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Clonidine Inhibits Phenylephrine-Induced Contraction of Rat Thoracic Aortae by Competitive Antagonism of α_1-Adrenoceptors Independent of α_2-Adrenoceptor Stimulation

Daisuke Chino, Mai Naramatsu, Keisuke Obara, Yoshio Tanaka*

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toho University, Chiba, Japan
Email: *yotanaka@phar.toho-u.ac.jp

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Abstract

Clonidine is a classically categorized α_2-adrenoceptor (α_2-AR) agonist that produces vascular contractions by stimulating arterial smooth muscle α_2-ARs. However, clonidine inhibits α_1-AR-mediated arterial contractions. Recently, it was suggested that repeated stimulation with clonidine induces desensitization of α_2-ARs, thus inhibiting noradrenaline-induced smooth muscle contractions. In the present study, we examined whether clonidine-mediated inhibition of α_1-AR contractions involves interactions with α_2-ARs in rat thoracic aortae. 1) Clonidine and guanfacine inhibited electrical field stimulation-induced contractions in a concentration-dependent, yohimbine-sensitive manner in isolated rat vas deferens preparations. 2) Clonidine almost completely suppressed phenylephrine-induced sustained contractions of rat thoracic aortae. 3) Clonidine competitively inhibited phenylephrine-induced contractions with a pA_2 value of 6.77 at concentrations between 10^{-7} and 10^{-6} M. At 10^{-5} M, clonidine inhibited phenylephrine-induced contractions and dramatically reduced maximum contractions. 4) In contrast, clonidine did not inhibit contractions produced by high KCl or prostaglandin $F_{2\alpha}$. 5) Inhibition of phenylephrine-induced sustained contractions by clonidine was also produced in the presence of yohimbine. However, guanfacine did not inhibit phenylephrine-induced sustained contractions. These findings suggest that clonidine inhibits phenylephrine-induced contraction of rat thoracic aortae by competitive antagonism of α_1-ARs, which is mediated through a mechanism independent of α_2-AR stimulation.

Keywords

Clonidine, α_2-Adrenoceptor (α_2-AR), α_1-Adrenoceptor (α_1-AR), Rat Aorta, Relaxation

1. Introduction

Clonidine is classically classified as a selective α_2-adrenoceptor (α_2-AR) agonist [1]. Clonidine has been used clinically to treat high blood pressure, particularly in severe cases in which other fundamental antihypertensive drugs such as calcium antagonists do not result in improved symptoms. In addition, clonidine is used as a pre-anesthetic medication for various surgeries. Underlying mechanisms of clonidine-induced hypotensive effects include reduction of efferent sympathetic nerve activities, attributable to clonidine-mediated stimulation of α_2-ARs in central nerves (vasomotor centers in bulbar), though α_2-ARs are also present peripherally [2].

Clonidine was first regarded as a presynaptic α_2-AR agonist that suppresses the release of noradrenaline (NA) from sympathetic nerve endings by stimulating presynaptic α_2-ARs [3] [4]. More recently, it has been shown that clonidine also targets postsynaptic α_2-ARs, and clonidine-induced vascular contractions are explained by the stimulation of vascular smooth muscle α_2-ARs [5] [6] [7]. Further, experimental evidence suggests possible interactions between clonidine and α_1-ARs, and it has been suggested that clonidine acts as a partial agonist for α_1-ARs. In vascular smooth muscles, clonidine causes contractile effects by directly stimulating α_1-ARs and competitive antagonistic effects against full agonists, including NA [5] [6] [8]. However, it is generally recognized that the effects of clonidine on α_1-ARs are generated through different mechanisms than those on α_2-ARs, and these effects are produced independently.

It has been suggested that α_1-ARs cross-talk with other drug receptors such as bradykinin B_2 receptors, endothelin-1 (ET_A) receptors, and lysophosphatidic acid (LPA) receptors [9]. Although there are few studies examining possible cross-talk between α_2-ARs and α_1-ARs, it has been shown that NA-induced contractions decrease as a result of clonidine-induced desensitization of α_2-ARs in testicular capsules [10]. Based on this background information, we hypothesized that clonidine inhibits α_1-AR-mediated contractions by stimulating α_2-ARs; clonidine-induced inhibition of α_1-AR-mediated contractions results from stimulation of α_2-ARs.

Clonidine strongly inhibits phenylephrine-induced contractions in isolated rat thoracic aorta smooth muscles; therefore, the present study was carried out to determine whether this is mediated through stimulation of α_2-ARs.

2. Materials and Methods

2.1. Drugs and Chemicals

The following drugs were used: clonidine and phenylephrine (Sigma-Aldrich Co., MO, USA); guanfacine (Enzo Life Sciences, NY, USA); NA and yohimbine (Wako Pure Chemical Industries, Osaka, Japan); prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Fuji Pharma Co., Ltd, Tokyo, Japan); acetylcholine (ACh) and tetrodotoxin (TTX) (Daiichi Sankyo, Tokyo, Japan). All other chemicals used in the present study were commercially available and of reagent grade.

All drugs were prepared as an aqueous solution and diluted with distilled water.

2.2. Animals

Male Wistar rats (8 - 9 weeks old, weighing 190 - 230 g, Sankyo Labo Service, Tokyo, Japan) were housed under controlled conditions (temperature 20˚C - 22˚C, relative air humidity 50% ± 5%, fixed 12 h-light [08:00 h to 20:00 h]/12 h-dark cycle). Food and water were available *ad libitum* to all animals. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Toho University School of Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.3. Preparation of Rat Aortic Rings

Wistar rats were sacrificed by cervical dislocation, and a section of the thoracic aorta from between the aortic arch and the diaphragm was isolated and placed in normal Tyrode's solution (158.3 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10.0 mM $NaHCO_3$, and 5.6 mM glucose). The aortae were cleaned of loosely adhering fat and connective tissue and cut into ring segments of approximately 2 mm in length. The aortic rings were stripped of endothelium by gently rubbing the intimal surface with an eyebrow brush.

2.4. Measurement of Tension Changes in Rat Aortae

The aortic rings were mounted using stainless steel hooks in 5-ml organ bath chambers (UC-5; UFER Medical Instrument, Kyoto, Japan) containing normal Tyrode's solution at 35˚C ± 1˚C (pH 7.4) and continuously bubbled with 95% (v/v) O_2 and 5% (v/v) CO_2. Changes in tension were isometrically measured with a force-displacement transducer (T7-8-240; Orientec, Tokyo, Japan) connected to a minipolygraph (Signal Conditioner: Model MSC-2; Primetech Corp., Tokyo, Japan). The data were recorded on a computer with PowerLab/ML-846™ and Chart™ (Version 7.0) software (ADInstruments Japan, Tokyo, Japan). During an equilibration period of 60 min, the bathing solution (normal Tyrode's solution) was changed every 20 min and the aortic rings were subjected to a tension of 1.0 g.

