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ORIGINAL ARTICLE

Propofol attenuates angiotensin II-induced vasoconstriction by inhibiting Ca^{2+}-dependent and PKC-mediated Ca^{2+} sensitization mechanisms

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Abstract
Purpose Angiotensin II (Ang II)-induced vascular contraction is mediated by Ca^{2+}-dependent mechanisms and Ca^{2+} sensitization mechanisms. The phosphorylation of protein kinase C (PKC) regulates myofilament Ca^{2+} sensitivity. We have previously demonstrated that sevoflurane inhibits Ang II-induced vasoconstriction by inhibiting PKC phosphorylation, whereas isoflurane inhibits Ang II-induced vasoconstriction by decreasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle. Propofol also induces vasodilation; however, the effect of propofol on PKC-mediated myofilament Ca^{2+} sensitivity is poorly understood. The aim of this study is to determine the mechanisms by which propofol inhibits Ang II-induced vascular contraction in rat aortic smooth muscle.
Methods An isometric force transducer was used to investigate the effect of propofol on vasoconstriction, a fluorometer was used to investigate the change in $[Ca^{2+}]_i$, and Western blot testing was used to analyze Ang II-induced PKC phosphorylation.
Results Ang II (10^{-7} M) elicited a transient contraction of rat aortic smooth muscle, which was associated with an elevation of $[Ca^{2+}]_i$. Propofol (10^{-6} M) inhibited Ang II-induced vascular contraction ($P < 0.01$) and increase in $[Ca^{2+}]_i$ ($P < 0.05$) in rat aortic smooth muscle. Ang II also induced a rapid increase in $[Ca^{2+}]_i$ in cultured vascular smooth muscle cells, which was suppressed by propofol ($P < 0.05$). Propofol (10^{-6} M) attenuated Ang II-stimulated PKC phosphorylation ($P < 0.05$).
Conclusion These results suggest that the inhibitory effect of propofol on Ang II-induced vascular contraction is mediated by the attenuation of a Ca^{2+}-dependent pathway and Ca^{2+} sensitivity through the PKC signaling pathway.

Keywords Artery · Calcium · Propofol · Protein kinase C

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Introduction

Angiotensin II (Ang II) plays an important role in regulating systemic vascular tone and in maintaining arterial blood pressure. Ang II-induced vascular contraction is mediated by Ca^{2+}-dependent mechanisms and Ca^{2+}-sensitization mechanisms [1]. Activation of the Ang II receptor causes an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through the release of Ca^{2+} from the intracellular stores and through the influx of Ca^{2+} from the extracellular space, which eventually facilitates phosphorylation of the myosin light chain (MLC) (Ca^{2+}-dependent mechanism). Ang II also stimulates the phosphorylation of protein kinase C (PKC). Activation of PKC can inhibit the dephosphorylation of phosphorylated MLC, thereby potentiating vascular contraction at a given $[Ca^{2+}]_i$ (Ca^{2+} sensitization mechanism) [2].

General anesthetics reduce arterial blood pressure, in part, by blood vessel dilation. One cause of hypotension during general anesthesia is the inhibition of vascular smooth muscle contraction induced by physiological humoral factors such as Ang II. We have previously demonstrated that volatile anesthetics including sevoflurane and isoflurane attenuate Ang II-induced vascular contraction by inhibiting Ca^{2+}-dependent mechanisms or Ca^{2+} sensitization mechanisms. The mechanisms behind

the inhibitory effect of these anesthetics differ [3, 4]. For example, sevoflurane inhibits Ang II-induced PKC phosphorylation without affecting $[Ca^{2+}]_i$ [3], whereas isoflurane mainly suppresses Ang II-induced increase in $[Ca^{2+}]_i$ [4].

Propofol (2,6-diisopropylphenol) is a widely used intravenous anesthetic that decreases arterial blood pressure, in part, through blood vessel relaxation and myocardial depression [5–7]. The exact mechanism underlying propofol-induced vasodilation is not fully understood. Samain et al. [8, 9] have shown that propofol inhibits Ang II-induced increases in muscle tension and in $[Ca^{2+}]_i$ in rat aortic smooth muscle. However, whether propofol influences the Ang II-induced PKC-mediated Ca^{2+} sensitization mechanisms in vascular smooth muscle remains unclear.

