First published June 10, 2005; doi:10.1152/ajpheart.00288.2005.

# Correlation between mRNA levels and functional role of $\alpha_1$-adrenoceptor subtypes in arteries: evidence of $\alpha_{1L}$ as a functional isoform of the $\alpha_{1A}$-adrenoceptor

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Submitted 23 March 2005; accepted in final form 26 May 2005

**Daniel Martí, Raquel Miquel, Khalid Ziani, Regina Gisbert, M. Dolores Ivorra, Elsa Anselmi, Lucrecia Moreno, Victoria Villagrasa, Domingo Barettino, and Pilar D'Ocon.** Correlation between mRNA levels and functional role of $\alpha_1$-adrenoceptor subtypes in arteries: evidence of $\alpha_{1L}$ as a functional isoform of the $\alpha_{1A}$-adrenoceptor. *Am J Physiol Heart Circ Physiol* 289: H1923–H1932, 2005. First published June 10, 2005; doi:10.1152/ajpheart.00288.2005.—The mRNA levels for the three $\alpha_1$-adrenoceptor subtypes, $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$, were quantified by real-time RT-PCR in arteries from Wistar rats. The $\alpha_{1D}$-adrenoceptor was prominent in both aorta (79.0%) and mesenteric artery (68.7%), $\alpha_{1A}$ predominated in tail (61.7%) and small mesenteric artery (73.3%), and both $\alpha_{1A}$- and $\alpha_{1D}$-subtypes were expressed at similar levels in iliac artery. The mRNA levels of the $\alpha_{1B}$-subtype were a minority in all vessels (1.7–11.1%). Concentration-response curves of contraction in response to phenylephrine or relaxation in response to $\alpha_1$-adrenoceptor antagonists on maximal sustained contraction induced by phenylephrine were constructed from control vessels and vessels pretreated with 100 μmol/l chloroethylclonidine (CEC) for 30 min. The significant decrease in the phenylephrine potency observed after CEC treatment together with the inhibitory potency displayed by 8-{2-[4-(2-methoxyphenyl)-1-piperazinyl]-8-azaspiro (4,5) decane-7-dionedihydrochloride} (BMY-7378, an $\alpha_{1D}$-adrenoceptor antagonist) confirm the relevant role of $\alpha_{1D}$-adrenoceptors in aorta and iliac and proximal mesenteric arteries. The potency of 5-methylurapidil (an $\alpha_{1A}$-adrenoceptor antagonist) and the changes in the potency of both BMY-7378 and 5-methylurapidil after CEC treatment provided evidence of a mixed population of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors in iliac and distal mesenteric arteries. The low potency of prazosin ($pIC_{50} < 9$) as well as the high 5-methylurapidil potency in tail and small mesenteric arteries suggest the main role of $\alpha_{1A}/\alpha_{1L}$-adrenoceptors with minor participation of the $\alpha_{1D}$-subtype. The mRNA levels and CEC treatment corroborated this pattern and confirmed that the $\alpha_{1L}$-adrenoceptor could be a functional isoform of the $\alpha_{1A}$-subtype.

chloroethylclonidine; prazosin; mesenteric arteries; adrenergic response

THE SYMPATHETIC NERVOUS SYSTEM plays an important role in regulating the contractile tone of vessels, and it has been clearly shown that $\alpha_1$-adrenoceptors mediate vasoconstriction in the peripheral blood circulation. Radioligand binding, molecular cloning studies, and isolated tissue experiments have identified three $\alpha_1$-adrenoceptor subtypes that are designated $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$ (16, 19). These three subtypes display high subnanomolar affinities for prazosin. Furthermore, functional studies have provided evidence for the existence of a fourth $\alpha_1$-adrenoceptor, the $\alpha_{1L}$-adrenoceptor subtype, which displays a low affinity for prazosin ($pK_B < 9$) and some other $\alpha_1$-adrenoceptor antagonists (2, 9, 11, 33, 43). This $\alpha_{1L}$-adrenoceptor has no molecular correlate and seems to represent a functional phenotype of the $\alpha_{1A}$-adrenoceptor (5, 12, 25).

Previous studies have shown that the mRNAs encoding the three $\alpha_1$-adrenoceptor subtypes are expressed in different arteries (37). In addition, traditional organ bath studies in the vessels using selective antagonists have suggested that although only one $\alpha_1$-adrenoceptor subtype seems to be mainly responsible for the adrenergic response, the results are not consistent with competitive antagonism at a single site, which in turn suggests a heterogeneous population of functionally present subtypes (21, 22, 26, 27, 35, 43).

The present report shows that a methodology combining the quantification of the mRNA levels by real-time RT-PCR with the pharmacological characterization of the functional subtypes provides good correlation among the potencies calculated for each antagonist and the levels of mRNA for each subtype found in the vessel. Moreover, this methodology provides additional information on minority subtypes and confirms that the $\alpha_{1L}$-adrenoceptor could be a functional isoform of the $\alpha_{1A}$-subtype.

## MATERIALS AND METHODS

### *Real-Time Quantitative RT-PCR*

All protocols were approved by the University of Valencia Animal Ethics Committee (Faculty of Pharmacy Section). Aorta as well as tail, iliac, mesenteric, and small mesenteric arteries (SMAs) were dissected and rapidly frozen in liquid nitrogen. Pools of tissues from three animals were made for small-sized vessels to obtain RNA, but aortas were processed individually. The frozen tissues were ground to powder in a mortar and were dissolved in TriPure isolation reagent (Roche). Total RNA was obtained after chloroform extraction and isopropanol precipitation (following manufacturer's instructions) and was dissolved in diethyl pyrocarbonate (DEPC)-treated water. The integrity of the RNA samples was checked by electrophoresis in agarose gel, and RNA concentrations were estimated spectrophotometrically. Rat genomic DNA to be used as a standard in real-time PCR was obtained from a rat

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Table 1. *Oligonucleotide primers used in the study*

| Gene | Gene Symbol | Oligonucleotide Sequences | Position* | Product Size, bp |
|---|---|---|---|---|
| $\alpha_{1A}$-Adrenergic receptor | *Adra1a* | S: 5′-CGA ATC CAG TGT CTT CGC AG-3′ | 1059–1078 | 100 |
| | | AS: 5′-ACC ATG TCT CTG TGC TGT CCC-3′ | 1139–1159 | |
| $\alpha_{1B}$-Adrenergic receptor | *Adra1b* | S: 5′-GCT CCT TCT ACA TCC CGC TCG-3′† | 1018–1038 | 300 |
| | | AS: 5′-AGG GGA GCC AAC ATA AGA TGA-3′ | 1298–1318 | |
| $\alpha_{1D}$-Adrenergic receptor | *Adra1d* | S: 5′-GAA GGT GAT GGG TTA TGG TG-3′ | 2321–2340 | 151 |
| | | AS: 5′-GAA GCC ATA GCT GAA GCC T-3′ | 2454–2472 | |
| Glyceraldehyde-3-phosphate dehydrogenase | *Gapd* | S: 5′-GCA CCA CCA ACT GCT TAG CC-3′ | 1298–1317 | 100 |
| | | AS: 5′-CTG AGT GGC AGT GAT GGC AT-3′ | 1379–1398 | |

