Anesthesiology
1998; 89:731–40

Mechanisms of Isoflurane-increased Submaximum Ca^{2+}-activated Force in Rabbit Skinned Femoral Arterial Strips

Hiroshi Toda, M.D., and Judy Y. Su, Ph.D.†*

Background: **Isoflurane enhances contraction in isolated intact arterial rings by a protein kinase C (PKC) activator and also causes contracture in skinned arterial strips. This study investigated the mechanisms of this isoflurane activation of the contractile proteins of skinned strips.**

Methods: **The skinned strips, mounted on photodiode force transducers, were prepared from rabbit femoral arteries treated with saponin. The strips were activated by 1 μM Ca^{2+} (buffered with 7 mM EGTA) with or without inhibitors for PKC and calmodulin-dependent protein kinase II (CaM kinase II). When force reached steady state, isoflurane was administered and changes in force were observed. Another group of the strips was frozen to assay myosin light chain phosphorylation (MLC-p) using two-dimensional electrophoresis and immunoblotting. Analysis of variance was used to compare the results from test and control groups. Probability values <0.05 were significant.**

Results: **Isoflurane (1–5%) dose dependently increased (24–81%) the Ca^{2+}-activated force. At 1% and 5% isoflurane, MLC-p did not change either as the force increased or reached a new steady state level. However, with 3% isoflurane, MLC-p transiently decreased (29.1% and 17.1% of total MLC for 0% and 3% isoflurane, respectively). The 3% isoflurane-increased force was blocked by 10 μM bisindolymaleidmide, an inhibitor of PKC, but not by 10 μM Gö-6976, an inhibitor of Ca^{2+}-dependent PKC, and was enhanced 50% by 0.1 mM KN-62, an inhibitor of CaM kinase II.**

Conclusions: **Isoflurane increased submaximum Ca^{2+}-activated force in skinned femoral arterial strips by activating Ca^{2+}-independent PKC, possibly ε isoezyme. The isoflurane-decreased MLC-p may be caused by activation of CaM kinase II. (Key words: Calmodulin-dependent protein kinase II; myosin light chains; protein kinase C.)**

* Visiting Scientist. Current address: Department of Anesthesiology, Kyoto University, Kyoto, Japan.

† Research Professor.

Received from the Department of Anesthesiology, University of Washington, Seattle, Washington. Submitted for publication . Accepted for publication. Supported by grant GM48243 from the National Institutes of Health. Presented in part at the American Society for Anesthesiologists annual meeting, New Orleans, Louisiana, October 1996. Dr. Toda was a visiting scientist from the Department of Anesthesiology, Kyoto University, Kyoto, Japan.

Address reprint requests to Dr. Su: Research Professor, Department of Anesthesiology, Box 356540, University of Washington, Seattle, Washington 98195-6540. Address electronic mail to: jsu@u.washington.edu

UNDER isoflurane anesthesia, vasodilation and hypotension[1] are in part attributed to the direct action of isoflurane on vascular smooth muscle. Such vasodilation can be shown in isolated intact arteries activated by agonists.[2,3] The mechanisms of this isoflurane-induced relaxation could be the result of inhibiting the voltage-gated Ca^{2+} channel[4] and thus decreasing Ca^{2+} influx *via* the sarcolemma. In contrast, a direct Ca^{2+} release from the sarcoplasmic reticulum (SR) by isoflurane has been shown[5,6] or implicated[7–10] without tension generation in various arterial preparations. This small Ca^{2+} release may also contribute to the increase in the caffeine-induced transient tension increase by isoflurane that has been observed in skinned femoral arterial strips under low EGTA conditions of the load-release cycle.[3] Isoflurane (≥3%; without caffeine in the release phase) also directly induces a small transient tension increase followed by sustained contracture.[3] This isoflurane-induced transient tension increase corresponds with the small Ca^{2+} release from the SR,[5,6] whereas the sustained contracture suggests an increased sensitivity of the contractile proteins to Ca^{2+}. However, there is no direct evidence for isoflurane-increased sensitivity of the contractile proteins to Ca^{2+} and, if such action is present, the underlying mechanisms.

Smooth muscle contraction is regulated by myosin light chains (MLC) and may also be modulated by the thin-filament–associated proteins calponin and caldesmon.[11,12] The regulatory mechanism by MLC consists of Ca^{2+}-dependent activation of myosin light chain kinase, and Ca^{2+}-independent inhibition of MLC phosphatase[12] (thus increases in the ratio of MLC kinase to MLC phosphatase activity) or activation of Rho-associated protein kinase.[13] The increased activity of MLC kinase over MLC phosphatase or increased activity of Rho-associated kinase results in increased phosphorylation of MLC (MLC-p).[12,13] On the other hand, the contraction regulated by calponin or caldesmon is less well established and may be mediated by activation of protein kinase C or calm-

odulin-dependent protein kinase II.[12,14] A direct Ca^{2+}-independent activation by the ε isoenzyme of PKC (PKC-ε) has been demonstrated in sarcolemma-permealized aortic cells.[12]

Recently, Park and associates[15] showed that isoflurane enhances vasoconstriction by oleic acid (an activator of PKC) in rat coronary arteries, which suggests that isoflurane directly activates the contractile proteins. Whether this direct effect of isoflurane depends on the released Ca^{2+} is not known. If the isoflurane-enhanced force is independent of Ca^{2+}, then the vasoconstriction could be mediated either by activation of PKC-ε, by inhibition of MLC phosphatase, or by activation of Rho-associated kinase. The last two possibilities would be associated with increases in MLC-p.

Accordingly, this study was designed to determine whether, under constant free Ca^{2+} concentrations, isoflurane directly activates the contractile proteins in saponin-skinned rabbit femoral arterial strips. We further examined whether this increase in force caused by isoflurane was associated with increased phosphorylation of MLCs or was blocked by inhibitors of PKC or calmodulin-dependent protein kinase II.

Materials and Methods

This study was approved by the University of Washington Animal Care Committee. The preparation of and experimental procedures for use of isolated arterial skinned strips were the same as have been reported for halothane[16] and isoflurane[3] and are described briefly here.

Male New Zealand white rabbits (2.2 to 2.5 kg) were killed using a captive bolt pistol followed by exsanguination. Femoral arteries were rapidly and carefully isolated. Connective and fat tissues were trimmed free from the surface of the arteries. Arterial strips approximately 1 mm wide were placed in relaxing solution (containing free Ca^{2+} <10 nM, 7 mM EGTA, 2 mM $MgATP^{2-}$, 0.1 mM free Mg^{2+}, 15 mM creatine phosphate, 35 mM Na^{+}, 35 mM K^{+}, and 300 mOsm ionic strength, with *p*H 7 at 23°C). Each strip was mounted on two pairs of forceps with one end attached to a photodiode tension transducer. The strip was then stretched to a resting tension of 50 mg. The tension was recorded on a Quadra 950 (Apple Computer, Cupertino, CA) using a customized LabVIEW software program (National Instrument, Austin, TX).

