ELSEVIER

European Journal of Pharmacology 341 (1998) 95–103

ejp

Expression and function of α_1-adrenoceptor subtypes in the porcine renal artery

Yinbi Zhou, Junji Nishimura, Hiroshi Seguchi, Katsuya Hirano, Hideo Kanaide *

Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, Fukuoka 812-82, Japan

Received 17 September 1997; revised 17 October 1997; accepted 21 October 1997

Abstract

We investigated the expression and function of α_1-adrenoceptor subtypes in the porcine renal artery. Reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing indicated that the mRNAs for α_{1a}- and α_{1b}-adrenoceptors were expressed in the porcine renal artery. Chloroethylclonidine, an α_{1B}- and α_{1D}-adrenoceptor antagonist, partially inhibited the phenylephrine-induced contraction, while 3 nM BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), an α_{1D}-adrenoceptor antagonist, had no effect. In contrast, 5-methylurapidil, an α_{1A}-adrenoceptor antagonist, induced a rightward shift of the phenylephrine concentration–response curve. The simultaneous measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension revealed that chloroethylclonidine pretreatment abolished the phenylephrine-induced increases in $[Ca^{2+}]_i$ and tension in the Ca^{2+}-free solution. The application of 5-methylurapidil (3 nM) to the chloroethylclonidine-pretreated strips completely inhibited the 3 μM phenylephrine-induced $[Ca^{2+}]_i$ and tension increase in normal PSS. We concluded that both α_{1A}- and α_{1B}-adrenoceptors mediate the phenylephrine-induced contraction of the porcine renal artery accompanied by an increase in $[Ca^{2+}]_i$, and that α_{1A}-adrenoceptors cause Ca^{2+} influx whereas α_{1B}-adrenoceptors mainly mediate Ca^{2+} release. © 1998 Elsevier Science B.V.

Keywords: α_1-Adrenoceptor subtype; Contraction; Ca^{2+} concentration, cytosolic; Renal artery, porcine

1. Introduction

α_1-Adrenoceptors play a crucial role in the regulation of vascular tone and systemic blood pressure (Vargas and Gorman, 1995). At least three α_1-adrenoceptor subtypes have been identified by pharmacological studies and by molecular cloning. The α_1-adrenoceptors are subclassified into α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor subtypes based on their pharmacological properties. α_{1A}-adrenoceptors are selectively antagonized by 5-methylurapidil, while α_{1B}- and α_{1D}-adrenoceptors are sensitive to alkylation by chloroethylclonidine. In addition, α_{1D}-adrenoceptors can be selectively antagonized by BMY 7378 (8-[2-[4-(2-ethoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride) (Goetz et al., 1995; Vargas and Gorman, 1995). Although the nomenclature of these subtypes and the alignment of the cloned subtypes to the pharmacologically defined subtypes used to be confusing, a generally accepted classification system is now available. According to this new classification system, the lower case is used to represent the cloned subtypes, namely, α_{1a} (previous α_{1c}), α_{1b} (previous α_{1b}) and α_{1d} (previous α_{1a} or $\alpha_{1a/d}$), which show the same pharmacologic properties with the pharmacologically identified α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, respectively (Minneman and Esbenshade, 1994; Bylund et al., 1994; Michel et al., 1995; Vargas and Gorman, 1995). This classification system is therefore used throughout the article to avoid confusion.

α_1-Adrenoceptor subtypes are widely distributed in vascular smooth muscle, and more than one subtype is co-expressed in some vessels, as shown by functional studies, receptor binding studies and mRNA analysis. However, the distinct function of each α_1-adrenoceptor subtype in vascular smooth muscle has yet to be elucidated (for review see Vargas and Gorman, 1995; Michel et al., 1995). In cells of non-vascular tissues, it has been hypothesized that α_{1B}-adrenoceptors stimulate intracellular Ca^{2+} release whereas α_{1A}-adrenoceptors induce Ca^{2+} influx, as a result of coupling to different signal transduction pathways (Han et al., 1987, 1990b; Minneman, 1988; Tsujimoto et al., 1989; Wilson and Minneman, 1990; Nomura et al., 1993;

* Corresponding author. Tel.: +81-92-6425548; fax: +81-92-6425552; E-mail: kanaide@molcar.med.kyushu-u.ac.jp

Horie et al., 1994; Blue et al., 1994). In contrast, several studies with rat vascular smooth muscle cells have indicated that α_{1A}-adrenoceptors induce both intracellular Ca^{2+} release and Ca^{2+} influx (Oriowo et al., 1992, Oriowo and Ruffolo, 1992; Sayet et al., 1993; Lepretre et al., 1994). The findings of Suzuki et al. (1990) for the rabbit aorta, are consistent with the above hypothesis concerning non-vascular tissues. It therefore remains controversial as to whether or not this hypothesis is valid for vascular smooth muscle.

It should be noted that some of the controversy regarding the role of α_1-adrenoceptors in mobilizing Ca^{2+} in vascular smooth muscle might be due to the complexity of the vessels, which have been characterized by other researchers to contain both α_{1A}- and α_{1B}-adrenoceptors (Han et al., 1990a; Piascik et al., 1994). Another problem concerning this controversy is that the simultaneous measurement of $[Ca^{2+}]_i$ and tension of intact vascular strips has not been previously performed to test this hypothesis in vascular tissue. In one study, $[Ca^{2+}]_i$ was measured in enzymatically dispersed or cultured rat portal venous smooth muscle cells (Lepretre et al., 1994). We thus considered it to be essential to perform simultaneous measurement of $[Ca^{2+}]_i$ and tension on intact vascular strips, in which the α_1-adrenoceptor subtypes are well characterized by pharmacological and by molecular biological techniques.

In the present study, we examined the effects of various antagonists of α_1-adrenoceptors on the phenylephrine-induced contraction and determined the expression of mRNA specific for each α_1-adrenoceptor subtype by using reverse transcription polymerase chain reaction (RT-PCR), in order to characterize the function of α_1-adrenoceptor subtypes in the regulation of phenylephrine-induced contractions of porcine renal artery. The simultaneous measurement of $[Ca^{2+}]_i$ and tension was performed to directly show the role of each α_1-adrenoceptor subtype in Ca^{2+} mobilization. The obtained results were consistent with the hypothesis based on studies of non-vascular tissues. Namely, α_{1A}-adrenoceptors are suggested to induce Ca^{2+} influx, while α_{1B}-adrenoceptors are considered to stimulate intracellular Ca^{2+} release.

