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

Judy Y. Su, Ph.D., Anhkiet C. Vo, B.S.†*

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 Gö6976 for PKC, and KN-93 and the inhibitor protein (CKIINtide) 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. CKIINtide, 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 CKIINtide, 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 CKIINtide.**

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 isoflurane[2] or halothane[1] 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.[1–3] 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,[3] 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 artery[4] 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 channels[1] and the colocalization of CaMKII with IP_3 receptor on the SR membrane in intestinal mucosa cells[5] 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.

* Research Professor, † Research Technologist.

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Address reprint requests to Dr. Su: Department of Anesthesiology, Box 356540, University of Washington, Seattle, Washington 98195-6540. Address electronic mail to: jsu@u.washington.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

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 (CKIINtide)[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 Go6976 (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 described[1,2,10] 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 solutions[2] (1) to make the sarcolemma permeable with saponin (300 μg/ml in relaxing solution for 4.5 min); (2) to release Ca^{2+} from the SR (25 mM caffeine in relaxing solution); (3) to activate the contractile proteins (pCa 5.0 buffer); and finally (4) to wash Ca^{2+} 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 Ca^{2+} (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[2] 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.

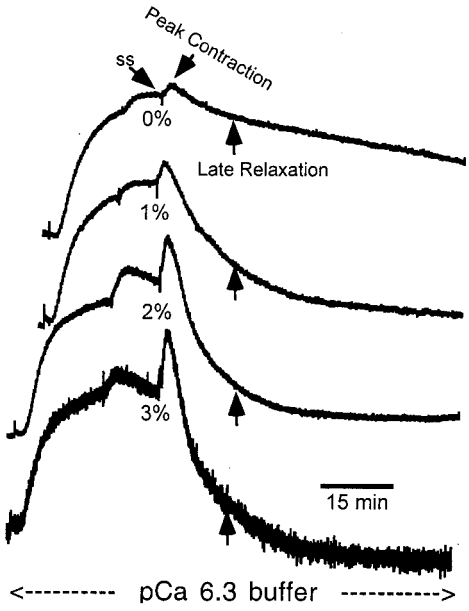

Fig. 1. Tracings for halothane-induced biphasic effect. pCa 6.3 buffer = −log [Ca^{2+}] (M) = 0.5 μM Ca^{2+} buffered with 7 mM EGTA; 0, 1, 2, and 3% = halothane concentrations; ss = control force at steady state before administration of halothane; peak contraction = peak force increased by halothane; late relaxation = force decreased by halothane after peak contraction. The tracings showed that halothane dose-dependently increased Ca^{2+}-activated force reaching peak within 2 min (peak contraction). At 15 min after administration of halothane (late relaxation), the force was decreased in a dose-dependent manner.

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 IC_{50} to an increment of 0.5 and 1 log unit.

Inhibitors included bisindolylmaleimide[8] and Gö6976[7] for PKC, and KN-93[9] and the inhibitor protein (CKIINtide)[6] for CaMKII. Gö6976 and KN-93 were applied only at 3% halothane to confirm the observations with bisindolylmaleimide and CKIINtide. 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–

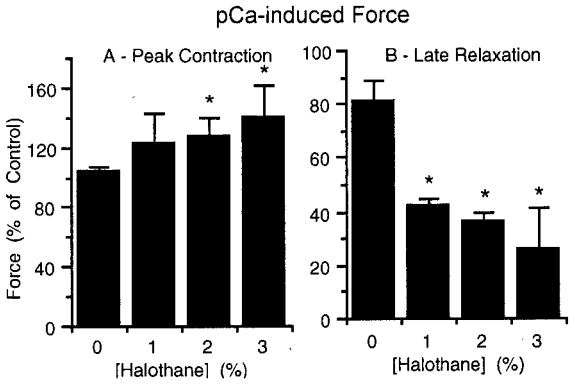

Fig. 2. Dose–response relationship of halothane-induced biphasic effects. Halothane (1, 2, and 3%) dose-dependently increased the pCa-induced force (*A*, peak contraction), which was significant at 2% and 3% halothane. Halothane, at all three concentrations, also significantly decreased the pCa-induced force in a dose-dependent manner.

output board with 16-bit resolution (NB-MIO-16XL; National Instrument, Austin, TX).

