Ca\(^{2+}\)-Calmodulin-dependent Protein Kinase II Plays a Major Role in Halothane-induced Dose-dependent Relaxation in the Skinned Pulmonary Artery

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Background: Previously, the authors have shown in Ca\(^{2+}\)-clamped skinned arterial strips that protein kinase C (PKC) plays a role in 3% halothane- or isoflurane-increased force. PKC in the pulmonary artery and Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII) in the femoral artery have been implicated in isoflurane-induced relaxation. For this study, the authors used clinical concentrations of halothane to examine the role of PKC and CaMKII in the halothane-induced biphasic effect on contraction in skinned pulmonary arterial strips.

Methods: Rabbit pulmonary arterial strips were mounted on force transducers and treated with saponin to make the sarcolemma permeable (“skinning”). Skinned strips were activated by low Ca\(^{2+}\) (pCa 6.3) buffered with 7 mM EGTA, or the PKC activator phorbol-12,13-dibutyrate (PDBu, 1 µM) until force reached a steady state (control). Halothane (1, 2, and 3%) was administered, and the force was observed at peak and 15 min (test results). Ca\(^{2+}\) ionophore (A23187, 10 µM) and inhibitors were preincubated in a relaxing solution and present in subsequent contracting solutions. Inhibitors were bisindolylmaleimide and G60976 for PKC, and KN-93 and the inhibitor protein (CKII\(\text{Ntide}\)) for CaMKII.

Results: Halothane (1–3%) dose-dependently caused an initial increase (18–35%) and a subsequent decrease (48–68%) in pCa 6.3-induced force. Bisindolylmaleimide, 3 and 10 µM, completely blocked the increase in force at 2% and 3% halothane, respectively. CKII\(\text{Ntide}\), 0.1 µM, reduced the force at 3% halothane. The decrease in force at 1% and 2% halothane was partially prevented by 0.01 µM bisindolylmaleimide, and at 1, 2, and 3% halothane by 0.01, 0.1, and 1 µM CKII\(\text{Ntide}\), respectively. At 3% halothane, the increased force was abolished by A23187. In PDBu-induced force, 3% halothane-induced relaxation was also partially prevented by lower concentrations of KN-93 and CKII\(\text{Ntide}\).

Conclusions: In skinned pulmonary arterial strips, the dose-dependent increase in force by halothane is associated with PKC activation, and that of decrease is associated with CaMKII activation.

PULMONARY vascular smooth muscle,1,2 compared to that from systemic vascular bed,3 has been shown to be differentially affected by the volatile anesthetics. Using plasma membrane-permeable (“skinned”) pulmonary arterial strips from rabbits, we have shown that isoflurane4 or halothane5 induces contraction and later relaxation under Ca\(^{2+}\)-clamped conditions. This Ca\(^{2+}\)-independent relaxation by the anesthetics may in part contribute to reduced pulmonary artery pressures observed in patients during induction and maintenance of anesthesia. Therefore, understanding the cellular signaling pathways leading to the anesthetic-induced biphasic effect in the pulmonary artery may provide important information in selective control of pulmonary hypertension.

Protein kinase C (PKC) and Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII) have been implicated in the volatile anesthetic-induced contraction and/or relaxation of vascular smooth muscle.1–4 Evidence of PKC activation by volatile anesthetics leading to contraction of vascular smooth muscle has been documented in various arterial types. Isoflurane enhances PKC-activated force in isolated intact coronary artery,4 and the anesthetic-increased force is reduced by a PKC inhibitor shown in the skinned strip of femoral and pulmonary arteries.1–5 However, mechanisms of anesthetic-induced relaxation have been shown to be dependent on arterial type. The isoflurane-increased force in skinned femoral arterial strips is further enhanced by a CaMKII inhibitor,5 suggesting that CaMKII activation causes relaxation. In skinned pulmonary arterial strips, the isoflurane-induced late relaxation is partially prevented by PKC inhibitors,2 suggesting that PKC activation can also lead to relaxation. It is not known whether the signaling pathways that result in halothane-induced relaxation are similar to those of isoflurane or whether the response varies with the arterial type.