The rings were challenged with high-KCl (8×10^{-2} M) Tyrode's solution, which was prepared by replacing the NaCl with an equimolar amount of KCl at least twice until a reproducible maximal contractile response was obtained. The absence of endothelium was confirmed by the lack of relaxation in response to acetylcholine ACh (10^{-5} M) in the aortic rings pre-contracted with NA (10^{-7} M). After this procedure, preparations without functional endothelium were left to equilibrate for at least 40 min.

2.5. Experimental Procedure

Aortic ring preparations were pre-contracted with phenylephrine (3×10^{-7} M), PGF_{2a} (10^{-5} M), or high-KCl (4×10^{-2} M) Tyrode's solution, which was prepared

by substituting NaCl with an equimolar amount of KCl to produce sustained contractions. After muscle contractions reached a steady-state level, clonidine (10^{-9} - 10^{-5} M) was cumulatively applied to the bath medium. Effects of the cumulative addition of guanfacine (10^{-9} - 10^{-5} M) on phenylephrine (3×10^{-7} M)-induced contractions were examined using separate preparations.

In a separate series of experiments, the antagonistic effects of clonidine on phenylephrine-induced contractions were examined. Specifically, clonidine (10^{-7}, 10^{-6}, or 10^{-5} M) was added to the bath medium 20 min before the cumulative addition of phenylephrine (10^{-9} - 10^{-4} M). Moreover, to investigate possible involvement of α_2-ARs in the antagonism of phenylephrine-induced contractions by a high concentration of clonidine, yohimbine (10^{-5} M) or its vehicle (distilled water) was added to the bath medium 20 min before phenylephrine (10^{-6} M). After the phenylephrine-induced muscle contractions reached a steady-state level, clonidine (10^{-5} M) was applied to the bath medium.

2.6. Measurement of Tension Changes in Response to Nerve Electrical Field Stimulation (EFS) in Rat Vas Deferens

Wistar rats were sacrificed by cervical dislocation, and both right and left vas deferens were isolated. Each vas deferens was mounted between stimulating electrodes made of platinum using a cotton thread, with an optimal resting tension of 1.0 g; samples were maintained in a 20-ml organ bath containing normal Tyrode's solution continuously gassed with 95% O_2, 5% CO_2, and kept at 35˚C ± 1.0˚C (pH = 7.4). Changes in vas deferens smooth muscle (VDSM) tension were isometrically recorded with a force-displacement transducer (TB-612T; Nihon Kohden, Tokyo, Japan) connected to an amplifier (AP-621G; Nihon Kohden, Tokyo, Japan) and recorded with PowerLab2/26™ and Chart™ (Version 7.0) software (ADInstruments Japan Inc., Nagoya, Japan). Before starting the experiment, the preparations were equilibrated for 60 min with fresh bathing solution (normal Tyrode's solution) that was exchanged every 20 min. To ensure that preparations were capable of generating normal contractile responses after equilibrating, they were contracted with high-KCl (8×10^{-2} M) Tyrode's solution and then contracted twice with NA (10^{-6} M), which was subsequently washed out when the maximum contraction was recorded. When VDSM tension returned to basal levels, a train of 100-μsec square pulses of supramaximal intensity (50 V) was applied transmurally at a frequency between 2 and 16 Hz for 5 sec. The stimulus pulses were delivered by an electronic stimulator (SEN-3301, Nihon Kohden Corporation, Tokyo, Japan). The interval between EFS pulses was at least 3 min. When frequency-dependent contractions to EFS were obtained, clonidine (10^{-8} - 10^{-7} M) or guanfacine (10^{-9} - 10^{-8} M) was cumulatively added at 10-min intervals before the next series of frequency-dependent EFS pulses. The antagonistic action of yohimbine (10^{-6} M) was assessed by examining its effect on clonidine- or guanfacine-induced inhibition. The neurogenic characteristics of constrictor responses to EFS were confirmed at the end of every experiment by the abolition of responses in the presence of TTX (3×10^{-7} M).

2.7. Evaluation and Statistical Analysis

The relative relaxation of α_2-AR agonists (clonidine and guanfacine) on sustained vascular contractions is expressed as percent relaxation; the tension level just before adding the agonists was considered 0% relaxation, and the basal tension level before applying vasoconstrictor stimulations (phenylephrine, $PGF_{2\alpha}$, or high KCl) was considered 100% relaxation. To construct control concentration-response curves (CRCs) for phenylephrine, percent contractions were calculated by using the tension level before the cumulative application of phenylephrine as 0% and the maximum contraction obtained with application of high KCl (8×10^{-2} M) as 100% for each preparation.

EC_{50} and E_{max} values were defined as the concentration required to produce a 50% response and maximal response induced by phenylephrine, respectively. EC_{50} values were converted to logarithmic values (pD_2, $-\log EC_{50}$) for statistical analysis. Competitive antagonistic potency is expressed as a pA_2 value determined from a Schild plot analysis of the results [11].

The data are expressed as means ± S.E.M. or 95% confidence intervals (CIs); n refers to the number of experiments. Statistical analysis was performed with the unpaired student's *t*-test or one-way analysis of variance (one-way ANOVA) followed by Dunnett's multiple comparison test using GraphPad Prism™ (version 7.00; GraphPad Software, San Diego, C.A., USA). A P value < 0.05 was considered significant in all cases.

3. Results

3.1. Inhibitory Effects of Clonidine on EFS-Induced Rat Vas Deferens Contractions and the Antagonistic Effects of Yohimbine vs. Clonidine

Figure 1 shows the effects of clonidine on EFS-induced contractions in rat vas deferens in the absence and presence of yohimbine. EFS evoked frequency (2 - 16 Hz)-dependent contractions in rat vas deferens (Figure 1 control). These contractions were neurogenic, but not myogenic—as they were completely abolished in the presence of TTX (3×10^{-7} M) (data not shown). Clonidine suppressed EFS-induced vas deferens contractions in a concentration-dependent manner (10^{-8} - 10^{-7} M); statistical significance was determined for 3×10^{-8} M vs. 2 Hz and 4 Hz contractions ($P < 0.05$) and 10^{-7} M vs. 2 to 16 Hz contractions ($P < 0.05$ for 2, 4, and 16 Hz and $P < 0.01$ for 8 Hz) (Figure 1(B)). In contrast, suppressed contractions attributable to clonidine (10^{-7} M) strongly recovered in the presence of yohimbine (10^{-6} M); thus, there were no statistical significances between control contractions and contractions in the presence of clonidine plus yohimbine.

