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Role of Protein Kinase C, Ca^{2+}/Calmodulin-dependent Protein Kinase II, and Mitogen-activated Protein Kinases in Volatile Anesthetic–induced Relaxation in Newborn Rabbit Pulmonary Artery

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Background: **This study examined the responsiveness of skinned pulmonary arteries from newborn rabbit to volatile anesthetics and the role of protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and the downstream effectors, mitogen-activated protein kinases (ERK1/2 and p38).**

Methods: **Pulmonary arterial strips from 9- to 12-day-old rabbits were mounted on force transducers and treated with saponin ("skinned" strips). The skinned strips were activated by pCa 6.3 until force reached a steady state (control). Isoflurane or halothane was then administered. The result (test) was expressed as a percentage of the control. Inhibitors included bisindolylmaleimide (Ca^{2+}-dependent and -independent PKC), Gö6976 (Ca^{2+}-dependent PKC), CKIINtide (CaMKII), KN-93 (CaMKII), PD98059 (MEK/ERK1/2), and SB203580 (p38).**

Results: **The anesthetics dose-dependently decreased pCa-induced force (4–32% for 1–5% isoflurane; 17–76% for 1–3% halothane). The inhibitors of PKC (bisindolylmaleimide and Gö6976) and MEK/ERK1/2 (PD98059) completely prevented the relaxation induced by 3% isoflurane and partially prevented that induced by 2% and 3% halothane with the same effective inhibitor concentrations. In contrast, the effective concentration of CaMKII inhibitors was a direct function of the anesthetic concentration for different inhibitors (KN-93 for isoflurane and CKIINtide for halothane), and that of the p38 inhibitor (SB20358) was a direct function of both anesthetics.**

Conclusions: **In Ca^{2+}-clamped skinned pulmonary arterial strips from newborn rabbits, the anesthetics induce relaxation, which is prevented by the PKC inhibitors MEK/ERK/12, CaMKII, and p38. It is proposed that the anesthetic-induced relaxation is *via* cPKC/MEK/ERK1/2 and CaMKII/p38 pathways and, in addition, *via* CaMKII-p/MLCK-p(−)/MLC-p(−) for halothane.**

ON the basis of clinical impressions, volatile anesthetics induce greater hypotension in a newborn than an adult. The anesthetic-induced hypotension could be attributed to a direct effect on cardiac or vascular smooth muscle, resulting in myocardial depression or vasodilation or both. In the isolated intact cardiac muscle, halothane has been shown to cause greater depression of myocardial contraction in the newborn than the adult rabbit.[1] However, halothane induces depression to a lesser degree on the contractile proteins of the myocardium from a newborn than that from an adult.[2,3] Little information is available regarding the effect of volatile anesthetics on vascular smooth muscle from the newborn.

In adult vascular smooth muscle under Ca^{2+}-clamped conditions, we have shown that the anesthetics have differential effects in different arterial types.[4–6] Isoflurane causes a sustained contraction in the femoral artery[4] and a biphasic effect (increases followed by decreases) in the pulmonary artery.[5] We show further that the anesthetic-increased force is primarily *via* protein kinase C (PKC) signaling, whereas the anesthetic-induced late relaxation by isoflurane is predominantly *via* PKC[6] and that by halothane *via* Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in an activity-dependent manner.[7] This positive correlation between dose-dependent-induced relaxation and activation of CaMKII by halothane[7] is consistent with the Ca^{2+} release from the ryanodine or IP_3 receptor of the sarcoplasmic reticulum Ca^{2+} release channel[5] and the localization of CaMKII adjacent to the ryanodine or IP_3 receptor.[8] The evidence that PKC is activated predominantly by isoflurane and CaMKII by halothane is consistent with the amount of Ca^{2+} release from the sarcoplasmic reticulum by the anesthetics (isoflurane < halothane) and the lower effective concentrations of Ca^{2+} to activate PKC (ED_{50} for Ca^{2+} ≈100 nM)[9] than CaMKII (ED_{50} for Ca^{2+} ≈500 nM).[10] Whether isoflurane-induced relaxation is also modulated by CaMKII remains to be investigated.

It is hypothesized that in pulmonary artery from newborn as well as that from adult[6,7] rabbits, isoflurane and halothane induced relaxation *via* PKC and CaMKII signaling pathways. Accordingly, we examined the effects of the anesthetics on force development in skinned pulmonary arterial strips from newborn rabbits under low-Ca^{2+}–clamped condition. We also tested the roles of PKC and CaMKII and their possible downstream effectors mitogen-activated protein (MAP) kinase members (ERK1/2 and p38) using selective inhibitors of the enzymes. We found that in skinned pulmonary arterial strips from newborn rabbits, both anesthetics induced relaxation, but halothane to a greater extent. The inhibitors completely prevented the relaxation induced by isoflurane and only partially prevented that by halothane.

