

## Role of PKC in Isoflurane-induced Biphasic Contraction in Skinned Pulmonary Arterial Strips

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**Background:** Activation or inhibition of protein kinase C (PKC) has been implicated in the anesthetic-induced contraction or relaxation of different types of arteries. In skinned pulmonary arterial strips, the initial halothane-induced contraction has been attributed to PKC activation, but the subsequent relaxation has not. Whether isoflurane has a similar biphasic effect is not known. This study examined the role of PKC and its isoforms in the effect of isoflurane on pulmonary artery.

**Methods:** Rabbit pulmonary arterial strips were mounted on force transducers and treated with saponin to make the sarcolemma permeable ("skinned" strips). Skinned strips were activated by low  $\text{Ca}^{2+}$  (pCa 6.5 or pCa 6.3 buffered with 7 mM EGTA) or the PKC activator phorbol-12,13-dibutyrate (1  $\mu\text{M}$ ) until force reached a steady state (control). Various concentrations of isoflurane (test) were administered, and force was observed at time intervals up to 60 min. The PKC inhibitors (bisindolylmaleimide and Go6976 from 0.1 to 10  $\mu\text{M}$ ) were preincubated in a relaxing solution and the subsequent contracting solutions. The results were expressed as a percentage of control, with  $P < 0.05$  considered significant for statistical comparison between the tests and time controls.

**Results:** In a dose-dependent fashion, isoflurane (1–5%) initially increased (5–40%) and then decreased (3–70%) the  $\text{Ca}^{2+}$ - or phorbol-12,13-dibutyrate (pCa 6.7 buffer)-activated force. The increased in force caused by isoflurane was partially reduced by 3 and 10  $\mu\text{M}$  bisindolylmaleimide, but not by Go6976. Isoflurane-induced relaxation was partially prevented by both inhibitors at 0.1 and 0.3  $\mu\text{M}$ .

**Conclusions:** Isoflurane causes biphasic effects in skinned pulmonary arterial strips that may be in part mediated by different isoforms of PKC.

PATIENTS with pulmonary hypertension present a challenge during anesthesia, in part because of the lack of commonly available selective dilators for the pulmonary vasculature. Control of pulmonary hypertension may therefore involve the use of agents with effects on the systemic circulation. *Via* a variety of mechanisms, induction and maintenance of anesthesia tend to reduce pulmonary artery pressures. One mechanism of at least some volatile anesthetics involves the direct effects of the agents on pulmonary vascular smooth muscle. A better understanding of the cellular pathways for the volatile agents on smooth muscle physiology might even-

tually yield clues on how to enhance pulmonary vasodilation selectively over systemic vasodilation.

Protein kinase C (PKC) plays an important role in contraction of vascular smooth muscle<sup>1</sup>; however, the exact role for PKC is poorly defined. PKC and its isoforms (for review, see Parekh *et al.*<sup>2</sup>) have been implicated in the action of volatile anesthetics. In isolated coronary artery activated by the PKC activator oleic acid, halothane decreases force, and isoflurane enhances force.<sup>3</sup> In the light of the putative role of PKC in smooth muscle, these data suggest that halothane inhibits PKC whereas isoflurane activates PKC. In contrast, isoflurane<sup>4</sup> and halothane<sup>5</sup> increase force that is activated by submaximum concentrations of  $\text{Ca}^{2+}$  in  $\text{Ca}^{2+}$ -clamped skinned femoral<sup>4</sup> and pulmonary<sup>5</sup> arterial strips. This force is blocked by high concentrations (10  $\mu\text{M}$ ) of bisindolylmaleimide, an inhibitor of both  $\text{Ca}^{2+}$ -dependent PKC (conventional PKC [cPKC] =  $\alpha$ ,  $\beta\text{I}$ ,  $\beta\text{II}$ , and  $\gamma$ ) and  $\text{Ca}^{2+}$ -independent PKC (novel PKC [nPKC] =  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ) but not by the cPKC inhibitor Go6976.<sup>4,5</sup> These observations led us to speculate that increased force by halothane<sup>5</sup> and isoflurane<sup>4</sup> results from activation of PKC. The differential effect of PKC inhibitors on different PKC isoforms suggests unique roles for the isoforms in smooth muscle contraction; however, their exact roles are unclear.

A dual role for PKC in smooth muscle contraction has been proposed (see Zhong and Su,<sup>6</sup> fig. 1). In tracheal smooth muscle, purified cPKC $\alpha$ , as well as  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase II (CaMKII), has been shown to phosphorylate myosin light chain (MLC) kinase (MLCK) at the same site.<sup>7</sup> Thus, cPKC $\alpha$ -phosphorylated MLCK would also result in decreased MLCK activity and decreased MLC phosphorylation as shown with CaMKII,<sup>7</sup> leading to relaxation. Halothane has also been shown to decrease the force after a short period of enhanced contraction in skinned pulmonary arterial strips.<sup>5</sup> This effect might be attributed to the cPKC $\alpha$ -MLCK-MLC signaling pathway. However, a role for cPKC in the relaxation of force has not been directly documented. In contrast, Turner *et al.*<sup>8</sup> have shown that PMA (a PKC activator) increases force but is associated with increased MLCK activity and MLC phosphorylation. This suggests that a different pathway could be involved in PKC activation resulting in force development, possibly *via* extracellular signal-related kinase (ERK)-MLCK-MLC signaling as observed in nonmuscle cells.<sup>9</sup> Kitazawa *et al.*<sup>10</sup> have shown another pathway for PKC-induced force *via* CPI-17 phosphorylation to inhibition of MLC phosphatase resulting in increased MLC phosphoryla-

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tion. Thus, PKC could play a dual role (contraction and relaxation) in smooth muscle, and this may also be mediated by different isoforms of this enzyme. This study was undertaken to determine whether isoflurane, like halothane, could induce biphasic effects in pulmonary arteries, and to examine the role of PKC isoforms in the isoflurane effect.