After mounting and stretching, the strips were immersed in a relaxing solution plus 0.3 mg/ml saponin for 5 min to permealize the sarcolemma (*i.e.*, they were "skinned"). The viability of the skinned strips was tested with a solution with the same ionic composition as the relaxing solution except that it contained 10 μM Ca^{2+} (produced about 70% of the maximum force).

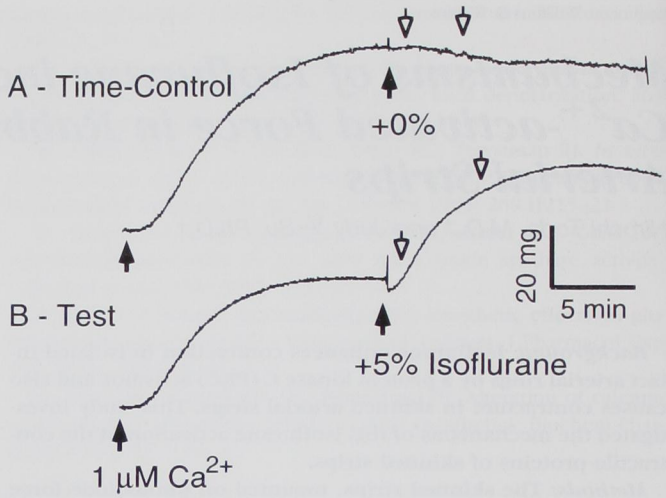

Fig. 1. Tracings showing the experimental protocol to study the effects of isoflurane on submaximum Ca^{2+}-activated force development in skinned femoral arterial strips and to collect strips to quantify myosin light chain isoforms. Tracings showed that two skinned strips were initially activated by a buffer solution containing 1 μM Ca^{2+} until steady state force developed. One strip (*A*; time control) was then tested in a fresh buffer solution containing no isoflurane (+0%). A different strip (*B*; test) was tested in a buffer containing 3% isoflurane (+3% isoflurane). The peak force after administration of the second buffer was compared with that of the control (before administration of the second buffer). In another group of experiments, the strips were frozen (open arrows) during force development (within 1 min) or at peak force (5 min) after administration of the second buffer solution without or with isoflurane to quantify myosin light chain isoforms.

Study of the Effect of Isoflurane on Submaximum Ca^{2+} Activation of the Contractile Protein

The skinned strips were allowed to equilibrate in relaxing solution and then activated submaximally with 1 μM Ca^{2+} (produced about 30% of the maximum force) until force development reached a steady state (10–15 min). Isoflurane (0%, 1%, 3%, 5%) was administered until the force reached a new steady state (fig. 1). The effect of isoflurane on this submaximum Ca^{2+}-activated force was measured up to 30 min after administration of isoflurane.

To quantify MLC phosphorylation, after administration of isoflurane, the skinned strips were quickly frozen in freon (−130°C) cooled with liquid nitrogen, within 1

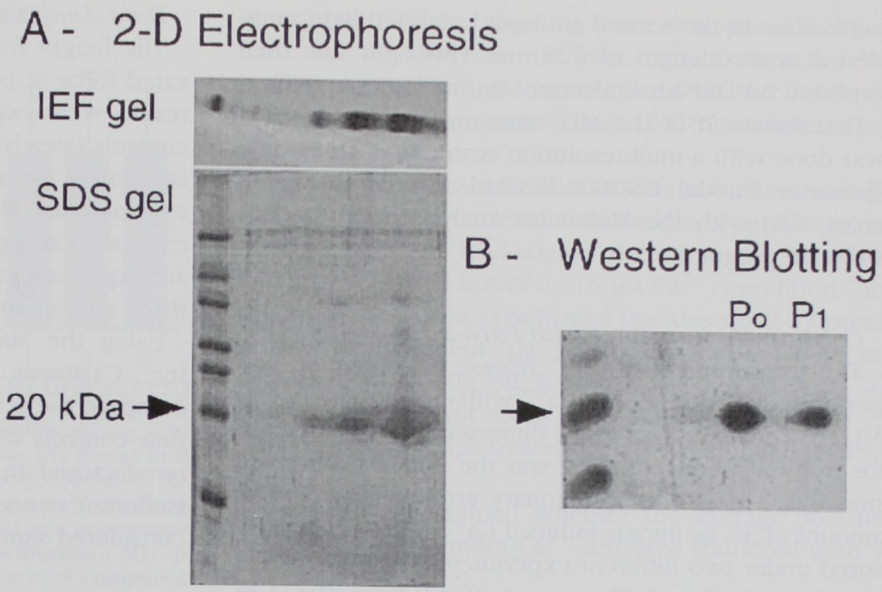

Fig. 2. Gels showing the sequence of the experimental procedure to quantify myosin light chain (MLC) isoforms. (*A*) Isoforms of MLCs in the strips were separated by two-dimensional electrophoresis. Initially MLC was separated on gels using isoelectric focusing (IEF) electrophoresis (*A*, upper panel; Coomassie blue staining) for the first dimension, which was followed by sodium dodecyl sulfate gel electrophoresis for the second dimension (*A*, lower panel; Coomassie blue staining; 20 kDa (arrows) = protein standard of 20-kDa molecular weight. (*B*) Western Blotting of MLC in the sodium dodecyle sulfate gel. Proteins were transferred to nitrocellulose membranes using trans-blot electrophoresis, and MLC isoforms were identified by a specific antibody using immunoblotting (Po = nonphosphorylated MLC; P1 = one phosphorylated MLC).

min or at 5 min (near steady state force; fig. 1. open arrows). The frozen tissues were transferred into a centrifuge tube containing 10% trichloroacetic acid-acetone mixture and kept at −70°C.

We further examined the mechanisms of isoflurane-enhanced submaximum Ca^{2+}-activated force (fig. 1) using inhibitors of PKC or calmodulin-dependent protein kinase II (CaM kinase II). In this study, 10 μM Gö-6976 (Go), which inhibits Ca^{2+}-dependent PKC (α, βI isoenzymes),[17] or bisindolylmaleimide I-HCl (Bim),[17,18] which inhibits Ca^{2+}-dependent (α, βI, βII, and γ isoenzymes)[17,18] and Ca^{2+}-independent (δ, ε, and ξ isoenzymes)[17] PKC, or 0.1 mM KN-62,[19] which inhibits CaM kinase II were present in all the solutions. The diluents of the inhibitors were used as time controls.