In contrast to isoflurane, halothane has been shown to inhibit the PKC-activated force in isolated coronary arteries,4 and it has been speculated by the authors that isoflurane activates and halothane inhibits PKC. However, CaMKII could also play a role in the halothane-induced relaxation in the coronary artery4 as shown with isoflurane in other arterial types.2,3 The observations of halothane-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) via both the ryanodine- and IP\(_3\)-receptor SR Ca\(^{2+}\) release channels1 and the colocalization of CaMKII with IP\(_3\) receptor on the SR membrane in intestinal mucosa cells5 also support the above speculation. Therefore, we hypothesized that halothane-induced relaxation in the pulmonary artery may be predominantly via CaMKII signaling. In the present study, we demonstrate in skinned pulmonary arterial strips that the increased force by halothane as well as relaxation by 1% halothane is predominantly via activation of PKC and that halothane-induced dose-dependent relaxation is a direct function of CaMKII activation.

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Methods and Materials

Materials

Thymol-free halothane was supplied by Mr. Peter Haines of Halocarbon Laboratories (Hackensack, New Jersey). The peptide of the inhibitor protein (CKIIIn tide)6 for CaMKII was supplied by Debra Brickey, Ph.D., and Thomas Soderling, Ph.D. (Vollum Research Institute, Portland, Oregon). Other inhibitors or activators of PKC and CaMKII, including Gö6976,7 bisindolylmaleimide HCl,8 KN-93,9 and phorbol-12,13-dibutyrate (PDBu), were purchased from Calbiochem (La Jolla, CA) or RBI (Natick, MA). Chemicals were analytical or reagent grade. Stock solutions of Gö6976 (1 mM) were made in 100% DMSO. Final concentrations of 0.1-1.0 μM Gö6976 contained 0.1% DMSO. The same concentrations of DMSO were used in parallel for time controls.

Skinned Arterial Strips

Male New Zealand white rabbits (2.2-2.5 kg) were killed using a captive bolt pistol. This method of euthanasia has been approved by the University of Washington Animal Care Committee (Seattle, Washington). The method of preparing skinned arterial strips has been described1,2,10 with slight modification. Right or left pulmonary arteries were quickly isolated and cut into rings of 0.3–0.5 mm width. The endothelium was gently rubbed with a glass rod, and the denuded rings were cut open. The strips were mounted on photodiode force transducers and stretched to 50 mg of resting tension. The strips were then immersed into a series of bathing solutions2 (1) to make the sarcolemma permeable with saponin (300 μg/ml in relaxing solution for 4.5 min); (2) to release Ca2+ from the SR (25 mM caffeine in relaxing solution); (3) to activate the contractile proteins (pCa 6.3 buffer); and finally (4) to wash Ca2+ away in the strips (relaxing solution). The skinned strips were then immersed into another series of bathing solutions for experimental studies.

Experimental Procedure

As described previously,2 the skinned strips were activated by either Ca2+ (pCa 6.3 buffer; for pCa-induced force) or PDBu in pCa 6.7 buffer. When the force reached steady state (control force [ss], fig. 1), a fresh solution with the same ionic composition containing one of four halothane concentrations (0, 1, 2, or 3%) was then administered (test), and the results were observed for up to 60 min (fig. 1). The test results at peak (contraction in the presence of halothane) were expressed as a percentage of the steady state force (ss, fig. 1) just prior to the addition of halothane. The force of late relaxation was defined as the force present at 15 min after the addition of halothane. The reason for choosing 15 min instead of 30 min for isoflurane was that halothane rapidly induced relaxation after peak contraction, which reached a dose-dependent manner at 15 min (late relaxation, fig. 1).

The force of late relaxation was also expressed as a percentage of the steady state force (ss, fig. 1) just prior to the addition of the volatile anesthetic. The response with 0% halothane was used as a time control.

The inhibitors of PKC or CaMKII were preincubated in a relaxing solution and present in the subsequent contracting solutions. The test conditions included (1) vehicle only (without inhibitor or halothane) as time control for halothane; (2) one of halothane concentrations plus vehicle (no inhibitor) as time control for inhibitors; and (3) one of halothane concentrations at various concentrations of an inhibitor. At least three different concentrations of the inhibitors were tested from as low as IC50 to an increment of 0.5 and 1 log unit.