Oligonucleotide sequence orientations are indicated as sense (S) and antisense (AS). *Positions depend on corresponding mRNA sequence in RefSeq NCBI database (38). †Oligonucleotide primer sequences for *Adra1b* gene are in accordance with Scofield et al. (41).

liver crude nuclear fraction. Freshly dissected rat liver tissue (~0.5 g) was homogenized using an Ultra-Turrax dispersing instrument (IKA) in 2 ml of solution that contained 10 mM Tris·HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40. After low-speed centrifugation (500 *g* for 3 min at 4°C), the crude nuclear fraction present in the pellet was resuspended in a solution of 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, and 10% glycerol. DNase-free RNase A (Sigma) was added to a concentration of 20 μg/ml, and after 10 min at room temperature, SDS was added to a concentration of 1%, proteinase K (Roche) was added to a concentration of 200 μg/ml, and digestion proceeded at 55°C for 16 h. After digestion, DNA was phenol extracted and precipitated with isopropanol. The DNA pellet was washed with 70% ethanol, briefly air dried, and resuspended in water. DNA concentration was estimated spectrophotometrically.

Total RNA (1–2 μg) and oligo$(dT)_{16}$ primer (250 ng) in DEPC-treated water were preheated to 70°C and cooled on ice for cDNA synthesis. Reactions (25 μl) contained 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 40 U of RNAsin (Promega), 2 mM of each deoxynucleoside triphosphate, and 300 U of Moloney murine leukemia virus reverse transcriptase, RNase H minus (Promega). The reactions were incubated at 42°C for 45 min. A quantitative analysis of the levels of the mRNAs encoding the three $\alpha_1$-adrenoceptor subtypes was performed by real-time RT-PCR with a GeneAmp 5700 sequence-detection system (Applied Biosystems). Oligonucleotide pairs were designed for $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors and for *Gapd* as an internal control. The sequences of the primer pair used for the $\alpha_{1B}$-adrenoceptor were those included in the report by Scofield et al. (41). The sequences of the oligonucleotide primers used in this study, their positions on the corresponding mRNA sequences, and the expected sizes for the PCR products are shown in Table 1. The three $\alpha_1$-adrenoceptor subtypes were assayed by real-time PCR on dilutions of the same RT reaction, and *Gapd* was used as an internal control to normalize for differences in the efficiency of reverse transcription among different samples. We analyzed (in duplicate reactions) a 10-fold dilution of the RT reaction of each vessel used for each gene tested; four serial dilutions of genomic DNA (ranging from the equivalent of 33 to 3,333 copies) were performed to obtain the standard plot. Water and a mock RT reaction made without reverse transcriptase were assayed as negative controls. Real-time PCR reactions were set in 25 μl with SYBR Green I PCR master mix (Applied Biosystems) including either 5 μl of diluted RT reaction or genomic DNA and 5 pmol of each primer. The PCR reaction took place with 40 cycles and consisted of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 20 s. After completion, the specificity of the reaction was checked by analysis of the thermal denaturation profile of the product. The threshold cycle values ($C_t$) obtained for each $\alpha_1$-adrenoceptor subtype in each RT reaction were interpolated in the standard plots generated with the genomic DNA, and the values of copy number per microgram of RNA were calculated using the GeneAmp 5700 sequence-detection system software (Applied Biosystems). These absolute values were normalized with the copy number values obtained for *Gapd*.

*Functional Study in an Isolated Organ Bath*

Rings of aorta, tail artery, iliac artery, and mesenteric artery (~3–5 mm in length) of female Wistar rats (200–220 g body wt) were denuded of endothelium by gentle rubbing and were suspended in a 10-ml organ bath that contained a physiological solution maintained at 37°C and gassed with 95% $O_2$-5% $CO_2$. An initial 1-g load was applied to each preparation and was maintained throughout a 75–90-min equilibration period. Tension was recorded isometrically from Grass FTO3 force-displacement transducers, and data were recorded on a computer disk (MacLab).

Mesenteric arterial trees were dissected and cleared of surrounding adipose tissue. A ring segment (2 mm in length) from the second branch of the arterial tree was mounted in a myograph (J. P. Trading; Aarhus, Denmark) with separate 6-ml organ baths that contained a physiological solution at 37°C and was gassed with 95% $O_2$-5% $CO_2$ as described previously (54). After a 30-min stabilization period, the internal diameter of each vessel was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure ($l_{100}$ = 90–180 μm) according to the standard procedure of Mulvany and Halpern (31). Tension was recorded isometrically, and data were recorded on a disk (MacLab).

The composition of the physiological solution was (in mmol/l) 118 NaCl, 4.75 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $KH_2PO_4$, 25 $NaHCO_3$, and 11 glucose.

After the equilibration period, a sustained contractile response to 10 μmol/l phenylephrine (Sigma) was elicited in all vessels, and the absence of a relaxant response to 10 μmol/l acetylcholine (Sigma) addition indicated the absence of a functional endothelium. The experimental procedures described below were followed after vessels were washed and values returned to baseline.

*Concentration-response curves for contraction in response to phenylephrine.* A single agonist curve was obtained by the cumulative addition of increasing concentrations of phenylephrine (0.1 nmol/l to 100 μmol/l) until a sustained maximal contractile response ($E_{max}$) was obtained in each tissue. The concentration needed to obtain this maximal response was 1 μmol/l in aorta, 10 μmol/l in iliac, tail, and mesenteric arteries, and 30 μmol/l in SMAs. After rings were washed and values had returned to baseline, the irreversible effect of the alkylating antagonist chloroethylclonidine (CEC; Sigma) was also evaluated. Rings were exposed to CEC (100 μmol/l) for 30 min and then washed for 60 min before a new cumulative addition of phenylephrine was carried out.

The concentration needed to obtain 50% of the maximal response (expressed as $pEC_{50}$) was calculated from a nonlinear regression plot (GraphPad Software; San Diego, California).