Isoflurane was delivered through a Verni-Trol vaporizer (Ohio Medical Products, Madison, WI) with 100% nitrogen. One set of solutions was saturated with a mixture of isoflurane and nitrogen as test solutions, and another set of solutions with 100% nitrogen as control solutions (0% isoflurane). The vapor concentrations (expressed as a percentage of 1 atmosphere [atm]) of isoflurane in the solutions were assayed by gas chromatography.[20]

Quantification of Myosin Light Chain Isoforms by Two-dimensional Electrophoresis and Immunoblotting

The method of extraction and separation of MLC isoforms from vascular smooth muscle was the same as that described by Kitazawa *et al.*[21] using two-dimensional electrophoresis (fig. 2) for its high resolution and sensitivity.[22] Briefly, MLC proteins were extracted from the frozen strips by homogenization in glycerol–sodium dodecyl sulfate solution followed by centrifugation. The MLC isoforms in the supernatant of the homogenate were first separated according to their charges (isoelectric point at 4.5 to 5.4) using isoelectric focusing polyacrylamide gel electrophoresis. After this electrophoresis, the phosphorylated MLCs were nicely separated from nonphosphorylated MLCs in the isoelectric focusing polyacrylamide gel in the first dimension. An appropriate portion of the gel containing the MLC isoforms was then laid horizontally onto the top of a sodium dodecyl sulfate–polyacrylamide minislab gel to separate the isoforms according to their mass (molecular weight at 20 kDa) in the second dimension.

Finally, immunoblotting was performed as described by Hathaway and Haeberle[23] by transferring MLC isoforms in the sodium dodecyl sulfate gel onto nitrocellulose membranes using trans-blot electrophoresis. The immunoblotting procedure was performed to increase the sensitivity and specificity of detecting MLC with polyclonal antibodies (supplied by Dr. Susan Gunst, Department of Physiology, Indiana University, Indianapolis, IN) for MLC. This was followed by exposure to a second antibody labeled with horseradish peroxidase (anti-rabbit IgG peroxidase conjugate, Sigma Chemical Co., St. Louis, MO) to react with the immobilized protein antigen (MLC). Finally, a DuPont western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA) was applied to the membrane in which the enzyme horseradish

peroxidase in the second antibody catalyzed light emission at a wavelength of 428 nm. This light was then captured on DuPont Reflection Autoradiography Film.

Determination of the MLC immunoblots on the film was done with a multiresolution scanning imaging densitometer (model GS-700; Bio-Rad Laboratories, Hercules, CA) with the Molecular Analyst image analysis software program.

Calcium Measurement with Fura-2 Fluorescent Dye

The measurement of Ca^{2+} release by isoflurane was performed to correlate the Ca^{2+} with changes in force or MLC-p. The method of fura-2 fluorescence measurement for free Ca^{2+} concentration was the same as described previously in skinned pulmonary arterial strips.[24] The amount of 3% isoflurane-induced Ca^{2+} release was measured under two different experimental conditions: (1) low EGTA buffer (0.05 mM to buffer 0.1 μM Ca^{2+}) in simple load-release cycling, and (2) high EGTA buffer (7 mM) in 1 μM Ca^{2+} activation.

The fluorescence of fura-2 (F340 and F380) was measured continuously by exposing the skinned strip in 100 μl quartz tissue bath alternately, at 50 Hz, to 340 ± 10 and 380 ± 10 nm excitation wavelengths. The fluorescence emitted at the 500 ± 20 nm wavelength was detected using a modified calcium analyzer (CAF-100, Japan Spectroscopic, Tokyo, Japan). The fluorescence emitted from excitation wavelengths of 340 and 380 nm were recorded on a PowerMac 7100 (Apple Computers, Cupertino, CA) at a sampling rate of 10 Hz using a customized LabVIEW software program interfaced with a multifunction I/O board with 16-bit resolution (NB-MIO-16XL; National Instruments, Austin, TX).

The amount of free Ca^{2+} released from the SR was estimated from the difference in Ca^{2+} between the baseline and the peak based on the equation of Ca^{2+} (in nanomoles) = Kd × (Sf2/Sb2) × [(Ri − Rmin)/(Rmax − Ri)],[25] using an apparent dissociation constant for fura-2/Ca^{2+} complex (Kd) = 421.8 nM ± 40.5 (n = 16), Sf2/Sb2 = 3.6 ± 0.4 (n = 34), Rmin = 0.101 ± 0.006 (n = 25), and Rmax = 0.708 ± 0.15 (n = 23) estimated in skinned femoral arteries. Where Sf2 is the fluorescence emitted at 380 nm excitation wavelength (F380) at 0 Ca^{2+} under which fura-2 was in free form, Sb2 is F380 at 0.1 mM Ca^{2+} under which fura-2 was in Ca^{2+} bound form, Ri is the ratio of fluorescence emitted at 340 nm excitation wavelength (F340) to F380 at various free Ca^{2+} concentrations, Rmin is the F340 to F380 ratio at 0 Ca^{2+}, and Rmax is the F340:F380 ratio at 0.1 mM Ca^{2+}.

Data Analysis

The height from baseline to submaximum Ca^{2+}-activated force at peak or steady state was calculated. The results were expressed as a percentage of those of the controls (steady state force development before administration of isoflurane) for both the test and time-control experiments. The amount of phosphorylated MLC was expressed as a percentage of the total MLC. The results are expressed in mean and SDs of the mean from at least three arterial strips and three rabbits.

Using the StatVIEW software program (BrainPower Inc., Calabasas, CA), the Student's *t* test was used to compare the results from the test experiments and the time-controls within each isoflurane concentration, and two-factorial analysis of variance was used to compare isoflurane concentrations. Probability values <0.05 were considered significant.[26]

Materials

Isoflurane was supplied by OHMEDA Inc. (Liberty Corner, NJ). Fura-2 was purchased from Molecular Probes, Inc. (Eugene, OR). Antiserum for MLCs was supplied by Dr. Susan Gunst, University of Indiana, Indianapolis, Indiana. Inhibitors for PKC [Gö-6976 = [2-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a(pyrrolo)3,4-c(-carbazole)] and bisindolylmaleimide I-HCl], and for calmodulin-dependent protein kinase II {KN-62 = 1-[N,O-bis(5-Isoquinolinessulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine} were purchased from Calbiochem (La Jolla, CA). Other chemicals were analytical or reagent grade. Stock solutions of 1 mM for Gö-6976 or 10 mM KN-62 were made in 100% dimethyl sulfoxide (DMSO). In time-control experiments, the solutions contained the same concentrations of DMSO present in the test solutions (containing the inhibitors).