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 between halothane with or without various concentrations of the inhibitors. A *P* value of less than 0.05 was considered significant.[11]

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 107.1.8 ± 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 0.01 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

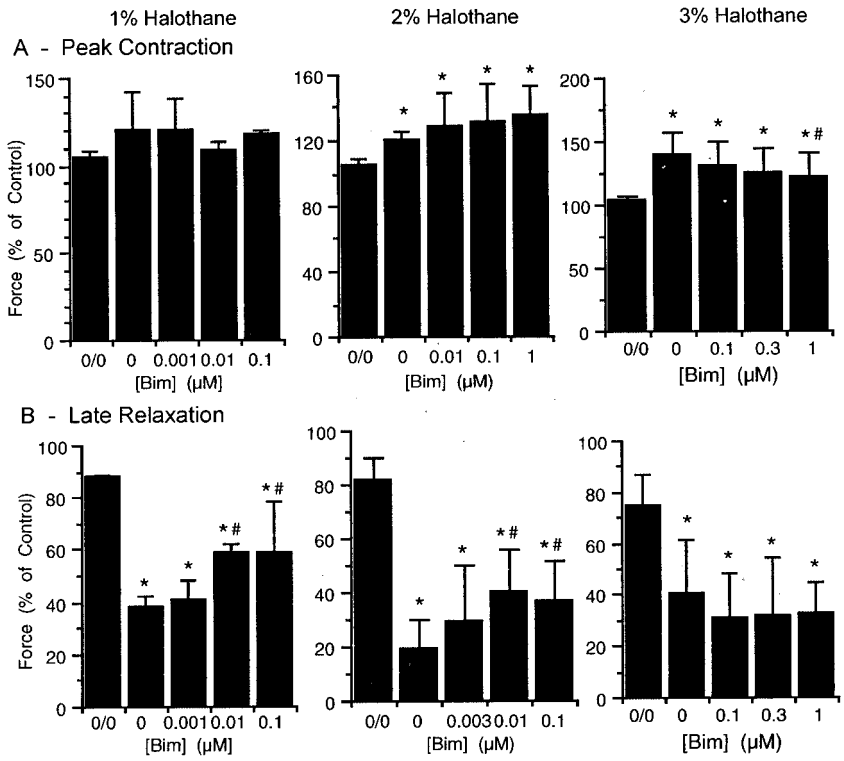

Fig. 3. Influence of c/nPKC inhibitor bisindolylmaleimide on biphasic effect of halothane on pCa-induced force. 0/0 = time controls (without halothane or bisindolylmaleimide); Force = pCa 6.3–induced force expressed as a percentage of the control force (steady state force before the administration of halothane) in mean ± SD (n = 3–9); [Bim] = concentrations of bisindolylmaleimide where 0/0 = without halothane or bisindolylmaleimide; 0 or [Bim]i = 0 μM or a specific bisindolylmaleimide concentration up to 1 μM tested plus one of the halothane concentrations (1, 2, or 3%); * = $P < 0.05$ compared to no halothane or bisindolylmaleimide (0/0); # = compared to 0 μM in the presence of halothane (0). The increased force (0 *vs.* 0/0, *A*) by 2% halothane was not significantly affected by bisindolylmaleimide up to 1 μM, and by 3% halothane was reduced by 1 μM bisindolylmaleimide. In contrast, the decreased force (0/0 *vs.* 0, *B*) was partially prevented by 0.01 and 0.1 μM bisindolylmaleimide at 1% and 2% halothane but was not affected by bisindolylmaleimide up to 1 μM at 3% halothane.