*Concentration-response curves for relaxation in response to selective $\alpha_1$-adrenoceptor antagonists.* Concentration-response curves for relaxation (CRCR) were performed by the addition of cumulative concentrations of prazosin (0.001 nmol/l to 1 μmol/l; Sigma), 5-methylurapidil (0.001 nmol/l to 10 μmol/l; RBI), cyclazosin (0.001 nmol/l to 1 μmol/l; Sigma), and 8-{2-[4-(2-methoxyphenyl)-1-pipera-

Table 2. *Expression levels of three $\alpha_1$-adrenoceptor subtypes in different vessels*

| Artery | Expression of Adrenoceptor Subtype, copies/μg of RNA | | | Total Expression, copies/μg of RNA |
|---|---|---|---|---|
| | $\alpha_{1A}$ | $\alpha_{1B}$ | $\alpha_{1D}$ | |
| Aorta | 29,451.3±14,970.8 | 10,659.5±3,562.3 | 151,627.7±93,152.4 | 191,738.5 |
| Tail | 206,922.30±50,342.9 | 37,357.5±12,020.8 | 91,493.7±8,187.9 | 335,773.5 |
| Iliac | 22,115.1±4,212.0 | 4,586.5±1,107.9 | 20,747.2±2,626.7 | 47,448.8 |
| Mesenteric | 19,257.9±7,249.2 | 7,102.4±3,827.6 | 57,797.9±5,776.4 | 84,158.2 |
| Small mesenteric | 22,839.5±4,611.8 | 524.8±124.5 | 7,790.4±776.6 | 31,154.7 |

Values (which are normalized) are means ± SE for 3–5 determinations performed in duplicate.

zinyl]-8-azaspiro (4,5) decane-7-dionedihydrochloride} (BMY-7378; 0.001 nmol/l to 10 μmol/l; RBI) to tissues in which sustained contractions had been induced by a maximal concentration of phenylephrine. Relaxations were expressed as a percentage of the maximum increment in tension obtained by the agonist addition.

In some experiments, the activity of antagonists after incubation with the alkylating agent CEC was evaluated. Rings from tail, iliac, and mesenteric arteries and SMAs were exposed to CEC (100 μmol/l) for 30 min and then washed for 60 min before a new addition of the maximal concentration of phenylephrine was carried out. CRCRs in response to selective $\alpha_1$-adrenoceptor antagonists were obtained by the addition of cumulative concentrations of each compound on this sustained maximal contraction elicited by phenylephrine after CEC treatment.

The concentration [−log(mol/l)] needed to produce 50% relaxation ($pIC_{50}$) was obtained from a nonlinear regression plot, and the data were fitted to one- and two-site models. If residual sums of squares were statistically less for a two-site fit of data than for a one-site fit as determined by an *F*-test comparison, then the two-site model was accepted (GraphPad Software).

## RESULTS

### *Analysis of $\alpha_1$-Adrenoceptor Subtype mRNA Levels by Real-Time Quantitative RT-PCR*

A two-step real-time RT-PCR assay was used to quantify the mRNA levels of the three $\alpha_1$-adrenoceptor subtypes in the different vessels assayed. In the first step, first-strand cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and oligo$(dT)_{16}$ as primer. The product of that RT reaction was used in the second step as a template for real-time PCR. All four transcripts (the three $\alpha_1$-adrenoceptor subtypes and *Gapd* as a normalization control) were analyzed on dilutions of the same RT reaction. In addition, negative control reactions in which a mock RT reaction was made without reverse transcriptase or in which the RT reaction was omitted were analyzed in parallel. The amplification plot did not reach the threshold value in these negative-control reactions. Serial dilutions of rat genomic DNA were amplified in parallel reactions to construct the corresponding standard plots. The standard plots in which the logarithm of the calculated copy number was related to the $C_t$ value showed slopes close to the theoretical value of 3.33 and correlation coefficients of $r > 0.99$. The $C_t$ values obtained for each gene were interpolated on the corresponding standard plot to obtain the absolute copy number per microgram of RNA, and those crude values were normalized with the values obtained for *Gapd*. The normalized values obtained for each $\alpha_1$-adrenoceptor subtype in the different vessels are shown in Table 2. The results indicate that all three $\alpha_1$-subtypes were expressed in the vessels analyzed, although there were significant differences in expression levels. The mRNA for $\alpha_{1D}$-adrenoceptor predominated in aorta and mesenteric arteries, whereas $\alpha_{1A}$-adrenoceptor was prominent in tail and SMAs. Both receptor subtypes were expressed to similar levels in iliac artery. Finally, the mRNA for the $\alpha_{1B}$-adrenoceptor subtype was a minority in all tissues. The relative fractions of the three $\alpha_1$-adrenoceptor subtypes in each of the vessels analyzed are displayed in Fig. 1.

### *Functional Study in an Isolated Organ Bath*

*Concentration-response curves of contraction in response to phenylephrine.* A cumulative addition of increasing concentrations of phenylephrine to each tissue gave a concentration-response curve of contraction with $E_{max}$ and $pEC_{50}$ values as summarized in Table 3 and displayed in Fig. 2. This curve could be reproduced with no significant changes after rings

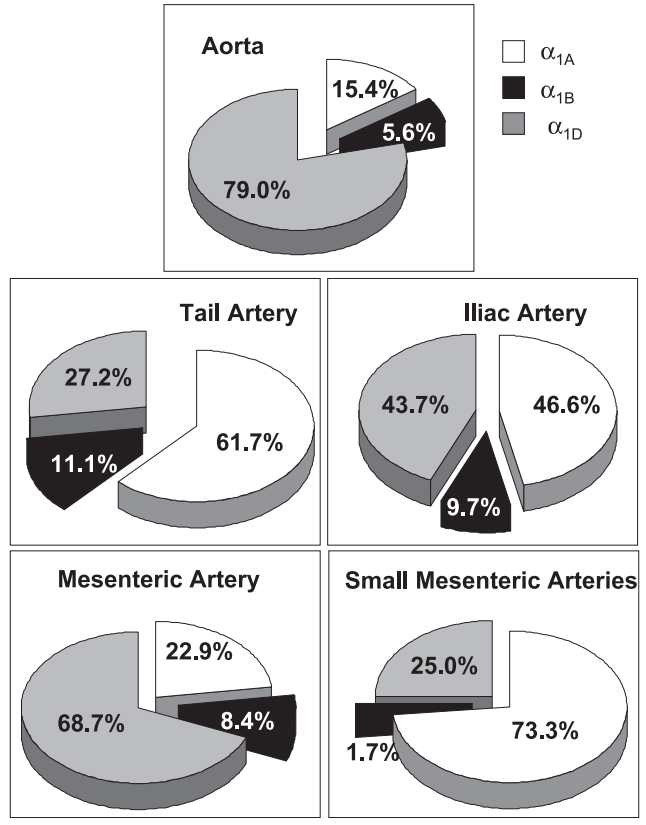


Fig. 1. Relative $\alpha_1$-adrenoceptor subtype composition in the different vessels studied. Relative levels (in percentages) of the mRNAs for each subtype are shown with regard to the $\alpha_1$-adrenoceptor mRNA total copy number.

