Propofol Increases Pulmonary Artery Smooth Muscle Myofilament Calcium Sensitivity

Role of Protein Kinase C

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Background: Vascular smooth muscle tone is regulated by changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and myofilament Ca^{2+} sensitivity. These cellular mechanisms could serve as targets for anesthetic agents that alter vasomotor tone. This study tested the hypothesis that propofol increases myofilament Ca^{2+} sensitivity in pulmonary artery smooth muscle (PASM) *via* the protein kinase C (PKC) signaling pathway.

Methods: Canine PASM strips were denuded of endothelium, loaded with fura-2/AM, and suspended in modified Krebs–Ringer's buffer at 37°C for simultaneous measurement of isometric tension and $[Ca^{2+}]_i$.

Results: The KCl (30 mM) induced monotonic increases in $[Ca^{2+}]_i$ and tension. Verapamil, an L-type Ca^{2+} channel blocker, attenuated KCl-induced increases in $[Ca^{2+}]_i$ and tension to an equal extent. In contrast, propofol attenuated KCl-induced increases in $[Ca^{2+}]_i$ to a greater extent than concomitant changes in tension and caused an upward shift in the peak tension–$[Ca^{2+}]_i$ relation. Increasing extracellular Ca^{2+} in the presence of 30 mM KCl resulted in similar increases in $[Ca^{2+}]_i$ in control and propofol-pretreated strips, whereas concomitant increases in tension were greater during propofol administration. The Ca^{2+} ionophore, ionomycin (0.1 μM), increased $[Ca^{2+}]_i$ to approximately 50% of the value induced by 60 mM KCl. Under these conditions, propofol (10, 100 μM) caused increases in tension equivalent to 11 ± 2 and 28 ± 3% of the increases in tension in response to 60 mM KCl, whereas $[Ca^{2+}]_i$ was slightly decreased. Similar effects were observed in response to the PKC activator, phorbol 12-myristate 13-acetate (PMA, 1 μM). Specific inhibition of PKC with bisindolylmaleimide I before ionomycin administration decreased the propofol- and PMA-induced increases in tension and abolished the propofol- and PMA-induced decreases in $[Ca^{2+}]_i$. Selective inhibition of Ca^{2+}-dependent PKC isoforms with Gö 6976 also attenuated propofol-induced increases in tension.

Conclusion: These results suggest that propofol increases myofilament Ca^{2+} sensitivity in PASM, and this effect involves the PKC signaling pathway.

VASCULAR smooth muscle tone is regulated by changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and myofilament Ca^{2+} sensitivity. One consequence of increasing $[Ca^{2+}]_i$ is activation of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and phosphorylation of the 20-kd myosin light chain (LC20), which results in an increase in cross-bridge cycling and tension development. Parallel to this process, other mechanisms can either modulate the regulation of myosin activity (thick filament-based regulation) or regulate pathways terminating on actin-binding proteins (thin filament-based regulation), resulting in alterations in myofilament Ca^{2+} sensitivity. Cellular mechanisms that regulate $[Ca^{2+}]_i$ and/or myofilament Ca^{2+} sensitivity could serve as targets for anesthetic agents that alter vasomotor tone.

Propofol is a widely used intravenous anesthetic for cardiac and noncardiac surgical cases. Our laboratory is systematically investigating the effects of propofol on pulmonary vascular regulation. We have previously observed that propofol potentiates the pulmonary vasoconstrictor responses to both hypoxia[1] and α adrenoreceptor activation[2] in dogs that were instrumented long-term. Propofol potentiated hypoxic pulmonary vasoconstriction by inhibiting K^+_{ATP}-mediated pulmonary vasodilation.[1] Propofol potentiated α adrenoreceptor–mediated pulmonary vasoconstriction by inhibiting the concomitant production of a vasodilator metabolite of the cyclooxygenase pathway.[3] In the present *in vitro* study, we tested the hypothesis that propofol may also have a direct effect on pulmonary vascular smooth muscle tone. Specifically, we tested the hypothesis that propofol increases myofilament Ca^{2+} sensitivity in isolated canine pulmonary artery smooth muscle (PASM). Simultaneous measurement of $[Ca^{2+}]_i$ and tension allowed us to assess changes in PASM myofilament Ca^{2+} sensitivity. We also tested the hypothesis that a propofol-induced increase in myofilament Ca^{2+} sensitivity is mediated *via* the protein kinase C (PKC) signaling pathway.

Materials and Methods

All experimental procedures and protocols were approved by the Cleveland Clinic Foundation Institutional Animal Care and Use Committee (Cleveland, Ohio).

Preparation of Pulmonary Arterial Smooth Muscle Strips

Healthy male mongrel dogs weighing 24–32 kg were anesthetized with pentobarbital sodium (30 mg/kg, intravenous) and fentanyl citrate (15 μg/kg, intravenous). After tracheal intubation, the lungs were mechanically ventilated. A catheter was placed in the right femoral

artery, and the dogs were killed by controlled hemorrhage. A left lateral thoracotomy was performed through the fifth intercostal space, and the heart was arrested with induced ventricular fibrillation. The heart and lungs were removed from the thorax *en bloc*, and the lower right and left lung lobes were dissected free. Intralobar pulmonary arteries (2–4 mm ID) were dissected carefully and immersed in cold modified Krebs–Ringer's bicarbonate (KRB) solution. The arteries were cleaned of connective tissue and cut into strips (2 × 8 mm). The endothelium was removed by gently rubbing the intimal surface with a cotton swab. Endothelial denudation was later verified by the absence of a vasorelaxant response to acetylcholine (10^{-6} M).

Simultaneous Measurement of Tension and Intracellular Ca^{2+} Concentration

Pulmonary arterial strips without endothelium were loaded with 5×10^{-6} M acetoxymethyl ester of fura-2 (fura-2/AM) solution, as described previously.[3] A noncytotoxic detergent, 0.05% Cremophor EL (Sigma Chemical, St. Louis, MO), was added to solubilize the fura-2/AM in the solution. After fura-2 loading, the arterial strips were washed with KRB buffer to remove uncleaved fura-2/AM and mounted between two stainless steel hooks in a temperature-controlled (37°C) 3-ml cuvette. The strips were continuously perfused at 12 ml/min with the KRB solution bubbled with 95% air and 5% CO_2 (pH 7.4). One hook was anchored and the other was connected to a strain gauge transducer (Grass FTO3, Grass Instrument Co., Quincy, MA) to measure isometric tension. The resting tension was adjusted to 4.0 g, which was determined in preliminary studies to be optimal for achieving a maximum contractile response to 30 mM KCl. Fluorescence measurements were performed using a dual-wavelength spectrofluorometer (Deltascan RFK6002, Photon Technology International, Lawrenceville, NJ) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Because calculations of absolute concentrations of $[Ca^{2+}]_i$ rely on a number of assumptions, the 340 to 380 fluorescence ratio (340/380 ratio) was used as a measure of $[Ca^{2+}]_i$. The individual 340 and 380 signals were measured in all experiments, and the signals were observed to change in opposite directions in response to the various interventions. Because each PASM strip served as its own control, background fluorescence was assumed to be constant and was not subtracted from the calculated 340/380 ratio. The temperature of all solutions was maintained at 37°C in a water bath. Fura-2 fluorescence signals (340 and 380 nm and 340/380 ratio) and tension were measured at a sampling frequency of 2 Hz, and collected with a software package from Photon Technology International.