Materials and Methods

Materials

Thymol-free halothane was supplied by Peter Haines, B.S. (director of medical marketing, Halocarbon Labora-

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tories, Hackensack, NJ) and isoflurane from OHMEDA Inc. (Liberty Corner, NJ). The peptide of the inhibitor protein (CKIINtide)[11] for CaMKII was supplied by Debra Brickey, Ph.D. (research scientist), and Thomas Soderling, Ph.D. (professor), of Vollum Research Institute (Portland, OR). Inhibitors of PKC (Gö69769[12] and bisindolylmaleimide hydrochloride[13]), CaMKII (KN-93[14]), p38 (SB203580 hydrochloride[15]), and MEK/ERK1/2 (PD98059[16]) were purchased from Calbiochem (La Jolla, CA), RBI (Natick, MA), or Cell Signaling Technology (New England Biolabs, England, UK). Chemicals were analytical or reagent grade. Stock solutions of Gö6976 and PD98059 were made in 100% dimethyl sulfoxide, and the final concentrations of the inhibitors contained 0.1% dimethyl sulfoxide. The same concentrations of dimethyl sulfoxide were used in parallel for time controls.

Skinned Arterial Strips

The method of preparing skinned arterial strips has been described previously.[4–7,17] Briefly, newborn New Zealand White rabbits (9–12 days old) were killed with a decapitator. This method of euthanasia has been approved by University of Washington Animal Care Committee. The procedure for making the sarcolemma of pulmonary arterial strips to be permeable (300 μg/ml saponin in relaxing solution for 4 min) and the composition of the bathing solution (pCa buffer) were as described previously,[2] except that 10 mM EGTA was used. Isometric tension of the skinned strips was recorded on a computer (Tower Macintosh G3; Apple Computer, Inc., Cupertino, CA) with a customized LabVIEW software program interfaced with a multifunction I/O board with 16-bit resolution (NB-MIO-16XL; National Instrument, Austin, TX). The experiments were performed at room temperature (23 ± 2°C).

Experimental Procedure

The experimental procedure was the same as described previously.[4–7] The skinned strips were activated by Ca^{2+} buffered with 10 mM EGTA (pCa 6.3 or 6.0 buffer). When the force reached steady state (control force), a fresh solution with the same ionic composition containing one of the concentrations of halothane (1, 2, or 3%) (fig. 1) or isoflurane (1, 3, or 5%) was then administered, and the results were observed for up to 30 min (test force). At the end of each experiment, the maximum force of the strips was tested with pCa 4.0 buffer, which was used to estimate the control force as a percentage of the maximum force. The inhibitors were preincubated in a relaxing solution and were present in the subsequent contracting solutions. Time-control experiments (without anesthetic and inhibitor, or at one of the concentrations of anesthetic without inhibitor) were performed in parallel. The test results were analyzed at 15 or 30 min and were expressed as a percentage of the control.

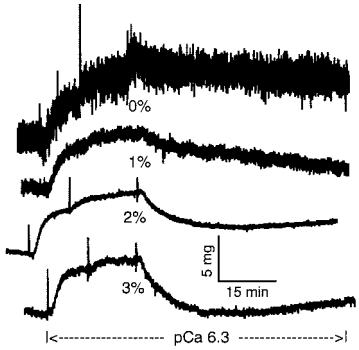

Fig. 1. Tracings showing the effect of different concentrations of halothane on pCa 6.3–induced force. *0%, 1%, 2%,* and *3%* = concentrations of halothane from four different arterial strips. Each preparation was activated with pCa 6.3 buffer, which was changed once (shown with an exchange artifact). When the force generated by pCa 6.3 reached a steady state (control), another fresh pCa 6.3 buffer saturated with one of the halothane concentrations was administered, and the result was observed for up to 60 min (test). The tracings show that halothane decreases pCa 6.3–induced force, which is increased with increased halothane concentrations from 1 to 3%.

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 conditions included (1) vehicle only (no inhibitor or anesthetic) as time control for anesthetic, (2) one of the anesthetic concentrations plus vehicle (no inhibitor) as time control for inhibitors, and (3) one of the anesthetic concentrations plus one of the inhibitor concentrations. The test results were expressed as a percentage of the control. Means and standard deviations of the mean were obtained from at least three arterial strips and three animals.