Table 3. *Parameters of concentration-response curves of contraction in response to phenylephrine in different arterial vessels*

| Artery | Phenylephrine | | Phenylephrine + CEC | |
|---|---|---|---|---|
| | $E_{max}$, mg | $pEC_{50}$ | $E_{max}$, mg | $pEC_{50}$ |
| Aorta | 941.7±116.3 | 7.84±0.04 | 54.8±21.25‡ | |
| Tail | 1,091.7±215.9 | 5.95±0.08 | 1,063.6±211.8 | 5.65±0.09* |
| Iliac | 595.6±93.7 | 6.52±0.16 | 429.01±105.8 | 5.03±0.11† |
| Mesenteric | 719.1±64.6 | 6.93±0.10 | 343.7±59.5† | 5.13±0.21† |
| Small mesenteric | 862.0±69.2 | 5.95±0.08 | 679.8±95.6* | 5.49±0.13† |

Values are means ± SE of 4–9 experiments. CEC, chloroethylclonidine. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.01$ vs. control.

were washed and values returned to baseline (results not shown). The higher $pEC_{50}$ value for phenylephrine in the aorta indicates that the $\alpha_{1D}$-subtype plays a fundamental role in this tissue according to the higher potency and affinity of agonists for this subtype (30). After CEC treatment, only higher concentrations of phenylephrine yielded a contractile response in aorta; significant decreases in $E_{max}$ and $pEC_{50}$ were observed in mesenteric arteries, and slight but insignificant changes in $E_{max}$ and a decrease in $pEC_{50}$ were observed in tail and iliac arteries (Table 3 and Fig. 2).

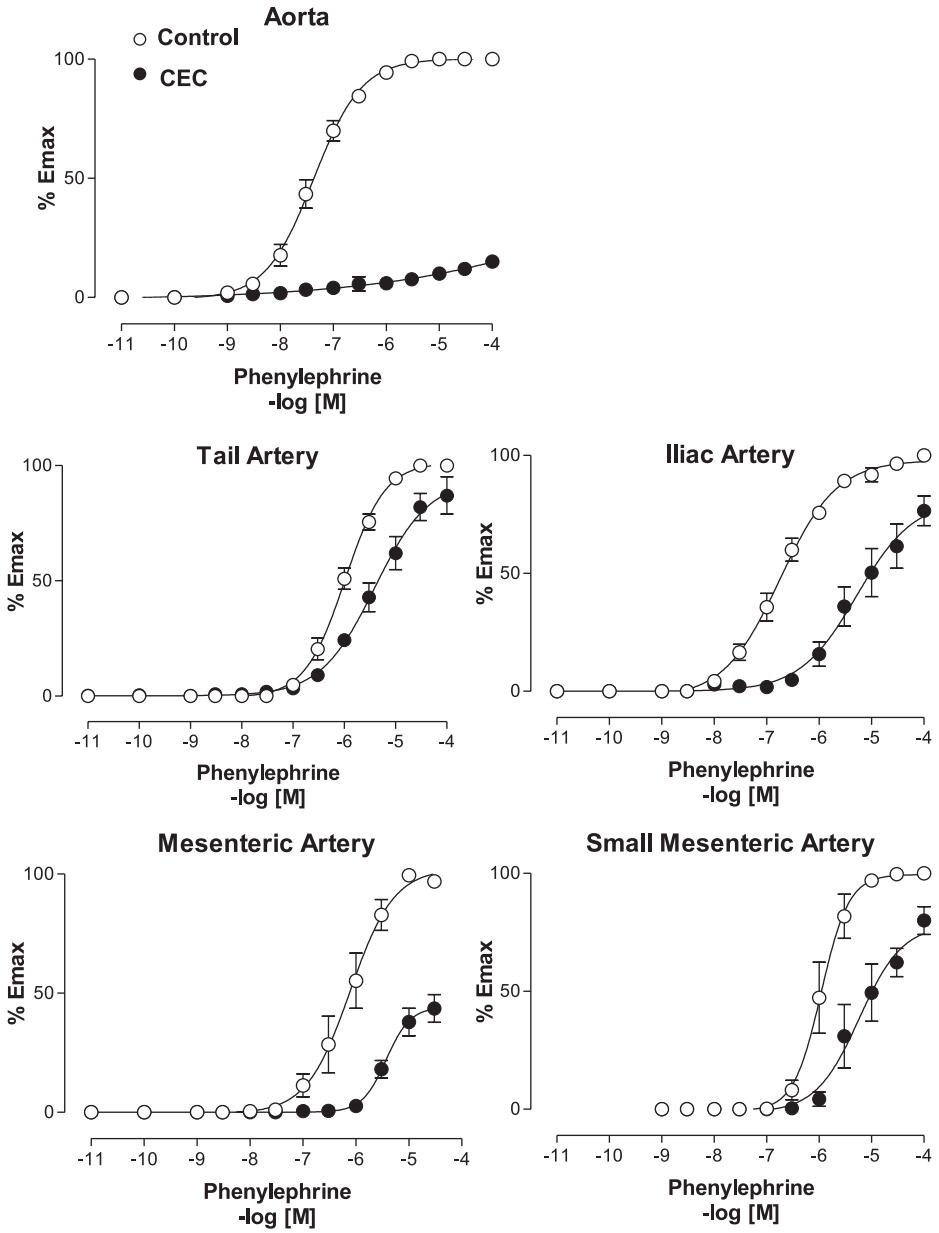


Fig. 2. Effects of chloroethylclonidine (CEC) pretreatment on the concentration-response curve of contraction in response to phenylephrine in isolated rings.

Table 4. *Values of pIC50 for agents tested on maximal sustained contractile responses induced by phenylephrine in rat aorta*

| $\alpha_1$-Adrenoceptor Antagonist | $pIC_{50}$ | *n* |
|---|---|---|
| Prazosin | 9.68±0.15 | 10 |
| BMY-7378 | 8.09±0.06 | 6 |
| Cyclazosin | 7.92±0.1 | 6 |
| 5-Methylurapidil | 7.1±0.07 | 6 |

Values are means ± SE; *n,* no. of experiments. BMY-7378, 8-{2-[4-(2-methoxyphenyl)-1-piperazinyl]-8-azaspiro (4,5) decane-7-dionedihydrochloride}. Maximal contraction was induced with 1 μmol/l phenylephrine.

*CRCRs in response to selective $\alpha_1$-adrenoceptor antagonists.* CRCRs in response to prazosin (an $\alpha_1$-adrenoceptor antagonist that discriminates for the $\alpha_{1L}$-subtype), 5-methylurapidil (an $\alpha_{1A}$-selective antagonist), cyclazosin (an $\alpha_{1B}$-selective antagonist; Ref. 13), and BMY-7378 (an $\alpha_{1D}$-selective antagonist) were obtained by adding cumulative concentrations of the compounds to tissues in which sustained contractions had been induced by concentration of phenylephrine that had elicited maximal responses. The concentrations of phenylephrine needed to obtain a maximal response in each vessel were as follows: 1 μM in aorta, 10 μM in iliac and mesenteric arteries, and 30 μM in tail and SMAs (Fig. 2). The potency ($pIC_{50}$) of the fitted curves of relaxation obtained for each antagonist in the different vessels is summarized in Tables 4 (for aorta), 5 (for tail artery), 6 (for iliac artery), 7 (for mesenteric artery), and 8 (for second branch of the mesenteric tree; SMA).