3.2. Inhibitory Effects of Guanfacine on EFS-Induced Vas Deferens Contractions and the Antagonistic Effects of Yohimbine vs. Guanfacine

Figure 2 shows the effects of guanfacine on EFS-induced contractions in rat vas

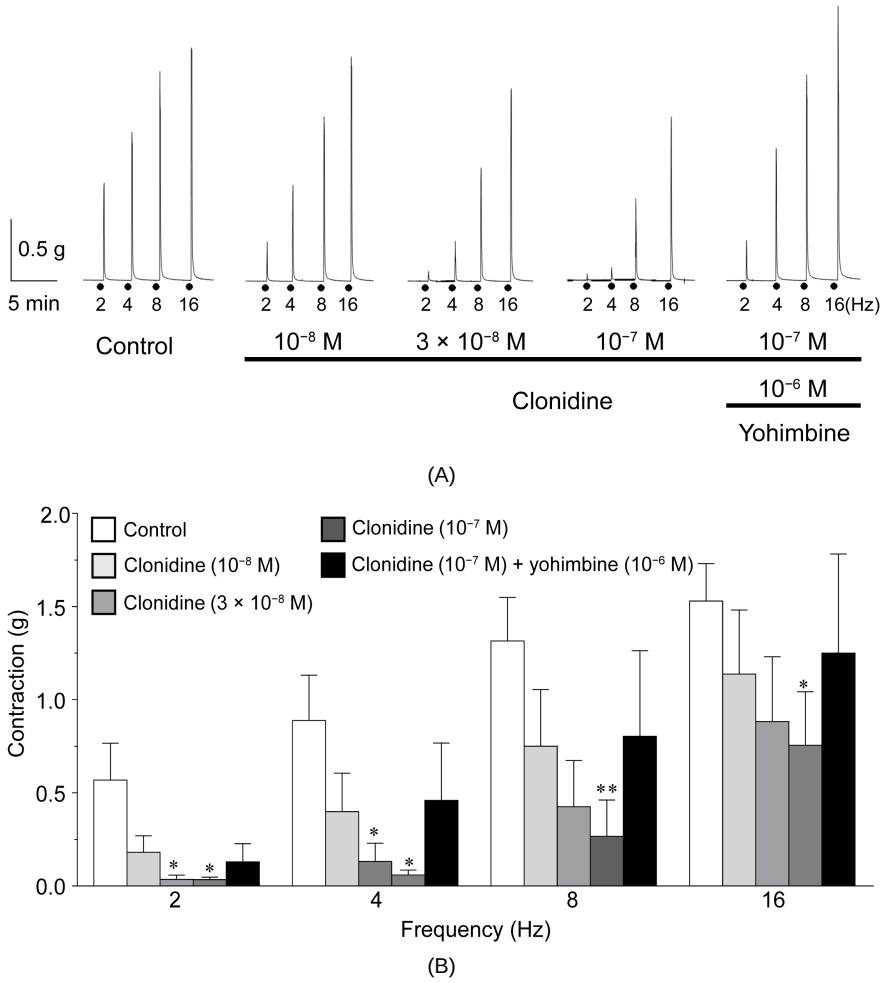

Figure 1. The effects of clonidine and subsequently-applied yohimbine on electrical field stimulation (EFS)-induced contractions in rat vas deferens. (A): A typical trace showing EFS-induced contractions and the effects of clonidine (10^{-8} - 10^{-7} M) and yohimbine (10^{-6} M). EFS was transmurally applied at a frequency between 2 and 16 Hz for 5 sec with a train of 100-μsec square pulses of supramaximal intensity (50 V). (B): Summarized data showing the results illustrated in **Figure 1(A)**. Data are shown as mean values ± S.E.M. (n = 3 for each). *$P < 0.05$, **$P < 0.01$ vs. control.

deferens in absence and presence of yohimbine. Similar to clonidine, guanfacine suppressed EFS-induced vas deferens contractions (2 - 16 Hz) in a concentration-dependent manner (10^{-9} - 10^{-8} M); however, the inhibitory potency of guanfacine was greater than that of clonidine. Suppressed contractions recovered in the presence of yohimbine (10^{-6} M) and guanfacine (10^{-8} M).

3.3. Inhibitory Effects of Clonidine on Phenylephrine-Induced Contractions

Figure 3 shows the effects of cumulative clonidine applications on phenylephrine-induced sustained contractions of rat thoracic aortae. Clonidine (10^{-9} - 10^{-5} M) inhibited phenylephrine (3×10^{-7} M)-induced contractions in a concentration-dependent manner (**Figure 3(A)**). The Emax and pD_2 values for clonidine were calculated as 97.9% ± 0.6% and 6.88 ± 0.08 (n = 4) (**Figure 3(B)**).