The 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 by Student *t* test. Two-factorial ANOVA was used to compare among anesthetic concentrations or various concentrations of the inhibitors. A value of $P < 0.05$ was considered statistically significant.[18]

Results

pCa–Tension Relationship in Skinned Pulmonary Arterial Strips from Newborn Rabbits

We found that pCa 6.3 (or 0.5 μM free Ca^{2+}) generated approximately [mean ± SD (n)] 13.7 ± 3.7 mg (3) or 43.0 ± 9.3% (3) of the maximum force generated by pCa 4.0 [31.3 ± 1.2 mg (3)], and pCa 6.0 (or 1 μM free Ca^{2+}) generated 20.1 ± 2.8 mg (3) or 63.2 ± 5.5% (3) of the maximum force from three different arterial strips and

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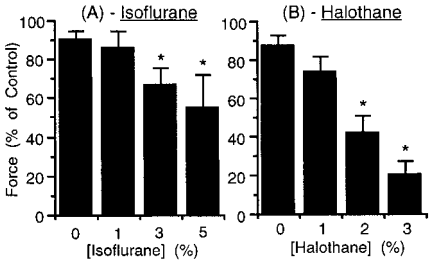

Fig. 2. Dose–response relationship of isoflurane and halothane. Force expressed as a percentage of the control in mean ± SD (n = 3–24). Isoflurane at 3 and 5% (*A*) and halothane at 1, 2, and 3% (*B*) decreased pCa-induced force in a dose-dependent manner. However, the decrease was not statistically significant between 3 and 5% isoflurane.

three newborn rabbits. We found that isoflurane caused greater relaxation in pCa 6.3– than pCa 6.0–activated force, but the relaxation induced by halothane was not significantly different between these pCa values. Therefore, pCa 6.0 was chosen to study the effects of halothane in skinned strips, which generated a force lower than 10 mg at pCa 6.3.

Effects of Isoflurane and Halothane on pCa-induced Force

As shown in figures 1 and 2, isoflurane and halothane decreased the pCa-induced force. The force decreased by 3 and 5% isoflurane was statistically significantly different from that of 0% isoflurane (fig. 2, *A*) [% of time control as 100% (0%, fig. 2, *A*); 96.3, 76.7, and 68% for 1, 3, and 5% isoflurane, respectively]. However, the relaxation induced by isoflurane was not statistically significantly different between 3 and 5% isoflurane. The pCa-induced force was dose-dependently decreased by halothane (fig. 2, *B*) [82.5, 45.2, and 24% of the time control as 100% (0%, fig. 2, *B*) for 1, 2, and 3% halothane, respectively]. In contrast to the observations in the adult rabbits,[6,7] however, the anesthetics did not induce a transient increase in force.

Influence of PKC Inhibitors on the Anesthetic-induced Relaxation

The relaxation induced by 3% isoflurane was prevented by bisindolylmaleimide (3 and 30 nM) in a dose-dependent manner and was completely prevented by 30 nM bisindolylmaleimide (3%, fig. 3, *A*), which is similar to the concentration to inhibit 50% of Ca^{2+}-dependent PKC (cPKC) activity *in vitro*.[13] In contrast, the relaxation induced by 5% isoflurane was not prevented but rather enhanced by 30 nM bisindolylmaleimide (5%, fig. 3, *A*). Conversely, the relaxation induced by halothane was partially prevented by higher concentrations of bisindolylmaleimide and in a dose-dependent manner (0.3 and 1 μM, fig. 3, *B*) for both 2% and 3% halothane.

The role of cPKC in the anesthetic-induced relaxation was examined further by use of a specific cPKC inhibitor, Gö6976.[12] We found that the relaxation induced by isoflurane was also prevented by Gö6976 up to 0.01 μM in a dose-dependent manner and was completely prevented by 0.01 μM Gö6976 for both 3 and 5% isoflurane (fig. 4, *A*). The relaxation induced by halothane was partially prevented by a higher dose range of Gö6976 (0.001–0.1 μM), and the maximum effective concentration of Gö6976 was 0.1 μM for both 2 and 3% halothane (fig. 4, *B*).

Influence of the Inhibitors of CaMKII on the Anesthetic-induced Relaxation

Because halothane-induced relaxation was partially prevented by the PKC inhibitors, we hypothesized that the halothane-induced relaxation was mediated in part by a CaMKII signaling pathway, as shown in the adult rabbits.[7] We used the CaMKII inhibitor KN-93 by competing with the calmodulin binding site[14] or the peptide of the inhibitor protein (CKIINtide) by noncompetitive binding to the catalytic site.[11]

We found that the isoflurane-induced relaxation was again completely prevented by 10 nM CKIINtide for 3 and 5% isoflurane (fig. 5, *A*) and that of halothane was partially prevented by CKIINtide (10 and 30 nM for 2% halothane and 30 nM for 3% halothane; fig. 5, *B*). In contrast, KN-93 dose-dependently and completely prevented the relaxation induced by 3% (0.1 μM KN-93) and 5% (1 μM KN-93) isoflurane (fig. 6, *A*). However, 2 and 3% halothane-induced relaxation was partially prevented by 0.01 μM KN-93 for 2% halothane and 0.01, 0.1, and 0.3 μM KN-93, reaching a plateau at 0.01 μM (fig. 6, *B*).