Using the present methodology on rat aorta, we compared the potency of each antagonist (see Table 4) with the $pK_i$ obtained on cloned $\alpha_1$-adrenoceptors (Table 9), and we concluded that the $pIC_{50}$ showed by prazosin (>9) excluded the participation of the $\alpha_{1L}$-subtype in this vessel. The high potency of BMY-7378 indicated the major role of the $\alpha_{1D}$-subtype, which was confirmed by the lower potency of 5-methylurapidil and cyclazosin. In this vessel, the lack of an adequate response to phenylephrine after CEC treatment did not allow us to analyze the selective antagonist activity in CEC-pretreated aortas.

Table 5. *Values of pIC50 for agents tested on maximal sustained contractile responses induced by phenylephrine in rat tail artery*

| $\alpha_1$-Adrenoceptor Antagonist | Tail | | | Tail + CEC | |
|---|---|---|---|---|---|
| | *Site 1* $pIC_{50}$ | *Site 2* $pIC_{50}$ | *n* | $pIC_{50}$ | *n* |
| Prazosin | 8.80±0.12 | | 7 | ND | |
| BMY 7378 | 6.40±0.10 | | 7 | ND | |
| Cyclazosin | 8.22±0.11 | | 6 | 8.34±0.26 | 5 |
| 5-Methylurapidil | 8.73±0.10 | 5.88±0.26 | 9 | 7.97±0.10 | 6 |

Values are means ± SE; *n,* no. of experiments. *Site 1* $pIC_{50}$ represents values of agents tested on 10 μmol/l phenylephrine induced maximal contraction when curve fitting for one site was statistically significant or for first site when curve fitting for two different sites was statistically significant (see data analysis). *Site 2* $pIC_{50}$ represents value obtained for a second site when curve fitting for two different sites was statistically significant (see data analysis). Tail + CEC, tail artery pretreated with 100 μmol/l chloroethylclonidine for 30 min and subsequent washing of CEC. ND, not determined.

Table 6. *Values of pIC50 for agents tested on maximal sustained contractile responses induced by phenylephrine in rat iliac artery*

| $\alpha_1$-Adrenoceptor Antagonist | Iliac Artery | | | Iliac Artery + CEC | |
|---|---|---|---|---|---|
| | *Site 1* $pIC_{50}$ | *Site 2* $pIC_{50}$ | *n* | $pIC_{50}$ | *n* |
| Prazosin | 9.11±0.11 | | 6 | ND | |
| BMY-7378 | 7.61±0.14 | | 5 | 6.59±0.22* | 4 |
| Cyclazosin | 8.85±0.10 | | 6 | 8.40±0.13 | 5 |
| 5-Methylurapidil | 8.68±0.27 | 6.76±0.03 | 4 | 8.66±0.10 | 4 |

Values are means ± SE. *$P < 0.01$.

In tail artery, the $pIC_{50}$ obtained with prazosin (see Table 5) was consistently lower than the affinity reported for cloned $\alpha_{1A}$-, $\alpha_{1B}$-, and $\alpha_{1D}$-subtypes (see Table 9), and it was also lower than the $pIC_{50}$ obtained for prazosin in aorta or iliac or mesenteric arteries (see Tables 4, 6, and 7). The prazosin potency in tail artery correlated well to the values described for the functionally defined $\alpha_{1L}$-adrenoceptors (2, 9, 11, 33, 43). Concentration-response curves for 5-methylurapidil discriminated for two different sites ($pIC_{50}$ *Site 1* and $pIC_{50}$ *Site 2*; see Table 5), one of which ($pIC_{50}$ *Site 2*) disappeared after CEC pretreatment as Fig. 3 shows. The 5-methylurapidil potency after CEC treatment was similar to that observed by *site 1* in untreated vessels and correlated to the $pA_2$ of this antagonist for the $\alpha_{1A}/\alpha_{1L}$-subtype (2). The cyclazosin potency was not affected by CEC pretreatment at that time, and we concluded that the participation of the $\alpha_{1B}$-subtype was not evident. In accordance with this and the low tissue sensitivity to CEC treatment, a major role of the $\alpha_{1A}/\alpha_{1L}$-subtype in tail artery and a minor role of another CEC-sensitive subtype (probably $\alpha_{1D}$) were suggested.

High prazosin potency in iliac artery excluded the functional role of $\alpha_{1L}$-adrenoceptors (see Table 6). The concentration-response curve for 5-methylurapidil provided a significant fit for two different sites, one of which ($pIC_{50}$ *Site 1*) correlated well with the presence of the $\alpha_{1A}$-subtype in the vessel (see $pK_i$ values in Table 9). A comparison of the $pIC_{50}$ value for BMY-7378 with its $pK_i$ value, which was obtained in competition experiments on cloned $\alpha_1$-adrenoceptor subtypes, suggested the partial role of $\alpha_{1D}$-adrenoceptors together with (an) other subtype(s). One of the two affinity sites evidenced by 5-methylurapidil in nontreated iliac arteries disappeared after

Table 7. *Values of pIC50 for agents tested on maximal sustained contractile responses induced by phenylephrine in rat proximal or distal mesenteric arteries*

| $\alpha_1$-Adrenoceptor Antagonist | Mesenteric Artery | | | Mesenteric Artery + CEC | |
|---|---|---|---|---|---|
| | *Site 1* $pIC_{50}$ | *Site 2* $pIC_{50}$ | *n* | $pIC_{50}$ | *n* |
| Prazosin | P: 10.2±0.08* | | 6 | ND | |
| | D: 9.62±0.12 | | | | |
| BMY-7378 | P: 9.81±0.13 | P: 7.53±0.11 | 4 | ND | |
| | D: 6.97±0.09 | | 8 | | |
| Cyclazosin | P: 10.23±0.10* | | 6 | 8.89±0.20 | 6 |
| | D: 9.32±0.10 | | | | |
| 5-Methylurapidil | P: 10.3±0.29 | P: 7.46±0.34 | 4 | 9.54±0.08 | 4 |
| | D: 9.34±0.39 | D: 7.06±0.19 | 5 | | |

Values are means ± SE; *n*, no. of experiments. Region of mesenteric artery is described as a proximal (P) or distal (D) section with regard to aorta. *Data from Ziani et al. (54).

Table 8. *Values of pIC50 for agents tested on maximal sustained contractile responses induced by phenylephrine in rat small mesenteric artery*

| $\alpha_1$-Adrenoceptor Antagonist | Small Mesenteric Artery | | Small Mesenteric Artery + CEC | |
|---|---|---|---|---|
| | $pIC_{50}$ | $n$ | $pIC_{50}$ | $n$ |
| Prazosin | 8.48±0.28 | 6 | | |
| BMY-7378 | 6.30±0.14 | 8 | | |
| Cyclazosin | 9.36±0.20 | 6 | 8.92±0.22 | 6 |
| 5-Methylurapidil | 7.98±0.11 | 7 | 8.15±0.08 | 7 |

Values are means ± SE for agents tested on 30 μmol/l phenylephrine-induced maximal contraction; $n$, no. of experiments.