Inhibitors included bisindolylmaleimide8 and Gö69767 for PKC, and KN-939 and the inhibitor protein (CKIIIn tide)6 for CaMKII. Gö6976 and KN-93 were applied only at 3% halothane to confirm the observations with bisindolymaleimide and CKIIIn tide. Muscle strips not exposed to inhibitor or halothane were used to judge the response of the strips exposed to halothane (with or without the inhibitor).

Experiments were performed at room temperature (21 ± 2°C). The tension was recorded on a G3 Apple Computer (Cupertino, CA) with a customized LabVIEW software program interfaced with a multifunction input-
was considered significant. Approximately 29.0 ± 9.5 mg from a pool of 96 experimental data, which was induced by pCa 6.3 (sic effect: an increase followed by a decrease in force/H11006).

Results
Effects of Halothane on pCa 6.3-induced Force
This study was to examine whether halothane would have a more pronounced effect at a lower free Ca$^{2+}$ buffer (pCa 6.3) than had been previously observed$^1$ in pCa 6.0. The force induced by pCa 6.3 was 20.4 ± 9.5 mg from a pool of 96 experimental data, which was approximately 29.0 ± 11.0% of the maximum tension (pCa 4.0).

Figure 2 demonstrates that halothane induced a biphasic effect: an increase followed by a decrease in force induced by pCa 6.3 (fig. 1) in a dose-dependent manner. Compared to the response at 0% halothane, the mean increases were 18, 22, and 34% for 1, 2, and 3% halothane, respectively, and were statistically significant at 2% and 3% halothane (fig. 2A). The increased force by 3% halothane in pCa 6.3-induced force was comparable to the 30% increase previously observed in pCa 6.0-induced force.$^1$ The decreases in force with late relaxation, compared to the response with 0% halothane, were 48, 56, and 68% for 1, 2, and 3% halothane, respectively (fig. 2B), which is more than the 20-40% decreases previously observed with pCa 6.0.$^1$

Influence of PKC Inhibitors on Halothane-induced Biphasic Effect on pCa-induced Force
As shown in figure 3, bisindolylmaleimide (0.01–1 μM), an inhibitor of cPKC (IC$_{50}$ = 0.01 μM) and nPKC (IC$_{50}$ = 0.13 μM),$^8$ did not significantly affect the halothane-increased force at 1% and 2% halothane (fig. 3A). However, bisindolylmaleimide at 1 μM significantly reduced the increased force at 3% halothane (fig. 3A). In a different group of experiments, higher concentrations of bisindolylmaleimide completely blocked the increased force by halothane. The response with no halothane or inhibitor was 105.8 ± 5.8 (n = 6) in comparison to 139.3 ± 32.2 (n = 6) with halothane but without inhibitor, and 114.4 ± 11.9 (n = 8) with 2% halothane and 3 μM bisindolylmaleimide. At 3% halothane, the response with no halothane or inhibitor was 107.1 ± 6.9 (n = 4), 150.1 ± 29.8 (n = 4) with halothane but no inhibitor, 125.0 ± 20.2 (n = 4) with halothane and 3 μM bisindolylmaleimide, and 105.2 ± 9.6 (n = 5) with halothane and 10 μM bisindolylmaleimide. This complete inhibition of 3% halothane-increased force by 10 μM bisindolylmaleimide was also observed with pCa 6.0–induced force.$^1$

To confirm the effect of bisindolylmaleimide, we used another PKC inhibitor, Go6976, a specific inhibitor for cPKC (IC$_{50}$ = 0.002–0.02 μM).$^7$ We found that Go6976 (0.1–1 μM) dissolved in 0.1% DMSO did not affect the 3% halothane-increased force (data not shown).

In contrast, the relaxation induced by 1% and 2% halothane was partially prevented by 0.1 μM bisindolylmaleimide, but relaxation induced by 3% halothane was not significantly prevented by bisindolylmaleimide (fig. 3B) or Go6976 (tested up to 1 μM).

Influence of CaMKII Inhibitors on Halothane-induced Biphasic Effect on pCa-induced Force

We further tested whether CaMKII plays a role in halothane-induced biphasic effect. Two inhibitors were used. The peptide of the inhibitor protein (CKIINtide, IC$_{50}$ = 0.05 μM)$^6$ noncompetitively binds to the catalytic site of CaMKII, whereas KN-93 (IC$_{50}$ = 0.37 μM)$^9$ competitively binds to the calmodulin binding site of CaMKII.

