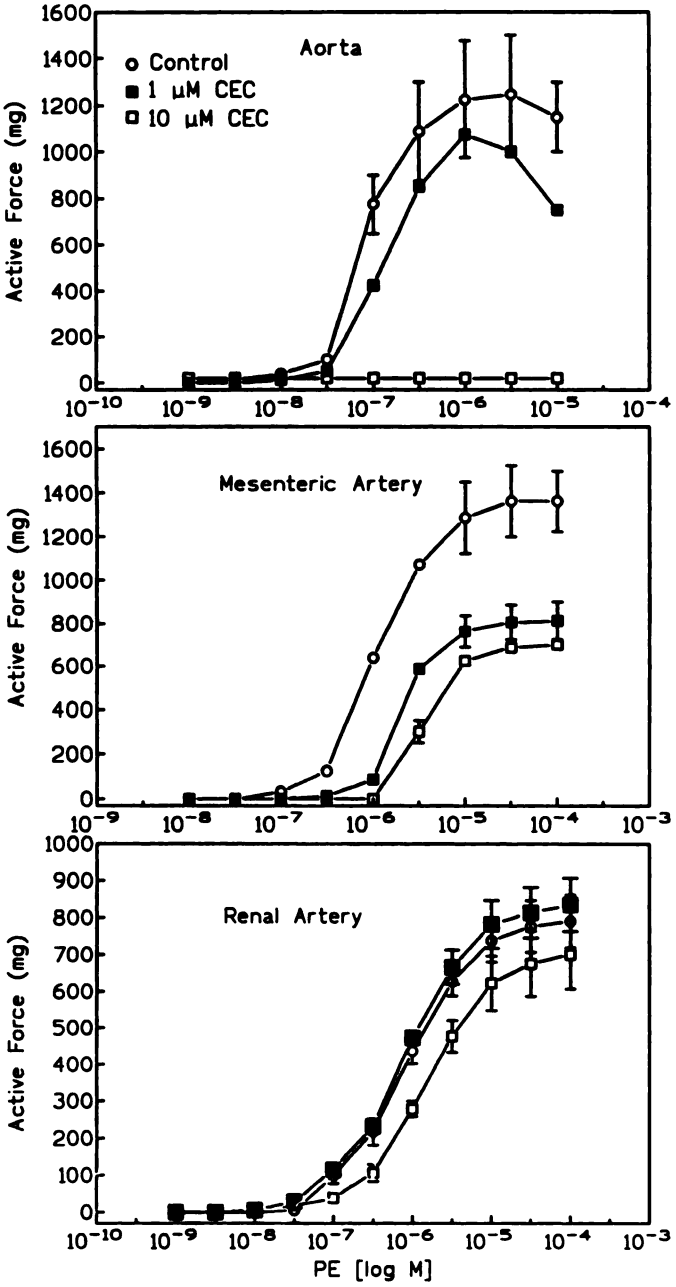

Fig. 10. Phenylephrine responsiveness of the aorta, mesenteric resistance arteries, and renal arteries in the presence and absence of CEC. Cumulative concentration-response curves for phenylephrine (*PE*) were prepared before and after treatment with the indicated concentrations of CEC, as described in Materials and Methods. Each *point* represents the mean ± standard error of experiments on three to six rings, each from a different animal.

Using Northern blot analysis, Schwinn *et al.* (6, 7) reported that the α_{1C}-adrenoceptor could not be localized to rat peripheral tissue. It is known that messages for the α_1-adrenoceptors are rare. Using the more sensitive technique of *in situ* hybridization histochemistry, we provide the first evidence that the α_{1C}-adrenoceptor can in fact be detected in rat peripheral blood vessels. Recently, Bailey *et al.* (26), in abstract form, showed that the α_{1C} mRNA could be detected in hearts. Furthermore, our ongoing studies with both *in situ* hybridization and ribonuclease protection assays show that the α_{1C}-adrenoceptor enjoys a wide distribution in rat periphery.[1] Demonstration that its mRNA is localized to the cardiovascular system is suggestive evidence that the α_{1C}-adrenoceptor participates in the regulation of cardiovascular function.