CEC treatment, and the site that remained sensitive to 5-methylurapidil antagonism correlated well with the $\alpha_{1A}$-adrenoceptor subtype (see Table 6 and Fig. 3). In addition, the lower potency shown by BMY-7378 after CEC treatment suggested that the $\alpha_{1D}$-adrenoceptors were irreversibly alkylated by CEC (see Table 6 and Fig. 3). The cyclazosin potency did not significantly decrease after CEC treatment. Therefore, the functional role of the $\alpha_{1B}$-adrenoceptor was not clearly shown by this antagonist. According to these results, a mixed population of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors were functionally active in iliac artery.

The high prazosin potency in mesenteric artery (see Table 7) excluded the functional role of $\alpha_{1L}$-adrenoceptors. CRCRs for BMY-7378 showed marked differences depending on the portion of the artery used. BMY-7378 discriminates for two sites in the section close to aorta (proximal section); one of them, which corresponded to the $\alpha_{1D}$-subtype, was not evident in the distal section of the vessel (see Table 7). That the concentration-response curves for 5-methylurapidil provided the best fit to the two-site model suggests the participation of the $\alpha_{1A}$-subtype and another subtype in the functional response to phenylephrine. Pretreatment of mesenteric artery with CEC destroyed this adrenoceptor population, and posterior concentration-response curves for 5-methylurapidil yielded a value of $pIC_{50}$ for one site (see Table 7 and Fig. 3) that correlated well with the $pK_i$ obtained on cloned $\alpha_{1A}$-adrenoceptors (see Table 9). The potency shown by cyclazosin after CEC treatment was not significantly lower than the value obtained from nontreated arteries (see Table 7); thus the role of $\alpha_{1B}$-adrenoceptors was not confirmed by this antagonist. According to these results, a mixed population of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors were functionally active in this artery, where the role of $\alpha_{1D}$-adrenoceptors was seen to be greater in the portion of mesenteric artery close to aorta.

The $pIC_{50}$ value obtained for prazosin in SMA (see Table 8) correlated well with its affinity for the $\alpha_{1L}$-subtype ($pA_2 < 9$). BMY-7378 inhibited phenylephrine-induced contraction with a low potency that excluded the major participation of the $\alpha_{1D}$-subtype in this vessel. The $pIC_{50}$ value obtained with 5-methylurapidil corroborated that the $\alpha_{1A}/\alpha_{1L}$-subtypes were mainly responsible for the adrenergic response in this tissue. No direct evidence for the participation of the $\alpha_{1B}$-subtype was obtained when cyclazosin was used as an antagonist.

## DISCUSSION

In the present work, we propose a new methodology that not only simplifies the pharmacological analysis of the population of $\alpha_1$-adrenoceptor subtypes that are functionally active in a vessel but also provides additional information about the nature of the other subtypes involved in the adrenergic response.

Our method includes the quantification of mRNA levels for each subtype by real-time quantitative RT-PCR and the realization of concentration-response curves of contraction in response to cumulative concentrations of phenylephrine before and after CEC treatment. Changes in the parameters of these curves ($E_{max}$ and $pEC_{50}$) offer valuable information that is complemented by the CRCRs of selective antagonists on maximal phenylephrine-induced contraction before and after CEC treatment.

The present state of knowledge with respect to CEC-irreversible inactivation of $\alpha_1$-adrenoceptor subtypes is that the $\alpha_{1A}$-subtype is "insensitive," the $\alpha_{1B}$-subtype is "sensitive," and the $\alpha_{1D}$-subtype is "partially sensitive" to this alkylating agent. However, it is now clear that CEC is able to inactivate all of the $\alpha_1$-adrenoceptors (37), and differences between subtypes may be due to *1*) the alkylation rate, which is lower in the $\alpha_{1A}$-subtype (slowly alkylated) than in the $\alpha_{1B}$- or $\alpha_{1D}$-subtypes (rapidly alkylated) according to Xiao and Jeffries (52); and *2*) the subcellular localization of subtypes; namely, CEC preferentially alkylates the accessible cell surface of $\alpha_1$-adrenoceptors (20, 47). In this way, recent studies have shown major differences in the subcellular distribution of the $\alpha_1$-subtypes in that $\alpha_{1B}$-adrenoceptors are located at the cell membrane and $\alpha_{1A}$-adrenoceptors are also located (but intracellularly so) at the cell membrane, whereas very little surface expression of the $\alpha_{1D}$-adrenoceptors was detected, and the main localization of this subtype is in a perinuclear orientation (4, 10, 18, 20, 28, 37, 47).

Table 9. *Published antagonist $pK_i$ values calculated by use of measurements with cloned receptor subtypes*

| Receptor Subtype | $pK_i$ Values | | |
|---|---|---|---|
| | Prazosin | BMY-7378 | 5-Methylurapidil |
| $\alpha_{1A}$ | 9.9,[1] 9.7,[2] 9.5,[3] 9.37,[4] 9.72/9.88,[5] 9.9/9.3,[6] 9.14[7] | 6.1,[8] 6.2,[2] 6.54/6.8,[5] 6.57[9] | 9.2,[1] 8.5,[2] 8.6,[3] 8.65,[4] 9.12/9,[5] 8.5/8.7,[6] 8.69[7] |
| $\alpha_{1B}$ | 9.9[1] 9.6,[2] 9.8,[3] 10.44,[4] 9.42/10.08,[5] 9.5/9.9,[6] 9.34[7] | 6.2,[8] 6.7,[2] 7.36,[4] 6.24/7.25,[5] 6.77[9] | 7.7,[1]6.8,[2] 6.9,[3] 7.22,[4] 7/7.5,[5] 6.6/6.5,[6] 5.98[7] |
| $\alpha_{1D}$ | 9.9,[1] 9.5,[2] 9.6,[3] 10.03,[4] 9.46/10.04,[5] 10.4/10.1,[6] 8.71[7] | 8.2,[8] 8.2,[2] 9.00,[4] 8.16/9.39,[5] 8.94[9] | 8.0,[1] 7.8,[2] 7.3,[3] 7.59,[4] 7.3/7.92,[5] 7.2/7.1,[6] 6.30[7] |

Values are from [1]Ford et al. (12), [2]Kenny et al. (26), [3]Michel et al. (29), [4]Muramatsu et al. (32), [5]Saussy et al. (39), [6]Schwinn et al. (40), [7]Testa et al. (46), [8]Goetz et al. (17), and [9]Yang et al. (53).