## Methods and Materials

### Materials

Isoflurane was supplied by OHMEDA Inc. (Liberty Corner, NJ). Go6976 and bisindolylmaleimide I-HCl (PKC inhibitors) and phorbol-12,13-dibutyrate (PDBu) (a PKC activator) were purchased from Calbiochem (La Jolla, CA) or RBI (Natick, MA). Other chemicals were of analytical or reagent grade. Stock solutions of Go6976 (1 mM) were made in 100% dimethyl sulfoxide (DMSO). Final concentrations of 0.1–1.0  $\mu\text{M}$  Go6976 contained 0.1% DMSO, and 10  $\mu\text{M}$  Go6976 contained 10% DMSO. The same concentrations of DMSO were used in parallel for time-controls.

### Skinning Procedure

The method of preparing skinned arterial strips has been described.<sup>5,11</sup> Male New Zealand white rabbits (2.2–2.5 kg) were killed using a captive bolt pistol. This method has been approved by University of Washington Animal Care Committee (Seattle, WA).

Right pulmonary arteries were rapidly isolated. The arterial rings, approximately  $0.7 \pm 0.2$  mm in width, were placed in relaxing solution (contained no added  $\text{Ca}^{2+}$  in basic solution) and denuded by gently rubbing the endothelium with a glass rod. Rings were opened, mounted on two pairs of forceps with one end attached to a photodiode tension transducer, and then stretched to a resting tension of 50 mg. The strips were immersed sequentially in the following solutions: (1) saponin (300  $\mu\text{g}/\text{ml}$ ) in relaxing solution for 4.5 min to make the sarcolemma permeable ("skinned"); (2) caffeine (25 mM) in relaxing solution to release  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR); (3) 10  $\mu\text{M}$   $\text{Ca}^{2+}$  buffered with 7 mM EGTA to activate the contractile proteins; and (4) relaxing solution to wash away  $\text{Ca}^{2+}$  in the strips.

Experiments were performed at room temperature ( $21 \pm 2^\circ\text{C}$ ). The tension was recorded on a G3 Apple Computer (Cupertino, CA) with a customized LabVIEW software program interfaced with a multifunction input/output board with 16-bit resolution (NB-MIO-16XL; National Instrument, Austin, TX).

Basic solution contained varying concentrations of free  $\text{Ca}^{2+}$  (or  $\text{pCa} = -\log [\text{Ca}^{2+}]$  (M)) buffered with 7 mM EGTA, 2 mM  $\text{MgATP}^{2-}$ , 0.1 mM free  $\text{Mg}^{2+}$ , 15 mM creatine phosphate, 70 mM,  $\text{K}^+$  plus  $\text{Na}^+$  (from  $\text{Na}_2\text{ATP}$  and  $\text{Na}_2\text{CP}$ ), 20 mM methanesulfonate (major anion), and

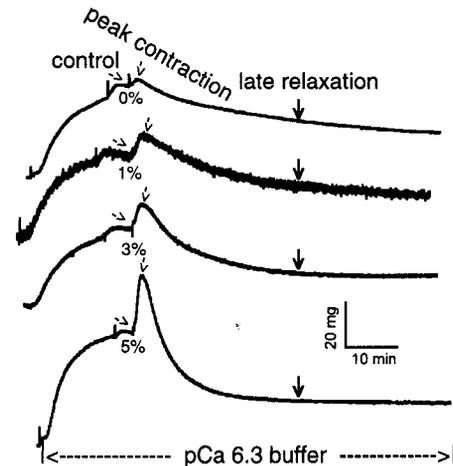


Fig. 1. Experimental protocol showing tracings of effects of isoflurane on pCa-induced force. pCa 6.3 buffer =  $-\log [\text{Ca}^{2+}]$  (M) = 0.5  $\mu\text{M}$   $\text{Ca}^{2+}$  buffered with 7 mM EGTA; control = steady state force before administration of isoflurane; 0, 1, 3, and 5% = isoflurane concentrations administered; peak contraction = peak force increased by isoflurane; late relaxation = force decreased by isoflurane after peak contraction. The tracings showed that isoflurane dose-dependently increased  $\text{Ca}^{2+}$ -activated force, reaching peak within 5 min (peak contraction). At 30 min after administration of isoflurane (late relaxation), the force was decreased by isoflurane in a dose-dependent manner.