Experimental Protocols

We measured tension and $[Ca^{2+}]_i$ simultaneously to investigate how propofol alters the relation between tension and $[Ca^{2+}]_i$ in pulmonary artery smooth muscle. In protocol 1, time control experiments were performed to assess the reproducibility of KCl-induced increases in $[Ca^{2+}]_i$ and tension. The PASM strips (n = 6) were treated with 30 mM KCl. After changes in $[Ca^{2+}]_i$ and tension had reached new steady state values (10–15 min), the strips were washed with fresh KRB solution and $[Ca^{2+}]_i$ and tension returned to baseline. After a return to baseline, a period of 10–15 min was allowed before the second application of 30 mM KCl. This same procedure was followed for the third application of 30 mM KCl.

In protocol 2, we tested the hypothesis that the Ca^{2+} channel antagonist, verapamil, and the intravenous anesthetic propofol would have differential effects on KCl-induced changes in $[Ca^{2+}]_i$ and tension. Once again the experimental design was similar to protocol 1. After the first application of KCl, the PASM strips (n = 6, each) were pretreated with either propofol (10 μM) or verapamil (0.1 μM) for 10 min. This was followed by the second application of 30 mM KCl. The strips were then washed with fresh KRB buffer. After a return to baseline values, the strips were pretreated with either propofol (100 μM) or verapamil (0.3 μM) for 10 min. This was followed by the third application of 30 mM KCl. In additional PASM strips (n = 5), a similar procedure was followed to assess the effects of higher concentrations of verapamil (1 and 10 μM) on KCl-induced increases in $[Ca^{2+}]_i$ and tension.

In protocol 3, we investigated the effects of propofol on the $[Ca^{2+}]_i$–tension relation by increasing the extracellular Ca^{2+} concentration. In each PASM strip (n = 5), the response to 30 mM KCl was assessed first. After washout, the strips were treated with a Ca^{2+}-free buffer containing 2 mM EGTA for 10 min. This solution was replaced with a Ca^{2+}-free solution that did not contain EGTA. After 10 min, this solution was replaced with a Ca^{2+}-free solution containing 30 mM KCl. Finally, after 10 min, the extracellular Ca^{2+} concentration was increased in control and propofol-pretreated (100 μM) strips in an incremental fashion from 0 mM to 0.25, 0.5, 1, and 3 mM. In protocols 1–3, changes in tension and $[Ca^{2+}]_i$ are expressed as a percentage of the response to the first application of 30 mM KCl.

In protocol 4, we eliminated the possible inhibitory effect of propofol on increases in $[Ca^{2+}]_i$ in response to KCl-induced depolarization,[4] which could mask a propofol-induced increase in myofilament Ca^{2+} sensitivity. To do this, we performed experiments where $[Ca^{2+}]_i$ was increased using the Ca^{2+} ionophore ionomycin (0.1 μM). First, increases in tension and $[Ca^{2+}]_i$ in response to 60 mM KCl were obtained. We used 60 mM KCl as our reference response in this protocol because in prelimi-

nary experiments we observed that ionomycin increased [Ca^{2+}]$_i$ to approximately 50% of the value induced by 60 mM KCl. The strips were then washed with fresh KRB solution. After tension and [Ca^{2+}]$_i$ had returned to baseline, ionomycin (0.1 μM) was administered. This concentration of ionomycin had essentially no effect on tension, whereas it increased [Ca^{2+}]$_i$ to approximately 50% of the value induced by 60 mM KCl. In this setting, propofol (1, 10, 100 μM) was administered cumulatively to the perfusion solution. The same procedure was repeated in strips pretreated with bisindolylmaleimide I (BIS, 1 μM), a specific PKC inhibitor,[5] or Gö 6976 (10 μM), a selective inhibitor of Ca^{2+}-dependent PKC isoforms.[6] We also investigated the effects of phorbol 12-myristate 13-acetate (PMA, 1 μM), a specific PKC activator, on tension and [Ca^{2+}]$_i$ in ionomycin-treated strips in the presence and absence of BIS. To determine whether an increase in [Ca^{2+}]$_i$ is required for propofol-induced changes in tension, propofol (1, 10, 100 μM) was administered to PASM strips (n = 3) in the absence of extracellular Ca^{2+} (Ca^{2+}-free solution containing 2 mM EGTA) and after pretreatment with the IP$_3$-receptor antagonist 2-aminoethoxydiphenyl borate (2-APB, 100 μM, Calbiochem, La Jolla, CA), to inhibit propofol-induced changes in Ca^{2+} release from the sarcoplasmic reticulum.[4] In protocol 4, changes in tension and [Ca^{2+}]$_i$ are expressed as a percentage of the response to 60 mM KCl.

Solutions and Chemicals

The KRB solution had the following composition: 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$, 25 mM NaHCO$_3$, 0.016 mM Ca-EDTA, and 11.1 mM glucose, gassed with 95% air and 5% CO$_2$ at 37°C, pH 7.4. The KCl solutions were prepared in an isotonic fashion by replacing NaCl with equimolar KCl. The following chemicals were used: fura-2/AM (Texas Fluorescence Labs, Austin, TX), acetylcholine chloride, ionomycin, BIS, PMA, Cremophor EL, Gö 6976, and 2-APB. The BIS, Gö 6976, and ionomycin were dissolved in dimethyl sulfoxide and diluted with distilled water. The final concentration of dimethyl sulfoxide in the organ bath was less than 0.1% (vol/vol). In control experiments, none of the agents or solutions used in this study caused significant shifts in the fluorescence signals in fura-2 nonloaded PASM strips, suggesting that changes in fluorescence of endogenous substances are negligible.

Data Analysis

All values are expressed as mean ± SEM. Statistical analysis used analysis of variance, Student paired t test, and the Bonferroni test for multiple comparisons. The peak tension–[Ca^{2+}]$_i$ relations were compared using linear regression analysis. A P value of less than 0.05 was considered statistically significant. In all experiments,
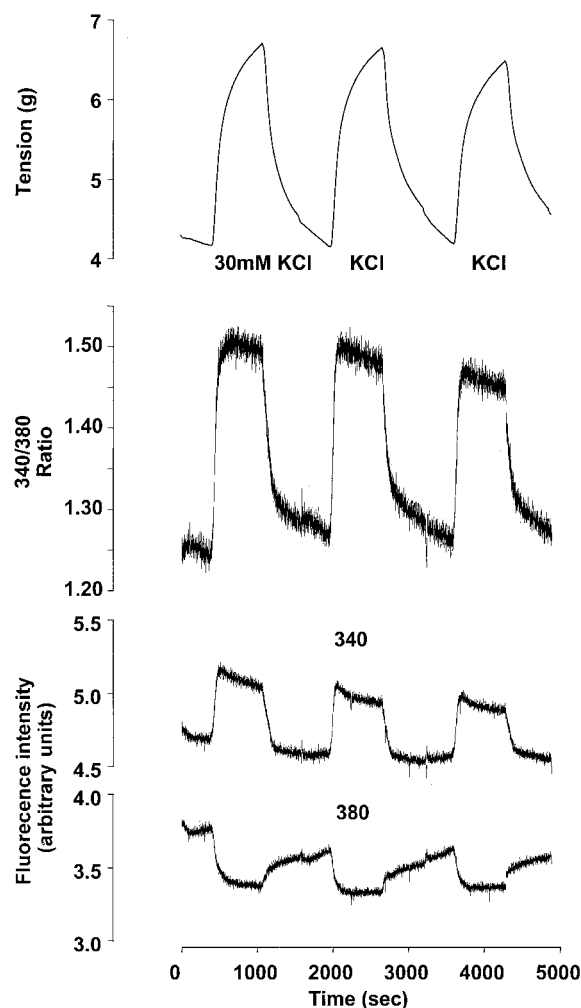
Fig. 1. Original tracings demonstrating the effects of three consecutive applications of 30 mM KCl on tension, intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) (340/380 ratio), as well as the 340 and 380 fluorescent signals in an endothelium-denuded pulmonary artery smooth muscle (PASM) strip.

n equals the number of dogs from which the PASM strips were obtained. For each protocol, multiple strips from the same dog were averaged, so that all dogs were weighted equally in the analysis.