Another measure of expression of the mRNAs detected in the aorta and mesenteric resistance arteries involves studying contractility. Phenylephrine induced a concentration-dependent increase in developed force of segments of the aorta, renal arteries, and mesenteric resistance arteries. CEC differed in its ability to inhibit contraction of these blood vessels. These observations were similar to those of Han *et al.* (12). Reconciling these contractile data with our results from other experimental models is difficult. There are several reasons for this. It appears that all receptors are colocalized in the peripheral vascular system. The lack of a specific antagonist (reversible or irreversible) for any of these receptors makes discrete identification by functional or binding assays nearly impossible. Finally, many of the ligands used to define α_1-adrenoceptor subtypes exhibit similar affinities for these receptors. Nonetheless, certain observations can be made regarding the regulatory functions of the individual members of the α_1-adrenoceptor family. The α_{1B}-adrenoceptor can be completely inactivated by CEC. Our previous binding data have shown that the aorta contains a large number of CEC-sensitive α_1-adrenoceptors (>85%) (25). The contractile response to phenylephrine is also completely sensitive to this ligand. These data suggest that the α_{1B} is the major regulatory α_1-adrenoceptor subtype in the aorta. This raises the rather perplexing question of why the α_{1A}-adrenoceptor (detected via binding studies) and the mRNA for the α_{1C}- and α_{1D}-adrenoceptors are colocalized in the aorta. In contrast, the renal artery is almost completely resistant to the inhibitory effects of CEC. This suggests that, whereas the mRNA for the three CEC-sensitive receptors (α_{1B}-, α_{1C}-, and α_{1D}-adrenoceptors) can be detected in the renal artery, the α_{1A}-adrenoceptor is the predominant regulatory receptor; this is consistent with the work of Elhawary *et al.* (10), who studied renal blood flow in intact animals. Incidentally, Elhawary *et al.* (10) showed that, although the α_{1A}-adrenoceptor is the major regulatory receptor in the renal artery, CEC-sensitive sites were detected in homogenates prepared from this tissue.

In contrast to the situation in aorta and renal arteries, the mesenteric resistance artery was found to be intermediate in sensitivity to CEC, with both CEC-sensitive and -resistant components for the contractile activity of this vessel. This is consistent with our previous ligand binding studies. We showed that approximately 40–50% of the α_1-adrenoceptors in the mesentery were sensitive to CEC (25). It seems reasonable to suggest that the receptor modulating phenylephrine action after CEC treatment is the α_{1A}-adrenoceptor. The nature of the CEC-sensitive receptor is problematic, considering that the mRNAs for three CEC-sensitive receptors (α_{1B}, α_{1C}, and α_{1D}) have been detected in the mesentery. Therefore, it may be difficult to identify which receptors are inactivated by this ligand.

Our results also suggest that the contribution of members of the α_1-adrenoceptor subtype family to the overall process of vascular smooth muscle contraction is different within different blood vessels. The α_{1A}-adrenoceptor appears to exert a major

[1] D. M. Perez, J.-L. Chen, M. T. Piascik, N. Malik, R. Gaivin, and R. M. Graham. Cloning, expression, and tissue distribution of the rat α_{1C}-adrenergic receptor provide evidence for its classification as the α_{1A} subtype. Manuscript in preparation.

regulatory function in resistance vessels such as the mesenteric and renal arteries. The α_{1B}-adrenoceptor appears to be important in regulating the function of the aorta. Although we could detect mRNAs for the α_{1C}- and α_{1D}-adrenoceptors in all test arteries, these receptors do not appear to function in the regulation of the aorta or renal artery. Their roles in the regulation of the mesenteric resistance artery cannot be determined without the development of specific antagonists.

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