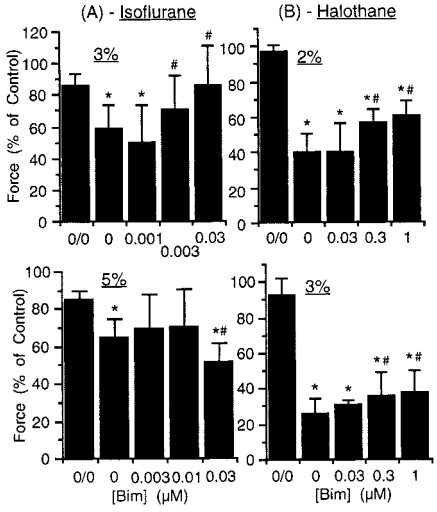

Fig. 3. Influence of c/nPKC inhibitor bisindolylmaleimide on the anesthetic-induced relaxation. Force is expressed as a percentage of control (steady-state force before the administration of the anesthetic) in mean ± SD (n = 3–7); [Bim] (μM) = concentrations of c/nPKC inhibitor bisindolylmaleimide II HCl in μM; one of the anesthetic concentrations was present except at 0/0 (time control), in which neither anesthetic nor Bim was present; *$P < 0.05$ compared with that of the time control (0/0); #$P < 0.05$ compared with that of the anesthetic in the absence of Bim (0 μM).

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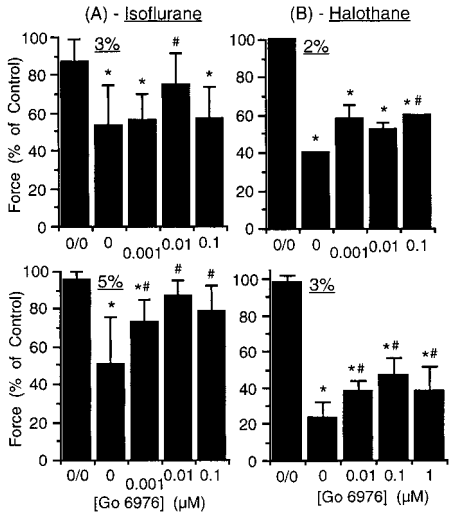

Fig. 4. Influence of cPKC inhibitor Gö6976 on the anesthetic-induced relaxation. Force is expressed as a percentage of the control (steady-state force before the administration of the anesthetic) in mean ± SD (n = 3–6); [Gö6976] (μm) = concentration of cPKC inhibitor Gö6976 in μM; one of the anesthetic concentrations was present except at 0/0 (time control), in which neither anesthetic nor Gö6976 was present; *P < 0.05 compared with that of the time control (0/0); #P < 0.05 compared with that of the anesthetic in the absence of Gö6976 (0 μM).

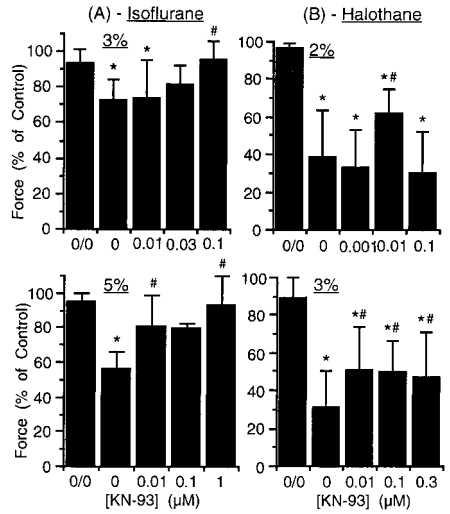

Fig. 6. Influence of the CaMKII inhibitor KN-93 on the anesthetic-induced relaxation. Force expressed as a percentage of the control in mean± SD (n = 3–9); [KN-93] (μM) = concentrations of CaMKII inhibitor KN-93 in μM; one of the anesthetic concentrations was present except at 0/0 (time control) in which neither anesthetic nor KN-93 was present; *P < 0.05 compared with that of the time control (0/0); #P < 0.05 compared with that of the anesthetic in the absence of KN-93 (0 μM).