We found that halothane-increased force was not significantly affected by CKIINtide at 1% and 2% halothane but was partially reduced by 0.1 μM CKIINtide at 3% halothane (fig. 4A). However, the increased force by 3% halothane was not significantly affected by KN-93 (maximum 10 μM; data not shown).

In contrast, late relaxation was partially prevented by CKIINtide in a dose-dependent manner at different dose concentrations of the inhibitors. A $^*$statistical significant at $^*$$^*$0.05.

### Data Analysis

The height from baseline to steady state force (control) and force at various time intervals (test) after administration of the test solutions were analyzed. The test results were expressed as a percentage of the control. Mean and SD of the mean were obtained from at least three arterial strips of three separate rabbits.

StatVIEW software program (BrainPower, Inc., Calabasas, CA) was used for statistical analysis. Results from the strips treated with the inhibitors and the time controls for the inhibitors were compared using the Student $t$ test. Two-factorial analysis of variance was used to compare halothane with or without various concentrations of the inhibitors. A $P$ value of less than 0.05 was considered significant.$^{11}$

### Results

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In contrast, the relaxation induced by 1% and 2% halothane was partially prevented by 0.1 μM bisindolylmaleimide, but relaxation induced by 3% halothane was not significantly prevented by bisindolylmaleimide (fig. 3B) or Go6976 (tested up to 1 μM).

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We found that halothane-increased force was not significantly affected by CKIINtide at 1% and 2% halothane but was partially reduced by 0.1 μM CKIINtide at 3% halothane (fig. 4A). However, the increased force by 3% halothane was not significantly affected by KN-93 (maximum 10 μM; data not shown).

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ranges (0.01 and 0.1 μM for 1% halothane, 0.1 and 1 μM for 2% halothane, and 1 μM for 3% halothane; fig. 4B).

The relaxation induced by 3% halothane was also partially prevented by KN-93 at 3 and 10 μM (n = 3–10; 83.4 ± 4.7, 34.3 ± 14.5, 35.4 ± 20.2, 60.2 ± 24.6, and 65.6 ± 24.9 for no halothane and no inhibitor, 0, 1, 3, and 10 μM KN-93 with halothane, respectively). 

Effects of Halothane and the Influence of CaMKII Inhibitors on PDBu-induced Force.

The preceding results suggested that halothane-induced relaxation involved both CaMKII and PKC at 1% and 2% halothane but only CaMKII at 3% halothane. It was hypothesized that PKC was initially activated and later inhibited by a high concentration of halothane.

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Fig. 5. Dose–response relationship of halothane on phorbol-12,13-dibutyrate (PDBu)-induced force. Mean ± SD (n = 3); PDBu = a PKC activator in the presence of pCa 6.7; PDBu-induced force after administration of halothane at peak force (A, peak contraction) or 30 min (B, late relaxation) expressed as percent of the control force (steady state force before the administration of halothane): * = P < 0.05 compared to 0% halothane. Halothane, at 3%, significantly increased the PDBu-induced force (A, peak contraction) and dose-dependently decreased the force (B, late relaxation) up to 2% halothane. No further decrease in PDBu-induced force was observed by 3% halothane.

Fig. 6. Influence of Ca2+ ionophore A23178 on halothane-induced biphasic effects on Ca2+-activated force.

The above results suggested that halothane-increased force was predominantly via PKC and that of relaxation was predominantly via CaMKII. We further examined the role of intracellular Ca2+ stores in the halothane-induced biphasic effects using nonspecific Ca2+ ionophore A23187 to deplete Ca2+ from the SR. We found that halothane-increased force was completely blocked by 10 μM A23187 (peak contraction, fig. 6). Surprisingly, the halothane-decreased force was significantly enhanced by A23187 (late relaxation, fig. 6).

Discussion

The most significant findings of this study are as follows. (1) The dose-dependent increases in contraction by 2% and 3% halothane are completely blocked by the PKC inhibitor bisindolylmaleimide at 3 and 10 μM, respectively, and the increased force by 3% halothane is also reduced by 0.1 μM CKIINtide (fig. 7) and completely blocked by Ca2+ ionophore A23187 treatment. (2) The relaxation induced by 1% and 2% halothane is partially prevented by 0.01 μM bisindolylmaleimide, and the dose-dependent relaxation by 1, 2, and 3% halothane is also partially prevented by increasing effective concentrations (0.01, 0.1, and 1 μM, respectively) of the peptide of the inhibitor protein (CKIINtide) of CaMKII (fig. 7).