2. Materials and methods

2.1. Tension studies and $[Ca^{2+}]_i$ measurement

2.1.1. Tissue preparation

Kidneys were obtained from pigs of either sex from a local slaughterhouse. They were brought to the laboratory in a pre-aerated normal physiological salt solution (PSS) immediately after the animals were killed. The third branch of the porcine renal artery was isolated and the fat and adventitia were carefully removed by dissection under a binocular microscope. The vessel was then cut open longitudinally and the endothelium was removed by rubbing the luminal surface with a cotton swab to obtain medial strips (approximately $1 \times 3 \times 0.1$ mm). All procedures were done in normal PSS aerated by mixed gas containing 5% CO_2 and 95% O_2.

2.1.2. Simultaneous measurement of $[Ca^{2+}]_i$ and tension

The simultaneous measurement of $[Ca^{2+}]_i$ and tension of the porcine renal artery was done as previously reported (Seguchi et al., 1996). In brief, to load fura-2 in the form of acetoxymethyl ester (fura-2/AM), the strips were incubated in Dulbecco-modified Eagle's medium containing 25 μM fura-2/AM dissolved in dimethyl sulphoxide and 5% fetal bovine serum for 4 h at 37°C. After being loaded with fura-2, the strips were rinsed with normal PSS to remove the dye before starting the measurements. The strips were mounted vertically in a quartz organ bath with one end connected to a force-displacement transducer (strain gauge TB-612T, Nihon Koden, Japan). Before the specific protocol, the strips were stimulated with 118 mM K^+ for 10 min, every 15 min, until a stable contraction was obtained. The resting tension was increased stepwise until the maximal contraction was achieved. The resting tension thus obtained was about 350 mg. Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were monitored by using a front-surface fluorometer specifically designed for fura-2 fluorometry (model CAM-OF) (Abe et al., 1990; Hirano et al., 1990). The ratio of the fura-2 fluorescence intensities at 340 nm excitation to those at 380 nm excitation, which were collected at 500 nm, was then monitored to indicate the level of $[Ca^{2+}]_i$. The ratio and tension were expressed as a percentage, taking the values at rest in normal PSS solution (5.9 mM K^+) and in depolarization solution (118 mM K^+) to be 0% and 100%, respectively.

2.1.3. Drug application

The competitive antagonists 5-methylurapidil and BMY 7378 were applied 10 min before the application of phenylephrine unless otherwise indicated. The chloroethylclonidine pretreatment involved incubating the strips in PSS solutions containing a given concentration of chloroethylclonidine at 37°C for 40 min, followed by washing with PSS repeatedly every 10 min, for an additional 30 min. The same procedure was done in the controls except that the antagonist was not added to eliminate any possible time-related differences. The cumulative dose–response curves of phenylephrine were made separately in the presence or absence of antagonist and in chloroethylclonidine-pretreated or -untreated strips. In the $[Ca^{2+}]_i$ measurement protocol, one submaximal dose of phenylephrine was applied.

2.2. RT-PCR and sequencing of PCR product

Total RNA was isolated from renal arterial smooth muscle cells according to the method described by Chom-

czynski and Sacchi (1987). Care was taken not to include either the endothelium or the adventitia. Possible contaminating genomic DNA was digested by DNase. Since the cDNA sequences for pig α_{1a}- or α_{1b}-adrenoceptors have yet to be described, we designed the primers from the conserved region of the known cDNA sequences for α_{1a} or α_{1b}, based on the rat sequence. The oligonucleotides used for the primers are as follows. For α_{1a}, 5′-ACTGAGGGAGATGGTGTGGA-3′ (RT primer), 5′-AAGTGACGCTCCGCATC CA-3′ (PCR sense primer) and 5′-GATACCCGAGCCAAAATACTATTTT-3′ (PCR antisense primer). For α_{1b}, 5′-TCTTTTCCCTGGAGAACTTA-3′ (RT primer), 5′-ACCGGCCACAACACATCAGC-3′ (PCR sense primer) and 5′-CTGCCCAGAT GTCACAGAAG-3′ (PCR antisense primer). RT-PCR was done as previously described (Nishimura et al., 1996). In brief, the total RNA (2 μg) from the porcine renal artery was used for the RT reaction in a total volume of 20 μl. An aliquot (1 μl) of RT product was amplified by PCR in a total volume of 11 μl. The thermal cycle used in this study was (1) denaturation for 30 s at 94°C, (2) annealing of primers for 90 s at 55°C, (3) extension of primers for 30 s at 72°C. The PCR amplifications were performed for 40 cycles. A portion (10 μl) of the PCR mixture was electrophoresed in 3% agarose gel in a TAE buffer [40 mM Tris(hydroxymethyl)aminomethane-acetate (pH 7.5–7.8), 1 mM EDTA]. The gel was stained with ethidium bromide and photographed. The expected size of the product for α_{1a}-adrenoceptors was thus 258 bp while that for α_{1b}-adrenoceptors was 349 bp, according to the sequence of rat. The nucleotide sequences of the PCR products for porcine α_{1a}- and α_{1b}-adrenoceptors were directly determined by Sawady Technology (Tokyo), using an Autosequencer (373S, ABI, Foster City, CA).

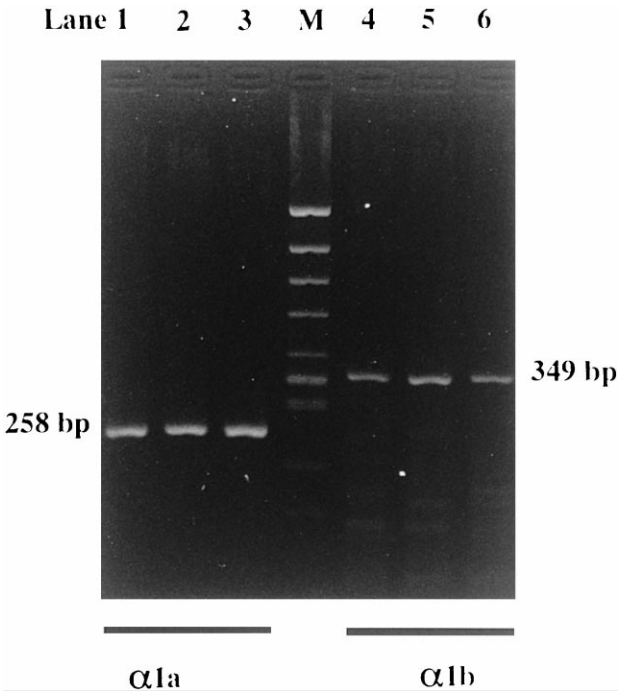

Fig. 1. Detection of α_{1a}- and α_{1b}-adrenoceptor mRNAs by RT-PCR in the porcine renal artery. PCR amplification of α_{1a}- and α_{1b}-adrenoceptor was performed for 40 cycles. The predicted sizes of these PCR products are shown on the left (α_{1a}) or right (α_{1b}) of the photograph. The experiments were done with the total RNA from 3 different pigs. The lane marked 'M' represents the DNA size marker (ϕX174/Hinc II digest). The size of each band is, from top to bottom, 1057, 770, 612, 495, 392, 345+341+335, 297+291, 210 and 162 bp.