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

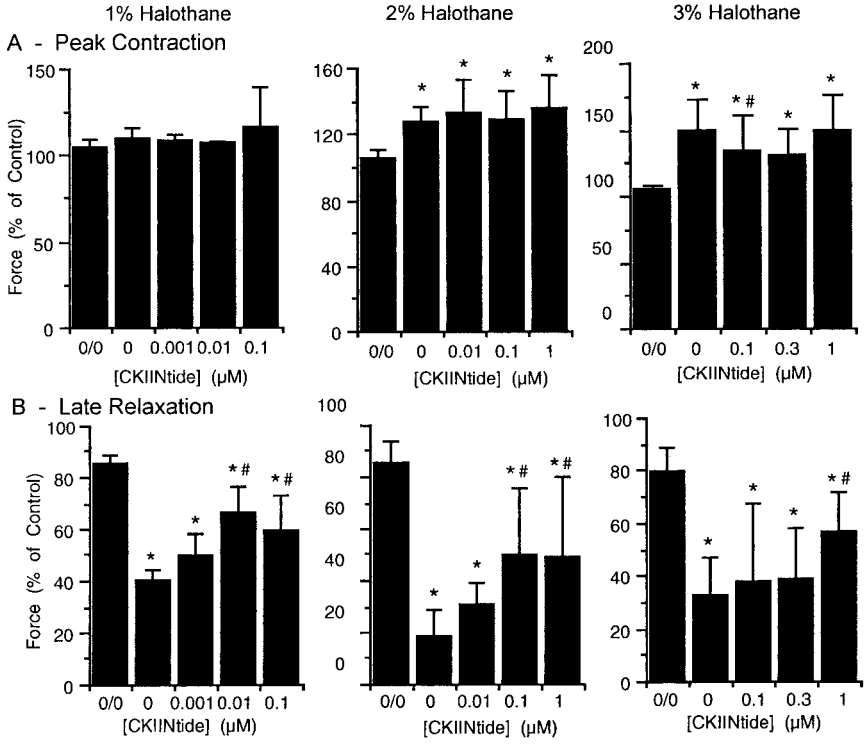

Fig. 4. Influence of the peptide of the inhibitor protein (CKIINtide) of CaMKII on halothane-induced biphasic effects on pCa-induced force. 0/0 = time controls (without halothane or CKIINtide); Force = pCa 6.3–induced force expressed as a percentage of the control force (steady state force before the administration of halothane) in mean ± SD (n = 3–6); [CKIINtide] = concentrations of CKIINtide where 0/0 = without halothane or CKIINtide; 0 or [CKIINtide]i = 0 μM or a specific CKIINtide concentration up 1 μM plus one of the halothane concentrations (1, 2, or 3%); * = $P < 0.05$ compared to no halothane or bisindolylmaleimide (0/0); # = compared to 0 μM in the presence of halothane (0). The increased force (0 *vs.* 0/0, *A*) by 2% halothane was not significantly affected by CKIINtide, and by 3% halothane was reduced by 0.1 μM CKIINtide. In contrast, the decreased force (0/0 *vs.* 0, *B*) was partially prevented by 0.01 and 0.1 mM CKIINtide at 1% halothane, by 0.1 and 1 μM CKIINtide at 2% halothane, and by 1 μM at 3% halothane.

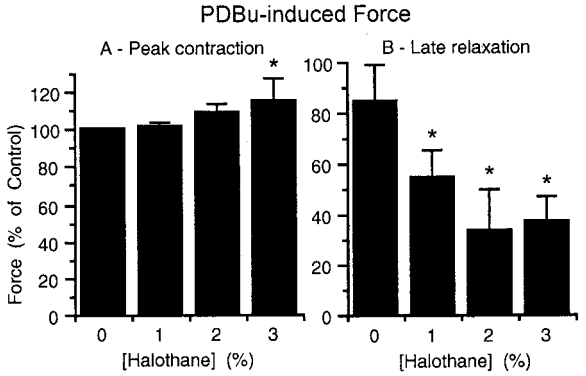

Fig. 5. Dose–response relationship of halothane on phorbol-12,13-dibutyrate (PDBu)-induced force. Mean ± SD (n = 3); PDBu = a PKC activator in the presence of pCa 6.7; PDBu-induced force after administration of halothane at peak force (*A*, peak contraction) or 30 min (*B*, late relaxation) expressed 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.

(3%), as has been observed with enflurane,[12] but was too low to be detected by the inhibitor. To further test this hypothesis, we used PDBu, a phorbol ester, to activate PKC. If the hypothesis was correct, we would expect that 3% halothane would again induce relaxation, but to a lesser extent because in this circumstance only CaMKII would be involved with relaxation. This halothane-induced relaxation would also be partially prevented by the CaMKII inhibitors.