50 mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid] dipotassium), 300 mOsm, with a pH of  $7.00 \pm 0.02$  ( $21^\circ\text{C}$ ).

pCa buffer, containing free  $\text{Ca}^{2+}$  concentrations of 0.316  $\mu\text{M}$  (pCa 6.5 buffer) or 0.5  $\mu\text{M}$  (pCa 6.3 buffer), was used to induce a minimum of 10 mg force (approximately 10–30% of maximum force based on previously established pCa-tension curves). We found that there was no significant difference in the responsiveness of the skinned strips to the effect of isoflurane with either pCa 6.5- or pCa 6.3-induced force. Isoflurane was saturated in pCa buffer, and the partial pressure of isoflurane in the buffer, expressed as a percentage of 1 atmosphere, was assayed by gas chromatography.<sup>12</sup>

### Experimental Procedure

**Effects of Isoflurane on pCa- or PDBu-induced Force.** Skinned strips were activated by either pCa 6.3 or PDBu in pCa 6.5 until force reached a steady state (control result). A fresh solution saturated with various concentrations of isoflurane (0, 1, 3, or 5%) was then substituted (test solution). The force (test result) after isoflurane administration was recorded for up to 60 min (fig. 1).

**Influence of PKC Inhibitors in the Effect of 3% Isoflurane on pCa- or PDBu-induced Force.** Protein kinase C inhibitors (Go6976 for cPKC<sup>13</sup> and bisindolylmaleimide for c/nPKC<sup>14</sup>) from 0.01 to 10  $\mu\text{M}$  were pre-incubated in a relaxing solution, the subsequent pCa or PDBu contracting solutions, and the contracting solu-

tions with isoflurane. When Go6976 was tested, parallel experiments were performed with DMSO (0.1 and 1%) to serve as a time-control. The test results were observed at various time intervals up to 60 min and expressed as a percentage of the control.

#### Data Analysis

The change in force was measured from baseline to steady state force (control), or to the force present after various time intervals up to 60 min after administration of isoflurane either in the pCa 6.3–6.5 or the PDBu solutions. For the study of isoflurane dose–response relation, test solutions included various isoflurane concentrations. When the effects of the PKC inhibitors were tested, three responses were examined: (1) 0% isoflurane and 0  $\mu\text{M}$  inhibitor (time control for isoflurane); (2) 3% isoflurane and 0  $\mu\text{M}$  inhibitor (time control for each concentration of the inhibitor); and (3) various concentrations of the inhibitor and 3% isoflurane for the study of the inhibitor.

The test force was expressed as a percentage of the control force. Mean and SD from at least three arterial strips and three separate animals were expressed. Results from the strips treated with the inhibitors and the time controls were compared by Student *t* test for paired data using the StatVIEW software program (BrainPower, Inc., Calabasas, CA), and two-factorial analysis of variance was used to compare different concentrations of inhibitors or isoflurane. A *P* value of less than 0.05 was considered significant.<sup>15</sup>

**Definitions of “Peak Contraction” and “Late Relaxation.”** We found that isoflurane increased (within 5 min) and decreased (between 15 and 60 min) the  $\text{Ca}^{2+}$ - or PDBu-induced force (fig. 1), depending on experimental conditions (isoflurane concentrations, PDBu, pCa buffer, PKC inhibitors with or without DMSO). However, the isoflurane effects were relatively consistent within each animal. Therefore, the results were expressed as “peak contraction” for isoflurane-induced peak force and “late relaxation” for isoflurane-induced decreased force at a later phase when time controls reached approximately 50–70% of control force within 30 min for most experiments. However, the test results (isoflurane with or without the inhibitors) for late relaxation were analyzed at the same time periods (e.g., 30 min, fig. 1) as those of the time control, within a series of experiments in each animal.

## Results

#### Effects of Isoflurane on pCa-induced Force

We found that isoflurane initially increased and later decreased the pCa-induced force (fig. 1). Isoflurane-increased force peaked between 5 and 10 min, and the decrease in force peaked between 15 and 60 min. As

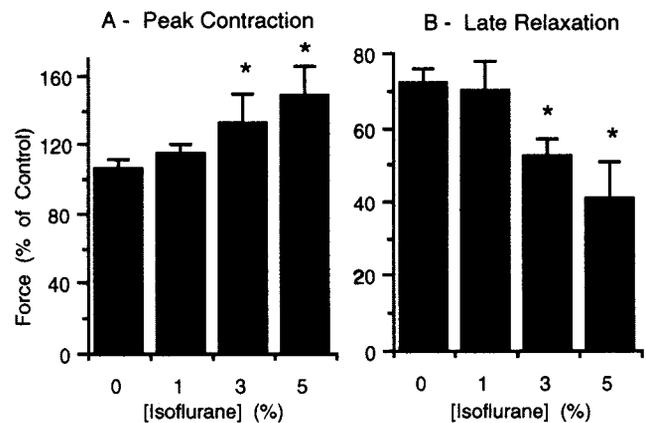


Fig. 2. Dose–response relation of isoflurane on pCa-induced force. Mean  $\pm$  SD ( $n = 3$ ); force at peak contraction (A) or late relaxation (B; at 15 and 30 min) after administration of isoflurane is expressed as percent of the control force (steady state force before the administration of isoflurane). \**P* < 0.05 compared with that of time controls (0%). Isoflurane dose-dependently increased (peak contraction) and then decreased (late relaxation) the submaximum  $\text{Ca}^{2+}$ -activated force.