A

```
5' cgg aaa aac gcc ccg gtc gga ggc agc ggg gtg acc
   Arg Lys Asn Ala Pro Val Gly Gly Ser Gly Val Thr

agc gcc aag aac aag acg cac ttc tcc gtg aga ctc ctc
Ser Ala Lys Asn Lys Thr His Phe Ser Val Arg Leu Leu

aag ttt tcc cga gag aag aaa gcg gcc aag aca ctg ggc
Lys Phe Ser Arg Glu Lys Lys Ala Ala Lys Thr Leu Gly

ata gtg gtc ggc tgc ttc gtc ctc tgc tgg ctg cct ttt
Ile Val Val Gly Cys Phe Val Leu Cys Trp Leu Pro Phe

ttc tta gtg atg ccc att ggg tct ttc ttt cct gat ttc
Phe Leu Val Met Pro Ile Gly Ser Phe Phe Pro Asp Phe

agg ccc tca gaa aca gtt ttt 3'
Arg Pro Ser Glu Thr Val Phe
```

B

```
5' gcc cac tgg gga gag ttg aaa aat gcc aac ttc act
   Ala His Trp Gly Glu Leu Lys Asn Ala Asn Phe Thr

ggc ccc aac cag acc tcg agc aac tcc aca ctg ccc cag
Gly pro Asn Gln Thr Ser Ser Asn Ser Thr Leu Pro Gln

ctg gac atc acc agg gcc atc tcc gtg ggc ctg gtg ctg
Leu Asp Ile Thr Arg Ala Ile Ser Val Gly Leu Val Leu

ggt gcc ttc atc ctc ttt gcc atc gtg ggc aac atc ctg
Gly Ala Phe Ile Leu Phe Ala Ile Val Gly Asn Ile Leu

gtc atc ttg tct gtg gcc tgc aac cgt cac ctg cgg acg
Val Ile Leu Ser Val Ala Cys Asn Arg His Leu Arg Thr

ccc acc aac tac ttc atc gtc aac ctg gcc att gcc gac
Pro Thr Asn Tyr phe Ile Val Asn Leu Ala Ile Ala Asp

ctg ctg cta agc ttc act gtg ctg ccc ttc tca gcg gcc
Leu Leu Leu Ser Phe Ser Val Leu Pro Phe Ser Ala Ala

ctg gag gtg ctg ggc tac tgg gtg ctg g 3'
Leu Glu Val Leu Gly Tyr Trp Val Leu
```

Fig. 2. The partial nucleotide (lower case) and deduced amino acid (upper case) sequences for pig α_{1a}- (A) and α_{1b}- (B) adrenoceptors. The nucleotide sequences of the PCR products for α_{1a}- and α_{1b}-adrenoceptors of porcine renal artery sample were determined by direct sequencing.

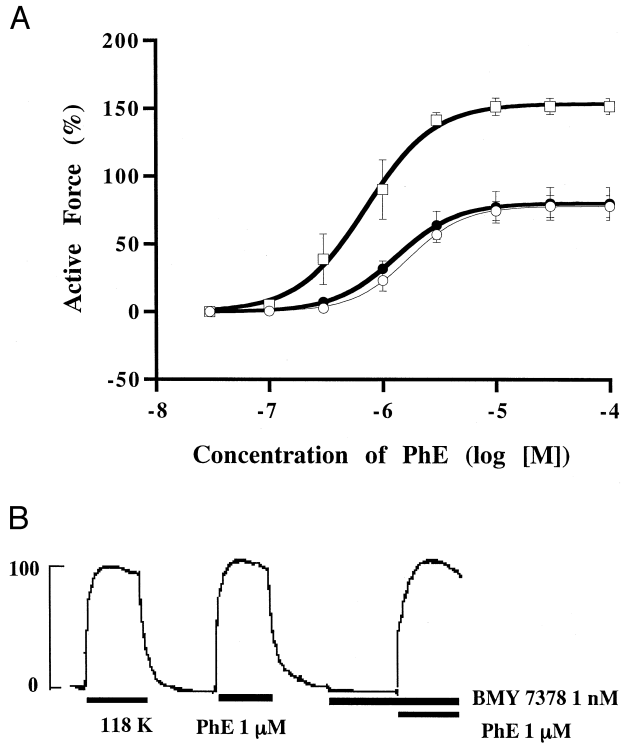

Fig. 3. (A) The effects of various concentrations of chloroethylclonidine (□; 0: control, ○; 30 μM; ●; 100 μM) pretreatment on the concentration–response curve for phenylephrine. The tension development was expressed as a percentage of the 118 mM K^+-induced contraction. The values were expressed as the means ± S.E. ($n = 4$). (B) A representative recording of the effect of 3 nM BMY 7378 on the contraction induced by 1 μM phenylephrine. The trace shown is representative of three independent experiments.

2.3. Chemicals and solution

Phenylephrine was purchased from the Sigma Chemical (St. Louis, MA). 5-methylurapidil, BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), and chloroethylclonidine were purchased from Research Biochemicals, (Natick, MA). Fura-2/AM was obtained from Dojindo (Kumamoto). Moloney Murine Leukemia Virus reverse transcriptase, 5 × RT buffer, and 0.1 M dithiothreitol were purchased from BRL (Gaithersburg). dNTPs (dATP, dCTP, dTTP, dGTP) were from TaKaRa (Kyoto). RNase inhibitor was purchased from TOYOBO (Osaka). Taq DNA polymerase was from Pharmacia Biotech (Uppsala). RQ1 RNase-free DNase was from Promega (Madison, WI). All other chemicals were of the highest grade commercially available. The oligonucleotides for primers were synthesized by Sawady Technology (Tokyo).