Results

Effects of Sequential Application of KCl on KCl-induced Increases in [Ca^{2+}]$_i$ and Tension

Figure 1 illustrates changes in tension and the 340, 380, and 340/380 fura-2 fluorescence signals in a PASM strip in response to three sequential applications of 30 mM KCl. Increases in [Ca^{2+}]$_i$ and tension in response to the second and third application of 30 mM KCl are summarized as a percentage of the first KCl response. Changes in tension (108 ± 5 and 99 ± 4%) and [Ca^{2+}]$_i$ (96 ± 2 and 90 ± 3%) in response to sequential treatments of 30 mM KCl were highly reproducible.
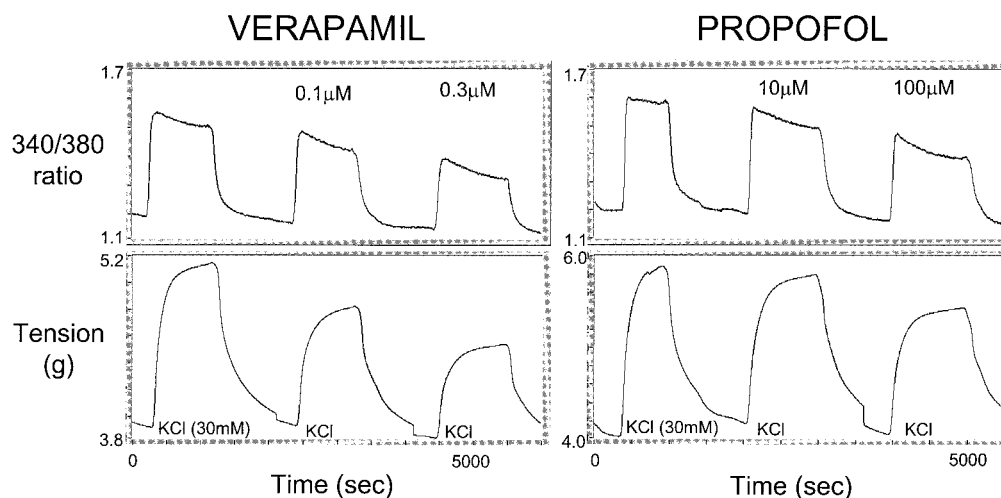
Fig. 2. Original tracings demonstrating effects of verapamil and propofol on increases in tension and $[Ca^{2+}]_i$ (340/380 ratio) in response to 30 mM KCl in pulmonary artery smooth muscle (PASM) strips. Sudden decreases in tension after washout are artifacts due to pauses in data acquisition.

Effects of Verapamil and Propofol on KCl-induced Increases in $[Ca^{2+}]_i$ and Tension

Figure 2 illustrates the effects of verapamil and propofol on original recordings of changes in $[Ca^{2+}]_i$ and tension induced by 30 mM KCl. The KCl caused a monotonic increase in tension that was maintained at a peak value as well as a monotonic increase in $[Ca^{2+}]_i$ that slightly decreased from a peak value. Inhibition of L-type Ca^{2+} channels with verapamil (0.1 and 0.3 μM) caused dose-dependent decreases in $[Ca^{2+}]_i$ and tension of equal magnitude. Propofol (10 and 100 μM) also caused dose-dependent decreases in $[Ca^{2+}]_i$. However, tension was relatively well preserved in the presence of propo-

fol. To further contrast the effects of verapamil and propofol on KCl-induced increases in $[Ca^{2+}]_i$ and tension, the data presented in figure 2 are plotted as phase–plane loops showing the continuous $[Ca^{2+}]_i$–tension relation (fig. 3). The dashed arrows connect the points where the maximum tension and the corresponding $[Ca^{2+}]_i$ were achieved. Verapamil attenuated both peak tension and $[Ca^{2+}]_i$ to a similar extent, as indicated by the slope of the arrow approximately passing through unity. In contrast, propofol caused an upward shift in the relation between peak tension and $[Ca^{2+}]_i$ compared with verapamil. Propofol significantly decreased the slope of the peak tension–$[Ca^{2+}]_i$ relation compared
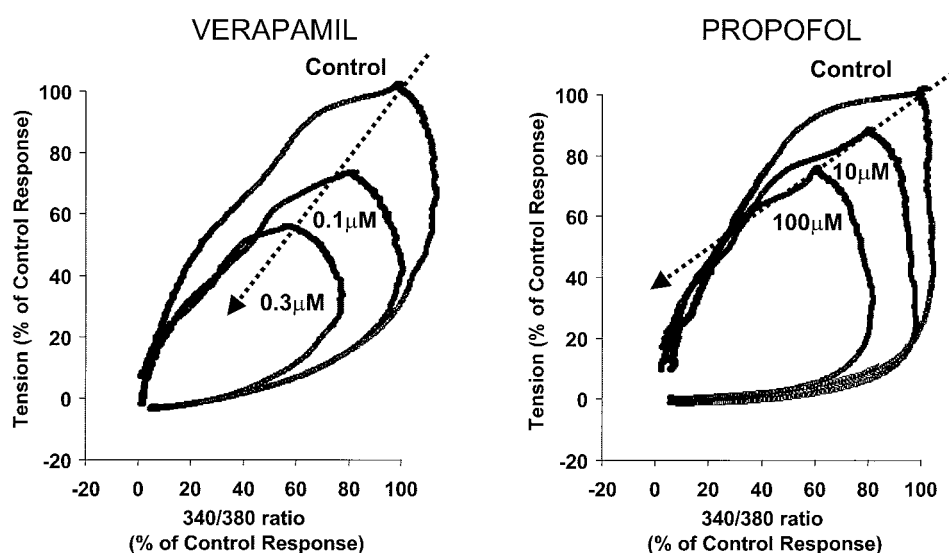

Fig. 3. Phase–plane plots demonstrating effects of verapamil and propofol on changes in tension and $[Ca^{2+}]_i$ (340/380 ratio) in response to 30 mM KCl in pulmonary artery smooth muscle (PASM) strips. Data were acquired at 2 Hz and were generated by replotting the tracings shown in figure 2. Propofol caused an upward shift in the slope of the KCl-induced peak tension–$[Ca^{2+}]_i$ relation compared to verapamil. The dashed arrows connect the points where the maximum tension and the corresponding $[Ca^{2+}]_i$ were achieved.

with verapamil (0.64 ± 0.08 *vs.* 1.07 ± 0.08, respectively, $P < 0.05$). When higher concentrations of verapamil (1 and 10 μM) were used to further reduce KCl-induced increases in tension and $[Ca^{2+}]_i$, the slope of the peak tension–$[Ca^{2+}]_i$ relation (1.17 ± 0.10) was similar to that observed with the lower concentrations of verapamil and significantly higher ($P < 0.05$) than that observed with propofol. Moreover, PKC inhibition with BIS completely reversed the propofol-induced decrease in the slope of the peak tension–$[Ca^{2+}]_i$ relation in response to KCl depolarization (BIS plus propofol, 100 μM, slope = 1.26 ± 0.10). These results suggest that propofol increases myofilament Ca^{2+} sensitivity in PASM *via* a PKC-sensitive pathway.