shown in figure 2, isoflurane dose-dependently increased force in pCa-activated strips, reaching a peak at approximately 5 min (mean  $\pm$  SD;  $n = 3$ ;  $105.7 \pm 6.4$ ,  $115.2 \pm 4.9$ ,  $132.7 \pm 16.6$ , and  $148.1 \pm 17.0$  for 0, 1, 3, and 5% isoflurane, respectively). The increase in force was statistically significant at 3 and 5% isoflurane. However, 30 min after isoflurane administration, the force was significantly decreased at 3 and 5% isoflurane compared with that of the time controls ( $n = 3$ ;  $71.7 \pm 4.3$ ,  $69.7 \pm 8.2$ ,  $52.0 \pm 4.9$ , and  $40.7 \pm 9.9$  for 0, 1, 3, and 5% isoflurane, respectively), and the decrease was dose dependent (fig. 2).

#### Influence of PKC Inhibitors on the pCa-induced Force Affected by Isoflurane

We next tested the hypothesis that the biphasic effect of isoflurane on pCa-induced force was due to activation of PKC *via* different signaling pathways. PKC inhibitors were used at concentrations from 0.1 to 10  $\mu\text{M}$ , which were 10- to 1,000-fold the concentration (< 10 nM) required to inhibit 50% of PKC *in vitro*.<sup>13,14</sup>

We found that isoflurane-induced decrease in force was significantly prevented by 0.3  $\mu\text{M}$  bisindolylmaleimide compared with that of 0  $\mu\text{M}$  bisindolylmaleimide ( $n = 5$ ;  $63.4 \pm 7.8$ ,  $27.4 \pm 10.1$ ,  $42.8 \pm 23.0$ ,  $54.4 \pm 30.4$ , and  $40.0 \pm 13.0$  for time control [0  $\mu\text{M}$  bisindolylmaleimide] without isoflurane, 0, 0.1, 0.3, and 1  $\mu\text{M}$  bisindolylmaleimide in the presence of 3% isoflurane, respectively; fig. 3A). The isoflurane-induced late relaxation was also significantly blocked by 0.1 and 0.3  $\mu\text{M}$  Go6976 compared with that of 0  $\mu\text{M}$  Go6976 ( $n = 4$ ;  $55.7 \pm 9.2$  for time control [0% isoflurane and 0  $\mu\text{M}$  Go6976],  $28.3 \pm 4.4$ ,  $41.3 \pm 7.8$ ,  $41.9 \pm 6.8$ , and  $31.5 \pm 16.6$  for 0, 0.1, 0.3, and 1  $\mu\text{M}$  Go6976 in the presence of 3% isoflurane, respectively; fig. 3B).

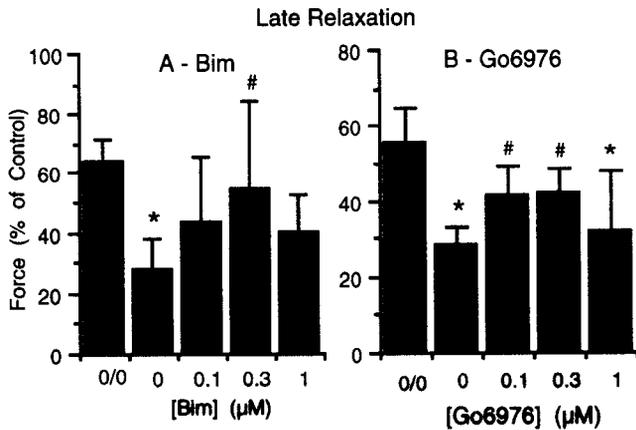


Fig. 3. Influence of low concentrations of protein kinase C inhibitors on decreased pCa-induced force by isoflurane. Bim = bisindolylmaleimide I HCl, a protein kinase C inhibitor of conventional-novel protein kinase C; Go6976 = a conventional protein kinase C inhibitor; 0/0 = time controls without isoflurane and inhibitors. Isoflurane, 3%, was present in 0, 0.1, 0.3, and 1.0  $\mu\text{M}$  bisindolylmaleimide or Go6976. Mean  $\pm$  SD ( $n = 4-5$ ). Force after administration of isoflurane at late relaxation is expressed as percent of control force (steady state force before the administration of isoflurane). \* $P < 0.05$  compared with that of the time control (0/0); #compared with that of isoflurane in the absence of bisindolylmaleimide or Go6976 (0  $\mu\text{M}$ ). Isoflurane-decreased force was significantly prevented at 0.3  $\mu\text{M}$  bisindolylmaleimide (A) and at 0.1 and 0.3  $\mu\text{M}$  Go6976 (B) but not by higher concentration (1  $\mu\text{M}$ ) of the inhibitors.