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induced force (3 μM) is threefold higher than that in PDBu-induced force (1 μM).

Influence of Ca2+ Ionophore A23178 on Halothane-Induced Biphasic Effects on Ca2+-activated Force

KN-93 at 1 μM also partially prevented the 3% halothane-induced relaxation (n = 3 to 4; 76.7 ± 13.4, 26.0 ± 21.4, 35.6 ± 22.0, 37.8 ± 12.1, and 56.5 ± 35.6 for no halothane/no inhibitor, 0, 0.05, 0.3, and 1 μM KN-93 with halothane, respectively). Thus, the effective concentration of KN-93 to partially prevent the relaxation induced by 3% halothane in pCa-

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creased Ca\(^{2+}\) be activated by the localized Ca\(^{2+}\). Force (open circles) = percent of the time control = halothane-induced increases (A) or decreases (B) in pCa-induced force normalized as a percentage of without halothane and inhibitor (0/0); Effective [Inhibitor] (\(\mu M\)) = effective inhibitor concentration in \(\mu M\) in blocking halothane-increased force (A) or halothane-decreased force (B) derived from figures 3 and 4. Bim (filled circles) = bisindolylmaleimide, a cPKC inhibitor; CKIINtide (filled triangles) = the peptide of the inhibitor protein of CaMKII. This figure shows that the force induced by halothane increases with increasing halothane concentrations at 2\% and 3\% (open circles, A), which is associated with the increases in effective concentration of bisindolylmaleimide (filled circles, A), but CKIINtide is effective only at 3\% halothane (filled triangles, A). In contrast, force decreased by halothane in a dose-dependent manner (open circles, B) is associated with increases in effective concentrations of CKIINtide (filled triangles, B), but that of bisindolylmaleimide (filled circles, B) remains constant at 1\% and 2\% halothane and is ineffective as low as 0.001 \(\mu M\) at 3\% halothane.

Using the effective concentration of the inhibitors to reflect the enzyme activity, i.e., the higher the effective concentration of the inhibitor required to block the halothane-induced biphasic effect, the higher the activity of the enzyme, our results can be interpreted as follows. The blockade of the halothane-induced dose-dependent increase in force by high (> 3 \(\mu M\)) and increasing effective concentrations of the PKC inhibitor bisindolylmaleimide (IC\(_{50} = 0.01 \mu M\) for cPKC and 0.13 \(\mu M\) for ePKC)\(^8\) suggests a high PKC activity, which plays a major role in halothane-increased force. A complete blockade of 3\% halothane-increased force has also been shown previously in pCa 6.0-induced force.\(^1\) In contrast, the increased force by 3\% halothane partially blocked by low concentration (0.1 \(\mu M\)) of CKIINtide (IC\(_{50} = 0.05 \mu M\))\(^6\) suggests a low CaMKII activity. The above evidence of increased force partially blocked by the CaMKII inhibitor as well as completely blocked by high concentrations of the PKC inhibitor suggests that a CaMKII signaling pathway leading to contraction is via PKC in 3\% halothane-increased force (fig. 8). In PDBu-induced force, the absence of force increased by 2\% halothane and that reduced by 3\% halothane compared to those of pCa-induced force further confirms that PKC plays a major role in the halothane-increased force.

The complete blockade of halothane-increased force after Ca\(^{2+}\) ionophore A23187 treatment suggests a Ca\(^{2+}\)-dependent mechanism, which is consistent with the halothane-induced Ca\(^{2+}\) release from the SR.\(^1\) This increased Ca\(^{2+}\) by halothane resulting in contraction, however, could not be due to activation of Ca\(^{2+}\)-calmodulin-dependent myosin light chain kinase since the increased Ca\(^{2+}\) would be buffered by high concentrations of EGTA under our experimental condition and since the increased force by halothane can be blocked by the inhibitors of PKC, or CaMKII. Thus, cPKC and CaMKII must be activated by the localized Ca\(^{2+}\) which is released by halothane from the intracellular stores.\(^1\) These facts cannot, however, be reconciled with the ineffectiveness of the specific cPKC inhibitor Go6976. It could be due to dimethyl sulfoxide used to dissolve Go6976, which induces Ca\(^{2+}\) release from the SR (data not shown), resulting in a higher basal level by Ca\(^{2+}\). Whether cPKC activity is modulated by basal activity and Ca\(^{2+}\) concentrations remains to be confirmed.