Results

Effects of Isoflurane on Submaximum Ca^{2+}-activated Force Development and Its Relation to Myosin Light Chain Phosphorylation

Skinned femoral arterial strips activated by 1 μM Ca^{2+} produced an average force of 45.6 mg ± 17.3 (n = 38) at steady state (approximate 15 min after activation; fig. 1), which is about 30% of the maximum force (0.1 mM Ca^{2+}). Isoflurane dose dependently increased the submaximum force (24%, 56%, and 81% increase from control for 1%, 3%, and 5% isoflurane, respectively; fig. 3A), which reached plateau at approximately 5–10 min (fig. 1B). The isoflurane-induced increases in submaximum

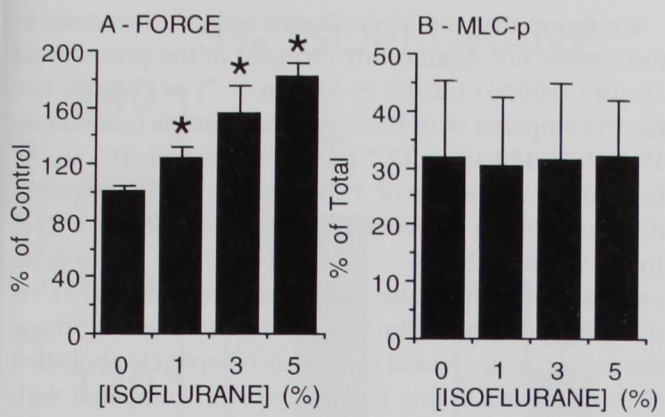

Fig. 3. Effects of various concentrations of isoflurane on submaximum Ca^{2+}-activated force at steady state (*A*, force) and myosin light chain phosphorylation (*B*, MLC-p). Mean ± SD (n = 6–18); force development activated by 1 μM Ca^{2+} at steady state after isoflurane administration expressed as a percentage of control force (before administration of isoflurane); MLC-p expressed as a percentage of total MLC. *$P < 0.05$ compared with that of time controls (0% isoflurane); isoflurane (1–5%) dose dependently increased the submaximum Ca^{2+}-activated force without changes in MLC-p.

force were relatively sustained (observed up to 30 min) with a slow decrease of approximate 1% per min of the control force generated by 1 μM Ca^{2+}.

The MLC phosphorylation (MLC-p) was examined at the peak (5 min) of the isoflurane-increased submaximum Ca^{2+}-activated force; isoflurane, however, did not significantly change the degree of MLC-p (% of total MLC at 31.8% ± 13.4 [n = 20], 30.2% ± 12.4 [n = 8], 31.3% ± 13.8 [n = 7], and 32.1% ± 10.1 [n = 7], for 0, 1, 3, and 5% isoflurane, respectively; fig. 3B). Surprisingly, MLC-p showed a trend toward a dose-dependent decrease during force development (1 min) induced by 1% and 3% isoflurane, which reached statistical significance at 3% isoflurane (29.1% ± 7.8 [n = 21], 23.3% ± 14.1 [n = 9], and 17.1% ± 7.6 [n = 6] of total MLC for 0%, 1%, and 3% isoflurane, respectively; fig. 4). However, at 5% isoflurane, MLC-p (26.8% ± 10.2 [n = 8] of total MLC) was not significantly decreased compared with that of 0% isoflurane (fig. 4).

Influence of Calcium Ionophore (A23187) on Isoflurane-increased Submaximum Ca^{2+}-activated Force and Direct Isoflurane-induced Transient Tension Increase

It is possible that increased force by isoflurane resulted in part from Ca^{2+} release from the SR by isoflurane. We treated the skinned strips with a calcium ionophore, A23187, to deplete SR Ca^{2+} stores. The presence of ionophore did not change the force in response to 1 μM Ca^{2+} but did decrease the force enhancement by 5% isoflurane (198.7% ± 9.2 [n = 6] and 152.2% ± 3.4 [n = 6] of control in untreated and A23187-treated strips, respectively; fig. 5).

Previously we observed that isoflurane induces a small transient tension increase followed by contracture in skinned strips in load-release cycling under the low EGTA (0.05 mM to buffer 0.316 μM Ca^{2+}) condition.[3] In this study, we further confirmed that this small transient tension development (fig. 6A) was associated with increases in MLC-p (fig. 6B; % of total MLC) of 23.5% ± 7.6 (n = 10) in control strips (0% isoflurane) to 37.8% ± 18.4 (n = 8) in strips treated with 3% isoflurane, respectively.

We further examined whether the average 56% increase in submaximum Ca^{2+}-activated (buffered with 7

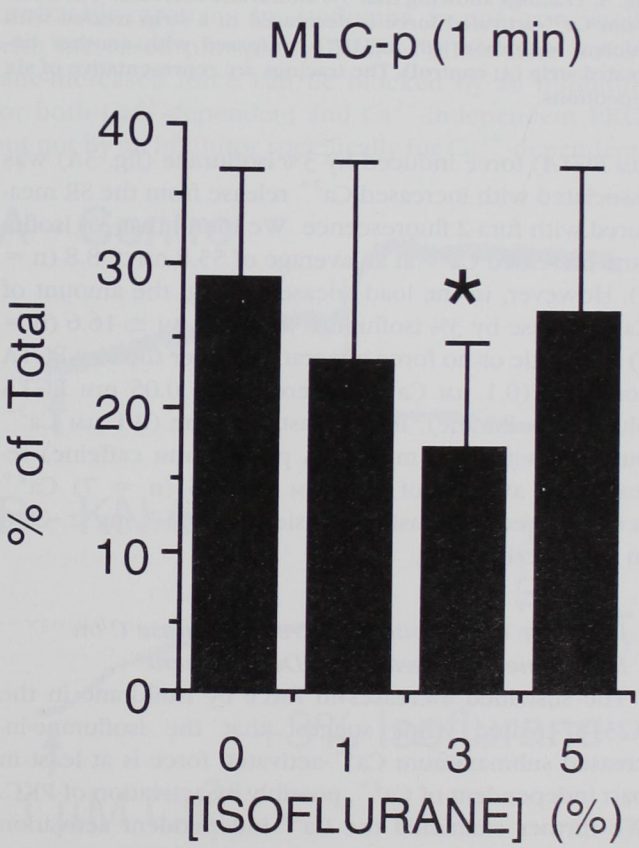

Fig. 4. Effects of various concentrates of isoflurane on myosin light chain phosphorylation (MLC-p) during isoflurane-induced increases in force development. Means ± SD (n = 6–21); MLC-p expressed as a percentage of total MLC. *$P < 0.05$ compared with that of time controls (0% isoflurane); isoflurane (1–3%) dose dependently decreased MLC-p and was significant at 3% isoflurane.