The aim of this study is to elucidate the cellular mechanism by which propofol inhibits Ang II-induced vasoconstriction. With an emphasis on the Ca^{2+}-dependent mechanisms and Ca^{2+} sensitization mechanisms, we measured $[Ca^{2+}]_i$ and used Western blot analysis to determine PKC phosphorylation.

Methods

All experimental protocols were approved by the Wakayama Medical University Animal Care and Use Committee.

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

Male Wistar rats weighing 250–350 g were anesthetized with halothane and killed by decapitation. The chest of each rat was opened and the descending thoracic aorta was isolated. Connective tissues and excessive fat were carefully removed. The endothelium was denuded by gently rubbing the luminal surface with a cotton swab. The prepared aorta was cut into strips approximately 5.0 mm in length and 3.5 mm in width. Two to three strips were harvested from each rat. The strips were treated with acetoxymethyl ester of fura-2 (Fura-2/AM, 10^{-5} M) solution for 6–9 h at room temperature (20–22 °C). A noncytotoxic detergent, cremophor (0.1 %), was added to the solution to increase the solubility of the acetoxymethyl ester. After the loading period, the preparations were washed three times with a Krebs bicarbonate solution (KBS). The constituents of KBS included 118.2 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.8 mM $NaHCO_3$, and 10 mM dextrose. Each aortic strip was held in a horizontal position in a 37 °C temperature-controlled organ bath that was continuously perfused with the KBS solution aerated with a mixture of 95 % O_2 and 5 % CO_2. To measure muscle tension, one end of a strip was connected to an isometric force transducer (NEC San-ei Instruments, Tokyo, Japan). A passive tension of 3.0 g was applied, and the muscle strip was allowed to equilibrate for 60 min before the start of the experiment. Fluorescence measurements were performed using a dual-wavelength spectrofluorometer (CAF-110; Japan Spectroscopic, Tokyo, Japan) at the fluorescence excitation wavelength of 340 and 380 nm and an emission wavelength of 510 nm. The fluorescence ratio of 340 to 380 nm was used as an indicator of $[Ca^{2+}]_i$.

Changes in isometric tension and the 340/380 ratio in response to KCl (30 mM) were recorded. These values were then used as a reference standard (100 %). After washing with a bathing solution, each strips was then incubated for 15 min in a bathing solution that either lacked or contained propofol at concentration of 10^{-7}, 3×10^{-7}, or 10^{-6} M, followed by the addition of Ang II (10^{-7} M) to the bathing solution. The Ang II concentration used in the current experiment is based upon our previous studies [3, 4, 10]. The changes in isometric tension and the 340/380 ratio were recorded and expressed as a percentage of the reference value. For this experiment, aortic strips from six different animals ($n = 6$) were used, and each strip was exposed to only one concentration of propofol.

Live cell calcium imaging

Rat thoracic aortic smooth muscle cells (A-10 cells, CRL-1476) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in a humidified atmosphere of 5 % CO_2 and 95 % O_2 at 37 °C in Dulbecco's modified Eagle's medium (D-MEM; American Type Culture Collection), which was supplemented with 10 % fetal calf serum, penicillin (100 unit/ml), and streptomycin (100 μg/ml). The cells were allowed to grow until they formed a confluent monolayer. The cells were then subcultured to glass slides designed for fluorescence microscopy. The culture medium was replaced with a serum-free medium 24 h before the experiment. The A-10 cells were washed with phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA), which contained 154 mM NaCl, 3 mM Na_2HPO_4, and 1 mM KH_2PO_4, adjusted to a pH of 7.4. The cells were incubated for 30 min at 37 °C in PBS containing Fluo-3/AM (10^{-5} M) and then washed twice with PBS. Culture slides containing the Fluo-3/AM loaded cells were placed in a 37 °C temperature-controlled chamber mounted on the stage of an Olympus FV300 laser scanning confocal microscope (Olympus, Tokyo, Japan); this enabled the collection of data of the real-time Ca^{2+} dynamics of individual cells in vitro. Dynamic changes in the fluorescence intensity of the $[Ca^{2+}]_i$ in response to Ang II (10^{-7} M) in the presence and absence of propofol (10^{-6} M) were measured at the excitation wavelength of 506 nm fluorescence and the emission wavelength of 526 nm. After the application of Ang II, Fluo-3 fluorescence signals from some definite cells were

scanned and continuously monitored at a sampling frequency of 5 Hz for 5 min. The ratio of the fluorescence intensity in response to Ang II (F) and the fluorescence intensity just before the addition of Ang II (F_0) were compared in the presence and absence of propofol.

