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Isoflurane Activates PKC and Ca^{2+}–Calmodulin-dependent Protein Kinase II via MAP Kinase Signaling in Cultured Vascular Smooth Muscle Cells

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Background: **Protein kinase C (PKC) and Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) have been implicated in isoflurane-increased force in skinned femoral arterial strips. The extracellular signal–regulated kinases (ERK1/2) of mitogen-activated protein kinase have been shown to be target effectors of PKC and CaMKII. This study examined the role of the ERK1/2 signaling pathway in isoflurane activation of PKC and CaMKII using cultured vascular smooth muscle cells.**

Methods: **Vascular smooth muscle cells were prepared by cell migration from isolated rabbit femoral arterial segments. Growth of passage of vascular smooth muscle cells (80–90% confluence, passage 5–10) was arrested for 48 h before experiments, during which time phorbol 1,3-diaceylester treatment was used to down-regulate PKC. Cells were treated for 30 min with one of the inhibitors of mitogen-activated protein kinase kinase (PD98059), PKC (Go6976 and bisindolylmaleimide), or CaMKII (KN-93 and KN-62) at 10 μM. After administration of isoflurane, vascular smooth muscle cells were frozen rapidly, homogenized, and centrifuged. The homogenates were used for identification of phosphorylated ERK1/2 or for further centrifugation to separate the membrane from the cytosol for identification of PKC isoforms (α and ε) by Western blotting.**

Results: **Isoflurane increased ERK1/2 phosphorylation in a dose-dependent manner and reached a plateau at 10 min. PD98059 or down-regulated PKC blocked the increase of phosphorylated ERK1/2 levels by isoflurane, and bisindolylmaleimide, KN-93, or KN-62, but not by Go6976 reduced levels of phosphorylated ERK1/2. The membrane fraction of PKCε but not of PKCα was increased by isoflurane.**

Conclusions: **ERK1/2 signaling is downstream of PKC and CaMKII activated by isoflurane in vascular smooth muscle cells.**

IN the mitogen-activated protein (MAP) kinase cascade, extracellular signal–regulated kinase (ERK1/2) is important for agonist-activated apoptosis, proliferation, differentiation, and migration of cells. MAP kinase communicates by the translocation of enzymes to target sites where they interact with other effectors (for review, see Cobb[1]). Protein kinase C (PKC) is an important initiator of ERK1/2 signaling (for review, see Schonwasser *et al.*[2]).

Studies indicate that PKC is activated or inhibited by volatile anesthetics in various tissue preparations. In synaptosomes, halothane and propofol cause translocation of PKC to the membrane fraction.[3,4] Park *et al.*[5] showed that halothane decreases and isoflurane enhances PKC-induced contraction in isolated intact coronary arterial rings. In skinned arterial strips, submaximum Ca^{2+}-activated force that is increased by isoflurane in femoral arteries[6] or halothane in pulmonary arteries[7] is blocked by the PKC inhibitor bisindolylmaleimide, which inhibits both Ca^{2+}-dependent PKC (or conventional PKC [cPKC], including α, βI, βII, and γ) and Ca^{2+}-independent PKC (or novel PKC [nPKC], including δ, ε, η, and θ) (for review, see Parekh *et al.*[8]). However, the anesthetic-increased force[6,7] is not affected by the specific cPKC inhibitor Go6976. These results suggest that the anesthetics activate isotype-specific PKC (*i.e.*, activation of nPKC), resulting in increased force in vascular smooth muscle. Whether this anesthetic-increased force (i, fig. 1) by activation of PKC is *via* ERK1/2 signaling pathway is not known.

The multifunctional Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) has been shown to have a role in the anesthetic effects on relaxation (ii, fig. 1) of vascular smooth muscle. Toda and Su[6] have shown that isoflurane-increased submaximum Ca^{2+}-activated force was further enhanced by the CaMKII inhibitor KN-62. Moreover, the isoflurane-decreased myosin light chain (MLC) phosphorylation was prevented by KN-62.[6] These observations[6] can be interpreted as isoflurane activates CaMKII, resulting in decreased MLC phosphorylation and relaxation. This is consistent with the observation in tracheal smooth muscle that CaMKII activation by ionomycin is associated with decreased myosin light chain kinase (MLCK) activity and decreased MLC phosphorylation,[9,10] which would result in decreases in force development. However, in cultured vascular smooth muscle, ionomycin-increased ERK1/2 phosphorylation is shown to be blocked by another CaMKII inhibitor, KN-93,[11,12] suggesting a different pathway for CaMKII, *i.e.*, by ERK1/2 signaling, which is associated with agonist-induced force development.[13,14]

This study sought to determine whether isoflurane activates CaMKII and specific isoforms of PKC *via* ERK1/2 signaling (ii, fig. 1). Therefore, we examined the effect of isoflurane on ERK1/2 phosphorylation and on the translocation of PKC (nPKCε or cPKCα), and the influence of inhibitors of PKC, MAP kinase kinase (MEK), or CaMKII on the isoflurane effect in smooth muscle cells cultured from rabbit femoral arteries.