Influence of Inhibitors of MAP Kinases (p38 and ERK1/2) on the Anesthetic-induced Relaxation

In cultured vascular smooth muscle cells from rabbit femoral arteries, we have shown that isoflurane may activate PKC and CaMKII *via* one of the MAP kinases, extracellular signal–regulated kinases (ERK1/2).[19] Another MAP kinase, p38 MAP kinase, has also been implicated in angiotensin II–induced vascular smooth muscle contraction.[20] Accordingly, we examined further the role of ERK1/2 and p38 in the anesthetic-induced relaxation using a selective inhibitor of MEK/ERK1/2 (PD98059,[15] noncompetitive with respect to ATP binding to MEK) or p38 (SB203580[16]).

As shown in figure 7, PD98059 at 1 μM completely prevented 3% isoflurane–induced relaxation (3%, fig. 7, *A*) but not that of 5% isoflurane (5%, fig. 7, *A*). The relaxation induced by 2 and 3% halothane was partially prevented by PD98059, which was statistically significant at 0.1 and 1 μM PD98059 for 2% halothane and at 1 μM PD98059 for 3% halothane (fig. 7, *B*).

An inhibitor of p38 mitogen-activated kinase, SB203580, fully prevented the relaxation induced by isoflurane (0.03 and 0.3 μM SB203580 for 3 and 5% isoflurane, respectively; fig. 8, *A*) and partially prevented that of halothane (3 and 10 nM SB203580 for 2% halothane peak at 3 nM SB203580; 10 and 30 nM SB203580 for 3% halothane peak at 30 nM SB203580; fig. 8, *B*).

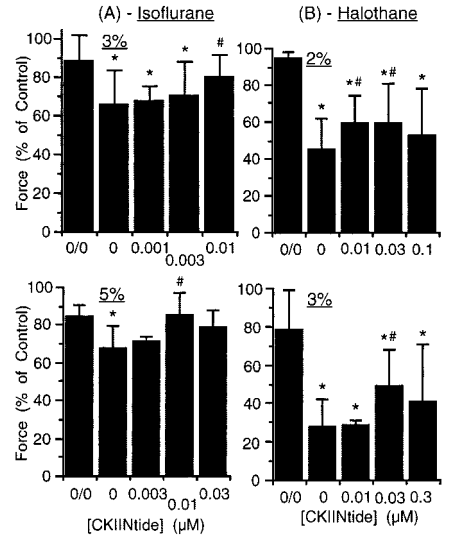

Fig. 5. Influence of the CaMKII inhibitor CKIINtide on the anesthetic-induced relaxation. Force is expressed as a percentage of the control in mean ± SD (n = 3–8); [CKIINtide] (μM) = concentrations of the peptide of the inhibitor protein of CaMKII in μM; one of the anesthetic concentrations was present except at 0/0 (time control), in which neither anesthetic nor CKIINtide was present; *P < 0.05 compared with that of the time control (0/0); #P < 0.05 compared with that of the anesthetic in the absence of CKIINtide (0 μM).

Discussion

The exciting findings and implications from this study in the skinned pulmonary arterial strip from the newborn rabbit under low-Ca^{2+}–clamped condition are that (1) isoflurane and halothane induce dose-dependent late relaxation; (2) the inhibitors of PKC, CaMKII or MAP kinases ERK1/2 and p38 completely prevents the relaxation caused by isoflurane but partially prevents that by halothane; (3) a similarity of the pattern of effective inhibitor concentrations of bisindolylmaleimide or

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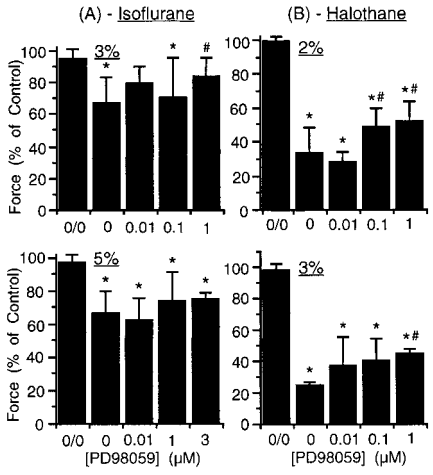

Fig. 7. Influence of the MEK/ERK1/2 MAP kinase inhibitor PD98059 on the anesthetic-induced relaxation. Force is expressed as a percentage of the control in mean ± SD (n = 3–9); [PD98059] (μM) = concentrations of the MEK inhibitor in μM; one of the anesthetic concentrations was present except at 0/0 (time control), in which neither anesthetic nor SB20358 was present; *$P < 0.05$ compared with that of the time control (0/0); #$P < 0.05$ compared with that of the anesthetic in the absence of PD98059 (0 μM).