Higher concentrations of bisindolylmaleimide ( $> 3 \mu\text{M}$ ) did not affect the isoflurane-induced relaxation (data not shown). In contrast, isoflurane did not induce relaxation in the presence of 1% DMSO and the force was significantly decreased by 10  $\mu\text{M}$  Go6976 (data not shown). However, peak force induced by isoflurane was significantly decreased by 3 and 10  $\mu\text{M}$  bisindolylmaleimide (fig. 4A) but not by 10  $\mu\text{M}$  Go6976 (fig. 4B).

#### Effects of Isoflurane on Phorbol-12,13-dibutyrate (PDBu)-induced Force

Based on our results, we further hypothesized that increased force by isoflurane results in part from activation of nPKC (figs. 4A and B), whereas decreased force results in part from activation of cPKC (figs. 3A and B). A differential activation of nPKC would prevent the isoflurane-induced increase in force, and the isoflurane-decreased force would readily be demonstrated. We used PDBu in pCa 6.7 buffer to activate PKC and generate force. Isoflurane at 3 and 5% again increased the PDBu-induced force (peak contraction, fig. 5A), which was decreased between 30 and 60 min (late relaxation, fig. 5B).

#### Effects of PKC Inhibitors on Phorbol-12,13-dibutyrate (PDBu)-induced Force

We further examined whether there was a differential activation of PKC isozymes in PDBu-induced force, using the c/nPKC inhibitor bisindolylmaleimide and the cPKC

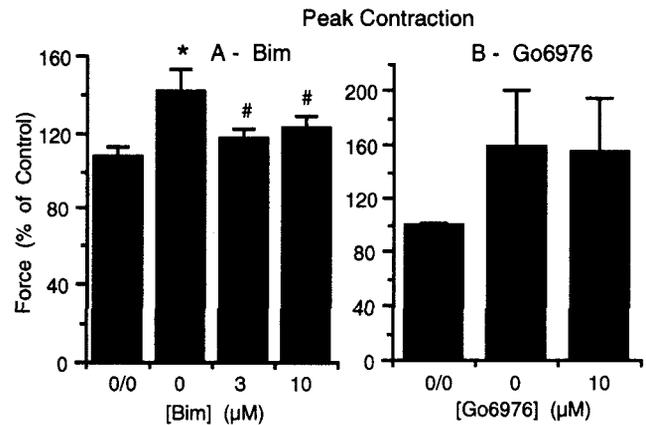


Fig. 4. Influence of high concentrations of protein kinase C inhibitors on increased pCa-induced force by isoflurane. Bim = bisindolylmaleimide I HCl, a protein kinase C inhibitor of conventional-novel protein kinase C; Go6976 = a conventional protein kinase C inhibitor; 0/0 = time controls without isoflurane and inhibitors. Isoflurane, 3%, was present in 0, 3, and 10  $\mu\text{M}$  bisindolylmaleimide or Go6976. Mean  $\pm$  SD ( $n = 3$ ). Peak force (peak contraction) after administration of isoflurane is expressed as percent of control force (steady state force before the administration of isoflurane). \* $P < 0.05$  compared with that of the time control (0/0); #compared with that of isoflurane in the absence of bisindolylmaleimide (0  $\mu\text{M}$ ). Isoflurane-increased force induced by pCa was completely blocked by 3 and 10  $\mu\text{M}$  bisindolylmaleimide (A). In contrast, isoflurane in the presence of 1% dimethyl sulfoxide (used as a vehicle for Go6976) did not increase the force (0  $\mu\text{M}$  vs. 0/0; B) which was not affected by Go6976 up to 10  $\mu\text{M}$  (10 vs. 0  $\mu\text{M}$ ; B).

inhibitor Go6976. As shown in figure 6, we found that bisindolylmaleimide dose-dependently inhibited the PDBu-induced force ( $n = 3-5$ ;  $92.2 \pm 3.4$ ,  $76.8 \pm 12.0$ ,  $33.6 \pm 16.1$ , and  $17.9 \pm 2.1$  for 0, 0.1, 3, and 10  $\mu\text{M}$  bisindolylmaleimide, respectively; fig. 6A), but Go6976

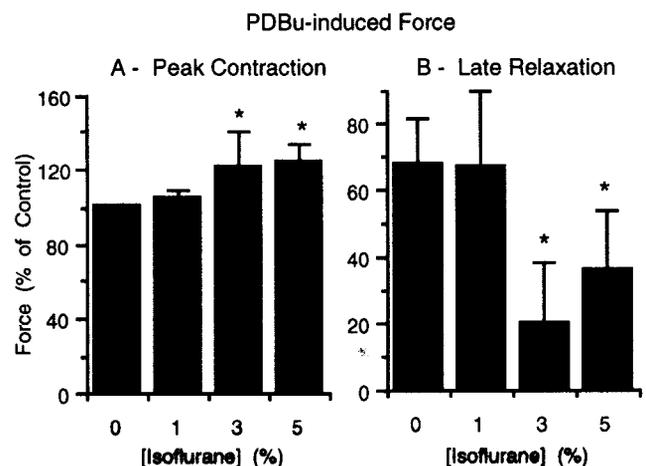


Fig. 5. Dose-response relation of isoflurane on phorbol-12,13-dibutyrate (PDBu)-induced force. Mean  $\pm$  SD ( $n = 3$ ). Force after administration of isoflurane is expressed as percent of control force (steady state PDBu-induced force before administration of isoflurane). Peak contraction = isoflurane-increased force; late relaxation = force at 60 min after administration of isoflurane. \* $P < 0.05$  compared with that of time control at 0% isoflurane. Isoflurane dose-dependently increased (A) and then decreased (B) the PDBu-induced force. However, there was no significant difference between 3% and 5% isoflurane.