Effects of Propofol on $[Ca^{2+}]_i$–tension Relation when Extracellular Ca2+ Is Increased

To more directly assess the effects of propofol on myofilament Ca^{2+} sensitivity, control and propofol-pretreated PASM strips bathed in a Ca^{2+}-free buffer containing 30 mM KCl were exposed to incremental increases in extracellular Ca^{2+} concentration. As summarized in figure 4, increasing extracellular Ca^{2+} concentration resulted in virtually identical increases in $[Ca^{2+}]_i$ in control and propofol-pretreated strips, whereas concomitant increases in tension were greater (*$P < 0.05$) in propofol-pretreated strips compared with control. This resulted in a shift in the $[Ca^{2+}]_i$–tension relation, such that for a given value of $[Ca^{2+}]_i$, tension was greater in the propofol-pretreated strips compared with the control.

Effects of Protein Kinase C Inhibition on Propofol-induced Increases in $[Ca^{2+}]_i$ and Tension in Ionomycin-pretreated Strips

Figure 5 illustrates original recordings of changes in $[Ca^{2+}]_i$ and tension induced by propofol in the presence or absence of the specific PKC inhibitor, BIS. To eliminate the inhibitory effect of propofol on increases in $[Ca^{2+}]_i$ in response to KCl-induced depolarization, which could mask a propofol-induced increase in myofilament Ca^{2+} sensitivity, the PASM strips were pretreated with the Ca^{2+} ionophore, ionomycin (0.1 μM), to increase $[Ca^{2+}]_i$. Ionomycin had essentially no effect on tension, whereas it increased $[Ca^{2+}]_i$ to approximately 50% of the value induced by 60 mM KCl (fig. 5). During these conditions, propofol caused a dose-dependent increase in tension but had little effect on $[Ca^{2+}]_i$. Pretreatment with BIS before ionomycin administration attenuated the increases in tension in response to propofol. Summarized data are shown in figure 6. Propofol significantly increased tension, whereas $[Ca^{2+}]_i$ was slightly decreased. Pretreatment with BIS attenuated the propofol-induced changes in tension and $[Ca^{2+}]_i$.

Effects of Protein Kinase C Inhibition on PMA-induced Increases in $[Ca^{2+}]_i$ and Tension in Ionomycin-pretreated Strips

Figure 7 summarizes the effects of the PKC activator PMA on $[Ca^{2+}]_i$ and tension in ionomycin-pretreated strips in the presence and absence of BIS. As observed with propofol, PMA increased tension, whereas $[Ca^{2+}]_i$ was slightly decreased. Pretreatment with BIS abolished the PMA-induced changes in tension and $[Ca^{2+}]_i$. These results indicate that increases in myofilament Ca^{2+} sensitivity in response to PMA involve the PKC signaling pathway.

Effects of Inhibition of Ca^{2+}-dependent Protein Kinase C Isoforms on Propofol-induced Increases in $[Ca^{2+}]_i$ and Tension in Ionomycin-pretreated Strips

As summarized in figure 8, pretreatment with Gö 6976, a selective inhibitor of Ca^{2+}-dependent PKC isoforms, also attenuated the propofol-induced increase in tension and the decrease in $[Ca^{2+}]_i$ in ionomycin-pretreated strips, though this effect was only apparent at the highest concentration of propofol. To determine whether an increase in $[Ca^{2+}]_i$ is required for the propofol-induced increase in tension, propofol (1, 10, and 100 μM) was administered to the PASM strips in the absence of extracellular Ca^{2+} and after pretreatment with the IP$_3$ receptor antagonist 2-APB to inhibit Ca^{2+} release from the sarcoplasmic reticulum. During these conditions, propofol had no effect on tension or $[Ca^{2+}]_i$.

Discussion

This is the first study to assess the effects of propofol on myofilament Ca^{2+} sensitivity in PASM. Our results demonstrate that propofol increases myofilament Ca^{2+} sensitivity. Moreover, this effect is attenuated by a specific inhibitor of PKC and an inhibitor of Ca^{2+}-dependent PKC isoforms. These results suggest that the propofol-induced increase in myofilament Ca^{2+} sensitivity involves the PKC signaling pathway.

The results from protocol 1 demonstrated that triplicate responses to KCl cause reproducible and consistent increases in $[Ca^{2+}]_i$ and tension. Our next goal was to determine whether propofol alters the relation between KCl-induced changes in tension and $[Ca^{2+}]_i$ in PASM. The KCl-induced increases in tension are mediated by Ca^{2+} influx through L-type, voltage-gated Ca^{2+} channels, which results in an increase in $[Ca^{2+}]_i$, phosphorylation of myosin light chain, and tension development. Thus, the L-type Ca^{2+} channel blocker verapamil would be expected to inhibit KCl-induced increases in tension and $[Ca^{2+}]_i$ to a similar extent, as was reported previously in isolated guinea pig aortae.[7] This assumes that verapamil has no effect on myofilament Ca^{2+} sensitivity, which was reported in canine coronary arteries.[8] In contrast to
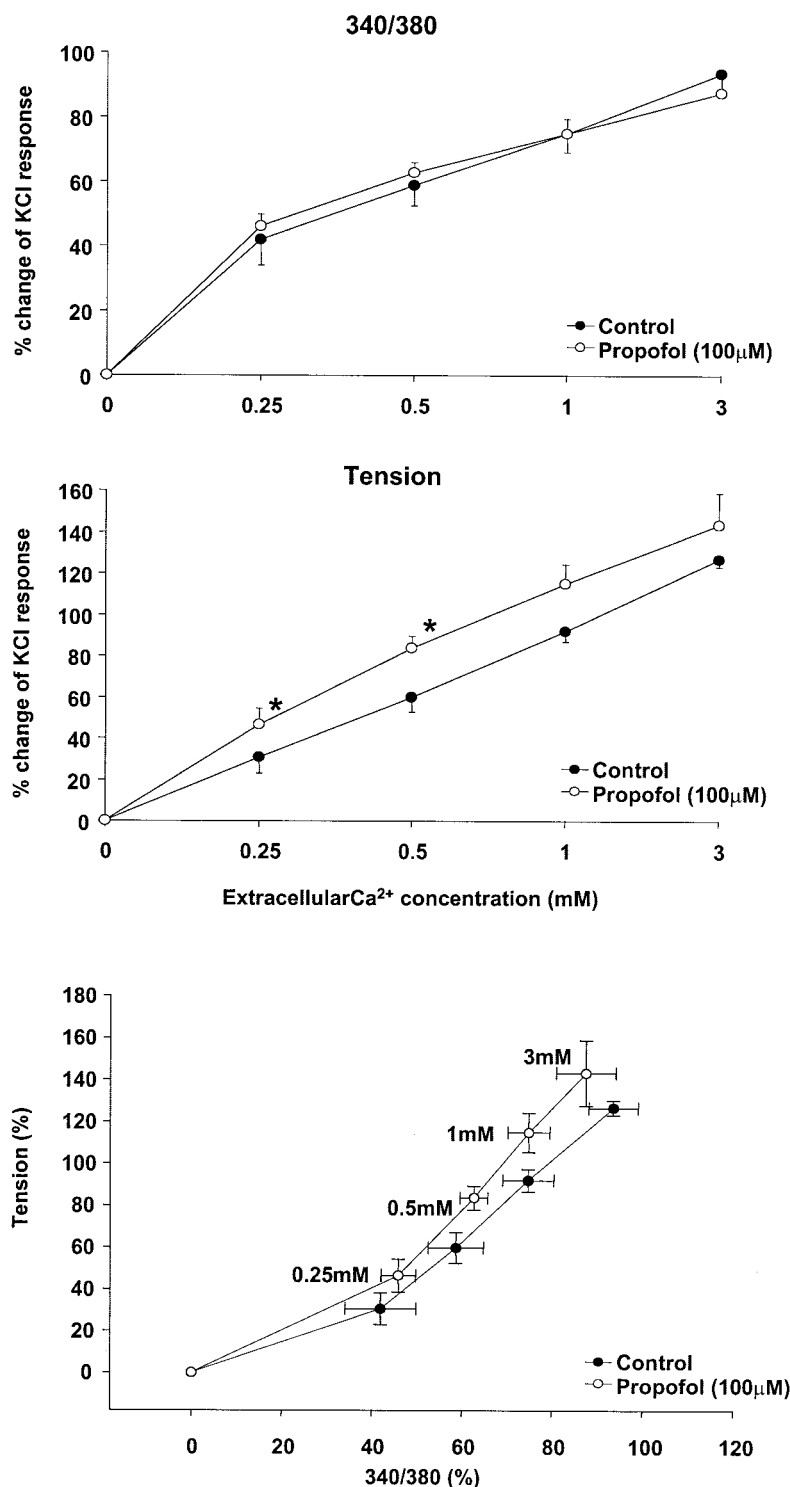
Fig. 4. Summarized data for the effects of increasing extracellular Ca^{2+} concentration in control and propofol-pretreated pulmonary artery smooth muscle (PASM) strips bathed in a Ca^{2+}-free buffer containing 30 mM KCl. Extracellular Ca^{2+} was increased incrementally from 0 to 3 mM while measuring $[Ca^{2+}]_i$ (340/380 ratio) and tension. (*Top*) Results are plotted as percentage changes relative to 30 mM KCl. (*Bottom*) Results are replotted to demonstrate the effects of propofol on the $[Ca^{2+}]_i$–tension relation. *$P < 0.05$ vs. control; n = 5.