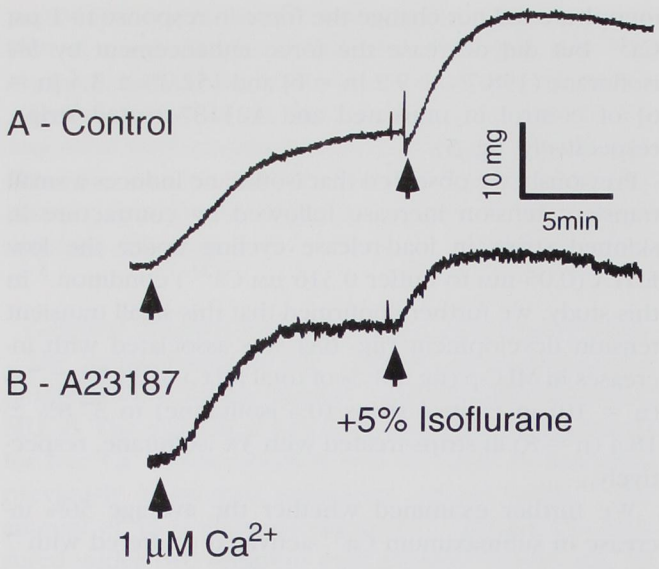

Fig. 5. Tracings showing that 5% isoflurane-increased submaximum Ca^{2+}-activated force is decreased in a strip treated with calcium ionophore (*B*; A23187) compared with another untreated strip (*A*; control). The tracings are representative of six repetitions.

mM EGTA) force induced by 3% isoflurane (fig. 3A) was associated with increased Ca^{2+} release from the SR measured with fura-2 fluorescence. We found that 3% isoflurane increased Ca^{2+} at an average of 55.8 nM ± 8.8 (n = 3). However, in the load-release cycling, the amount of Ca^{2+} release by 3% isoflurane was 43.3 nM ± 16.6 (n = 3) with little or no force generation under the low EGTA condition (0.1 μM Ca^{2+} buffered with 0.05 mM EGTA plus 3% isoflurane). In contrast, caffeine (0.1 μM Ca^{2+} buffered with 0.05 mM EGTA plus 10 mM caffeine) released an average of 51.4 nM ± 24.5 (n = 7) Ca^{2+} accompanied by tension transients of 54.7 mg ± 28.8 (n = 7) at the peak.

Influence of Inhibitors of Protein Kinase C on Isoflurane-increased Force Development

The sustained increases in force by isoflurane in the A23187-treated strips suggest that the isoflurane-increased submaximum Ca^{2+}-activated force is at least in part independent of Ca^{2+}, possibly by activation of PKC. We further examined the Ca^{2+}-independent activation of isoflurane by using two inhibitors of PKC: one was Gö-6976, an inhibitor of Ca^{2+}-dependent PKC (α, and βI isoenzymes),[17] and the other was bisindolylmaleimide, an inhibitor of both Ca^{2+}-dependent (α, βI, βII, and γ isoenzymes)[18] and Ca^{2+}-independent (δ, ε, and ξ isoenzymes)[17] PKC.

We found that the 3% isoflurane-induced increases in force were not significantly changed in the presence of 10 μM Gö-6976 (186.1% ± 33.1 [n = 7] of control, Go; fig. 7) compared with those of time-controls (containing 1% DMSO; 174.1% ± 17.7 [n = 7] of control, TC; fig. 7). In contrast, the effect of 3% isoflurane was significantly decreased in the presence of 10 μM Bim (158.4% ± 21.6 [n = 9] and 120.6% ± 14.5 [n = 3] of controls in untreated and Bim-treated strips, respectively; fig. 7). As the concentration of Bim was further increased to 30 μM, the isoflurane increased force was completely abolished (93% of control in one Bim-treated strip compared with 140% of control in one untreated strip).

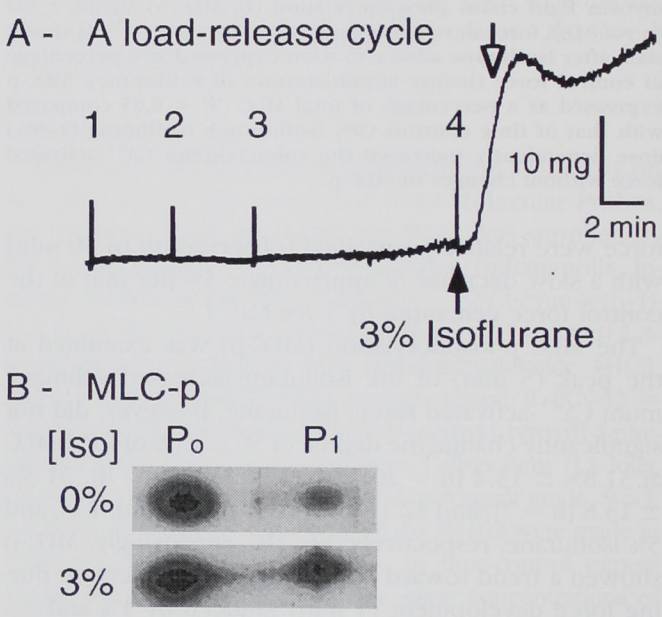

Fig. 6. Tracing showing that 3% isoflurane causes a transient tension increase in a load-release cycle (*A*), which is associated with increased myosin light chain phosphorylation (*B*, MLC-p) and the isoflurane-induced transient tension increase was followed by a sustained force development. (*A*) A load-release cycle[16]: 1 to 4 are solution artifacts. The skinned strip was immersed in four different solutions to load Ca^{2+} into and release Ca^{2+} from the SR; (1) Solution 1 contained 7 mM EGTA with no added Ca^{2+} to relax the strip; (2) solution 2 contained 0.316 μM Ca^{2+} buffered with 7 mM EGTA to load Ca^{2+} into the SR; (3) solution 3 contained 0.316 μM Ca^{2+} in 0.05 mM EGTA to reduce EGTA concentration, and (4) solution 4 contained 0.316 μM Ca^{2+} buffered with 0.05 mM EGTA, and 3% isoflurane to release Ca^{2+} from the SR resulting in a transient tension increase. (*B*) MLC-p: The skinned strips were frozen within 1 min during isoflurane-induced transient tension increase (arrow, A—A load-release cycle) and MLC-p was quantified using two-dimensional electrophoresis and western blotting. [Iso] = isoflurane concentration; 0% = 0% isoflurane; 3% = 3% isoflurane; Po = nonphosphorylated MLC; P1 = one phosphorylated MLC. The tracings are representative of 8–10 repetitions.