We found that halothane did not increased the PDBu-induced force (fig. 5A) until 3% halothane (114.5 ± 20.3% [n = 25]), which was significantly lower than with pCa-induced force (137.4 ± 18.6% (n = 26]). Halothane again induced relaxation in PDBu-induced force in a dose-dependent manner (1–3% halothane, fig. 5B) reaching maximum at 2% halothane (n = 3; 84.3 ± 14.5, 54.8 ± 10.2, 33.6 ± 16.5, and 37.5 ± 9.7% for 0, 1, 2, and 3% halothane, respectively; fig. 5B).

The relaxation induced by 3% halothane in PDBu-induced force was again partially prevented by CKIINtide at 0.1 μM (n = 3–12; 73.6 ± 12.3, 33.1 ± 20.5, 22.8 ± 22.6, 51.9 ± 26.1, and 36.7 ± 23.7 for no halothane/no inhibitor, and 0, 0.03, 0.1, and 0.3 μM CKIINtide with halothane, respectively). Thus, the effective concentration of CKIINtide to partially prevent 3% halothane–induced relaxation is 10-fold higher in pCa-induced force (1 μM, fig. 4B) than in PDBu-induced force (0.1 μM).

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

Influence of Ca^{2+} Ionophore A23178 on Halothane-induced Biphasic Effects on Ca^{2+}-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 Ca^{2+} stores in the halothane-induced biphasic effects using nonspecific Ca^{2+} ionophore A23187 to deplete Ca^{2+} 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 Ca^{2+} 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).

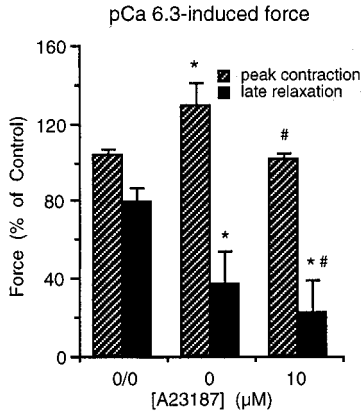

Fig. 6. Influence of A23178 on halothane-induced biphasic effects on pCa-induced force. A23178 = Ca^{2+} ionophore to deplete Ca^{2+} from the SR; 0/0 = without halothane or A23187; 3% halothane was present in 0 and 10 μM A23187; mean ± SD (n = 4–10); pCa-induced force after administration of halothane at peak force or 15 min expressed as percent of the control force (steady state force before the administration of halothane); * = $P < 0.05$ compared to no halothane or A23187 (0/0); # = compared to halothane in the absence of A23187 (0). Halothane-increased force was completely blocked by A2318 (peak contraction), and the decreased force was enhanced by A23187 (late relaxation).

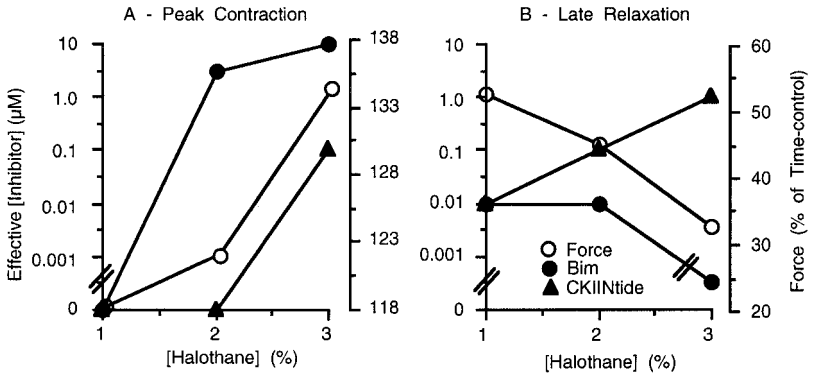

Fig. 7. Relationship between halothane concentrations, and effective concentrations of the inhibitors of PKC and CaMKII with respect to halothane-induced biphasic effects. Force (open circles) = percent of the time control = halothane-induced increases (*A*) or decreases (*B*) in pCa-induced force normalized as a percentage of without halothane and inhibitor (0/0); Effective [Inhibitor] (μM) = effective inhibitor concentration in μM in blocking halothane-increased force (*A*) or halothane-decreased force (*B*) derived from figures 3 and 4; Bim (filled circles) = bisindolylmaleimide, a c/nPKC 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 μM 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 μM) and increasing effective concentrations of the PKC inhibitor bisindolylmaleimide (IC_{50} = 0.01 μM for cPKC and 0.13 μM for ϵPKC)[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 μM) of CKIINtide (IC_{50} = 0.05 μM)[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 relaxation by the same effective concentration (0.01 μM)