Gö6976 and PD98059 with respect to anesthetic concentrations suggests that ERK1/2 is a downstream effector of PKC,[19,20] possibly cPKC; (4) the effective inhibitor concentrations of KN-93 (for isoflurane), CKIINtide (for halothane), or SB20358 as a direct function of the anesthetic concentrations suggest that p38 is a downstream effector of CaMKII[21,22]; (5) the isoflurane-induced relaxation completely prevented by the inhibitors suggests that there is cross talk between CaMKII and PKC,[23–25] between PKC and p38,[23] between CaMKII and ERK1/2,[26–28] and between ERK1/2 and p38[29]; and (6) a direct correlation between concentrations of CKIINtide and halothane suggests that CaMKII also acts *via* myosin light chain kinase.[30] Thus, the anesthetic-induced relaxation is *via* PKC, CaMKII, and MAP kinases in a Ca^{2+}-,[31] activity-, isozyme-, and time-dependent[32] manner.

The degree of relaxation induced by isoflurane or halothane (except at 1%) shown in this study in newborn rabbits is comparable to that observed in the adult rabbit[6,7] but without the transient increase in force. This lack of biphasic effects of the anesthetics in the newborn rabbit could be a result of low Ca^{2+} in the sarcoplasmic reticulum to be released by the anesthetics, resulting in lower activation of the PKC and CaMKII. Further investigation with respect to the Ca^{2+} release mechanisms would confirm the above speculation.

The effective concentrations of the inhibitors of PKC in preventing the relaxation, however, are not consistent between newborn and adult rabbits with respect to isoflurane and halothane. At 3% isoflurane, the relaxation prevented by the PKC inhibitor bisindolylmaleimide (0.03 μM, fig. 9, *A*) is lower than that observed in the adult (0.3 μM[6]). In contrast, the halothane-induced relaxation prevented by bisindolylmaleimide is higher in the newborn (1 μM, fig. 9, *B*) than in the adult (0.01 μM[7]). Using the effective inhibitor concentrations as an estimate of the enzyme activity, PKC would be activated by isoflurane to a lesser degree and by halothane to a greater degree in the newborn than the adult, suggesting that PKC plays a more important role in the halothane-induced relaxation in the newborn than the adult.

The pattern of the concentrations of inhibitors of PKC and MEK/ERK1/2 with respect to isoflurane (fig. 9, *A*) or halothane (fig. 9, *B*) suggests that MEK/ERK1/2 is a downstream effector of PKC resulting in anesthetic-induced relaxation. However, the lack of changes in effective inhibitor concentrations of PKC with respect to anesthetic concentrations [0.01 μM Gö6976 for 3 and 5% isoflurane (fig. 9, *A*); 0.1 μM Gö6976 or 1 μM bisindolylmaleimide for 2 and 3% halothane (fig. 9, *B*)] suggests activation of cPKC resulting in relaxation that is independent of PKC activity measured under our experimental conditions. The facts that relaxation induced by 5% isoflurane is enhanced by higher concentrations of bisindolylmaleimide [a cPKC/Ca^{2+}-independent (c/nPKC) inhibitor] and prevented by 0.01 μM Gö6976 (a cPKC inhibitor) suggest that nPKC plays a role in the contraction induced by isoflurane. Whether high isoflurane concentration (5%) has a direct inhibitory effect on PKC, like that reported in halothane,[33] remains to be examined. Thus, PKC could lead to relaxation *via* MEK/ERK1/2 (fig. 9, *C*)[19,20] or contraction possibly *via* CPI-17 (fig. 9, *D*).[34] A direct measurement of activity of PKC and MEK/ERK1/2 would confirm this speculation. The effectiveness of the inhibitors of PKC suggests that phospholipids (such as diacylglycerol) and Ca^{2+} (fig. 9C and D) contribute to activation of PKC.[35]

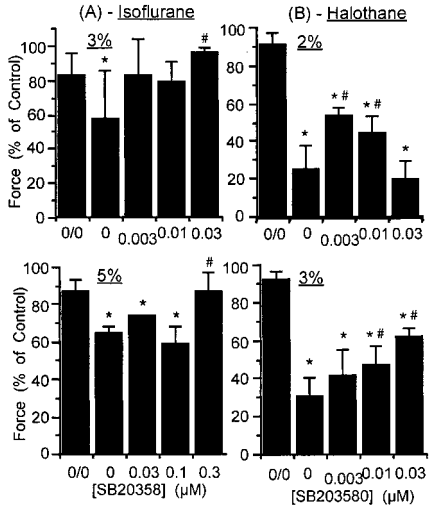

Fig. 8. Influence of the p38 MAP kinase inhibitor SB20358 on the anesthetic-induced relaxation. Force is expressed as a percentage of the control in mean ± SD (n = 3–7); [SB20358] (μM) = concentrations of p38 inhibitor in μM; one of the anesthetic concentrations was present except at 0/0 (time control), in which neither anesthetic nor SB20358 was present; *$P < 0.05$ compared with that of the time control (0/0); #$P < 0.05$ compared with that of the anesthetic in the absence of SB20358 (0 μM).