Western blot analysis

Isolated endothelium-denuded aortas about 3.5 cm in length were incubated in KBS that was bubbled with a mixture of 5 % CO_2 and 95 % O_2 for 60 min. One strip was obtained from each animal. To examine the effect of propofol on Ang II-induced PKC phosphorylation, 18 rat thoracic aorta strips were exposed to propofol at concentration of 0, 10^{-7} M, or 10^{-6} M for 15 min, followed by the addition of Ang II (10^{-7} M) into the bathing solution. Based upon the findings from our previous study [3, 4, 11], Ang II-induced PKC phosphorylation reached a peak level at 4 min after the application of Ang II. Therefore, the aortic strips were rapidly frozen on dry ice 4 min after the addition of Ang II. Another set of six strips from six different animals were not exposed to propofol; they were instead incubated with bisindolylmaleimide 1 (BIS 1, 10^{-5} M), a PKC inhibitor. The frozen aortas were cut into small pieces and homogenized in ice-cold lysis buffer [1 mM Tris–HCl, 5 mM $MgCl_2$, 100 mM NaCl, and 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5] containing 1 mM 4-(2-aminoethyl) benzonesulfonyl fluoride, 20 mg/ml leupeptin, and 20 mg/ml aprotinin. The homogenates were centrifuged at 13,000 g for 3 min at 4 °C, and the supernatant was collected. The protein concentration was determined by the bicinchoninic acid method [12].

Equal amounts of total protein were used for every sample in each experiment. Strips were treated with anti-β-action antibody (1:1,000) as a loading control. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was treated with anti-PKC (1:1,000) and anti-phospho-PKC antibodies (1:500) for 2 h, followed by 1 h of incubation with horseradish peroxidase-conjugated antibody (1:2,000). The density of the immunoreactive bands was detected using chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and assessed with image analysis software (NIH Image 1.62 National Institute of Health, Bethesda, MD, USA). The ratio of phosphorylated PKC to total PKC was used as an indicator of PKC activation.

Materials

All drugs were of the highest purity commercially available. Fura-2 and Fluo-3/AM were obtained from DOJINDO Laboratories (Kumamoto, Japan) and Carbiochem (Darmstadt, Germany), respectively. Polyclonal antibodies including anti-PKC, anti-phospho-PKC, anti-β-actin antibodies, and a secondary antibody labeled with horseradish peroxidase were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other drugs were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Propofol was dissolved in dimethyl sulfoxide and diluted in distilled water. The final concentration of dimethyl sulfoxide in the organ chamber was less than 0.1 % (vol/vol). This vehicle had no effect on tension or $[Ca^{2+}]_i$ at concentrations used in the study.

Data analysis

Data were presented as the median with the 25th and 75th percentiles and were evaluated with the Mann–Whitney U test for comparison between groups and the Kruskal–Wallis test, followed by the Newman–Keuls test for multiple comparisons. Statistical analyses were performed using the StatMate software program (Atoms, Tokyo, Japan). The sample size (n value) represented the number of rats from which aortic strips were obtained for simultaneous measurements of tension and $[Ca^{2+}]_i$. The number of aortas for Western blot analysis is equal to the number of the rats used in each protocol. For the live cell Ca^{2+} imaging experiment, the sample size represented the number of cells observed. $P < 0.05$ was considered statistically significant.