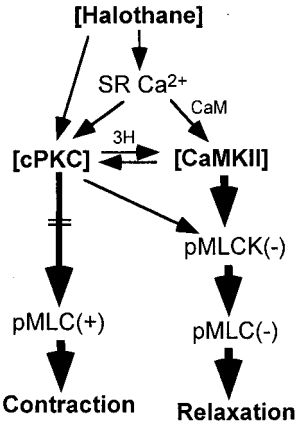

Fig. 8. Proposed signaling pathways in the pulmonary artery underlying halothane-induced contraction and relaxation. [Halothane] = changes in halothane concentrations; SR = sarcoplasmic reticulum; CaM = calmodulin; cPKC = Ca^{2+}-dependent protein kinase C; [CaMKII] = changes in activity of Ca^{2+}–calmodulin-dependent 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.

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 ($\leq$ 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.[13] 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.[14,15] 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)[6] 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,[12] may directly compete for both high- and low-affinity phorbol ester binding to PKC. At low anesthetic concentrations ($\geq$ 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.[16] Thus, the relaxation induced by halothane in PKC-activated force shown in isolated intact rat coronary artery[4] could also be *via* CaMKII, which remains to be confirmed.

This preferential activation of CaMKII over PKC by Ca^{2+} 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)[1] due to its colocalization with the IP_3 receptor on the SR membrane as shown in intestinal mucosal cells.[17] 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,[18] 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[13]; (2) phosphorylation of extracellular signal–regulated kinases (ERK1/2)[19–21] which then phosphorylates myosin light chain kinase (MLCK) resulting in increased MLCK activity and MLC phosphorylation[22] leading to increased force; (3) direct phosphorylation of MLCK resulting in decreased MLCK activity and decreased MLC phosphorylation[14,15] leading to relaxation; and (4) phosphorylation of CaMKII leading to relaxation. The possible signaling pathways for activated CaMKII would be *via* (1) ERK1/2[19,20] or p38[23] mitogen-activated protein kinase signaling resulting in contraction; and (2) p38 signaling,[24] and/or direct phosphorylation of MLCK resulting in decreased MLCK activity and decreased MLC phosphorylation[14,15,25] leading to relaxation.

The clinical implication of the results from this study is that halothane at clinical concentrations, as well as isoflurane,[2] 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-increased force is predominantly *via* PKC, and the relaxation is predominantly *via* CaMKII.

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References

1. Su JY, Tang LJ: Effects of halothane on the SR Ca^{2+} stores and the contractile proteins in rabbit pulmonary arteries. ANESTHESIOLOGY 1998; 88:1096–106

2. Su JY, Vo AC: Role of PKC in isoflurane-induced biphasic contraction in skinned pulmonary arterial strips. ANESTHESIOLOGY 2002; 96:155–61

3. Toda H, Su JY: Mechanisms of isoflurane-increased submaximum Ca^{2+}-activated force in rabbit skinned femoral arterial strips. ANESTHESIOLOGY 1998; 89:731–40

4. Park KW, Dai HB, Lowenstein E, Sellke FW: Protein kinase C-induced contraction is inhibited by halothane but enhanced by isoflurane in rat coronary arteries. Anesth Analg 1996; 83:286–90

5. Matovcik LM, Maranto AR, Soroka CJ, Gorelick FS, Smith J, Goldenring JR: Co-distribution of calmodulin-dependent protein kinase II and inositol trisphosphate receptors in an apical domain of gastrointestinal mucosa cells. J Histochem Cytochem 1996; 44:1243–1250

6. Chang BH, Mukherji S, Soderling TR: Characterization of a calmodulin kinase II inhibitor protein in brain. Proc Natl Acad Sci U S A 1998; 95:10890–5

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

8. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J: The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 1991; 266:15771–8