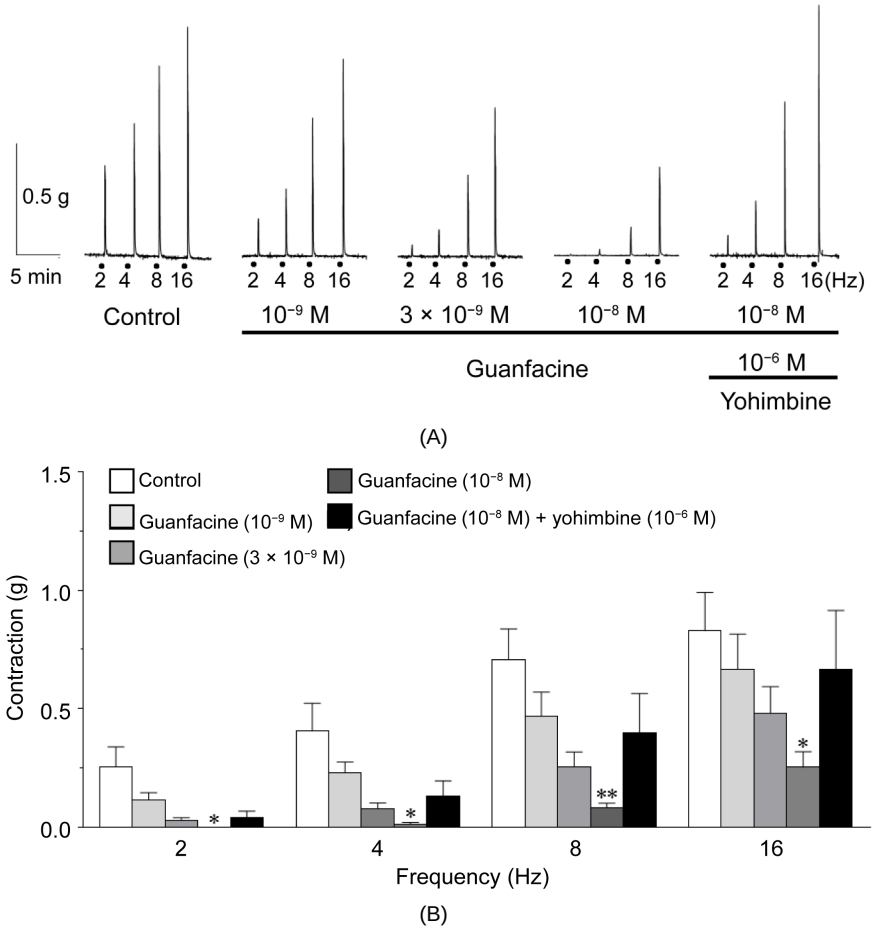

Figure 2. The effects of guanfacine and subsequently-applied yohimbine on electrical field stimulation (EFS)-induced contractions in rat vas deferens. (A): A typical trace showing EFS-induced contractions and the effects of guanfacine (10^{-9} - 10^{-8} M) and yohimbine (10^{-6} M). The EFS conditions were the same as **Figure 1**. (B): Summarized data showing the results illustrated in **Figure 2(A)**. Data are shown as mean values ± S.E.M. (n = 3 for each). *$P < 0.05$, **$P < 0.01$ vs. control.

Figure 4(A) shows the effects of clonidine on phenylephrine CRCs when applied cumulatively. Clonidine (10^{-7} - 10^{-6} M) resulted in a shift of the phenylephrine CRC to the right. The inhibitory effects of clonidine on the phenylephrine CRC are likely attributable to competitive antagonism since the regression line slope (1.01 ± 0.24) from the Schild plot analysis was not significantly different from that of unity (**Figure 4(B)**). The pA_2 value for clonidine was calculated as 6.77 (6.48 - 7.32, n = 4) (**Figure 4(B)**). In contrast, clonidine (10^{-5} M) dramatically shifted the phenylephrine CRC to the right and strongly reduced the Emax value from 96.9% ± 1.2% to 39.8% ± 5.5% (n = 4 for each, $P < 0.05$).

3.4. Inhibitory Effects of Clonidine on $PGF_{2\alpha}$ or 40 mM KCl-Induced Contractions

Figure 5(Aa), **Figure 5(Ab)** show the effects of clonidine on $PGF_{2\alpha}$-induced contractions. Clonidine did not inhibit $PGF_{2\alpha}$ (10^{-5} M)-induced contractions at concentrations up to 10^{-5} M. **Figure 5(Ba)**, **Figure 5(Bb)** shows the effects of

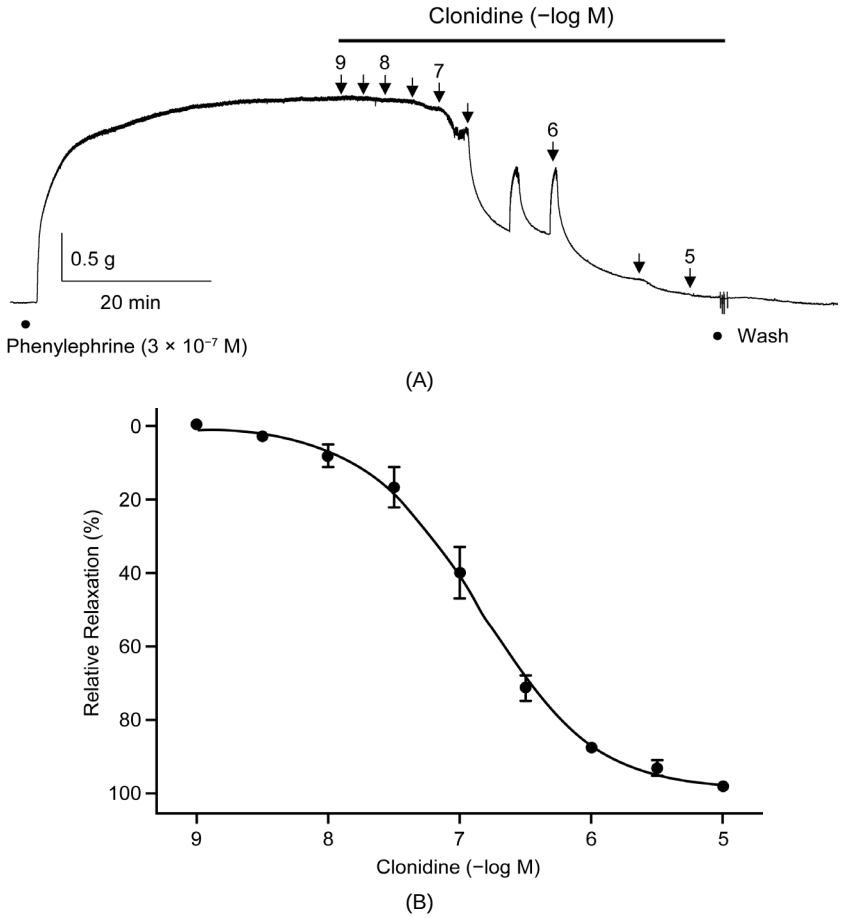

Figure 3. The inhibitory effects of clonidine on phenylephrine-induced contractions in rat thoracic aortae without endothelium. (A): A typical trace showing the effects of cumulatively applied clonidine (10^{-9} - 10^{-5} M) on a rat aorta precontracted with phenylephrine (3×10^{-7} M). Please note that Arabic numerals appearing in the figure are the negative logarithm of clonidine concentrations. (B): Concentration-response curves for the relaxant effects of clonidine on phenylephrine-induced contractions. Relative relaxation is expressed as percent reversal of phenylephrine-induced sustained tension development just before applying clonidine. Data are shown as mean values ± S.E.M. (n = 4 for each). When no error bar is shown, the error is smaller than the symbol.

clonidine on contractions induced by high concentrations of KCl (40 mM). Clonidine did not inhibit 40 mM KCl- induced contractions at concentrations up to 10^{-5} M. Clonidine produced additional tension development upon pre-contractions induced by $PGF_{2\alpha}$ (10^{-5} M) and high (40 mM)-KCl (Figure 5). The additional contractions induced by clonidine were speculated to be mediated through α_2-AR since they were abolished by yohimbine (data not shown). However, we did not analyze this phenomenon any further because this study focuses on the relaxant effects of clonidine.