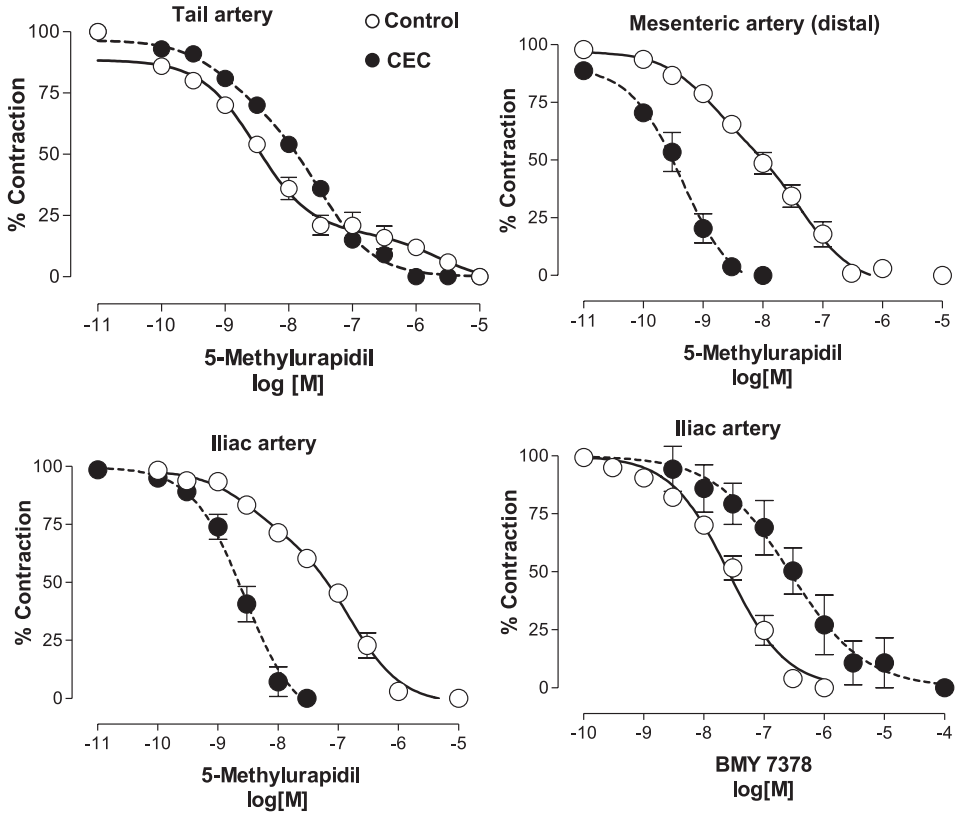


Fig. 3. Effects of CEC pretreatment on the concentration-response curves of relaxation by the $\alpha_1$-adrenoceptor antagonists in different vessels. Arterial rings were incubated with 100 μmol/l CEC for 30 min, extensively washed out, maximally contracted by phenylephrine, and exposed to cumulative concentrations of each antagonist.

If we are to take both considerations into account, we can understand why $\alpha_{1B}$-adrenoceptors are the more sensitive subtype to CEC alkylation and $\alpha_{1A}$-adrenoceptors are the more resistant subtype, and why the $\alpha_{1D}$-subtype shows a complex pattern of alkylation that leads to the designation of this receptor as "partially sensitive" to CEC depending on the percentage present in the membrane and, therefore, on its accessibility to the alkylating agent.

According to the aforementioned information, we can use CEC in functional studies to analyze the $\alpha_1$-adrenoceptor subtype that is responsible for the contractile response of a given vessel. However, great care must be taken with the experimental conditions with regard to the concentration of CEC employed and the time the alkylating procedure lasts. These two parameters determine whether one, two, or three subtypes are affected by the alkylating agent to a greater or lesser extent.

The most commonly used CEC concentration is 100 μmol/l, and a general agreement on this point exists. Lower concentrations give more confusing results. The alkylation time varies between 20, 30, and 45 min, but a 30-min time is mainly used since data in the literature demonstrate that most of the $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptors are destroyed with an alkylation time >30 min, but most of the $\alpha_{1A}$-adrenoceptors remain functionally active in isolated organ-bath studies (23, 27, 34). CEC at a concentration of 100 μmol/l for 30 min was used in this study in accordance with these previous data to mainly inactivate the $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptors and to avoid significantly affecting the $\alpha_{1A}$-subtype.

The major role of $\alpha_{1D}$-adrenoceptors in rat aorta previously reported (14, 15, 22, 26) is supported in this study by *1*) the higher levels of mRNA found for this subtype; *2*) the highest $pEC_{50}$ for phenylephrine observed in this tissue, which confirms the higher affinity for this agonist as shown by the $\alpha_{1D}$-subtype (30); and *3*) the fact that the response to phenylephrine dramatically decreased after CEC treatment according to the sensitivity of the $\alpha_{1D}$-subtype for the alkylating agent.

If the mRNA levels in tail artery and SMAs were considered, the most expressed subtype was $\alpha_{1A}$-adrenoceptors. The lower phenylephrine potency compared with aorta together with the minor sensitivity of these vessels to CEC treatment confirms the major presence of other subtypes different from $\alpha_{1D}$-adrenoceptors, although the minor participation of this subtype was suggested by the significant decrease in the phenylephrine potency after CEC treatment. That 5-methylurapidil discriminates for two different sites in the tail artery ($pIC_{50}$ values of 1 and 2; see Table 5) confirms the main role of $\alpha_{1A}$-adrenoceptors as other authors and ourselves have proposed (14, 27, 35, 36, 45). Nonetheless, this also suggests a minor role of another subtype, $\alpha_{1D}$-adrenoceptors, if the results obtained with the agonist are considered. These observations in functional studies correlate well with the levels of mRNA found for each subtype in this vessel, where the mRNA for $\alpha_{1A}$-adrenoceptors is the most expressed, although a significant level of mRNA for the $\alpha_{1D}$-subtype was also quantified in this tissue. The participation of the $\alpha_{1D}$-subtype observed in our experiments is not clearly shown by the classical Schild analysis, and using this analysis, it only becomes evident after reserpine treatment (45).

An interesting observation is that together with the higher mRNA levels for the $\alpha_{1A}$-subtype that we observed in tail and SMAs, the prazosin potency correlates well with its affinity for

the $\alpha_{1L}$-adrenoceptor. We can therefore propose that the $\alpha_{1L}$-subtype is mainly responsible for the adrenergic response in both vessels, which is in accordance with the proposal of Ford et al. (12) and Daniels et al. (5) regarding the nature of the $\alpha_{1L}$-adrenoceptor as a functional isoform of the $\alpha_{1A}$-subtype. This also confirms the main role of $\alpha_{1A}/\alpha_{1L}$-adrenoceptors in SMAs as described by other authors (43).

Functional experiments in iliac artery confirm the results obtained by real-time RT-PCR quantification where similar mRNA levels for the $\alpha_{1A}$- and $\alpha_{1D}$-subtypes were found that were consistent with previous studies that describe $\alpha_{1A}$-, $\alpha_{1B}$-, and $\alpha_{1D}$-mRNA in this tissue (35). The potency of each antagonist before and after CEC treatment also confirms the participation of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors in the functional response of iliac artery and can be summarized as follows: *1*) one of the two sites discriminated by 5-methylurapidil in non-CEC-treated tissues disappears after CEC treatment; the site that remains is the one for which 5-methylurapidil shows a higher potency, and this potency correlates well with its affinity for the cloned $\alpha_{1A}$-subtype; *2*) the potency of cyclazosin does not change after CEC treatment; *3*) BMY-7378 potency decreases in CEC-treated tissues with respect to non-treated tissues, and it correlates well with its affinity for the $\alpha_{1A}$- or $\alpha_{1B}$-subtypes. This could be interpreted as an irreversible alkylation by CEC of the $\alpha_{1D}$-adrenoceptor population present in the vessel.