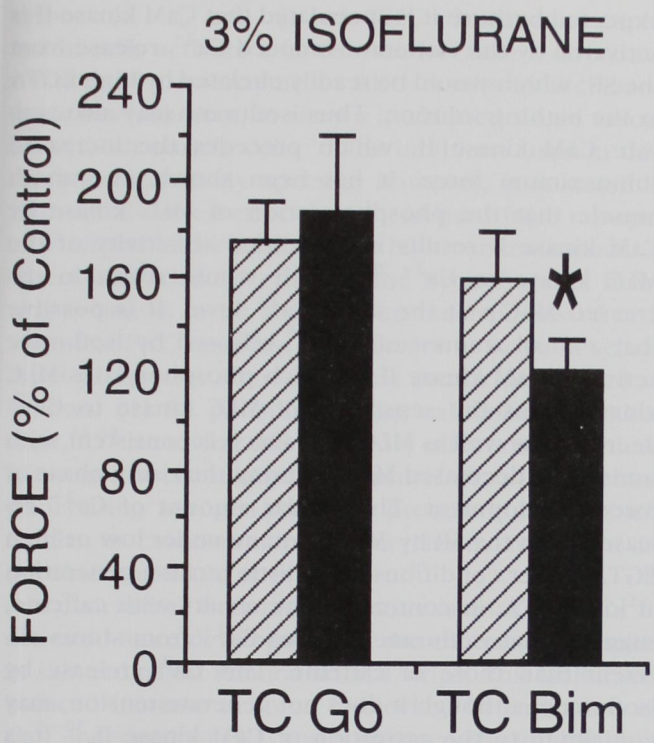

Fig. 7. The effect of inhibitors of protein kinase C on 3% isoflurane-induced increases in submaximum Ca^{2+}-activated force development. Means ± SD (n = 7–10); force development activated by 1 μM Ca^{2+} at steady state after isoflurane administration expressed as a percentage of control force (before administration of isoflurane); TC = time control; Go = Gö-6976 (10 μM), an inhibitor of Ca^{2+}-dependent protein kinase C; Bim = bisindolylmaleimide I-HCl (10 μM), an inhibitor of Ca^{2+}-dependent and Ca^{2+}-independent protein kinase C. *P < 0.05 compared with that of time control. The isoflurane-induced increases (TC) in force were blocked by Bim but not by Go.

Influence of Calmodulin-dependent Protein Kinase II Inhibitor on Isoflurane-increased Force

We further tested the hypothesis that isoflurane may activate calmodulin-dependent protein kinase II (CaM kinase II),[14] which resulted in the observed decrease in MLC-p (fig. 4). If the hypothesis is correct, by blocking CaM kinase II activity, we would expect a further increase in force development by isoflurane. In this study, KN-62,[19] a CaM kinase II inhibitor, was used at 0.1 mM in 15% DMSO. The mechanism of action of KN-62 is direct binding to the calmodulin-binding site of the enzyme.[19]

In the presence of 15% DMSO, we found that the 1 μM Ca^{2+}-activated force was significantly reduced with (fig. 8B) or without (fig. 8A) 0.1 mM KN-62. However, there was no significant difference between the control (containing 15% DMSO; 9.6 mg ± 7.1 [n = 7]) and 0.1 mM KN-62–treated strips (10.4 mg ± 3.2 [n = 7].

The isoflurane-increased submaximum Ca^{2+}-activated force was significantly enhanced in the presence of 0.1 mM KN-62 (tracing shown in fig. 8B; 266.9% ± 97.9 [n = 7]) compared with that of the control (213% ± 69.3 [n = 7]; in the presence of 15% DMSO; tracing shown in fig. 8A). The potentiation of the force induced by 3% isoflurane in the presence of KN-62 was associated with the blockade of the observed decreases in MLC-p. In other words, the effect of 3% isoflurane on MLC-p was no difference between strips treated with KN-62 (35.8% ± 13.4 [n = 4]) and those with 15% DMSO (diluent for KN-62; 32.3% ± 12.8 [n = 4]).

Discussion

The main findings of this study in skinned femoral arterial strips are that isoflurane, in a dose-dependent manner, directly increases the Ca^{2+}-activated force at submaximum levels of Ca^{2+}. This activation of the contractile proteins by isoflurane is not associated with increased phosphorylation of MLCs. The isoflurane-increased force can be blocked by an inhibitor for both Ca^{2+}-dependent and Ca^{2+}-independent PKC but not by an inhibitor specifically for Ca^{2+}-dependent

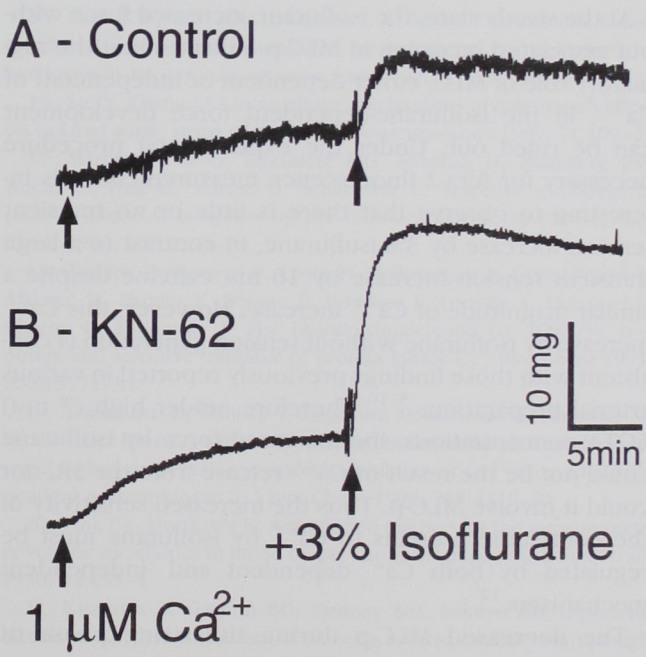

Fig. 8. Tracings showing that 3% isoflurane-induced increases in submaximum Ca^{2+}-activated force was enhanced by an inhibitor of calmodulin-dependent protein kinase II (KN-62). The tracings are representative of eight preparations. The strips were activated by 1 μM Ca^{2+} containing either 15% DMSO (*A*, control) or 0.1 mM KN-62 (*B*, KN-62) followed by solutions with the same compositions except saturated with 3% isoflurane.