3.5. Effects of Yohimbine at High Concentrations on Clonidine-Associated Suppression of Phenylephrine-Induced Contractions

In this series of experiments, we used phenylephrine at 10^{-6} M to obtain sustained

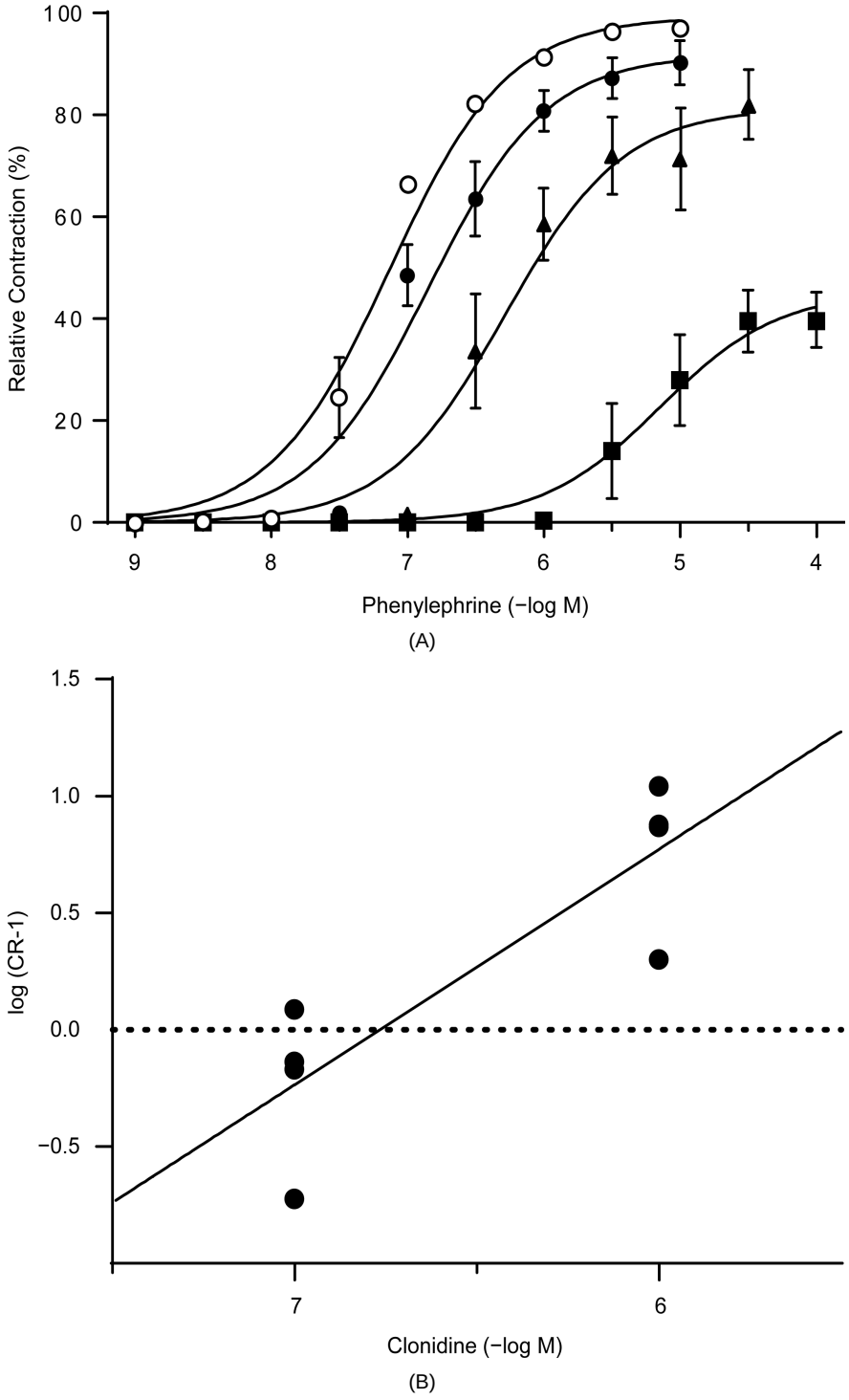

Figure 4. The antagonistic effects of clonidine on phenylephrine-induced contractions in rat thoracic aortae without endothelium. (A): Concentration-response curves for phenylephrine in the absence and presence of varied concentrations of clonidine; control (open circles), 10^{-7} M (closed circles), 10^{-6} M (closed triangles), and 10^{-5} M (closed squares). Contractile responses to phenylephrine are expressed as a percentage of phenylephrine (10^{-5} M)-induced contractions in the absence of clonidine. Data are shown as mean values ± S.E.M. (n = 4 for each). When no error bar is shown, the error is smaller than the symbol. (B): Schild plot analyses [11] carried out for clonidine against the effects of phenylephrine. Data are obtained from **Figure 4(A)**.