The partial blockade of 1\% and 2\% halothane-induced relaxed by the same effective concentration (0.01 \(\mu M\))

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![Figure 7. Relationship between halothane concentrations, and effective concentrations of the inhibitors of PKC and CaMKII with respect to halothane-induced biphasic effects. Force (open circles) = percent of the time control = halothane-induced increases (A) or decreases (B) in pCa-induced force normalized as a percentage of without halothane and inhibitor (0/0); Effective [Inhibitor] (\(\mu M\)) = effective inhibitor concentration in \(\mu M\) in blocking halothane-increased force (A) or halothane-decreased force (B) derived from figures 3 and 4. Bim (filled circles) = bisindolylmaleimide, a cPKC inhibitor; CKIINtide (filled triangles) = the peptide of the inhibitor protein of CaMKII. This figure shows that the force induced by halothane increases with increasing halothane concentrations at 2\% and 3\% (open circles, A), which is associated with the increases in effective concentration of bisindolylmaleimide (filled circles, A), but CKIINtide is effective only at 3\% halothane (filled triangles, A). In contrast, force decreased by halothane in a dose-dependent manner (open circles, B) is associated with increases in effective concentrations of CKIINtide (filled triangles, B), but that of bisindolylmaleimide (filled circles, B) remains constant at 1\% and 2\% halothane and is ineffective as low as 0.001 \(\mu M\) at 3\% halothane.]

![Figure 8. Proposed signaling pathways in the pulmonary artery underlying halothane-induced contraction and relaxation. [Halothane] = changes in halothane concentrations; SR = sarcolemmal reticulum; CaM = calmodulin; cPKC = Ca\(^{2+}\)-dependent protein kinase C; [CaMKII] = changes in activity of Ca\(^{2+}\)-calmodulin-dependent protein kinase II; 3H = 3\% halothane; pMLCK(−) = phosphorylated myosin light chain kinase at a specific site resulting in decreased activity; pMLC(+) = increased myosin light chain phosphorylation; pMLC(−) = decreased myosin light chain phosphorylation. The model describes that halothane induces Ca\(^{2+}\) release from the SR resulting in activation of cPKC and CaMKII, and halothane may also directly bind to cPKC at the phorbol ester binding site to activate PKC. The signaling pathway for activated PKC predominantly leads to contraction, and the signaling pathway for activated CaMKII predominantly leads to relaxation. However, at 3\% halothane, the increased force could also in part via CaMKII-PKC signaling pathway, and that of decreased force in part via PKC-CaMKII signaling.]

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of the PKC inhibitor bisindolylmaleimide suggests that PKC is activated to a similar degree, which would not account for the increased relaxation by 2% halothane compared with 1% halothane. Thus, the increased force blocked by high concentrations (≥ 3 μM) and the decreased force by low concentrations (0.01 μM) of bisindolylmaleimide suggest that high PKC activity would result in increased force and low PKC activity would result in relaxation. The PKC signaling pathway leading to contraction could be via CPI-17, phosphatase inhibition, and increased myosin light chain phosphorylation. On the other hand, a different PKC pathway would lead to relaxation by direct phosphorylation of myosin light chain kinase at the same site as that of cAMP kinase or CaMKII resulting in decreased myosin light chain kinase activity and decreased myosin light chain phosphorylation. These speculations remain to be confirmed.

The increasing relaxation by increasing halothane concentrations (1, 2, and 3%) associated with increasing effective concentrations (0.01, 0.1, and 1 μM, respectively) of the CaMKII inhibitor CKIINtide (IC_{50} = 0.05 μM) suggests increasing CaMKII activity associated with increasing relaxation. A comparison between the estimated relative activity of PKC and CaMKII (ratio of effective concentration to IC_{50}) reveals that PKC (0.01/0.01 μM) is approximately fivefold greater than CaMKII (0.01/0.05 μM) at 1% halothane, and CaMKII (0.1/0.05 μM) is approximately twofold greater than PKC (0.01/0.01 μM) at 2% halothane. This suggests that PKC plays a more important role than CaMKII in 1% halothane-induced relaxation. However, a direct correlation between relaxation and CaMKII activity suggests that CaMKII plays a major role in modulation of vascular smooth muscle relaxation.