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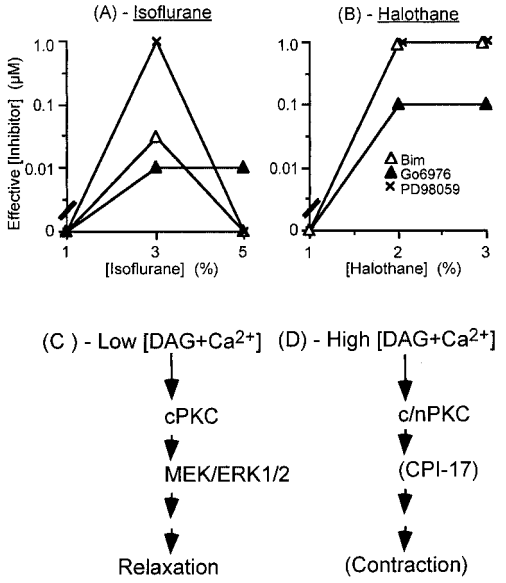

Fig. 9. Analysis of effective inhibitor concentrations of PKC and MEK/ERK1/2 with respect to anesthetic concentrations and proposed signaling pathways. Effective [Inhibitor] (μM) = effective inhibitor concentration in μM to prevent the relaxation induced by the anesthetics derived from figs. 3, 4, and 7; Bim (*open triangles*) = bisindolylmaleimide, a c/nPKC inhibitor; Gö6976 (*filled triangles*) = a cPKC inhibitor; PD98059 (*x*) = an inhibitor of MEK/ERK1/2; DAG = diacylglycerol; CPI-17 = a protein phosphorylated by PKC.[34] *A* and *B* show a similarity in patterns between inhibitors of cPKC (Gö6976 between isoflurane and halothane) and c/nPKC (Bim) and MEK/ERK1/2 (PD98059) with respect to isoflurane or halothane. Based on the similar pattern between the effective concentrations of the inhibitors, we propose that DAG at a lower level in the presence of Ca^{2+} activates cPKC *via* MEK/ERK1/2, leading to relaxation (*C*). At higher DAG associated with higher Ca^{2+} (*D*) (produced by 5% isoflurane), c/nPKC could be activated to a higher level *via* CPI-17 phosphorylation/myosin light chain phosphatase inhibition/increased myosin light chain phosphorylation, leading to force generation.[34]

The effective concentrations of KN-93 (competing with the calmodulin site of CaMKII[14]) but not that of CKIINtide (binding to the catalytic site of autophosphorylated CaMKII[11]) as a direct function of isoflurane suggest that CaMKII is activated by Ca^{2+}/calmodulin to a lower activity (0.01 μM CKIINtide in the autophosphorylated state; fig. 10, *A*). In contrast, a positive correlation between effective concentrations of CKIINtide (0.1 and 1 μM) and halothane (2 and 3%) but not that of KN-93 suggests that higher Ca^{2+} is released by halothane, which activates CaMKII, resulting in higher activity (0.03 μM CKIINtide in the autophosphorylated state; fig. 10, *B*).

The absence of increasing effectiveness of CKIINtide for isoflurane and that of KN-93 for halothane can be speculated on as follows. At low Ca^{2+} levels (released by isoflurane), CaMKII is initially activated by binding of Ca^{2+}/calmodulin, which is blocked by KN-93 by competing for the calmodulin binding site[14] in a dose-dependent manner, whereas CaMKII undergoes autophosphorylation[10] in a low-activity state blocked by CKIINtide (fig. 10, *A*). In contrast, at higher Ca^{2+} levels (released by halothane), the initial Ca^{2+}/calmodulin binding and activation of CaMKII (blocked by KN-93; fig. 10, *B*, *open circles*) result in a higher autophosphorylated state (or a higher activity) that increases with increases in Ca^{2+} levels (blocked by CKIINtide; fig. 10, *B, filled circles*).