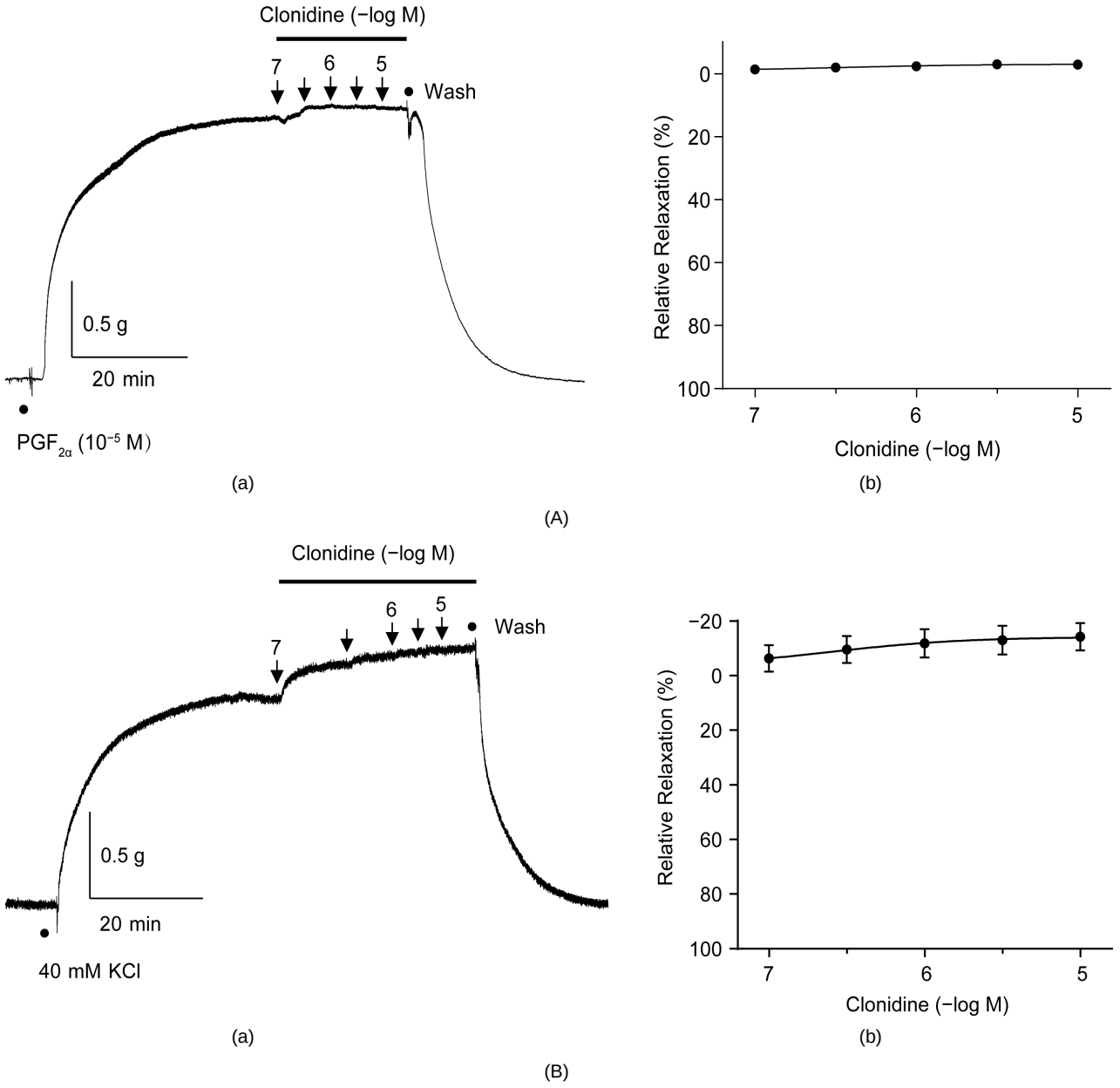

Figure 5. The effects of clonidine on $PGF_{2\alpha}$ (A)- or high KCl (B)-induced contractions in rat thoracic aortae without endothelium. (Aa), (Ba): A typical trace showing the effects of cumulatively adding clonidine (10^{-7} - 10^{-5} M) on the vasoconstriction elicited by $PGF_{2\alpha}$ (10^{-5} M) (Aa) or high (40 mM)-KCl (Ba). (Ab), (Bb): Concentration-response curves for clonidine-induced relaxation in pre-contracted thoracic aortae by $PGF_{2\alpha}$ (Ab) or high KCl (Bb). Relative relaxation is expressed as percent reversal of $PGF_{2\alpha}$- or high KCl-induced sustained tension development just before applying clonidine. Data are shown as mean values ± S.E.M. (n = 3 for each). When no error bar is shown, the error is smaller than the symbol. Please note that Arabic numerals appearing in the figure are the negative logarithm of clonidine concentrations.

pre-contractions; stable and sustained contractions were not generated by 3×10^{-7} M phenylephrine, attributable to its antagonistic actions against α_1-AR. Clonidine (10^{-5} M) almost completely suppressed phenylephrine-induced contractions both in the absence and presence of yohimbine (10^{-6} M) (Figure 6(A)); there were no statistically significant differences between the two responses (Figure 6(B)).

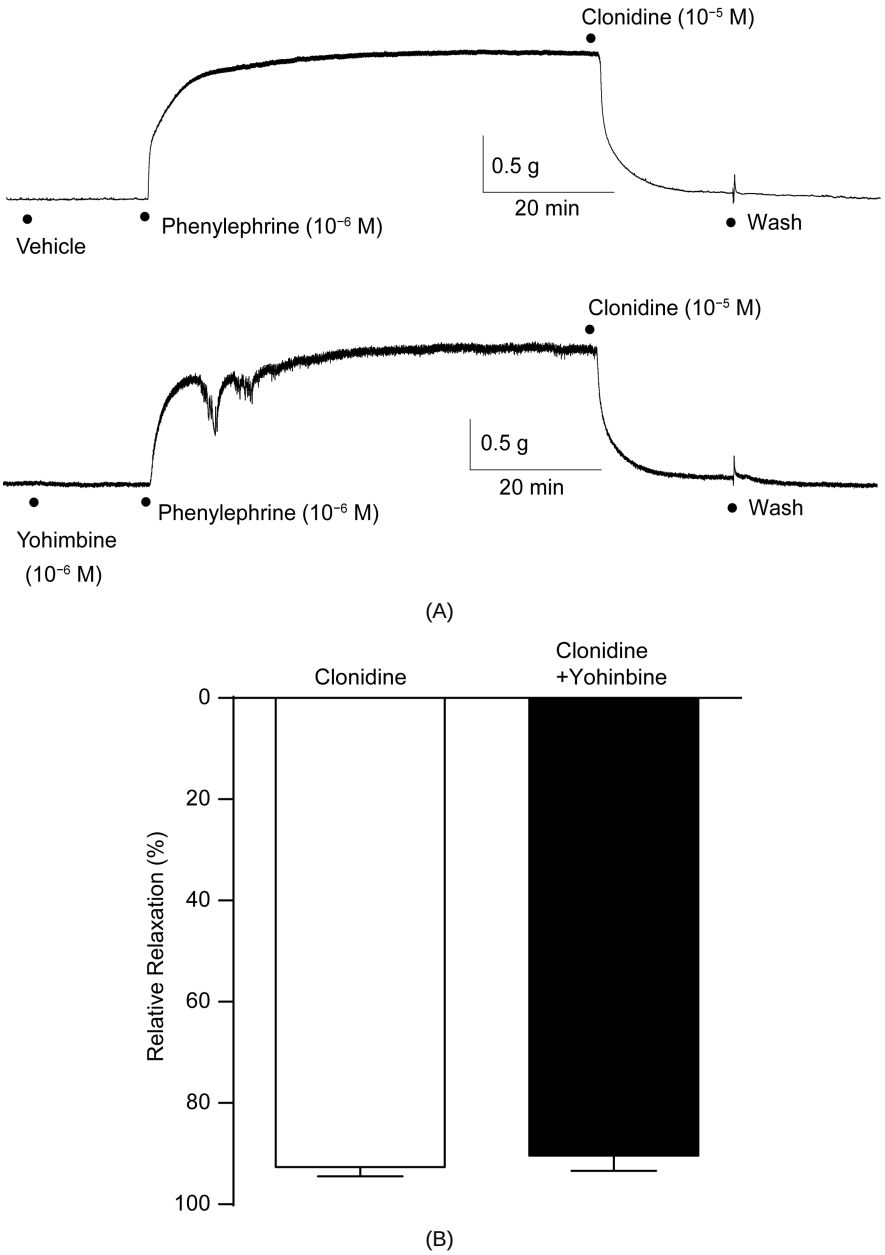

Figure 6. Pretreatment effects of yohimbine on the antagonistic effects of a high concentration of clonidine on phenylephrine-induced contractions in rat thoracic aortae without endothelium. (A): Typical traces showing the inhibitory effects of clonidine (10^{-5} M) on phenylephrine (10^{-6} M)-contracted aortae in the presence of yohimbine (10^{-6} M) (lower) or its vehicle (distilled water) (upper). (B): Summarized data showing the effects of yohimbine on the clonidine-induced relaxation illustrated in **Figure 6(A)**. Relative relaxation is expressed as percent reversal of phenylephrine-induced sustained tension development just before applying clonidine. Data are shown as mean values ± S.E.M. (n = 4 for each).