verapamil, propofol attenuated KCl-induced increases in $[Ca^{2+}]_i$ to a greater extent than concomitant increases in tension. This was reflected by an upward shift in the peak tension–$[Ca^{2+}]_i$ relation, suggesting an increase in myofilament Ca^{2+} sensitivity. It is important to note that this method to assess the effects of propofol on myofilament Ca^{2+} sensitivity has limitations (*e.g.*, unknown effects of propofol on neurotransmitter release) and

does not alone provide direct proof that propofol increases myofilament Ca^{2+} sensitivity. To more directly assess this possibility, we investigated the effects of propofol in PASM strips perfused with a Ca^{2+}-free solution containing 30 mM KCl (protocol 3). During these conditions, increasing extracellular Ca^{2+} concentration in an incremental fashion caused virtually identical increases in $[Ca^{2+}]_i$ in control and propofol-pretreated
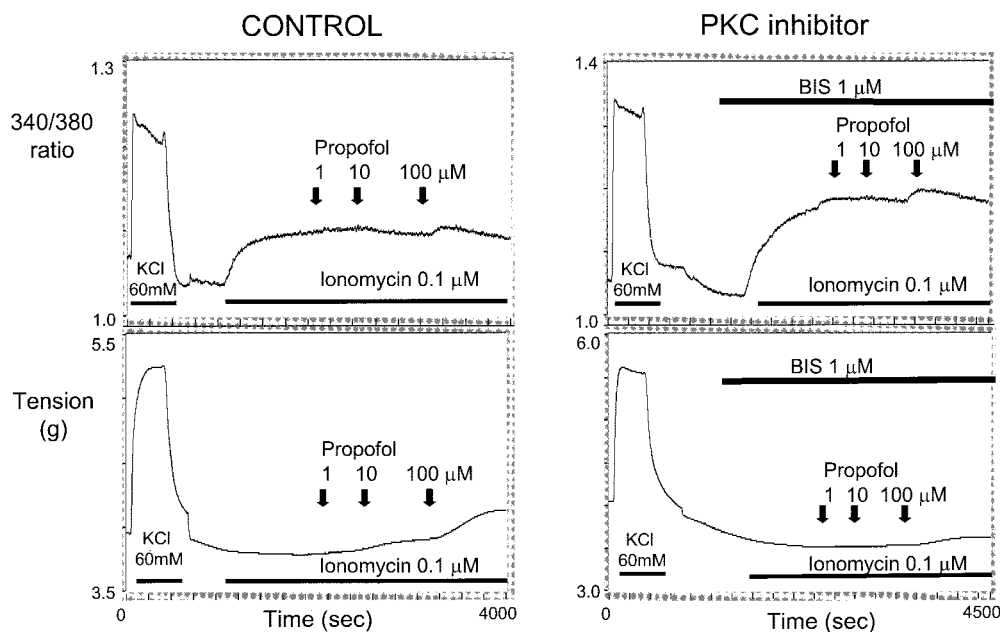
Fig. 5. Original tracings demonstrating the effects of the specific protein kinase C (PKC) inhibitor bisindolylmaleimide I (BIS) on propofol-induced changes in tension and [Ca^{2+}]$_i$ (340/380 ratio) in ionomycin-pretreated pulmonary artery smooth muscle (PASM) strips. Propofol increased tension in a dose-dependent manner without a concomitant increase in [Ca^{2+}]$_i$. Pretreatment with BIS before ionomycin administration attenuated the propofol-induced changes in tension and [Ca^{2+}]$_i$.

strips, whereas concomitant increases in tension were greater in propofol-pretreated strips. Thus, for a given value of [Ca^{2+}]$_i$, tension was greater during propofol compared with control; that is, propofol increased myofilament Ca^{2+} sensitivity. Taken together, our results suggest that propofol has at least two opposing effects on KCl-induced contraction. Propofol has been shown to inhibit L-type, voltage-gated Ca^{2+} channels in systemic vascular smooth muscle,[4,9] tracheal smooth muscle cells,[10] and myocardial cells.[11] Thus, it is likely that the inhibitory effect of propofol on KCl-induced increases in tension and [Ca^{2+}]$_i$ in PASM is due to inhibition of L-type, voltage-gated Ca^{2+} channels. However, this ef-

fect is partially offset by a concomitant propofol-induced increase in PASM myofilament Ca^{2+} sensitivity.