In contrast to PKC, the increased effective concentrations of the inhibitors of CaMKII or p38 as a direct function of anesthetic concentration (KN-93 for isoflurane, CKIINtide for halothane) observed in this study have also been reported in the adult rabbits with halothane.[7] Thus, a role for CaMKII/p38 signaling[21,22] in modulation of vascular smooth muscle relaxation (fig. 10, *C*) is further confirmed. The complete prevention of isoflurane-induced relaxation by the inhibition of PKC, CaMKII, p38, and ERK1/2 suggests cross talk among these protein kinases, possibly CaMKII/PKC,[23–25] PKC/p38,[23] CaMKII/ERK1/2,[19,26–28] and between ERK1/2 and p38[29] but not PKC/CaMKII.[36] The halothane-induced relaxation partially prevented by the inhibitors of CaMKII suggests that CaMKII/MLCK/MLC signaling (fig. 10, *D*)[30] may also play a role.

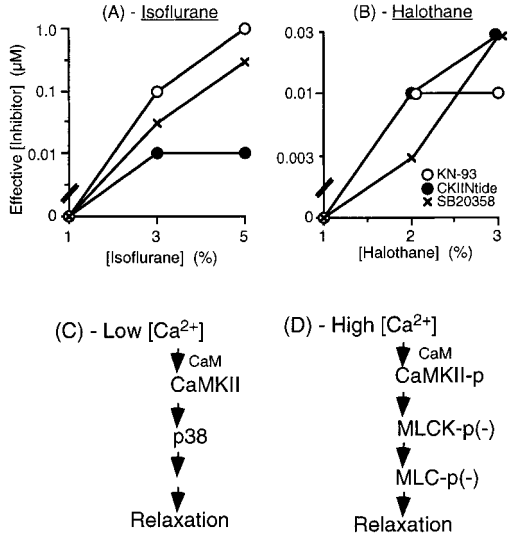

Fig. 10. Analysis of the effective inhibitor concentrations of CaMKII and mitogen-activated kinase p38 with respect to anesthetic concentrations and proposed signaling pathways. Effective [Inhibitor] (μM) = effective inhibitor concentration in μM to prevent the relaxation induced by the anesthetics derived from figs. 5, 6, and 8; KN-93 (*open circles*) = a CaMKII inhibitor competing at the calmodulin site; CKIINtide (*filled circles*) = the peptide of the inhibitor protein of CaMKII binding to the catalytic site of autophosphorylated CaMKII (CaMKII-p); SB20358 (*x*) = an inhibitor of p38 MAP kinase; MLCK-p(−) = phosphorylated myosin light chain kinase (MLCK-p), resulting in decreased MLCK activity; MLC-p(−) = decreased phosphorylated myosin light chains. *A* and *B* show a similar pattern of effective concentrations of KN-93 and SB20358 as a direct function of isoflurane (*A*), those of CKIINtide and SB20358 as a direct function of halothane (*B*). This similarity in patterns between inhibitors of CaMKII and p38 suggests that Ca^{2+} activates CaMKII *via* p38 signaling leading to relaxation (*C*). In contrast, at higher Ca^{2+} (released by halothane), CaMKII undergoes autophosphorylation to a higher CaMKII activity,[10] which phosphorylates MLCK, resulting in decreased MLCK activity and decreased myosin light chain phosphorylation,[30] leading to relaxation (*D*).

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The effectiveness of CaMKII inhibitors suggests that Ca^{2+} and calmodulin play a role in activation of CaMKII. Whether the anesthetics induce Ca^{2+} release from the sarcoplasmic reticulum in newborn pulmonary artery, as shown in the adult,[5] remains to be studied. Further investigations are needed as to factors such as phospholipids and Ca^{2+} levels,[37,38] Ca^{2+} release mechanisms by isoflurane and halothane, localization, or translocation between the membrane and the cytosol for PKC[32] and CaMKII resulting in differential effectiveness of the inhibitors and anesthetic- and time-dependent relaxation.

The similar degree of anesthetic-induced relaxation induced by the volatile anesthetics between newborn and adult rabbits suggests that the clinical impression of greater vasodilatation in the newborn than the adult may be attributed more to factors (such as endothelium, sensitivity of receptor on the plasma membrane, etc.) regulating vascular smooth muscle contraction than by the direct effects on the contractile proteins *via* intracellular signaling pathways shown in this study.

In summary, PKC, CaMKII, and the downstream effectors MAP kinases play a role in the anesthetic-induced relaxation in the pulmonary artery of newborn rabbits. CaMKII, in an activity-dependent manner, modulates concentration-dependent relaxation induced by both anesthetics, and PKC, depending on activity, modulates both relaxation and contraction in skinned pulmonary arterial strips of newborn rabbits.

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