The composition of the normal PSS was (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. The Ca^{2+}-free solution (Ca^{2+}-free PSS) contained 2 mM EGTA instead of 1.25 mM $CaCl_2$. High K^+ PSS was made by the equimolar substitution of KCl for NaCl.

2.4. Data analysis

The EC_{50} value, a concentration that increased tension to 50% of the maximum response, and the slope factor were determined from the cumulative concentration–response curves fitted according to a four-parameter logistic model (De Lean et al., 1987). The dissociation constant (pK_B) for an antagonist was derived from the equation (Arunlakshana and Schild, 1959):

$$pK_B = \log(\text{CR-1}) - \log(B)$$

where CR is the concentration ratio (ratio of EC_{50} value in the presence and absence of the antagonist) obtained with the concentration (B) of a given antagonist. The values are expressed as the means ± S.E. Student's t test was used to

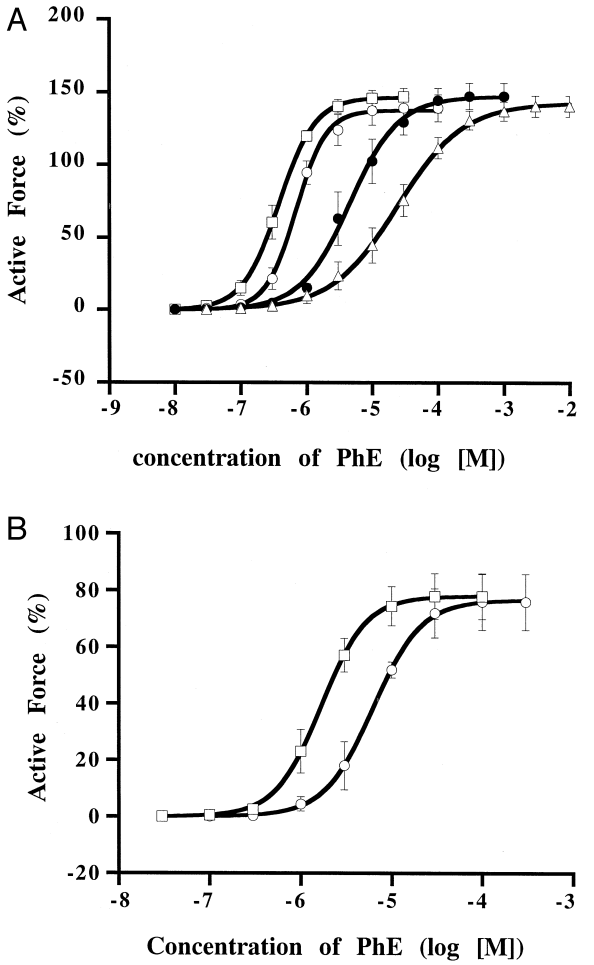

Fig. 4. (A) The effects of various concentrations of 5-methylurapidil (□; 0: control, ○; 3 nM, ●; 10 nM, △; 100 nM) on the concentration–response curve for phenylephrine. The developed tension was expressed as a percentage of the 118 mM K^+-induced contraction. The values were expressed as the means ± S.E. ($n = 4$ to 5). (B) The effects of 3 nM 5-methylurapidil (○) on the contraction induced by various concentrations of phenylephrine (□; 0: control) in the strips pretreated with 30 μM chloroethylclonidine. The developed tension was expressed as a percentage of the 118 mM K^+-induced contraction. The values were expressed as the means ± S.E. ($n = 4$).

determine statistically significant differences. $P < 0.05$ was considered to be of statistical significance.

3. Results

3.1. Detection of α_1-adrenoceptor subtype mRNAs in the porcine renal artery

Fig. 1 shows the expression of α_{1a}- and α_{1b}-adrenoceptor mRNAs in the porcine renal artery, determined by RT-PCR with total RNA prepared from porcine renal artery specimens and the primers for each mRNA. PCR amplification for both α_{1a} and α_{1b} was performed for 40 cycles. Bands of the expected sizes for α_{1a}- and α_{1b}-adrenoceptors were clearly seen in all samples from 3 different pigs. In order to confirm the specificity of RT-PCR for α_{1a}- and α_{1b}-adrenoceptors, the sequences of the PCR products thus obtained were determined by direct sequencing. The nucleotide and amino acid sequences for the PCR product for α_{1a}- and α_{1b}-adrenoceptors are shown in Fig. 2. As the sequences of pig α_{1a}- and α_{1b}-adrenoceptor mRNA have not yet been published, we compared them with the corresponding regions of the α_{1a}- and α_{1b}-adrenoceptor cDNA of other species. The similarities of the nucleotide sequences in comparison with the corresponding region of bovine, human, rabbit and rat α_{1a}-adrenoceptor cDNA sequences (obtained from Genbank data base) were 95.3, 92.5, 91.6 and 84.5%, respectively. In contrast, those for the amino acid sequences were 98.6, 94.4, 93.0 and 91.6%, respectively. The similarities in the nucleotide sequence of porcine α_{1b}-adrenoceptor to the corresponding region of human, hamster and rat α_{1b}-adrenoceptor cDNA (Genbank date base) were 96.0, 91.6 and 92.3%, respectively. Those of the amino acid sequence were 99.0%, 96.0% and 96.0%. These analyses thus