Finally, the results obtained in mesenteric artery suggest a main role for the $\alpha_{1D}$-subtype according to the mRNA levels, although its role is not as significant as in aorta. Furthermore, the $\alpha_{1A}$-subtype expression is increased with respect to aorta. In functional studies, BMY-7378 potency was different depending on the portion of the vessel considered, and sensitivity to CEC treatment was lower than in aorta. In fact, BMY-7378 and 5-methylurapidil discriminate for two different sites (*sites 1* and *2*; see Table 7) in mesenteric artery, but BMY-7378 only discriminates for the portion close to aorta, which suggests a predominant but not exclusive role for $\alpha_{1D}$-adrenoceptors in proximal segments. BMY-7378 potency was lower in the distal portion, which suggests minor $\alpha_{1D}$-adrenoceptor involvement in the responses of this portion to adrenergic stimulus. The above considerations suggest a mixed population of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors in mesenteric artery that changes according to an anatomical pattern related to the proximity to aorta. These results confirm previous studies reported by other authors who used the Schild analysis (1, 22, 49, 51). In this vessel, although differences in mRNA expression for the $\alpha_{1A}$- and $\alpha_{1D}$-subtypes were slight with respect to aorta, the functional role of the $\alpha_{1A}$-subtype is more evident than in aorta. If we consider that $\alpha_{1A}$-adrenoceptors are efficiently coupled to second-messenger production, whereas $\alpha_{1D}$- adrenoceptors are poorly coupled to it (14, 40), we can assume that slight increases in $\alpha_{1A}$-adrenoceptor expression together with slight decreases in $\alpha_{1D}$-adrenoceptor levels could have a repercussion for $\alpha_{1A}$ functionality in mesenteric artery. This would account for the lower sensitivity of this vessel to CEC treatment and also for the discrimination of BMY-7378 for two sites in mesenteric artery and not in aorta.

It is interesting to point out that the potency of the different antagonists calculated with our experimental procedure correlated well with the potency found by other authors and ourselves using the Schild plot analysis (we must compare present results to the p$A_2$ values obtained in previous studies and summarized in Table 10). There is an advantage in that our procedure facilitates the pharmacological analysis of a tissue, since the number of experiments and samples is considerably reduced (about four times) without a decrease in the accuracy of results. Another advantage this methodology offers is that the mathematical analysis of the inhibition curves allows us to statistically compare the adjustment to one- or two-site models, which is additional evidence of the minor participation of other subtypes.

When we applied this methodology, we found that either the $\alpha_{1D}$- or $\alpha_{1A}/\alpha_{1L}$-subtypes had a clear functional role in all vessels studied, yet a relevant role for the $\alpha_{1B}$-subtype was not found in any vessel, thus confirming previous observations (37). In the same way, the mRNA levels for this subtype were significantly lower than the other two in all the vessels. This observation suggests one role of the $\alpha_{1B}$-subtype as being a modulator of the functionality of the other subtypes as we have previously proposed for native receptors (8) and as other authors have studied in cells coexpressed with either the $\alpha_{1B}$- and $\alpha_{1A}$-subtypes (44) or the $\alpha_{1B}$- and $\alpha_{1D}$-subtypes (18).

The quantification of the mRNA levels corroborates the main functional role observed by $\alpha_{1D}$-adrenoceptors in conductance vessels and also by $\alpha_{1A}$-adrenoceptors in either distributing or resistance vessels. Considering that $\alpha_{1D}$-adrenoceptors are responsible for the slow appearance and slow disappearance of adrenergic responses, whereas $\alpha_{1A}$-adrenoceptors provide faster responses (8, 15, 54), the higher expression of $\alpha_{1D}$-adrenoceptors in poorly innervated conductance vessels avoids abrupt changes in vessel caliber and, consequently, in blood flow in response to the adrenergic stimulus. On the contrary, the higher expression of $\alpha_{1A}$-adrenoceptors in the densely innervated distributing vessels and particularly in the resistance vessels guarantees a faster contractile response to an adrenergic stimulus that is followed by a faster decrease in

Table 10. *Published antagonist p$A_2$/p$K_B$ values calculated from different rat vessels*

| Artery | p$A_2$/p$K_B$ Values | | |
|---|---|---|---|
| | Prazosin | 5-Methylurapidil | BMY-7378 |
| Aorta | 9.8,[1] 9.85,[2] 9.5,[3] 9.9,[4] 9.4[5] | 7.8,[1] 7.6,[2] 6.8,[5] 7.8,[6] 7.3,[7] 7.64,[8] 8.6[9] | 8.3,[5] 8.06,[7] 8.56,[8] 8.6,[9] 8.9,[10] 9.0,[11] 8.5,[12] |
| Tail | 8.4,[5] 8.9,[6] 9.3[9] | 8.0,[5] 9.0[6] | 5.8,[5] 6.3,[6] 6.5[12] |
| Iliac | 9.3[13] | 8.71[13] | 6.62,[13] 8.4[14] |
| Mesenteric | 9.9[6] | 7.9,[4] 8.05,[7] 7.7[12] | 8.8,[4] 6.6,[7] 8.7[12] |
| Small mesenteric | 8.5,[15] 8.3–8.8[16] | 9.1[17] | 6.16[16] |

Values are from [1]Kenny et al. (26), [2]Testa et al. (46), [3]Saussy et al. (39), [4]Hussain and Marshall (22), [5]Gisbert et al. (15), [6]Lachnit et al. (27), [7]Dhein et al. (7), [8]Asbun-Bojalil et al. (3), [9]Taki et al. (45), [10]Goetz et al. (17), [11]Deng et al. (6), [12]Arevalo-León et al. (1), [13]Shibano et al. (42) in mouse, [14]Villalobos-Molina et al. (50), [15]Van der Graaf et al. (48), [16]Stam et al. (43), and [17]Ipsen et al. (24).

tone after removal. These observations together with the different sensitivities to agonists exhibited by each subtype (lower for $\alpha_{1A}/\alpha_{1L}$ than for $\alpha_{1D}$) explain the specific distribution of each subtype through the arterial tree and allow for a fine adjustment of both contractile tone and blood flow to the adrenergic stimulus.

In conclusion, present data show that a combination of quantified mRNA levels along with functional studies including CEC treatment proves to be a useful tool to analyze the exact role of each $\alpha_1$-adrenoceptor subtype in a given vessel. In addition, the higher mRNA level for the $\alpha_{1A}$-subtype that is found in some vessels together with the functional role of $\alpha_{1L}$-adrenoceptors in these vessels provides additional evidence regarding the proposal that $\alpha_{1L}$-adrenoceptors are a functional isoform of the $\alpha_{1A}$-subtype.

## GRANTS

This work was supported by Research Grant SAF2001-2656 from the Spanish Comisión Interministerial de Ciencia y Tecnología. D. Martí Canet received a fellowship from Universidad Cardenal Herrera.

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