3.6. Effects of Guanfacine on Phenylephrine-Induced Contractions

Figure 7 shows the effects of guanfacine on phenylephrine-induced sustained contractions of rat thoracic aortae. Guanfacine did not inhibit phenylephrine (3×10^{-7} M)-induced contractions at concentration up to 10^{-5} M.

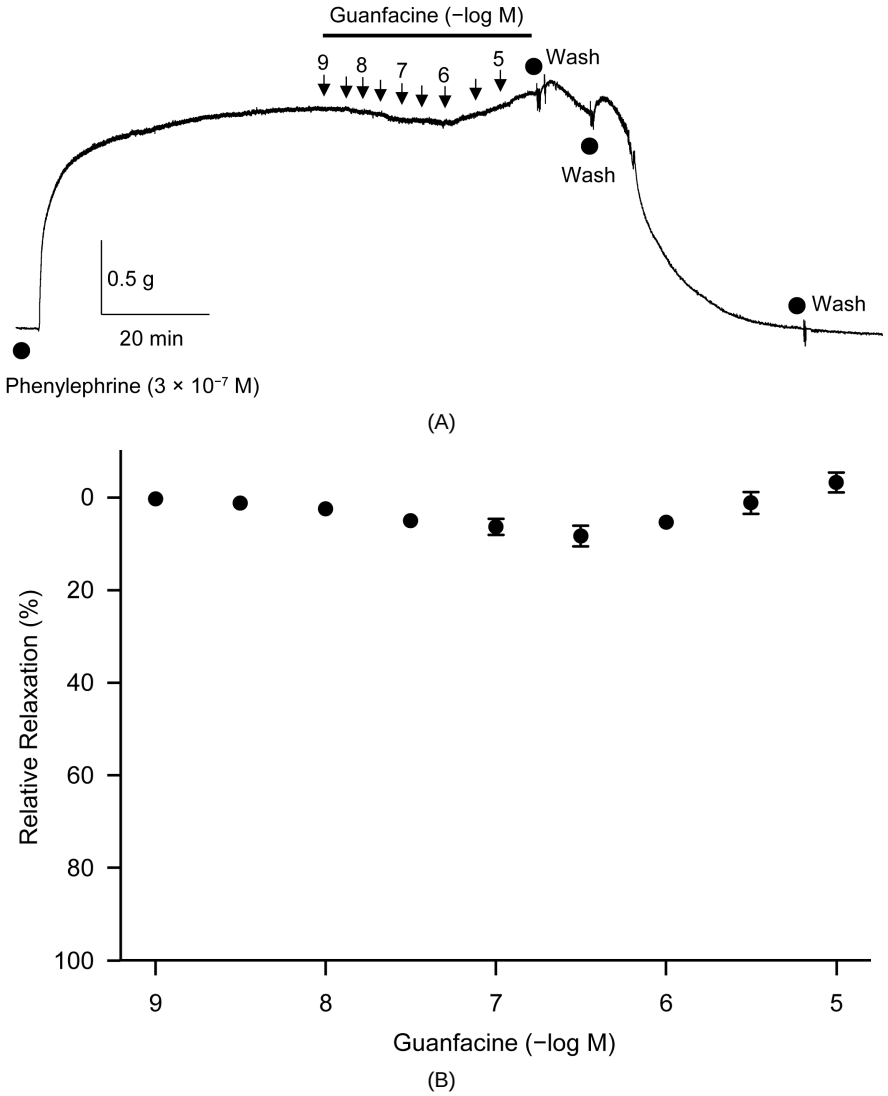

Figure 7. The effects of guanfacine on phenylephrine-induced contractions in rat thoracic aortae without endothelium. (A): A typical trace showing the effects of cumulatively adding guanfacine (10^{-9} - 10^{-5} M) on phenylephrine-elicited vasoconstriction (3×10^{-7} M). Please note that Arabic numerals appearing in the figure are the negative logarithm of guanfacine concentrations. (B): Concentration-response curves for guanfacine-mediated relaxation in thoracic aortae pre-contracted with phenylephrine. Relative relaxation is expressed as percent reversal of phenylephrine-induced sustained tension development just before applying clonidine. Data are shown as mean values ± S.E.M. (n = 4 for each). When no error bar is shown, the error is smaller than the symbol.

4. Discussion

This study examined the possibility that stimulation of α_2-ARs and subsequent interactions with α_1-ARs are involved in clonidine-associated inhibition of α_1-AR-mediated phenylephrine-induced contractions of rat thoracic aortae. Our results show that at high concentrations (10^{-5} M), clonidine suppresses phenylephrine-induced contractions in a non-competitive-like manner, while at lower concentration ranges (10^{-7} - 10^{-6} M), clonidine shows competitive antagonism against the effects of phenylephrine. However, clonidine-induced inhibition of

phenylephrine effects was produced under yohimbine inhibition of α_2-ARs and guanfacine, another α_2-AR inhibitor, did inhibit the effects of phenylephrine. Based on these findings, we suggest that stimulation of α_2-ARs and subsequent interactions with α_1-ARs are not mediated by the inhibitory effects of clonidine on α_1-AR-mediated contractions in rat aortae.

Clonidine, an α_2-AR stimulant, has been used as a central antihypertensive drug to treat hypertension. The hypotensive effects of clonidine are produced through suppression of sympathomimetic nerves and the relative superior activation of parasympathetic nerves resulting from stimulation of α_2-ARs in the pons and medulla oblongata [12]. Therefore, suppressed NA release from sympathetic nerves is thought to be insignificant. In contrast, clonidine produces contractile responses in peripheral smooth muscle tissues. A plausible explanation for clonidine-induced vascular contractions is that clonidine acts as a partial agonist on both α_1-ARs and α_2-ARs [5] [8]. As such, transient elevations in blood pressure by a bolus injection of clonidine can be explained by clonidine-stimulated α_2-ARs, α_1-ARs, or both receptor subtypes.

As mentioned above, clonidine inhibits α_1-AR-mediated contractions induced by NA or phenylephrine in endothelium-denuded artery preparations, while it produces arterial contractions through agonistic activity for α_1-ARs and α_2-ARs. These data suggest that clonidine acts as an α_1-AR partial agonist, which is likely the mechanism underlying the inhibitory effects of clonidine on α_1-AR-mediated arterial contractions [5] [6] [13]. Further, α_1-AR-mediated contractions are not completely inhibited by high concentrations (10^{-5} - 10^{-4} M) of clonidine. Rather, part of the contractile component remains unaffected, providing experimental evidence for the above explanation. In contrast to previous reports, our present study shows that clonidine inhibits phenylephrine-induced contractions of endothelium-denuded rat aortae in a concentration-dependent manner. Further, 10^{-5} M clonidine almost completely abolished the contractions (**Figure 3(B)**). This finding suggests that the inhibitory effects of clonidine on phenylephrine-induced contractions cannot be entirely explained by its partial agonist action on α_1-ARs, but that additional effects should be taken into account. Therefore, in the present study we continued to clarify other plausible factors contributing to the inhibitory effects of clonidine on phenylephrine-induced contractions in rat thoracic aortae.