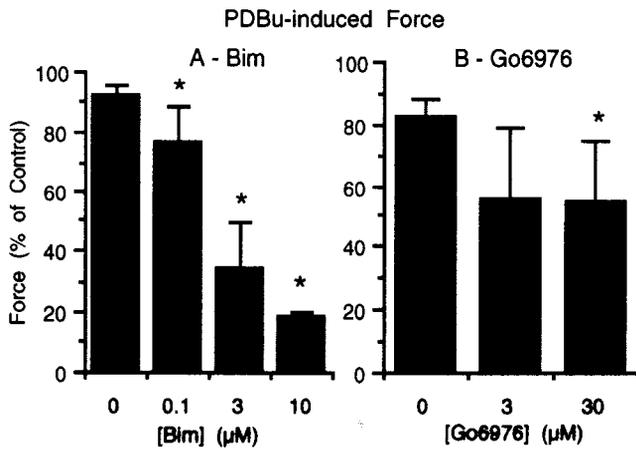


Fig. 6. Dose-response relation of protein kinase C inhibitors on phorbol-12,13-dibutyrate (PDBu)-induced force. Bim = bisindolylmaleimide I HCl, a protein kinase C inhibitor of conventional-novel protein kinase C; Go6976 = a conventional protein kinase C inhibitor. Mean  $\pm$  SD ( $n = 3$ ). Force at 15 min after administration of the inhibitor is expressed as percent of control force (steady state force before administration of the inhibitor). Dimethyl sulfoxide, 1%, was used to dissolve Go6976. \* $P < 0.05$  compared with that of time control at 0  $\mu\text{M}$  bisindolylmaleimide or Go6976. This figure shows that PDBu-induced force was dose-dependently decreased by bisindolylmaleimide (A), but not by Go6976, except at 30  $\mu\text{M}$  (B).

did not significantly inhibit PDBu-activated force, except at 30  $\mu\text{M}$  (fig. 6B).

#### Influence of PKC Inhibitors on Relaxation of Phorbol-12,13-dibutyrate (PDBu)-induced Force by Isoflurane

That Go6976 at low concentrations did not significantly affect the PDBu-induced force (fig. 6B) suggests that cPKC does not significantly contribute to the force generation. We next assessed whether isoflurane-induced relaxation was mediated by activation of cPKC by using the cPKC-inhibitors Go6976 and bisindolylmaleimide. In this study, pCa 6.5 buffer was used in PDBu-induced force because of consistency of the force generation. Under this experimental condition (PDBu in pCa 6.5 buffer), 3% isoflurane did not significantly affect the PDBu-induced force up to 60 min in the presence of 0.1% DMSO (control vehicle for Go6976). However, in the presence of isoflurane, the PDBu-induced force was significantly enhanced by 0.3  $\mu\text{M}$  Go6976 either at 5 min (peak contraction, fig. 7A) or 30 min (late relaxation, fig. 7B).

In the absence of 0.1% DMSO, we found that isoflurane also did not increase the PDBu-induced force. However, in these circumstances, force was also not significantly affected by the PKC inhibitor bisindolylmaleimide. Isoflurane did decrease the PDBu-induced force at a later phase ( $n = 3$ ;  $75.8 \pm 3.3$  and  $42.6 \pm 5.2$  for 0 and 3% isoflurane, respectively), which was again significantly prevented by 0.1  $\mu\text{M}$  bisindolylmaleimide ( $n = 3$ ;  $42.6 \pm 5.2$ ,  $35.6 \pm 8.6$ ,  $48.3 \pm 5.0$ , and

$56.7 \pm 5.0$  for 0, 0.01, 0.03, and 0.10  $\mu\text{M}$  bisindolylmaleimide, respectively).

## Discussion

The main findings of this study are as follows. (1) In skinned pulmonary arterial strips, isoflurane induced biphasic effects at clinically relevant concentrations. Therefore, isoflurane and halothane<sup>4</sup> have similar effects on pulmonary artery smooth muscle. These effects were characterized by increases followed by decreases in  $\text{Ca}^{2+}$ -activated or PDBu-induced force. (2) Isoflurane-induced increases in force were blocked by inhibition of c/nPKC bisindolylmaleimide but not by cPKC Go6976. (3) Isoflurane-induced decreases in force were prevented by both inhibitors.