9. Mamiya N, Goldenring JR, Tsunoda Y, Modlin IM, Yasui K, Usuda N, Ishikawa T, Natsume A, Hidaka H: Inhibition of acid secretion in gastric parietal cells by the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-93. Biochem Biophys Res Comm 1993; 195:608–15

10. Su JY, Zhang CC: Intracellular mechanisms of halothane's effect on isolated aortic strips of the rabbit. ANESTHESIOLOGY 1989; 71:409–17

11. Winer BJ: Statistical Principles in Experimental Design, 2nd edition. Edited by Maytham W, Shapiro A, Stern J. New York, McGraw-Hill, 1971, pp 77–104, 263–76

12. Slater SJ, Kelly MB, Larkin JD, Ho C, Mazurek A, Taddeo FJ, Yeager MD, Stubbs CD: Interaction of alcohols and anesthetics with protein kinase Calpha. J Biol Chem 1997; 272:6167–73

13. Kitazawa T, Takizawa N, Ikebe M, Eto M: Reconstitution of protein kinase C-induced contractile Ca^{2+} sensitization in Triton X-100-demembranated rabbit arterial smooth muscle. J Physiol 1999; 520:139–52

14. Stull JT, Hsu LC, Tansey MG, Kamm KE: Myosin light chain kinase phosphorylation in tracheal smooth muscle. J Biol Chem 1990; 265:16683–90

15. Tansey MG, Luby-Phelps K, Kamm KE, Stull JT: Ca^{2+}-dependent phosphorylation of myosin light chain kinase decreases the Ca^{2+} sensitivity of light chain phosphorylation within smooth muscle cells. J Biol Chem 1994; 269:9912–20

16. Capogrossi MC, Kaku T, Filburn CR, Pelto DJ, Hansford RG, Spergeon HA, Lakatta EG: Phorbol ester and dioctanoylglycerol stimulate membrane association of protein kinase C and have a negative inotropic effect mediated by changes in cytosolic Ca^{2+} in adult rat cardiac myocytes. Circ Res 1990; 66:1143–55

17. Matovcik LM, Maranto AR, Soroka CJ, Gorelick FS, Smith J, Goldenring JR: Co-distribution of calmodulin-dependent protein kinase II and inositol trisphosphate receptors in an apical domain of gastrointestinal mucosa cells. J Histochem Cytochem 1996; 44:1243–50

18. De Koninck P, Schulman H: Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. Science 1998; 279:227–30

19. Kim I, Hyun-Dong J, Gallant C, Zhan Q, Riper DV, Badwey JA, Singer HA, Morgan KG: Ca^{2+}-calmodulin-dependent protein kinase II-dependent activation of contractility in ferret aorta. J Physiol 2000; 526:367–74

20. Rokolya A, Singer HA: Inhibition of CaM kinase II activation and force maintenance by KN-93 in arterial smooth muscle. Am J Physiol 2000; 278: C537–45

21. Zhong L, Su JY: Isoflurane activates PKC and Ca^{2+}–calmodulin-dependent protein kinase II *via* MAP kinase signaling in smooth muscle cells cultured from rabbit femoral artery. ANESTHESIOLOGY 2002; 96:148–54

22. Nguyen DHD, Catling, AD, Web DJ, Sankovic M, Walker LA, Somlyo AV, Weber MJ, Gonias SL: Myosin light chain kinase functions downstream of Ras/ERK to promote migration of urokinase-type plasminogen activator-stimulated cells in an integrin-selective manner. J Cell Biol 1999; 146:149–64

23. Meloche S, Landry J, Huot J, Houle F, Marceau F, Giasson E: p38 MAP kinase pathway regulates angiotensin II-induced contraction of rat vascular smooth muscle. Am J Physiol Heart Circ Physiol 2000; 279:H741–51

24. Cao W, Medvedev AV, Daniel KW, Collins S: β-Adrenergic activation of p38 MAP kinase in adipocytes: cAMP induction of the uncoupling protein-1 (UCP1) gene requires p38 MAP kinase. J Biol Chem 2001; 276:27077–82

25. Turner JR, Angle JM, Black ED, Joyal JL, Sacks DB, Madara JL: PKC-dependent regulation of transepithelial resistance: Roles of MLC and MCL kinase. Am J Physiol 1999; 277(Cell Physiol 46):C554–62