Results

The effect of propofol on angiotensin II-induced increase in isometric force and $[Ca^{2+}]_i$

Ang II (10^{-7} M) induced a rapid and transient increase in muscle tension and in $[Ca^{2+}]_i$ of the rat aortic smooth muscle (Fig. 1). Ang II induced a maximal contraction that was 114 % (with a range of 103–128 %) of that induced by 30 mM KCl. Ang II induced a maximal elevation in $[Ca^{2+}]_i$ that was 139 % (123–155 %) of that induced by 30 mM KCl. Propofol inhibited Ang II-induced vasoconstriction in a concentration-dependent manner. This inhibition by propofol was significant at concentration of 10^{-6} M [42 % (32–58 %), $P < 0.01$]. Propofol also attenuated the Ang II-induced increase in $[Ca^{2+}]_i$. Inhibition by propofol reached statistical significance only at 10^{-6} M [92 % (69–100 %), $P < 0.05$] (Fig. 2).

Live cell calcium imaging

Addition of Ang II (10^{-7} M) to the culture medium induced a rapid increase in the F/F_0 ratio of the cultured

vascular smooth muscle cell. The F/F_0 ratio reached its maximum level of 2.1 (1.6–2.2) 5 s after the application of Ang II. Propofol itself did not influence fluorescence intensity. Propofol at concentrations of 10^{-7} M [1.3 (1.3–1.4), $P < 0.05$] and 10^{-6} M [1.3 (1.3–1.4), $P < 0.05$] inhibited the Ang II-induced increase in $[Ca^{2+}]_i$ in vascular smooth muscle cells (Fig. 3).

Effect of propofol on angiotensin II-induced PKC-phosphorylation

Ang II (10^{-7} M) stimulated the phosphorylation of PKC, with the ratio of phosphorylated PKC to total PKC ranging from 1.1 (1.0–1.2) to 1.6 (1.5–1.7). Propofol attenuated Ang II-induced PKC phosphorylation in a concentration-dependent manner. Propofol at a concentration of 10^{-6} M nearly abolished the PKC phosphorylation in response to Ang II (Fig. 4).

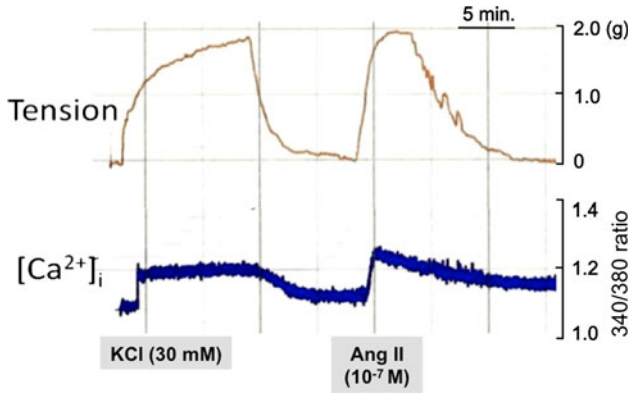

Fig. 1 An actual tracing demonstrating the simultaneous measurement of changes in isometric tension (*upper*) and in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (*lower*) in response to KCl (30 mM) and angiotensin II (10^{-7} M). The change in $[Ca^{2+}]_i$ is expressed as a ratio of fluorescence measurements at the excitation wavelengths of 340 and 380 nm

Discussion

The key findings of the current study were (1) propofol inhibited the Ang II-induced increase in muscle tension and in $[Ca^{2+}]_i$ in rat aortic smooth muscle; (2) propofol also suppressed the rapid increase in Ca^{2+} signal in cultured vascular smooth muscle cells after exposure to Ang II; and (3) propofol reduced Ang II-induced PKC phosphorylation, as revealed by Western blot analysis.

Ang II is a key component of the renin–angiotensin–aldosterone system that regulates vascular tension and arterial blood pressure. The Ang II-mediated signaling pathway in vascular tissue has been well established [1]. In brief, Ang II binds to the angiotensin type 1 receptor on the surface of vascular smooth muscle cells, where it rapidly activates phospholipase C (PLC), which generates inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG). IP_3 stimulates Ca^{2+} release from intracellular calcium stores and DG facilitates the phosphorylation of PKC. Ang II also activates the influx of Ca^{2+} from the extracellular space. PKC inhibits the action of myosin light chain phosphatase (MLCP), which reduces the dephosphorylation of phosphorylated MLC, thereby potentiating a contraction at a given $[Ca^{2+}]_i$ [2, 13]. Thus, Ang II-induced vasoconstriction is mediated by both Ca^{2+}-dependent mechanisms and Ca^{2+} sensitization (Ca^{2+}-independent) mechanisms.