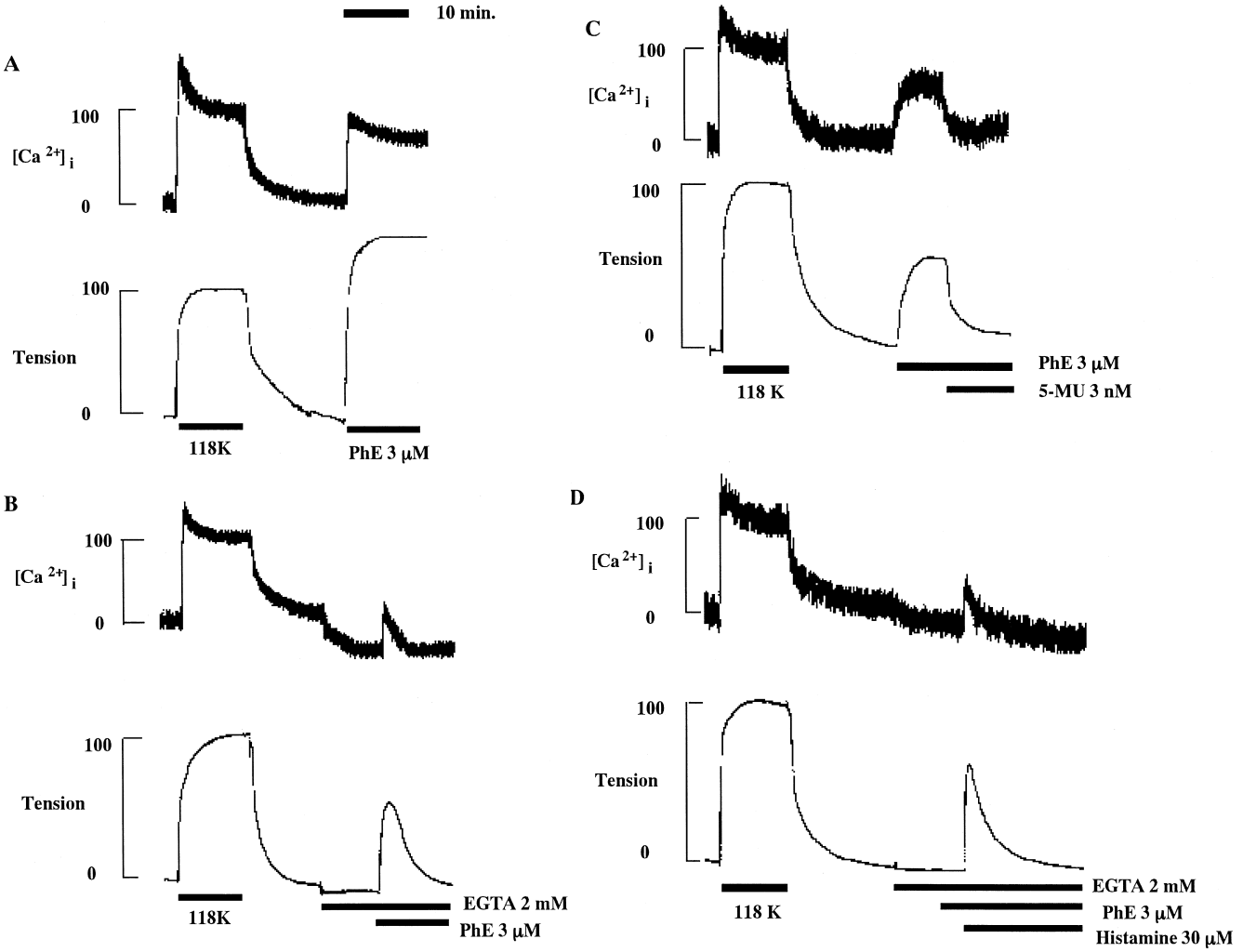

Fig. 5. Representative recordings showing the effect of 3 μM phenylephrine on $[Ca^{2+}]_i$ and tension in the presence (A and C) or absence (B and D) of extracellular Ca^{2+} in strips unpretreated (A and B) or pretreated (C and D) with 30 μM chloroethylclonidine. In (B) and (D), 3 μM phenylephrine was applied in the Ca^{2+}-free 2 mM EGTA solution. In (C), 3 nM 5-methylurapidil was added 3 min. after the contraction reached a steady state following the application of 3 μM phenylephrine in the PSS solution. In (D) 30 μM histamine was added after the application of 3 μM phenylephrine in the Ca^{2+}-free 2 mM EGTA solution. The traces shown are representative of three independent experiments.

strongly indicated that the RT-PCR performed in the present study specifically amplified and detected porcine α_{1a}- and α_{1b}-adrenoceptor mRNAs.

3.2. Pharmacological characterization of α_1-adrenoceptor subtypes in the porcine renal artery

As shown in Fig. 3A, the maximal phenylephrine-induced contraction was significantly inhibited by the pretreatment of the strips with 30 μM chloroethylclonidine, compared with the control contraction observed with no chloroethylclonidine treatment ($77.6 \pm 8.1\%$ vs. $151.4 \pm 5.9\%$ of 118 mM K^+-induced contraction, mean $\pm$ S.E.; $P < 0.001$; $n = 4$). However, the inhibitory effect was only partial, since the phenylephrine-induced contraction was not further inhibited by increasing the concentration of chloroethylclonidine to 100 μM ($80.0 \pm 12.0\%$, mean $\pm$ S.E., $n = 4$; Fig. 3A). Fig. 3B shows a representative recording of the effect of 3 nM BM Y7378, an α_{1D}-adrenoceptor antagonist, on the contraction induced by 1 μM phenylephrine. BMY 7378 (3 nM) had no effect on the contraction induced by 1 μM phenylephrine. Even when the phenylephrine concentration was reduced to 0.3 μM, BMY 7378 (3 nM) had no effect (data not shown).

The concentration–response curves for phenylephrine in the presence or absence of various concentrations of 5-methylurapidil, an α_{1A}-adrenoceptor antagonist, are shown in Fig. 4A. 5-methylurapidil at concentrations of 3, 10 and 100 nM significantly shifted the concentration–response curve for phenylephrine to the right, thus increasing the EC_{50} values of phenylephrine from 0.38 ± 0.07 to 0.70 ± 0.09 μM (mean $\pm$ S.E., $n = 4$; $P < 0.05$), 5.8 ± 2.5 μM (mean $\pm$ S.E., $n = 4$; $P < 0.05$) and 26.9 ± 7.9 μM (mean $\pm$ S.E., $n = 5$; $P < 0.01$), respectively. In addition, the slope of the curve made in the presence of 100 nM 5-methylurapidil became shallower than that of the control (0.96 ± 0.09 vs. 1.77 ± 0.21, $n = 5$; $P < 0.05$). After pretreatment of the strips with 30 μM chloroethylclonidine, 3 nM 5-methylurapidil caused a further rightward shift of the phenylephrine concentration–response curve (Fig. 4B), thus significantly increasing the EC_{50} values from 1.73 ± 0.50 to 6.0 ± 1.6 μM (mean $\pm$ S.E., $n = 4$; $P < 0.05$). The derived pK_B value for 5-methylurapidil under these conditions was 8.96 ± 0.04 (mean $\pm$ S.E., $n = 4$).