The consistent isoflurane-induced relaxation at the later phase suggests that the anesthetic-induced PKC signaling pathway is triggered by the initial anesthetic concentration, which was not significantly changed within 10 min. The isoflurane-induced relaxation prevented by a threefold lower concentration of Go6976 (0.1  $\mu\text{M}$ ) than bisindolylmaleimide (0.3  $\mu\text{M}$ ) agrees with the median inhibitory concentration ( $\text{IC}_{50}$ ) shown *in vitro* for cPKC (2.3–6.2 nM for Go6976<sup>13</sup> and 8.40–20 nM for bisindolylmaleimide<sup>14</sup>). That the isoflurane-induced relaxation is more sensitive to the effect of bisindolylmaleimide in PDBu (0.1  $\mu\text{M}$  bisindolylmaleimide) than that

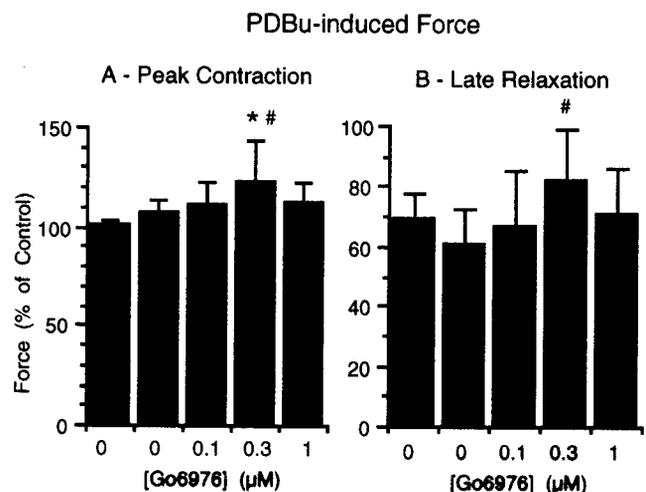


Fig. 7. Influence of conventional protein kinase C inhibitor Go6976 in isoflurane effect on phorbol-12,13-dibutyrate (PDBu)-induced force. Mean  $\pm$  SD ( $n = 4-7$ ). Force after administration of 3% isoflurane is expressed as percent of control force (steady state force before administration of isoflurane). \* $P < 0.05$  compared with that of the time control (0/0); #compared with that of isoflurane in the absence of Go6976 (0  $\mu\text{M}$ ). In the presence of 0.1% dimethyl sulfoxide (vehicle for Go6976), isoflurane did not significantly increase (0  $\mu\text{M}$  vs. 0/0; A) or decrease (0  $\mu\text{M}$  vs. 0/0; B) the PDBu-induced force. However, in the presence of 0.3  $\mu\text{M}$  Go6976, the force was significantly increased after administration of isoflurane either at 5 or 30 min.

in pCa (0.3  $\mu\text{M}$  bisindolylmaleimide)-induced force is in agreement with the suggestion of a predominant activation of nPKC by PDBu. This speculation is derived from the evidence of direct inhibition of PDBu-induced force with c/nPKC inhibitor (bisindolylmaleimide) but little with cPKC inhibitor (Go6976). In contrast, the higher concentrations of cPKC inhibitor, Go6976, are required to prevent the decreases in force by isoflurane in PDBu than in pCa-induced force. These findings could be due to the vehicle (DMSO) used in Go6976, possibly by DMSO inducing  $\text{Ca}^{2+}$  release from the SR (data not shown), resulting in higher activation of cPKC and thus less effectiveness of Go6976. This speculation remains to be confirmed.

The narrow range of effective concentrations of the PKC inhibitors could be due to the dual signaling pathways of PKC leading to contraction and relaxation as proposed previously (fig. 1)<sup>6</sup> and discussed later in this article. The partial prevention of relaxation by the PKC inhibitors suggests that mechanisms other than PKC also contribute to the isoflurane effects (e.g., CaMKII<sup>16</sup>).

The biphasic effects of isoflurane and the differential effect of the inhibitors from this study therefore suggest that PKC isoforms (nPKC and cPKC) have different roles in vascular smooth muscle contraction. nPKC and cPKC seem to be involved in contraction and relaxation of vascular smooth muscle, respectively. The biphasic responses of pulmonary arteries to isoflurane and halothane<sup>5</sup> were similar, suggesting anesthetics have comparable effects in the same arterial type. Whether halothane has the same mechanisms of action as isoflurane remains to be seen. In contrast, halothane and isoflurane have been shown to cause opposite effects on force induced by oleic acid (a cytosolic PKC agonist) in coronary arteries. In this case, halothane abrogates force, whereas isoflurane enhances force.<sup>3</sup> This apparent contradiction could be explained by differences in experimental conditions, arterial types, animal species, mechanisms, or by a differential role for PKC isoforms and requires further study.

A role for nPKC in vascular smooth muscle contraction is implied by the fact—shown in this and other studies—that peak force induced by isoflurane is blocked by high concentrations of bisindolylmaleimide, a c/nPKC inhibitor, but not by Go6976, an inhibitor of cPKC.<sup>4,5</sup> Furthermore, Horowitz *et al.*<sup>17</sup> have shown in single skinned vascular smooth muscle cells that the  $\epsilon$  isoenzyme of nPKC induces a  $\text{Ca}^{2+}$ -independent contraction. Kitazawa *et al.*<sup>10</sup> have also shown a component of  $\text{Ca}^{2+}$ -independent protein kinase C-induced contractile  $\text{Ca}^{2+}$  sensitization in Triton X-100-treated rabbit arterial smooth muscle.