Anesthetic-induced hypotension is partly attributable to the inhibition of the vascular contractile response induced by various humoral stimuli [14]. We have previously

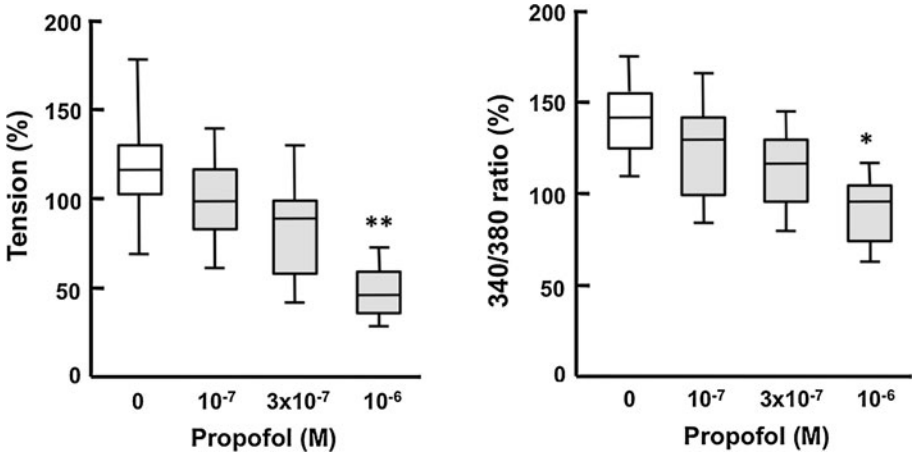

Fig. 2 Effects of propofol (10^{-7}, 3×10^{-7}, or 10^{-6} M) on Ang II (10^{-7} M)-induced vasoconstriction (*left*) and the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (*right*) in rat aortic strips. *Horizontal bars* represent medians, *boxes* represent the 25th and 75th percentile ranges, and *T bars* represent 5th and 95th percentile ranges. Changes in tension and $[Ca^{2+}]_i$ are expressed as the percentage relative to those induced by KCl (30 mM) ($n = 6$). $^*P < 0.05$, $^{**}P < 0.01$ versus control (absence of propofol)