3.3. Simultaneous measurements of $[Ca^{2+}]_i$ and tension

Fig. 5A and B show representative recordings of $[Ca^{2+}]_i$ and tension induced by 3 μM phenylephrine in the presence or absence of extracellular Ca^{2+}. In normal PSS, the phenylephrine-induced increase in $[Ca^{2+}]_i$ was composed of two phases, a transient phase followed by a plateau, and was also accompanied by a monophasic increase in tension (Fig. 5A). In the Ca^{2+}-free PSS, 3 μM phenylephrine induced transient increases in $[Ca^{2+}]_i$ and tension (Fig. 5B). Fig. 5C and D show the 3 μM phenylephrine-induced changes in $[Ca^{2+}]_i$ and tension of the strips pretreated with 30 μM chloroethylclonidine in normal PSS (C) or in Ca^{2+}-free PSS (D). In the normal PSS solution, 3 μM phenylephrine still induced sustained increases in $[Ca^{2+}]_i$ (54.1%) and tension (52.3%), although they were much smaller than those in the chloroethylclonidine-untreated strips (69.8% for $[Ca^{2+}]_i$ and 140.5% for tension; Fig. 5A). The application of 3 nM 5-methylurapidil induced decreases in $[Ca^{2+}]_i$ and tension, which nearly reached the baseline (Fig. 5C). In the Ca^{2+}-free PSS, the phenylephrine-induced transient increases in $[Ca^{2+}]_i$ and tension were completely abolished by the pretreatment with chloroethylclonidine. However, the next application of 30 μM histamine induced transient increases in $[Ca^{2+}]_i$ and tension (Fig. 5D), which were similar to those obtained without chloroethylclonidine pretreatment (data not shown).

4. Discussion

In the present study, we characterized the expression and contraction-regulating function of α_1-adrenoceptor subtypes in the porcine renal artery and tested the hypothesis that α_{1A}-adrenoceptors induce Ca^{2+} influx, while α_{1B}-adrenoceptors stimulate intracellular Ca^{2+} release. The major findings are that (1) the smooth muscle cells of the porcine renal artery expressed both α_{1A}- and α_{1B} (or α_{1a} and α_{1b})-adrenoceptors as evidenced by pharmacological analysis and mRNA analysis with RT-PCR, (2) α_{1A}-adrenoceptors only induced Ca^{2+} influx, while α_{1B}-adrenoceptors mainly stimulated intracellular Ca^{2+} release, as directly assessed by the simultaneous measurement of $[Ca^{2+}]_i$ and tension.

The RT-PCR analyses indicated that both α_{1a}- and α_{1b}-adrenoceptor mRNAs were expressed in the porcine renal artery (Fig. 1). The specificity of the RT-PCR was confirmed by the direct sequencing of each PCR product (Fig. 2). The co-expression of α_{1a}- and α_{1b}-adrenoceptor mRNAs in the same vascular smooth muscle cells is well documented (Vargas and Gorman, 1995). In addition, the mRNA of both receptor subtypes has also been detected in the rat renal artery by in situ hybridization (Piascik et al., 1994). The results of the present study are therefore consistent with those of previous reports.

We next examined whether or not both subtypes of α_1-adrenoceptor were involved in regulating the contraction of the porcine renal artery. In the functional study, we observed that pretreatment of the strips with 30 μM chloroethylclonidine significantly depressed the maximal phenylephrine-induced response (Fig. 3A). This result thus indicated that α_{1B}- and/or α_{1D}-adrenoceptors are involved in the contraction of the porcine renal artery, since chloroethylclonidine blocks both α_{1B}- and α_{1D}-adrenoceptors (Minneman and Esbenshade, 1994; Vargas and Gor-

man, 1995). However, BMY 7378, with pK_I values of 9.4, 7.2 and 6.6 for human α_{1d}-, α_{1b}- and α_{1a}-adrenoceptors, respectively (Goetz et al., 1995; Vargas and Gorman, 1995), failed to influence the phenylephrine-induced contraction at a concentration of 3 nM (Fig. 3B), suggesting that α_{1D}-adrenoceptors have, if any, negligible effects on the contraction of the porcine renal artery. However, α_{1B}-adrenoceptors may not be the only α_1-adrenoceptor subtype mediating contraction of the porcine renal artery, because 30 μM chloroethylclonidine pretreatment only depressed about half of the maximal phenylephrine-induced response and the effect was not significantly enhanced by increasing the chloroethylclonidine concentration up to 100 μM. The partial inhibition of the phenylephrine-induced contraction by chloroethylclonidine pretreatment could be explained by the presence of another α_1-adrenoceptor subtype which regulates contraction, namely α_{1A}-adrenoceptors. The existence of functioning α_{1A}-adrenoceptors in the porcine renal artery was confirmed by the inhibitory effect of 5-methylurapidil, a selective α_{1A}-adrenoceptor antagonist (Fig. 4). The effective concentrations of 5-methylurapidil (around 3 nM) closely agree with those of previous reports (Furukawa et al., 1995; Vargas and Gorman, 1995). In addition, the shallow curve slope caused by 100 nM 5-methylurapidil further supported the involvement of both α_1-adrenoceptor subtypes in regulating contraction, as 5-methylurapidil may also serve as an α_{1B}-adrenoceptor antagonist at higher concentrations (Michel et al., 1995; Vargas and Gorman, 1995). This was demonstrated when α_{1B} was inactivated by pretreatment with 30 μM chloroethylclonidine. Under this condition, 3 nM of 5-methylurapidil further caused a rightward shift of the phenylephrine concentration response curve, and yielded a pK_B value of 8.96 (Fig. 4B), which is characteristic of this antagonist for α_{1A}-adrenoceptors (Furukawa et al., 1995; Vargas and Gorman, 1995). This result, per se, favors that α_{1D}-adrenoceptors have, if any, negligible effects on the contraction of the porcine renal artery, since chloroethylclonidine only acts as a partial irreversible antagonist of α_{1D}-adrenoceptors (Vargas and Gorman, 1995). The above observations together indicated that both α_{1A}- and α_{1B}-adrenoceptors are present and functioning in the porcine renal artery.