PKC. During 3% isoflurane-induced force development, MLC-p initially decreased, whereas in the presence of an inhibitor of calmodulin-dependent protein kinase II (CaM kinase II), the isoflurane-increased force was enhanced and the reduced MLC-p was returned to that of time controls.

In this study, under the Ca^{2+}-clamped condition (1 μM Ca^{2+} in 7 mM EGTA), isoflurane increased force in a sustained manner. This observation is consistent with the isoflurane-induced contracture observed in the presence of low EGTA (0.05 mM).[3] This isoflurane-increased force also corresponds with that observed in isolated intact rings treated with the Ca^{2+} channel blocker, verapamil.[3] Direct activation of the contractile proteins by halothane and enflurane has also been shown in isolated intact canine mesenteric arterial rings.[27] This contraction is sustained independent of Ca^{2+}.[27] However, an inhibition of the contractile proteins by halothane and enflurane is also reported in membrane-permealized small rat mesenteric arteries.[28] Whether this discrepancy is due to differences in experimental conditions, the origin of vasculature (femoral artery *vs.* mesenteric artery), or animal species (rabbit, canine, or rat) remains to be shown.

At the steady state, the isoflurane-increased force without associated increases in MLC-p indicates that the regulatory role of MLC, either dependent or independent of Ca^{2+}, in the isoflurane-dependent force development can be ruled out. Under the experimental procedure necessary for fura-2 fluorescence measurement, it is interesting to observe that there is little or no transient tension increase by 3% isoflurane, in contrast to a large transient tension increase by 10 mM caffeine despite a similar magnitude of Ca^{2+} increase. However, this Ca^{2+} increase by isoflurane without tension generation is consistent with those findings previously reported in various arterial preparations.[5–10] Therefore, under high (7 mM) EGTA concentrations, the increased force by isoflurane could not be the result of Ca^{2+} release from the SR, nor could it involve MLC-p. Thus the increased sensitivity of the contractile proteins to Ca^{2+} by isoflurane must be regulated by both Ca^{2+}-dependent and -independent mechanisms.[12]

The decreased MLC-p during the rising phase of force development by isoflurane (3%) would cause a decrease in force. This speculation is consistent with the observation that when the decreased MLC-p by 3% isoflurane is blocked by an inhibitor of CaM kinase II, a further increased in isoflurane-induced force is observed. An increase in MLC-p by KN-62 would not be expected because it is speculated that CaM kinase II is activated by the isoflurane-induced Ca^{2+} release from the SR, which would be readily chelated by high EGTA in the bathing solution. Thus isoflurane may also activate CaM kinase II, which precedes the increased submaximum force. It has been shown in smooth muscle that the phosphorylation of MLC kinase by CaM kinase II results in decreased sensitivity of the MLC kinase to Ca^{2+},[29] which would result in decreased MLC-p at the same Ca^{2+} level. It is possible that a small amount of Ca^{2+}[5,6] released by isoflurane activates CaM kinase II,[30] which phosphorylates MLC kinase. Thus the sensitivity of MLC kinase to Ca^{2+} decreases as well as MLC-p,[29] which is consistent with transiently decreased MLC-p during the rising phase of force development. The similar amount of Ca^{2+} released from the SR by 3% isoflurane under low or high EGTA buffer conditions, with little tension generation at low EGTA, in contrast to the results with caffeine, suggests that isoflurane releases Ca^{2+} from stores different than those of caffeine. This Ca^{2+} release by isoflurane, although it does not generate tension, may contribute to the activation of CaM kinase. II.[30] It is possible that before chelation by 7 mM EGTA, the small amount of Ca^{2+} released from the SR by isoflurane[6] activates CaM kinase II localized on the SR membrane, which has been shown in striated muscles.[31] On the other hand, a larger amount of Ca^{2+} release by 5% isoflurane could activate both CaM kinase II and MLC kinase, resulting in no change in MLC-p. These speculations are confirmed by the reduction of isoflurane-increased force not only in strips treated with A23187 but also by increasing EGTA concentrations to 10 mM in the buffer tested with three isoflurane concentrations (from 124% to 116.3%, 156% to 132.7%, and 181% to 153.3%, for 1%, 3%, and 5% isoflurane for 7 mM and for 10 mM EGTA buffers, respectively). A direct isoflurane activation of CaM kinase II and its localization in the vascular smooth muscle remain to be examined.

The inhibition of isoflurane-increased submaximum force by the PKC inhibitor of both Ca^{2+}-dependent (α, β-I) and Ca^{2+}-independent (δ, ϵ, and ξ) isoenzymes (Bim)[17] is consistent with the observation made by Park *et al.*[15] that isoflurane enhances vasoconstriction induced by oleic acid (PKC activator) in rat coronary arteries. In addition to our observations of the absence of inhibition by the inhibitor of Ca^{2+}-dependent isoenzymes (α and β-I) of PKC (Gö-6976),[17] we could speculate that isoflurane activates Ca^{2+}-independent isoen-

zymes (δ, ϵ, and ξ) of PKC. Isoflurane could specifically activate ϵ isoenzyme because a direct activation of ϵ isoenzyme of PKC results in increased force development, as shown in skinned single vascular smooth muscle cells.[32] The inhibitors used in this study are relatively specific; however, it is possible that the observed effects result, in part, from nonspecific effects of the inhibitors at high concentrations. A direct activation of ϵ isoenzyme by isoflurane, however, remains to be shown. The mechanisms of activation of PKC resulting in increases in force development of vascular smooth muscle can be postulated as follows: (1) by phosphorylating calponin or caldesmon,[33,34] removing the inhibitory effect of these thin-filament associated regulatory proteins, or (2) by phosphorylating mitogen-activated protein kinase, which results in caldesmon phosphorylation, as shown in porcine carotid arteries *in vitro*.[35]

In conclusion, isoflurane increases submaximum Ca^{2+}-activated force development of the contractile proteins in skinned rabbit femoral arterial strips. This isoflurane-induced increase in force is blocked by inhibition of Ca^{2+}-independent PKC and is enhanced by inhibition of CaM kinase II. The isoflurane-increased submaximum Ca^{2+}-activated force is a net result of the combined transient activation of CaM kinase II, which decreases force, and PKC, which increases force.