The effects of propofol on myofilament Ca^{2+} sensitivity may depend on the species and/or the tissue type being investigated. Propofol had no effect on myofilament Ca^{2+} sensitivity in the absence or presence of muscarinic receptor stimulation in β-escin–permeabilized canine tracheal smooth muscle, even at supraclinical concentrations.[12] Propofol was reported to decrease myofilament Ca^{2+} sensitivity in rat ventricular myocytes at supraclinical concentrations.[13] In contrast, Nakae et al.[14] reported that clinically relevant concentrations of propofol increased myofilament Ca^{2+} sensitivity in iso-

Fig. 6. Summarized data for the effects of bisindolylmaleimide I (BIS) on propofol-induced changes in tension and [Ca^{2+}]$_i$ (340/380 ratio) in ionomycin-pretreated pulmonary artery smooth muscle (PASM) strips. Propofol increased tension in a dose-dependent manner with a slight concomitant decrease in [Ca^{2+}]$_i$ (*P < 0.05 vs. baseline). Pretreatment with BIS attenuated the propofol-induced increases in tension and decreases in [Ca^{2+}]$_i$ (†P < 0.05 vs. control). Results are expressed as percentage changes relative to response to 60 mM KCl. n = 6, each.
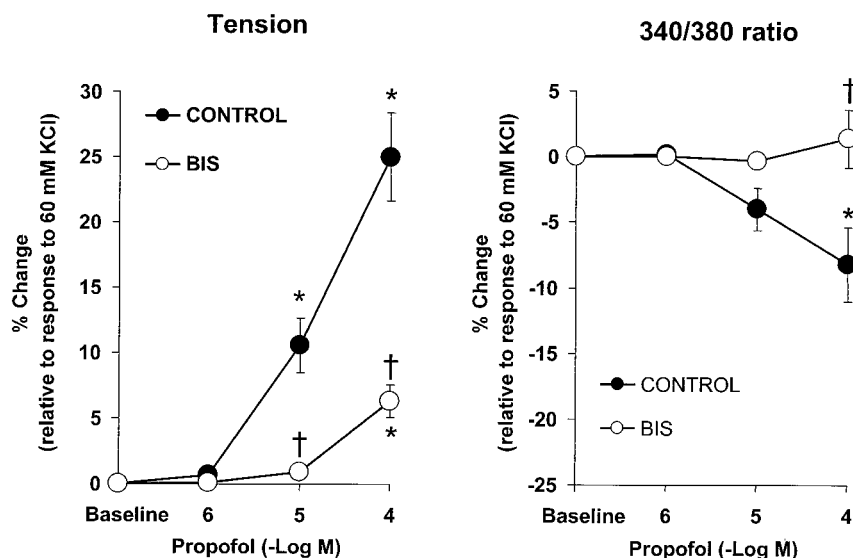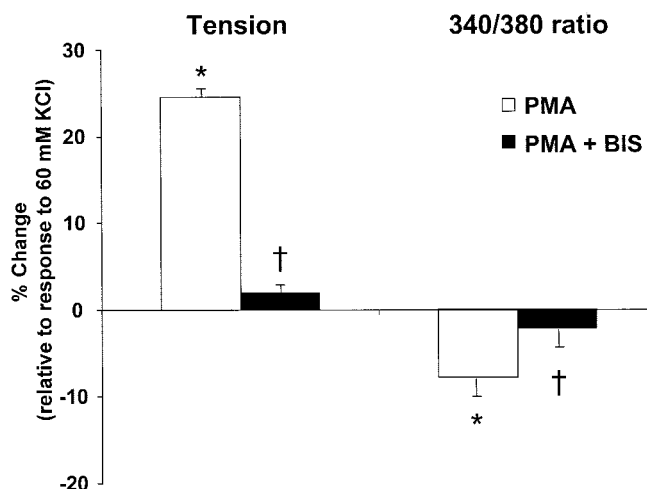
Fig. 7. Summarized data for the effects of bisindolylmaleimide I (BIS) on phorbol 12-myristate 13-acetate (PMA)–induced changes in tension and $[Ca^{2+}]_i$ (340/380 ratio) in ionomycin-pretreated pulmonary artery smooth muscle (PASM) strips. The PMA increased tension with a small concomitant decrease in $[Ca^{2+}]_i$ (*$P < 0.05$ vs. control). Pretreatment with BIS abolished the PMA-induced changes in tension and $[Ca^{2+}]_i$ (†$P < 0.05$ vs. PMA). Results are expressed as percentage changes relative to response to 60 mM KCl. n = 6, each.

lated guinea pig hearts. Our laboratory[15] has reported that propofol increases myofilament Ca^{2+} sensitivity in rat cardiomyocytes. Recently, we[16] demonstrated in cardiomyocytes that propofol increases the sensitivity of myofibrillar actomyosin adenosine triphosphatase to Ca^{2+} (*i.e.*, increases myofilament Ca^{2+} sensitivity), at least in part by increasing intracellular pH *via* a PKC-dependent activation of Na^+–H^+ exchange. In the present study, propofol caused an upward shift in the

PASM peak tension–$[Ca^{2+}]_i$ relation compared with verapamil-induced changes and a shift in the $[Ca^{2+}]_i$–tension relation when extracellular Ca^{2+} concentration was increased incrementally from a Ca^{2+}-free condition. Moreover, in ionomycin-pretreated PASM strips, propofol caused a dose-dependent increase in tension without a concomitant increase in $[Ca^{2+}]_i$. These results clearly indicate that propofol increases myofilament Ca^{2+} sensitivity in PASM.

Based on our previous results in cardiomyocytes,[16] we next tested the hypothesis that propofol increases PASM myofilament Ca^{2+} sensitivity *via* activation of the PKC signaling pathway. In the ionomycin-pretreated PASM strips, the propofol-induced increases in tension and decreases in $[Ca^{2+}]_i$ were attenuated by BIS, a specific inhibitor of PKC. Moreover, the PKC activator PMA mimicked the effects of propofol in the ionomycin-pretreated PASM strips; that is, PMA also increased tension and decreased $[Ca^{2+}]_i$. These PMA-induced changes were abolished by BIS. Taken together, these results suggest that the propofol-induced increase in myofilament Ca^{2+} sensitivity and the concomitant decrease in $[Ca^{2+}]_i$ involve PKC activation.

The primary mechanisms of contraction in vascular smooth muscle involve Ca^{2+}/calmodulin-dependent, MLCK–dependent phosphorylation of LC20,[17,18] and myofilament Ca^{2+} sensitization.[19] The PKC has been implicated in the signaling pathway that regulates myofilament Ca^{2+} sensitivity.[20] Some reports[21,22] indicate that PKC-mediated contraction is Ca^{2+} dependent,[23–25] whereas other studies suggest that it is Ca^{2+} independent. We recently identified six isoforms of PKC
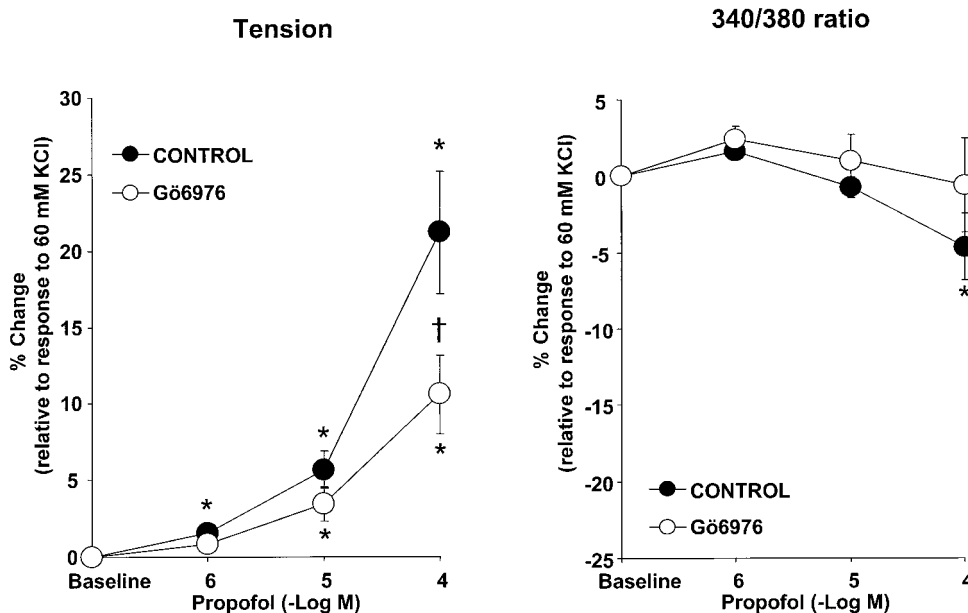
Fig. 8. Summarized data for the effects of Gö 6976, a selective inhibitor of Ca^{2+}-dependent protein kinase C (PKC) isoforms, on propofol-induced changes in tension and $[Ca^{2+}]_i$ (340/380 ratio) in ionomycin-pretreated pulmonary artery smooth muscle (PASM) strips. Pretreatment with Gö 6976 attenuated (†$P < 0.05$ vs. control) the propofol-induced increases (*$P < 0.05$ vs. baseline) in tension and decreases in $[Ca^{2+}]_i$. Results are expressed as percentage changes relative to response to 60 mM KCl. n = 7, each.