After establishing that the porcine renal artery has both α_{1A}- and α_{1B}-adrenoceptors, we tested the above-mentioned hypothesis by simultaneously measuring $[Ca^{2+}]_i$ and tension. As shown in Fig. 5A and B, the phenylephrine-induced increase in $[Ca^{2+}]_i$ in the normal PSS solution consisted of two components, namely, intracellular Ca^{2+} release and Ca^{2+} influx dependent on the presence of extracellular Ca^{2+}. The intracellular Ca^{2+} release in the absence of extracellular Ca^{2+} was completely abolished by the pretreatment of the strips with 30 μM chloroethylclonidine (Fig. 5D), thus indicating that intracellular Ca^{2+} release is mediated exclusively by the α_{1B}-adrenoceptor subtype. The possibility that chloroethylclonidine might have a non-specific inhibitory effect on intracellular Ca^{2+} release was ruled out, because the intracellular Ca^{2+} release induced by 30 μM histamine was not inhibited (Fig. 5D). The increases in $[Ca^{2+}]_i$ and tension induced by phenylephrine in the chloroethylclonidine-treated strips in the normal PSS were considered to be the result of Ca^{2+} influx, because the intracellular Ca^{2+} release mediated by α_{1B}-adrenoceptors was completely inhibited by chloroethylclonidine treatment, as mentioned above. This Ca^{2+} influx component must thus have been mediated by α_{1A}-adrenoceptors, since this component was completely inhibited by 3 nM 5-methylurapidil (Fig. 5C). The simultaneous measurement of $[Ca^{2+}]_i$ and tension therefore identified the α_1-adrenoceptors that mediate Ca^{2+} mobilization in the intact porcine renal artery, and the results closely correlated with the results reported for non-vascular tissue specimens (Han et al., 1987, 1992; Tsujimoto et al., 1989; Horie et al., 1994; Blue et al., 1994) and for a tension study with the rabbit aorta (Suzuki et al., 1990).

This is the first study to show direct evidence of differences in the α_1-adrenoceptor subtype involved in Ca^{2+} mobilization in intact vascular smooth muscle. These differences have been suggested to be due to different, yet still to be defined, signal transduction pathways. It has been assumed that α_{1A}-adrenoceptors are directly coupled to the Ca^{2+} channel and that α_{1B}-adrenoceptors activate phospholipase C and induce formation of inositol 1,4,5-triphosphate (IP_3) (Han et al., 1987, 1992; Minneman, 1988), or that each of the above subtypes is coupled to a different G protein (Han et al., 1990b). It has been also reported that α_{1B}-adrenoceptors stimulate Ca^{2+} influx by an agonist-operated Ca^{2+} channel (Nomura et al., 1993; Blue et al., 1994; Esbenshade et al., 1994). In the present study, we were unable to confirm this because of the existence of both α_{1A}- and α_{1B}-adrenoceptors and the lack of an irreversible antagonist for α_{1A}-adrenoceptors. However, we speculate that α_{1B}-adrenoceptors, besides causing Ca^{2+} release, may also be partly responsible for Ca^{2+} influx, since the level of the phenylephrine-induced increase in $[Ca^{2+}]_i$ after the treatment with chloroethylclonidine was much smaller than that after treatment with vehicle (Fig. 5D).

Contrary to the findings of the present study, other reports have demonstrated no difference in Ca^{2+} mobilization between the signal transduction mechanism activated by two α_1-adrenoceptor subtypes (Oriowo et al., 1992; Oriowo and Ruffolo, 1992; Hara et al., 1993; Sayet et al., 1993; Lepretre et al., 1994). Such a discrepancy might arise for the following two reasons: (1) Some difficulties exist in the characterization of the α_1-adrenoceptor subtypes in intact tissues. In the rat portal vein myocytes for example, Sayet et al. (1993) and Lepretre et al. (1994) reported that α_{1A}-adrenoceptors were expressed, while Han et al. (1990a) reported that both α_{1A}- and α_{1B}-adrenoceptors were expressed. In addition, regarding the rat

aorta, three different results have been reported, namely, only one α_1-adrenoceptor subtype (Oriowo et al., 1992; Oriowo and Ruffolo, 1992), one α_{1B}-adrenoceptor subtype (Testa et al., 1995), or both the α_{1A}- and α_{1B}-adrenoceptors subtypes (Vargas and Gorman, 1995). (2) There are so far only limited data available on the direct measurement of $[Ca^{2+}]_i$ in relation to the α_1-adrenoceptor subtypes, as mentioned in Section 1. We thus used both RT-PCR to examine mRNA expression and pharmacological methods to determine the α_1-adrenoceptor subtypes involved. The function of each subtype in Ca^{2+} mobilization was thus assessed by the direct measurement of $[Ca^{2+}]_i$ in intact vascular strips.

In summary, the porcine renal artery expresses both α_{1A}- and α_{1B}-adrenoceptors, as evidenced by RT-PCR and direct sequencing, and both subtypes are involved in the regulation of the porcine renal artery, as evidenced by the findings of the pharmacological study. In addition, by simultaneously measuring $[Ca^{2+}]_i$ and tension, we confirmed the different role of α_{1A}- and α_{1B}-adrenoceptors in mediating Ca^{2+} mobilization in vascular smooth muscle.

Acknowledgements

We thank Mr. Brian Quinn for reading the manuscript. This study was supported in part by Grants-in-aid for Scientific Research on General Scientific Research (07407022, 07833008), for Developmental Scientific Research (06557045), and for Creative Basic Research Studies of the Intracellular Signaling Networks, Ministry of Education, Science, Sports and Culture, Japan, and by a Grant from ''The Vehicle Racing Commemorative Foundation''.

References

Abe, S., Kanaide, H., Nakamura, M., 1990. Front-surface fluorometry with fura-2 and effects of nitroglycerin on cytosolic calcium concentrations and on tension in the coronary artery of the pig. Br. J. Pharmacol. 101, 545–552.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. Chemother. 14, 48–58.

Blue, D.J., Craig, D.A., Ransom, J.T., Camacho, J.A., Insel, P.A., Clarke, D.E., 1994. Characterization of the alpha-1 adrenoceptor subtype mediating [^{3}H]-arachidonic acid release and calcium mobilization in Madin-Darby canine kidney cells. J. Pharmacol. Exp. Ther. 268, 1588–1596.

Bylund, D.B., Eikenberg, D.C., Hieble, J.P., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., Molinoff, P.B., Ruffolo, R.J., Trendelenburg, U., 1994. International Union of Pharmacology nomenclature of adrenoceptors. Pharmacol. Rev. 46, 121–136.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

De Lean, A., Munson, P.J., Rodbard, D., 1987. Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose–response curves. Am. J. Physiol 235, E97–102.