The authors thank Dr. Alec Rooke for discussions, Barbara Pearson for editorial assistance, Luo-Jia Tang for technical assistance in assaying Ca^{2+}/fura-2 fluorescence, and OHMEDA Inc. (Murray Hill, NJ) for supplying isoflurane.

References

1. Eger EI II: Isoflurane: A review. ANESTHESIOLOGY 1981; 55:559–76

2. Hatano Y, Nakamura K, Yakushiji T, Nishiwada M, Mori K, Anaes FC: Comparison of the direct effects of halothane and isoflurane on large and small coronary arteries isolated from dogs. ANESTHESIOLOGY 1990; 73:513–7

3. Su JY: Mechanisms of action of isoflurane on contraction of rabbit conduit artery. Anesth Analg 1996; 82:837–42

4. Buljubasic N, Flynn NM, Marijic J, Rusch NJ, Kampine JP, Bosnjak ZJ: Effects of isoflurane on K^+ and Ca^{2+} conductance in isolated smooth muscle cells of canine cerebral arteries. Anesth Analg 1992; 75:590–6

5. Iaizzo PA: The effects of halothane and isoflurane on intracellular Ca^{2+} regulation in cultured cells with characteristics of vascular smooth muscle. Cell Calcium 1992; 13:513–20

6. Tsuchida H, Namba H, Yamakage M, Fujita S, Notsuki E, Namiki A: Effects of halothane and isoflurane on cytosolic calcium ion concentrations and contraction in the vascular smooth muscle of the rat aorta. ANESTHESIOLOGY 1993; 78:531–40

7. Stadnicka A, Flynn NM, Bosnjak ZJ, Kampine JP: Enflurane, halothane, and isoflurane attenuate contractile responses to exogenous and endogenous norepinephrine in isolated small mesenteric veins of the rabbit. ANESTHESIOLOGY 1993; 78:326–34

8. Sill JC, Eskuri S, Nelson R, Tarara J, Van Dyke RA: The volatile anesthetic isoflurane attenuates Ca^{++} mobilization in cultured vascular smooth muscle cells. J Pharmacol Exp Ther 1993; 265:74–80

9. Tsuchida H, Namba H, Seki S, Fujita S, Tanaka S, Namiki A: Role of intracellular Ca^{2+} pools in the effects of halothane and isoflurane on vascular smooth muscle contraction. Anesth Analg 1994; 78: 1067–76

10. Ozhan M, Sill JC, Atagunduz P, Martin R, Katusi'c ZS: Volatile anesthetics and agonist-induced contractions in porcine coronary artery smooth muscle and Ca^{2+} mobilization in cultured immortalized vascular smooth muscle cells. ANESTHESIOLOGY 1994; 80: 1102–13

11. Gerthoffer WT, Pohl J: Caldesmon and calponin phosphorylation in regulation of smooth muscle contraction. Can J Physiol Pharmacol 1994; 72:1410–4

12. Horowitz A, Menice CB, Laporte R, Morgan KG: Mechanisms of smooth muscle contraction. Physiol Rev 1996; 76:967–1003a

13. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M: Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem 1997; 272:12257–60

14. Walsh MP, Andrea JE, Allen BG, Clement-Chomienne O, Collins EM, Morgan KG: Smooth muscle protein kinase C. Can J Physiol Pharmacol 1994; 72:1392–9

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

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

17. 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

18. 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; 266:15771–81

19. Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M, Hidaka H: KN-62, 1-[N, o-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem 1990; 265:4315–20

20. Fink BR, Morikawa K: A simplified method for the measurement of volatile anesthetics in blood by gas chromatography. ANESTHESIOLOGY 1970; 32:451–5

21. Kitazawa T, Gaylinn BD, Denney GH, Somlyo AP: G-protein-mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem 1991; 266:1708–15

22. O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975; 250:4007–21

23. Hathaway DR, Haeberle JR: A radioimmunoblotting method for measuring myosin light chain phosphorylation levels in smooth muscle. Am J Physiol 1985; 249:C345–51

24. Su JY, Tang LJ: Effects of halothane on the sarcoplasmic reticu-

lum Ca^{2+} stores and contractile proteins in rabbit pulmonary arteries. ANESTHESIOLOGY 1998; 88:1096–106

25. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicator with greatly improved fluorescence properties. J Biol Chem 1985; 260:3440–50

26. Winer BJ: Statistical Principles in Experimental Design. McGraw-Hill, New York, 1971

27. Kakuyama M, Hatano Y, Nakamura K, Toda H, Terasako K, Nishiwada M, Mori K: Halothane and enflurane constrict canine mesenteric arteries by releasing Ca^{2+} from intracellular Ca^{2+} stores. ANESTHESIOLOGY 1994; 80:1120–7

28. Akata T, Boyle III WA: Volatile anesthetic actions on contractile proteins in membrane permeabilized small mesenteric arteries. ANESTHESIOLOGY 1995; 82:700–12

29. Tansey MG, Word RA, Hidaka H, Singer HA, Schworer CM, Kamm KE, Stull JT: Phosphorylation of myosin light chain kinase by the multifunctional calmodulin-dependent protein kinase II in smooth muscle cells. J Biol Chem 1992; 267:12511–6

30. Colbran RJ, Soderling TR: Calcium/calmodulin-dependent protein kinase II. Current Topics in Cellular Regulation 1990; 31: 181–221

31. Xu A, Hawkins C, Narayanan N: Ontogeny of sarcoplasmic reticulum protein phosphorylation by Ca^{2+}-calmodulin-dependent protein kinase. J Mol Cell Cardiol 1997; 29:405–18

32. Horowitz A, Clement-Chomienne O, Walsh MP, Morgan KG: ϵ-Isoenzyme of protein kinase C induces a Ca^{2+}-independent contraction in vascular smooth muscle. Am J Physiol 1996; 271:C589–94b

33. Winder SJ, Walsh MP: Smooth muscle calponin: Inhibition of actomyosin MgATPase and regulation by phosphorylation. J Biol Chem 1990; 265:10148–55

34. Mino T, Yuasa U, Naka M, Tanaka T: Phosphorylation of calponin mediated by protein kinase C in association with contraction in porcine coronary artery. Biochem Biophys Res Commun 1995; 208: 397–404

35. Adam LP, Franklin MT, Raff GJ, Hathaway DR: Activation of mitogen-activated protein kinase in porcine carotid arteries. Circ Res 1995; 76:183–90