We examined the effects of clonidine on phenylephrine CRCs and carried out Schild plot analyses. The inhibitory effects of clonidine at concentrations of 10^{-7} - 10^{-6} M on phenylephrine activity are attributable to competitive antagonism on α_1-ARs, as demonstrated by no significant differences between the regression line slope (1.01) from the Schild plot analysis and that of unity. Further, the pA_2 value was calculated to be 6.77 (**Figure 4(B)**), which is consistent with a previously reported value (6.7) against α_1-ARs (α_{1B}-ARs) [14]. Therefore, the inhibitory effects of clonidine at concentrations of 10^{-7} - 10^{-6} M are likely mediated through competitive antagonism of phenylephrine on α_{1B}-ARs. In contrast, clonidine (10^{-5} M) inhibited phenylephrine-induced contractions with strong sup-

pression of the maximum response, suggesting the possible involvement of non-competitive antagonism-like inhibitory mechanisms. However, clonidine did not show any inhibitory effects against high KCl- and $PGF_{2\alpha}$-induced contractions. Therefore, the inhibitory effects of clonidine at high concentrations (10^{-5} M) are not likely mediated through non-specific actions such as phosphodiesterase inhibition, but rather through inhibitory effects associated with α-AR (α_1-ARs, α_2-ARs, or both $\alpha_{1/2}$-ARs) mechanisms.

Although several mechanisms appear to account for the inhibitory effects of clonidine at high concentration (10^{-5} M) on the effects of phenylephrine, we suggest that clonidine desensitizes α_1-ARs, thus suppressing phenylephrine-induced contractions.

The α_{1B}-AR and α_{1D}-AR subtypes primarily mediate arterial contractions induced by endogenous catecholamines, and chemically synthesized α_1-AR agonists such as phenylephrine are primarily mediated by these α_1-AR subtypes [15] [16]. Further, α_{1B}-ARs are reportedly desensitized by phosphorylation through cross-talk upon stimulation of other drug receptors with bradykinin, endothelin, transforming growth factor (TGF)-β, and LPA [9]. Among these drug receptors, the LPA receptor is classified as a Gi-protein-coupled receptor and induces desensitization of α_{1B}-ARs when phosphorylated through activation of phosphatidylinositol-3 kinase (PI3K) and protein kinase C (PKC) [9]. The α_2-ARs expressed in rat aortic smooth muscle cells [17] are also Gi-protein coupled receptors and stimulation causes activation of PI3K and PKC [18]. Therefore, it is plausible that stimulation of α_2-ARs with clonidine desensitizes α_{1B}-ARs through cross-talk between α_{1B}-ARs and α_2-ARs. Based on these studies, we hypothesized that stimulation of α_2-ARs with high concentrations of clonidine would induce desensitization of α_{1B}-ARs, consequently inhibiting phenylephrine-induced contractions in rat thoracic aortae.

To verify this hypothesis, we examined whether the inhibitory effects of high concentrations of clonidine on phenylephrine-induced contractions could be attenuated by inhibiting α_2-ARs with yohimbine. Previously, we confirmed the stimulating effects of clonidine and guanfacine on α_2-ARs and the inhibitory effects of yohimbine on α_2-ARs using a rat vas deferens preparation. Our results show that clonidine (10^{-8} - 10^{-7} M) and guanfacine (10^{-9} - 10^{-8} M) inhibit EFS-induced contraction of rat vas deferens in a concentration-dependent manner, and their inhibitory effects are reduced in the presence of yohimbine (10^{-6} M) (**Figure 1**, **Figure 2**). Therefore, the α_2-AR-stimulating effects of clonidine and guanfacine, as well as the α_2-AR-inhibitory effects of yohimbine (10^{-6} M) were verified. In contrast, clonidine (10^{-5} M) inhibited phenylephrine-induced contractions in both the absence and presence of yohimbine (**Figure 6(A)**, **Figure 6(B)**). Furthermore, guanfacine, another α_2-AR agonist, did not inhibit phenylephrine- induced contractions (**Figure 7(A)**, **Figure 7(B)**). Based on these findings, it is likely that inhibition of phenylephrine-induced contractions of rat thoracic aortae by high concentrations (10^{-5} M) of clonidine is not mediated through α_2-ARs. Therefore, it seems unlikely that stimulation of α_2-ARs induces

desensitization of α_1 (α_{1B})-ARs through cross-talk in arterial smooth muscle.

The mechanisms by which high concentrations of clonidine inhibit phenylephrine-induced contractions in a noncompetitive manner remain to be determined. Nevertheless, it is thought that the inhibitory mechanisms are selective for α-ARs (α_1-AR, α_2-AR, or both α-ARs) and the related processes, since high concentrations (10^{-5} M) of clonidine do not inhibit high KCl- and $PGF_{2\alpha}$-induced contractions. It is possible that high (10^{-5} M) concentrations of clonidine inhibit phenylephrine-induced contractions by directly desensitizing α_1-ARs without mediating α_2-AR activity. However, if clonidine is solely an α_1-AR antagonist, high concentrations would not desensitize the α_1-ARs. As such, clonidine at high concentrations (10^{-5} M) likely has dual actions as an α_1-AR agonist and antagonist.

Finally, results of this study are clinically significant. Guanfacine is not used for hypertension in Japan, whereas clonidine is still employed as an antihypertensive drug—though it is not currently ranked as high as it has been in the past. The clinical usefulness of clonidine is supported by the idea that it acts as an α_1-AR competitive antagonist, whereas guanfacine does not, as shown in this study. Clonidine is still used as an α_2-AR agonist in basic pharmacological studies; however, our study indicates that studies using relatively high concentrations of clonidine should be very carefully interpreted, as it inhibits α_1-AR-mediated contractions at concentrations as low as 10^{-7} M.

5. Conclusion

In this study, we examined whether clonidine-mediated inhibition of phenylephrine-induced contractions in rat thoracic aortae involves stimulation of α_2-ARs and the subsequent inhibition of α_1-ARs. However, no evidence was observed to indicate that stimulation of α_2-ARs and subsequent interactions with α_1-ARs mediates the inhibitory effects of clonidine on phenylephrine activity. The inhibitory effects of clonidine on phenylephrine activity in rat aortae are unlikely to involve mediation of α_2-ARs; however, these effects may be produced by direct inhibitory action on α_1-ARs.

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Competing Interests

The authors declare that they have no competing interests.

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