in canine PASM cells,[26] representing the classic, novel, and atypical PKC isoform groups. Two of the isoforms (PKCα and PKCδ) underwent translocation in response to the PKC activator dioctanoylglycerol and in response to angiotensin II. PKCα is a member of the classic group A PKC isoforms, which are Ca^{2+} dependent. PKCβ and PKCγ are also members of the classic group A PKC isoforms but are not present in PASM.[26] PKCδ is a member of the novel group B PKC isoforms, which are Ca^{2+} independent. In the present study, selective inhibition of Ca^{2+}-dependent PKC isoforms with Gö 6976 attenuated the propofol-induced increases in tension and decreases in [Ca^{2+}]$_i$. Also, Gö 6976 was shown to inhibit the vasoconstrictor responses to angiotensin II[27] and hypoxia[28] in intact lungs. Moreover, our observation that propofol had no effect on tension during experimental conditions that prevented an increase in [Ca^{2+}]$_i$ (*i.e.*, absence of extracellular Ca^{2+} and after IP$_3$–receptor block) underscores the Ca^{2+} dependence of the propofol-induced, PKC-mediated increase in tension. Taken together, our results suggest that the propofol-induced increase in tension and decrease in [Ca^{2+}]$_i$ involves activation of PKCα.

The cellular mechanisms by which propofol causes PKC-dependent changes in [Ca^{2+}]$_i$ and tension have yet to be identified. The effect of propofol on [Ca^{2+}]$_i$ could be due to either a PKC-mediated decrease in Ca^{2+} influx (*e.g.*, *via* L-type, voltage-gated Ca^{2+} channels) or an increase in Ca^{2+} extrusion. PKC is known to activate Na$^+$–Ca^{2+} exchange[29] and/or Ca^{2+} pumps.[30] Recently, we[31] demonstrated that propofol inhibits capacitative Ca^{2+} entry in PASM cells *via* a PKC-dependent pathway. It is possible that the small decreases in [Ca^{2+}]$_i$ in response to propofol and PMA may be due to inhibition of capacitative Ca^{2+} entry *via* the PKC signaling pathway. PKC has been postulated to increase myofilament Ca^{2+} sensitivity *via* several mechanisms, including phosphorylation of thin-filament accessory proteins (*e.g.*, caldesmon, calponin), inhibition of myosin light chain phosphatase, phosphorylation of myosin light chain, and activation of Na$^+$–H$^+$ exchange, resulting in intracellular alkalinization.[19,32] In contrast, PKC was not found to play an important role in norepinephrine-induced increases in myofilament Ca^{2+} sensitivity in PASM.[33] A possible limitation of that study is that [Ca^{2+}]$_i$ and tension were not measured simultaneously in the same tissue.[33] Moreover, the study by Janssen *et al.*[33] used endothelium-intact pulmonary arterial rings that were not pretreated to inhibit the β-agonist effect of norepinephrine. These factors could confound interpretation of norepinephrine-induced changes in tension. Finally, it should be noted that neither BIS nor Gö 6976 abolished the propofol-induced increases in PASM tension. This suggests that other signaling mechanisms that regulate myofilament Ca^{2+} sensitivity (*e.g.*, tyrosine kinases, Rho-kinase) could be involved.

Ionomycin is a Ca^{2+} ionophore that increases [Ca^{2+}]$_i$, both *via* an increase in sarcolemmal Ca^{2+} influx and *via* Ca^{2+} release from intracellular stores. This latter effect could in turn lead to activation of capacitative Ca^{2+} entry. As previously reported,[34] we observed that ionomycin induced a smaller contraction than that predicted from the concomitant increase in [Ca^{2+}]$_i$. The precise mechanism underlying this ionomycin-induced dissociation between tension and [Ca^{2+}]$_i$ is unknown. One possible mechanism is that, because ionomycin can inhibit mitochondrial activity,[35,36] decreased adenosine triphosphate production may dissociate contraction from increases in [Ca^{2+}]$_i$. Alternatively, ionomycin itself may decrease myofilament Ca^{2+} sensitivity. This possibility needs to be considered when interpreting the effects of propofol in the ionomycin-pretreated PASM strips.

The plasma concentration of propofol in patients during maintenance of general anesthesia was reported to be in the range of 10^{-5} to 10^{-4} M.[37] Because 97–98% of propofol is bound to plasma proteins,[38] the free concentration of propofol is approximately 10^{-6} to 10^{-5} M. However, protein binding of propofol *in vivo* is unlikely to be instantaneous, so the free drug concentration associated with a bolus injection would be higher than the steady state value. Moreover, it was demonstrated recently that 28% of propofol is taken up by the lung during a single passage through the lung, and most of the propofol that undergoes pulmonary uptake is released back into the circulation by back diffusion.[39] This results in a higher concentration of propofol in the pulmonary artery than in the radial artery.[40] In this study, propofol concentrations of 10^{-5} M and higher significantly increased tension without increasing [Ca^{2+}]$_i$ in ionomycin-treated pulmonary arterial strips. This result indicates that propofol can increase PASM myofilament Ca^{2+} sensitivity at clinically relevant concentrations.

In conclusion, propofol has a direct inhibitory effect on PASM contraction that is mediated by a decrease in the availability of [Ca^{2+}]$_i$. Propofol also increases PASM myofilament Ca^{2+} sensitivity, and this effect is at least partially mediated by the PKC signaling pathway. Indirect evidence suggests an involvement of PKCα. Future studies will be required to determine whether propofol also increases myofilament Ca^{2+} sensitivity during receptor activation.

References

1. Nakayama M, Murray PA: Ketamine preserves and propofol potentiates hypoxic pulmonary vasoconstriction compared to the conscious state in chronically instrumented dogs. ANESTHESIOLOGY 1999; 91:760–71

2. Kondo U, Kim S, Nakayama M, Murray PA: Pulmonary vascular effects of propofol at baseline, during elevated vasomotor tone and in response to sympathetic α and β adrenoreceptor activation. ANESTHESIOLOGY 2001; 94:815–23

3. Ogawa K, Tanaka S, Murray PA: Propofol potentiates phenylephrine-induced contraction via cyclooxygenase inhibition in pulmonary artery smooth muscle. ANESTHESIOLOGY 2001; 94:833–9

4. Imura N, Shiraishi Y, Katsuya H, Itoh T: Effect of propofol on norepinephrine-induced increases in [Ca^{2+}]$_i$ and force in smooth muscle of the rabbit mesenteric resistance artery. ANESTHESIOLOGY 1998; 88:1566–78

5. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F: The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 1991; 266:15771–81

6. Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C: Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö-6976. J Biol Chem 1993; 268:9194–7