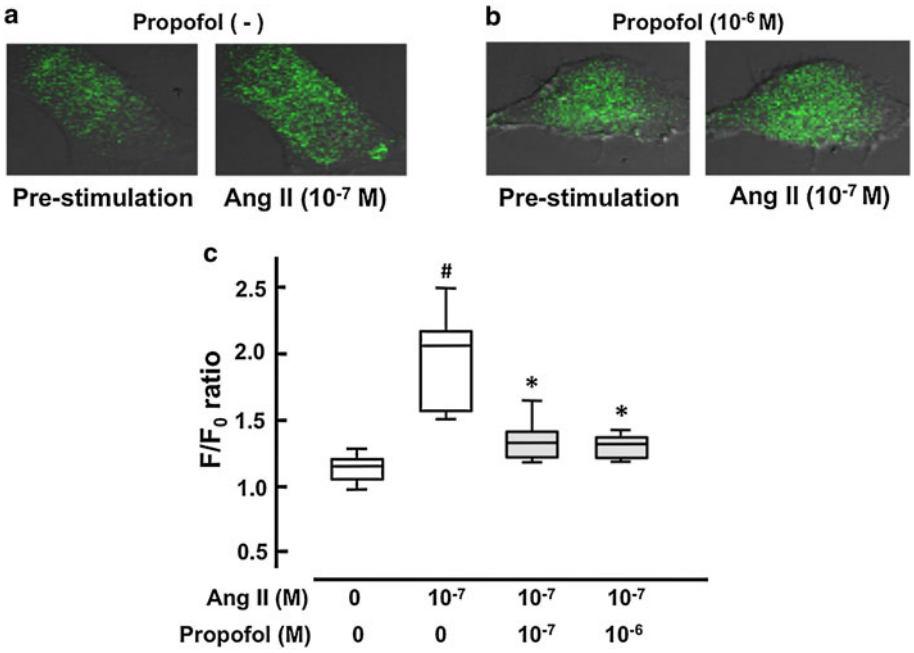

Fig. 3 Changes in calcium fluorescence imaging in response to Ang II (10^{-7} M) in a cultured rat aortic smooth muscle cell in the absence (**a**) and the presence (**b**) of propofol (10^{-6} M). Ang II induces a significant increase in the F/F_0 ratio, which is almost abolished (**c**) in the presence of propofol at concentrations of 10^{-7} and 10^{-6} M. *Horizontal bars* represent medians, *boxes* represent the 25th and 75th percentile ranges, and *T bars* represent 5th and 95th percentile ranges. $^{*}P < 0.05$ versus in the absence of propofol; $^{\#}P < 0.05$ versus the value before stimulation by Ang II

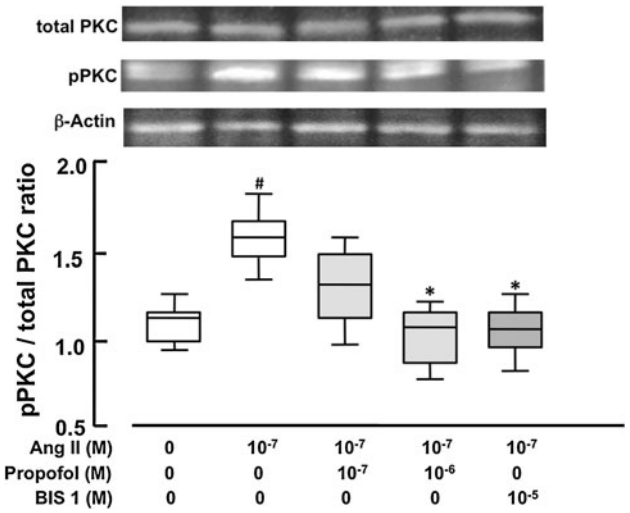

Fig. 4 The effects of propofol on Ang II-stimulated phosphorylation of protein kinase C (PKC). Aortic strips were exposed to propofol (10^{-7} or 10^{-6} M) or bisindolylmaleimide 1 (BIS 1, 10^{-5} M) for 15 min and were frozen 4 min after the addition of Ang II (10^{-7} M). PKC activation is indicated by the ratio of phosphorylated PKC (pPKC) to total PKC. *Horizontal bars* represent medians, *boxes* represent the 25th and 75th percentile ranges, and *T bars* represent 5th and 95th percentile ranges. $^{*}P < 0.05$ versus in the absence of propofol, $^{\#}P < 0.05$ versus the value before the exposure to Ang II

demonstrated that volatile anesthetics inhibit Ang II-induced vascular contraction by reducing $[Ca^{2+}]_i$ or by reducing myofilament Ca^{2+} sensitivity, and we have shown that the cellular mechanisms underlying the inhibitory effect of volatile anesthetics on Ang II-induced vascular contraction are not uniform [3, 4]. For example, sevoflurane inhibits Ang II-induced vascular contractile response by attenuating PKC phosphorylation but without affecting $[Ca^{2+}]_i$ [3]; by contrast, the inhibitory effect of isoflurane on Ang II-induced vascular contraction is mainly mediated by a decrease in $[Ca^{2+}]_i$ [4].

Propofol induces smooth muscle relaxation and decreases systemic blood pressure [5, 7, 8]. It has been reported that the vasodilatory property is mediated through a Ca^{2+}-dependent pathway [8, 9, 15, 16]. In the current study, propofol-induced vascular contraction was accompanied by a decrease in $[Ca^{2+}]_i$ in the rat aorta. The findings from the live cell imaging experiments also indicated that propofol decreased the $[Ca^{2+}]_i$ in a single cell. These findings are consistent with the previous studies that used aortic tissue and cultured smooth muscle cells [8, 9]. It has also been demonstrated that propofol decreases Ca^{2+} influx from the extracellular space without altering the release of Ca^{2+} from the internal stores [9]. However, other investigators have shown that propofol inhibits both Ca^{2+} entry and Ca^{2+} release from intracellular stores in mouse pituitary cells [17]. Although the mechanism by which propofol reduced $[Ca^{2+}]_i$ remains controversial, there is no doubt that propofol possesses the ability to reduce $[Ca^{2+}]_i$.