The fact that 3% halothane-induced relaxation is not prevented but enhanced by Ca^{2+} ionophore A23187 treatment suggests a Ca^{2+}-independent mechanism. It is also possible that Ca^{2+} in the SR stores is reduced by Ca^{2+} ionophore A23187; thus, less Ca^{2+} would be released by halothane, resulting in lower CaMKII or PKC activation leading to relaxation. However, halothane, as well as enflurane on purified cPKC, may directly compete for both high- and low-affinity phorbol ester binding to PKC. At low anesthetic concentrations (≥ 2% halothane), halothane binds to the high-affinity site, resulting in increased PKC activity, and at high concentrations (3% halothane), halothane also binds to the low-affinity, resulting in decreased PKC activity, which is too low to be detected by the PKC inhibitor. This speculation is supported by the evidence under PKC-activated condition (PDBu-induced force) that 3% halothane, compared to 2% halothane, induces no further relaxation, and decreased effective inhibitor concentration of CaMKII (CKIINtide, or KN-93) suggests lower CaMKII activity. Thus, PKC may also play a role in 3% halothane-induced relaxation, possibly via a PKC-CaMKII signaling pathway (fig. 8). However, the lower CaMKII activity could also be the result of reduced Ca^{2+} released by halothane in PDBu-treated strips since phorbol ester has been shown to reduce Ca^{2+} transient induced by electrical stimulation in cardiac myocytes. Thus, the relaxation induced by halothane in PKC-activated force shown in isolated intact rat coronary artery could also be via CaMKII, which remains to be confirmed.

This preferential activation of CaMKII over PKC by 2% halothane released by halothane could be due to halothane-induced Ca^{2+} release via the specific SR Ca^{2+} release channels (the IP_{3} and ryanodine receptors) due to its colocalization with the IP_{3} receptor on the SR membrane as shown in intestinal mucosal cells. This speculation, however, remains to be shown in vascular smooth muscle. The regulation of CaMKII and PKC by Ca^{2+} concentrations, as shown in vitro with CaMKII, and the modulation of vascular smooth muscle contraction remain to be investigated.

The mechanisms of the biphasic effects of halothane from this study can be speculated as follows (fig. 8). Halothane induces Ca^{2+} release from the SR. This increased Ca^{2+} results in activation of cPKC and CaMKII. Halothane also binds to the phorbol ester binding site, resulting in increased PKC activity at low concentrations and decreased activity at high concentrations. Activated PKC could undergo the following signaling pathways: (1) phosphorylation of CPI-17 which inhibits myosin light chain (MLC) phosphatase resulting in increased MLC phosphorylation leading to increased force; (2) phosphorylation of extracellular signal-regulated kinases (ERK1/2) which then phosphorylates myosin light chain kinase (MLCK) resulting in increased MLCK activity and MLC phosphorylation leading to increased force; (3) direct phosphorylation of MLCK resulting in decreased MLCK activity and decreased MLC phosphorylation leading to relaxation; and (4) phosphorylation of CaMKII leading to relaxation. The possible signaling pathways for activated CaMKII would be via (1) ERK1/2, or p38 mitogen-activated protein kinase signaling resulting in contraction; and (2) p38 signal, and/or direct phosphorylation of MLCK resulting in decreased MLCK activity and decreased MLC phosphorylation leading to relaxation.

The clinical implication of the results from this study is that halothane at clinical concentrations, as well as isoflurane, but to a greater extent, may cause pulmonary vasodilation in patients at resting state. The vasodilation may be sustained in those patients with low SR Ca^{2+} stores, or with low PKC activity, which may be beneficial to patients with pulmonary hypertension.

In summary, both PKC and CaMKII may play a dual role in halothane-induced contraction and relaxation. The halothane-induced force is predominantly via PKC, and the relaxation is predominantly via CaMKII.
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