Esbenshade, T.A., Theroux, T.L., Minneman, K.P., 1994. Increased voltage-dependent calcium influx produced by α_{1B}-adrenergic receptor activation in rat medullary thyroid carcinoma 6-23 cells. Mol. Pharmacol. 45, 591–598.

Furukawa, K., Rosario, D.J., Smith, D.J., Chapple, C.R., Uchiyama, T., Chess, W.R., 1995. α_{1A}-adrenoceptor-mediated contractile responses of the human vas deferens. Br. J. Pharmacol. 116, 1605–1610.

Goetz, A.S., King, H.K., Ward, S.D., True, T.A., Rimele, T.J., Saussy, D.J., 1995. BMY 7378 is a selective antagonist of the D subtype of α_1-adrenoceptors. Eur. J. Pharmacol. 272, R5–6.

Han, C., Abel, P.W., Minneman, K.P., 1987. α_1-adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. Nature 329, 333–335.

Han, C., Li, J., Minneman, K.P., 1990a. Subtypes of α_1-adrenoceptors in rat blood vessels. Eur. J. Pharmacol. 190, 97–104.

Han, C., Wilson, K.M., Minneman, K.P., 1990b. α_1-adrenoceptor subtypes and formation of inositol phosphates in dispersed hepatocytes and renal cells. Mol. Pharmacol. 37, 903–910.

Han, C., Esbenshade, T.A., Minneman, K.P., 1992. Subtypes of α_1-adrenoceptors in DDT1 MF-2 and BC3H-1 clonal cell lines. Eur. J. Pharmacol. 226, 141–148.

Hara, N., Ichinose, M., Sawada, M., Maeno, T., 1993. The alpha 1A-adrenoceptor couples to Ca^{2+} mobilization in mouse peritoneal macrophages. Life Sci. 53, PL135–140.

Hirano, K., Kanaide, H., Abe, S., Nakamura, M., 1990. Effects of diltiazem on calcium concentrations in the cytosol and on force of contractions in porcine coronary arterial strips. Br. J. Pharmacol. 101, 273–280.

Horie, K., Hirasawa, A., Tsujimoto, G., 1994. The pharmacological profile of cloned and stably expressed α_{1b}-adrenoceptor in CHO cells. Eur. J. Pharmacol. 268, 399–407.

Lepretre, N., Mironneau, J., Arnaudeau, S., Tanfin, Z., Harbon, S., Guillon, G., Ibarrondo, J., 1994. Activation of alpha-1A adrenoceptors mobilizes calcium from the intracellular stores in myocytes from rat portal vein. J. Pharmacol. Exp. Ther. 268, 167–174.

Michel, M.C., Kenny, B., Schwinn, D.A., 1995. Classification of α_1-adrenoceptor subtypes. Naunyn-Schmiedeberg's Arch. Pharmacol. 352, 1–10.

Minneman, K.P., 1988. α_1-adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. Pharmacol. Rev. 40, 87–119.

Minneman, K.P., Esbenshade, T.A., 1994. α_1-adrenergic receptor subtypes. Ann. Rev. Pharmacol. Toxicol. 34, 117–133.

Nishimura, J., Sakihara, C., Zhou, Y., Kanaide, H., 1996. Expression of rho A and rho kinase mRNA in porcine vascular smooth muscle. Biochem. Biophys. Res. Commun. 227, 750–754.

Nomura, T., Kondo, H., Hasegawa, S., Watanabe, T., Yokoyama, R., Ukai, K., Tachibana, M., Sumi, I.C., Nomura, H., Hagino, Y., 1993. α_{1B}-adrenoceptor-mediated stimulation of Ca^{2+} mobilization and cAMP accumulation in isolated rat hepatocytes. Eur. J. Pharmacol. 246, 113–120.

Oriowo, M.A., Ruffolo, R.J., 1992. Activation of a single alpha-1-adrenoceptor subtype in rat aorta mobilizes intracellular and extracellular pools of calcium. Pharmacology 44, 139–149.

Oriowo, M.A., Nichols, A.J., Ruffolo, R.J., 1992. Receptor protection studies with phenoxybenzamine indicate that a single alpha 1-adrenoceptor may be coupled to two signal transduction processes in vascular smooth muscle. Pharmacology 45, 17–26.

Piascik, M.T., Smith, M.S., Soltis, E.E., Perez, D.M., 1994. Identification of the mRNA for the novel α_{1D}-adrenoceptor and two other α_1-adrenoceptors in vascular smooth muscle. Mol. Pharmacol. 46, 30–40.

Sayet, I., Neuilly, G., Rakotoarisoa, L., Mironneau, C., Mironneau, J., 1993. Relation between α_1-adrenoceptor subtypes and noradrenaline-induced contraction in rat portal vein smooth muscle. Br. J. Pharmacol. 110, 207–212.

Seguchi, H., Nishimura, J., Toyofuku, K., Kobayashi, S., Kumazawa, J., Kanaide, H., 1996. The mechanism of relaxation induced by atrial

natriuretic peptide in the porcine renal artery. Br. J. Pharmacol. 118, 343.

Suzuki, E., Tsujimoto, G., Tamura, K., Hashimoto, K., 1990. Two pharmacologically distinct α_1-adrenoceptor subtypes in the contraction of rabbit aorta: Each subtype couples with a different Ca^{2+} signalling mechanism and plays a different physiological role. Mol. Pharmacol. 38, 725–736.

Testa, R., Guarneri, L., Poggesi, E., Simonazzi, I., Taddei, C., Leonardi, A., 1995. Mediation of noradrenaline-induced contractions of rat aorta by the α_{1B}-adrenoceptor subtype. Br. J. Pharmacol. 114, 745–750.

Tsujimoto, G., Tsujimoto, A., Suzuki, E., Hashimoto, K., 1989. Glycogen phosphorylase activation by two different alpha 1-adrenergic receptor subtypes: Methoxamine selectively stimulates a putative α_1-adrenergic receptor subtype (α_{1a}) that couples with Ca^{2+} influx. Mol. Pharmacol. 36, 166–176.

Vargas, H.M., Gorman, A.J., 1995. Vascular alpha-1 adrenergic receptor subtypes in the regulation of arterial pressure. Life Sci. 57, 2291–2308.

Wilson, K.M., Minneman, K.P., 1990. Different pathways of [^{3}H]inositol phosphate formation mediated by α_{1a}- and alpha α_{1b}-adrenergic receptors. J. Biol. Chem. 265, 17601–17606.