On the other hand, whether a PKC-mediated Ca^{2+} sensitization mechanism is involved in propofol-induced vasodilation is poorly understood. Through Western blot analysis, we have revealed that using propofol (10^{-6} M) has a potent inhibitory effect on Ang II-stimulated PKC phosphorylation. The extent of the inhibition is similar to that induced by BIS 1, a specific PKC inhibitor. We previously reported that propofol at a concentration as used in the current study suppressed vasoconstriction and the phosphorylation of PKC induced by phorbol 12,

13-dibutyrate, a potent activator of PKC [18]. These findings, taken together, show that the inhibition of a PKC-mediated Ca^{2+} sensitization mechanism, in addition to the Ca^{2+}-dependent mechanism, contributes to the propofol-induced relaxation of blood vessels. However, the exact mechanism by which propofol inhibits PKC-mediated Ca^{2+} sensitization is still unclear.

Some putative mechanisms in cardiomyocytes have been proposed. Propofol attenuates Ang II-induced hypertrophy of cultured cardiomyocytes and fibroblast proliferation. It does this by reducing the production of reactive oxygen species and the phosphorylation of extracellular signal-regulated kinase [19, 20]. Other researchers have investigated the effect of propofol on the contractility of cardiomyocytes obtained from drug-induced diabetic rats and found that propofol attenuated myofilament Ca^{2+} sensitivity via PKC and a nitric oxide-dependent pathway [21]. However, no information is available regarding the mechanisms by which propofol alters PKC phosphorylation and Ca^{2+} sensitivity in vascular smooth muscle. Furthermore, we did not determine which subtype of PKC was inhibited by propofol. Although PKC-α but not PKC-ε was phosphorylated by Ang II in our previous study using rat aortic tissue [3], propofol might modulate the vascular reactivity through an activation of other PKC subtypes. Further study is needed to clarify this issue.

The plasma concentration of propofol in patients during the induction and maintenance of general anesthesia is reported to be in the range of 2–5 μg/ml, which corresponds to approximately 10^{-5} to 3×10^{-5} M [22, 23]. Considering that 97–99 % of propofol is bound to plasma proteins [23], the free concentration of propofol is estimated to be 10^{-7}–10^{-6} M. The concentration used in the current study is within the limit of the clinical range. Propofol may thus, induce vasodilation by reducing $[Ca^{2+}]_i$ and myofilament Ca^{2+} sensitivity at a clinically relevant concentration.

A major limitation of the present study is that we used endothelium-denuded vascular tissue and cultured smooth muscle cells. The endothelium plays a crucial role in the regulating the underlying vascular smooth muscle tone. An endothelium-dependent mechanism contributes to vasorelaxation induced by some anesthetics. Propofol has been demonstrated to induce vasodilation in an endothelium-dependent and an endothelium-independent manner [24, 25]. Propofol furthermore has both stimulatory [24] and inhibitory [26] effects on agonist-induced, endothelium-dependent relaxation, which suggests that the presence of intact endothelium might have influenced the results of the present study. Because the primary goal of the present study was to elucidate the effect of propofol on Ang II-induced change in Ca^{2+} sensitivity in vascular smooth muscle, we used vascular preparations without the endothelium. Although propofol was demonstrated to induce vasodilation through the $[Ca^{2+}]_i$ and the myofilament Ca^{2+} sensitivity mechanisms in rat aortic smooth muscle, the present findings obtained from the current study cannot be directly extrapolated to a clinical situation.

In summary, we have demonstrated that, at a clinically relevant concentration, propofol attenuates Ang II-induced vasoconstriction by inhibiting an increase in $[Ca^{2+}]_i$ and by inhibiting the phosphorylation of PKC that regulates myofilament Ca^{2+} sensitivity.

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Conflict of interest None of the authors has any conflicts of interest.

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