Inhibition of isoflurane-induced relaxation by the c/nPKC (bisindolylmaleimide) or specific cPKC (Go6976) inhibitor at low concentrations in both pCa and PDBu-induced force suggests that relaxation is me-

diated through activation of cPKC by isoflurane. This possibility is consistent with evidence that in tracheal smooth muscle, purified cPKC $\alpha$ , as well as  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase II, phosphorylates MLCK, which would result in decreased MLCK activity and MLC phosphorylation<sup>7,16</sup> leading to decreased force.

The effectiveness of low concentrations (0.1–1  $\mu\text{M}$ ) of PKC inhibitors in preventing isoflurane-induced relaxation also suggests the specificity of the inhibition because *in vitro*, their  $\text{IC}_{50}$  is near 10 nM.<sup>13,14</sup> It is also possible that PKC at low activity induces relaxation that is sensitive to lower concentrations of the inhibitors. However, PKC at high activity induces contraction *via* a different pathway (see Zhong and Su,<sup>6</sup> fig. 1) (discussed in the following two paragraphs) that can only be blocked by high concentrations of the inhibitor. The ineffectiveness of higher concentrations of PKC inhibitors ( $\leq 1 \mu\text{M}$ ) in preventing the isoflurane-induced relaxation (fig. 3) could be that both relaxation and increased force (fig. 4A) by isoflurane are affected by the high concentrations of PKC inhibitors, resulting in no change in isoflurane-induced relaxation.

The enhanced isoflurane-induced relaxation by 10  $\mu\text{M}$  Go6976 observed in this study could be due to inhibition of both pathways (see Zhong and Su,<sup>6</sup> fig. 1), resulting in further decreases in force. However, this speculation cannot reconcile with the lack of inhibition of the isoflurane-increased force by Go6976. Whether DMSO (the vehicle for Go6976)-induced  $\text{Ca}^{2+}$  release from the SR (data not known) has a role remains to be examined.

The results of this study suggest the biphasic effects of isoflurane can be viewed within the framework of the following model (see Zhong and Su,<sup>6</sup> fig. 1).<sup>1</sup> Isoflurane directly activates PKC (nPKC). This is followed by translocation of nPKC from the cytosol to the membrane shown in cultured cells.<sup>6</sup> The translocated nPKC would then initiate signals, either by CPI-17<sup>10</sup> or by ERK–MLCK–MLC<sup>18,19</sup> signaling pathways, resulting in force generation (e.g., the isoflurane-increased force). Whether isoflurane induces nPKC translocation *via* ERK–MLCK–MLC signaling pathways remains to be determined.<sup>2</sup> Isoflurane induces  $\text{Ca}^{2+}$  release from the SR. This increased  $\text{Ca}^{2+}$  activates  $\text{Ca}^{2+}$ -dependent protein kinases, including cPKC. The increased cPKC activity would then directly phosphorylates MLCK, decreasing MLCK activity and decreasing MLC phosphorylation,<sup>7</sup> resulting in decreased force (isoflurane-induced relaxation) (see Zhong and Su,<sup>6</sup> fig. 1). This is consistent with the observations that halothane increases purified cPKC activity<sup>20</sup> and cytosolic cPKC activity in rat cerebrocortical synaptosomes.<sup>21</sup>

Alternately, cPKC signaling could also be *via* another pathway, ERK–MLCK–MLC signaling<sup>8,9,18,19</sup> resulting in increased force (isoflurane-increased force) (see Zhong and Su,<sup>6</sup> fig. 1). The fact that isoflurane increases PDBu-induced force under pCa 6.7 (fig. 6A) but not under pCa 6.5 (fig. 7A) also supports this speculation. It is specu-

lated that under low free  $\text{Ca}^{2+}$  buffer (pCa 6.7), PDBu activates predominantly nPKC so that further activation of cPKC by isoflurane would increase the PDBu-induced force (shown with isoflurane-induced peak contraction). However, under higher free  $\text{Ca}^{2+}$  buffer (pCa 6.5), cPKC would be partially activated by PDBu so that further activation of cPKC by isoflurane would result only in relaxation. If isoflurane-increased force were the result of nPKC activation, as suggested from the preferential inhibitory effect of bisindolylmaleimide (c/nPKC inhibitor) but not Go6976 (cPKC inhibitor),<sup>4,5,6</sup> the PDBu-induced force increased by isoflurane would be independent of pCa in the buffer. Thus, activation of cPKC by isoflurane-induced  $\text{Ca}^{2+}$  release from the SR could be *via* two different pathways: ERK-MLCK-MLC signaling leading to contraction and MLCK-MLC signaling leading to relaxation. This speculation remains to be examined.

The clinical implication of the results from this study is that isoflurane may cause transient pulmonary vasoconstriction followed by vasodilation in patients. Isoflurane may induce sustained pulmonary vasodilation in those patients with low SR  $\text{Ca}^{2+}$  stores (e.g., newborns) or with low PKC activity. If patients with pulmonary hypertension have low PKC activity, isoflurane could have some beneficial effects on pulmonary artery pressures.

In summary, isoflurane causes effects similar to those of halothane in pulmonary arteries. Isoflurane results in increased  $\text{Ca}^{2+}$ -activated force, followed later by decreased force, which may be in part mediated by activation of nPKC and cPKC, respectively.

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