7. Ozaki H, Abe AUY, Kinoshita M, Hori M, Mitsui-Saito M, Karaki H: Effects of a prostaglandin I $_2$ analog iloprost on cytoplasmic Ca^{2+} levels and muscle contraction in isolated guinea pig aorta. Jpn J Pharmacol 1996; 71:231–7

8. Yanagisawa T, Kawada M, Taira N: Nitroglycerin relaxes canine coronary arterial smooth muscle without reducing intracellular Ca^{2+} concentrations measured with fura-2. Br J Pharmacol 1989; 98:46 9–82

9. Xuan Y-T, Glass P: Propofol regulation of calcium entry pathways in cultured A10 and rat aortic smooth muscle cells. Br J Pharmacol 1996; 117:5–12

10. Yamakage M, Hirshman CA, Croxton TL: Inhibitory effects of thiopental, ketamine, and propofol on voltage-dependent Ca^{2+} channels in porcine tracheal smooth muscle cells. ANESTHESIOLOGY 1995; 83:1274–82

11. Buljubasic N, Marijic J, Berczi V, Supan DF, Kampine JP, Bosnjak ZJ: Differential effects of etomidate, propofol, and midazolam on calcium and potassium channel currents in canine myocardial cells. ANESTHESIOLOGY 1996; 85:1092–9

12. Hanazaki M, Jones KA, Warner DO: Effects of intravenous anesthetics on Ca^{2+} sensitivity in canine tracheal smooth muscle. ANESTHESIOLOGY 2000; 92:133–9

13. Hamilton DL, Boyett MR, Harrison SM, Davies LA, Hopkins PM: The concentration-dependent effects of propofol on rat ventricular myocytes. Anesth Analg 2000; 91:276–82

14. Nakae Y, Fujita S, Namiki A: Propofol inhibits Ca^{2+} transients but not contraction in intact beating guinea pig hearts. Anesth Analg 2000; 90:1286–92

15. Kanaya N, Murray PA, Damron DS: Propofol and ketamine only inhibit intracellular Ca^{2+} transients and contraction in rat ventricular myocytes at supraclinical concentrations. ANESTHESIOLOGY 1998; 88:781–91

16. Kanaya N, Murray PA, Damron DS: Propofol increases myofilament Ca^{2+} sensitivity and intracellular pH via activation of Na^+-H^+ exchange in rat ventricular myocytes. ANESTHESIOLOGY 2001; 94:1096–104

17. Silver PJ, Stull JT: Phosphorylation of myosin light chain and phosphorylase in tracheal smooth muscle in response to KCl and carbachol. Mol Pharmacol 1984; 25:267–74

18. Murphy RA, Aksoy MO, Dillon PF, Gerthoffer WT, Kamm KE: The role of myosin light chain phosphorylation in regulation of the cross-bridge cycle. Fed Proc 1983; 42:51–6

19. Somlyo AP, Somlyo AV: Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol 2001; 522:177–85

20. Morgan KG, Leinweber BD: PKC-dependent signalling mechanisms in differentiated smooth muscle. Acta Physiol Scand 1998; 164:495–505

21. Rembold CM, Murphy RA: [Ca^{2+}]-dependent myosin phosphorylation in phorbol-diester-stimulated smooth muscle contraction. Am J Physiol 1988; 255:C719–23

22. Gleason MM, Flaim SF: Phorbol ester contracts rabbit thoracic aorta by increasing intracellular calcium and by activating calcium influx. Biochem Biophys Res Commun 1986; 138:1362–9

23. Orton EC, Raffestin B, McMurtry IF: Protein kinase C influences rat pulmonary vascular reactivity. Am Rev Respir Dis 1990; 141:654–8

24. Jiang MJ, Morgan KG: Agonist-specific myosin phosphorylation and intracellular calcium during isometric contractions of arterial smooth muscle. Pflügers Arch 1989; 413:637–43

25. Sybertz EJ, Desiderio DM, Tetzloff G, Chiu PJ: Phorbol dibutyrate contractions in rabbit aorta: Calcium dependence and sensitivity to nitrovasodilators and 8-BR-cyclic GMP. J Pharmacol Exp Ther 1986; 239:78–83

26. Damron DS, Nadim HS, Hong S-J, Darvish A, Murray PA: Intracellular translocation of protein kinase C isoforms in canine pulmonary artery smooth muscle cells by angiotensin II. Am J Physiol 1998; 274:L278-L288

27. DeWitt BJ, Kaye AD, Ibrahim IN, Bivalacqua TJ, D'Souza FM, Banister RE, Arif AS, Nossaman BD: Effects of PKC isozyme inhibitors on constrictor responses in the feline pulmonary vascular bed. Am J Physiol Lung Cell Mol Physiol 2001; 280:L50–7

28. Barman SA: Effect of protein kinase C inhibition on hypoxic pulmonary vasoconstriction. Am J Physiol Lung Cell Mol Physiol 2001; 280:L888–95

29. Iwamoto T, Pan Y, Wakabayashi S, Imagawa T, Yamanaka HI, Shigekawa M: Phosphorylation-dependent regulation of cardiac Na^+/Ca^{2+} exchanger via protein kinase C. J Biol Chem 1996; 271:13609–15

30. Verma AK, Paszty K, Filoteo AG, Penniston JT, Enyedi A: Protein kinase C phosphoryates plasma membrane Ca^{2+} pump isoform 4a at its calmodulin binding domain. J Biol Chem 1999; 274:527–31

31. Horibe M, Kondo I, Damron DS, Murray PA: Propofol attenuates capacitative calcium entry in individual pulmonary artery smooth muscle cells. ANESTHESIOLOGY 2001; 95:605–13

32. Krampetz IK, Rhoades RA: Intracellular pH: Effect on pulmonary arterial smooth muscle. Am J Physiol 1991; 260:L516–21

33. Janssen LJ, Lu-Chao H, Netherton S: Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho kinase. Am J Physiol Lung Cell Mol Physiol 2001; 280:L666–74

34. Bruschi G, Bruschi ME, Regolisti G, Borghetti A: Myoplasmic Ca^{2+}-force relationship studied with fura-2 during stimulation of rat aortic smooth muscle. Am J Physiol 1988; 254:H840–54

35. Gmitter D, Brostrom CO, Brostrom MA: Translational suppression by Ca^{2+} ionophores: Reversibility and roles of Ca^{2+} mobilization, Ca^{2+} influx, and nucleotide depletion. Cell Biol Toxicol 1996; 12:101–13

36. Zobell RL, Hertelendy F, Fischer VW: The effects of ionophores on steroidogenesis and morphology of avian granulosa cells. Cell Tissue Res 1987; 248:551–8

37. Smith C, McEwan AI, Jhaveri R, Wilkinson M, Goodman D, Smith LR, Canada AT, Glass PSA: The interaction of fentanyl on the Cp $_{50}$ of propofol for loss of consciousness and skin incision. ANESTHESIOLOGY 1994; 81:820–8

38. Servin F, Desmonts JM, Haberer JP, Cockshott ID, Plummer GF, Farinotti R: Pharmacokinetics and protein binding of propofol in patients with cirrhosis. ANESTHESIOLOGY 1988; 69:887–91

39. He YL, Ueyama H, Tashiro C, Mashimo T, Yoshiya I: Pulmonary disposition of propofol in surgical patients. ANESTHESIOLOGY 2000; 93:986–91

40. Dawidowicz AL, Fornal E, Mardarowicz M, Fijalkowska A: The role of human lungs in the biotransformation of propofol. ANESTHESIOLOGY 2000; 93:992–7