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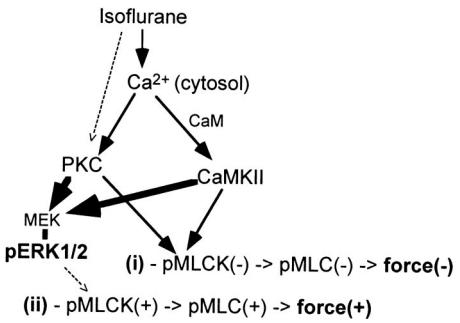

Fig. 1. Schematic diagram of signaling pathways affected by isoflurane in vascular smooth muscle. The diagram describes that isoflurane releases Ca^{2+} from the sarcoplasmic reticulum, which activates Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) or protein kinase C (PKC). CaM = calmodulin; MEK = mitogen-activated protein kinase kinase. Two possible signaling pathways for CaMKII and PKC are as follows: (*i*) direct phosphorylation of myosin light chain kinase (pMLCK) resulting in decreased activity (pMLCK(−)), to decreased phosphorylation of myosin light chains (pMLC(−)), and to decreased force (force(−)); and (*ii*) *via* activation of extracellular signal–regulated kinase (pERK1/2) signaling pathway, which may phosphorylate MLCK at a different site, resulting in increased activity (pMLCK(+)), to increased MLC phosphorylation (pMLC(+)), and to increased force (force(+)).

Materials and Methods

Materials

The following chemicals were supplied by RBI/Sigma (Natick, MA) or Calbiochem-Novabiochem (San Diego, CA): KN-62 and KN-93 (competitive inhibitors of CaMKII at the calmodulin site); PD98059 (a MEK inhibitor); bisindolylmaleimide I-HCl, an inhibitor of Ca^{2+}-dependent PKC (cPKC) and Ca^{2+}-independent PKC (nPKC); Go-6976 (an inhibitor of cPKC); PKC activators, including phorbol-12-myristate-13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu); and protease inhibitors. Polyclonal antibodies against phosphorylated or nonphosphorylated ERK1/2, cPKCα, βI, and βII, and nPKCε were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For Western blots, secondary anti-rabbit or anti-goat antibodies labeled with horseradish peroxidase (Sigma, St. Louis, MO) were detected by luminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Dulbecco's modified Eagle media (DMEM) were from Life Technologies (Rockville, MD). Other reagents were of analytical grade.

Cell Culture

Cultured vascular smooth muscle cells (VSMCs) were derived from rabbit femoral arteries using cell migration.[15] Male New Zealand white rabbits (1.5–2.0 kg) were killed using a captive bolt pistol, followed by exsanguination. Handling and killing of rabbits were approved by the University of Washington Animal Care Committee (Seattle, WA). Femoral arteries were isolated, cut into segments of 1–2 mm, placed in DMEM with 20% fetal bovine serum, and incubated at 37°C in 5% CO_2. VSMCs began to migrate from the suspended explants and adhere in 5–10 days. Cells were maintained in DMEM containing 10% fetal bovine serum and passaged every 3–5 days. VSMCs were identified by immunohistochemical method with antibody to smooth muscle cell-specific α actin (DAKO EPOS anti-human smooth muscle actin/HRP; DAKO A/S, Carpinteria, CA) and desmin (monoclonal mouse anti-human desmin; DAKO A/S) (fig. 2). Cells from the second week of migration (> 99% smooth muscle cells; fig. 2) were used for subsequent passages.

Experimental Procedure

For experimental studies, VSMCs from the 5th–10th passages were grown to 80–90% confluence and starved in 0.05% fetal bovine serum–DMEM for 48 h to arrest growth. Before experimentation, the VSMCs in 35- or 100-mm culture dishes were placed at room temperature for 30 min.

The effects of isoflurane on the quiescent VSMCs were studied in four experimental procedures. In the first procedure, VSMCs at room temperature were immersed in fresh DMEM containing 3% isoflurane for 30 s and 1, 2, 5, 10, 30, and 60 min (time course) or at various concentrations of 0, 1, 3, and 5% isoflurane for 10 min (dose response). In the second procedure, VSMCs were treated with 1 μM PDBu during the 48-h starvation pe-

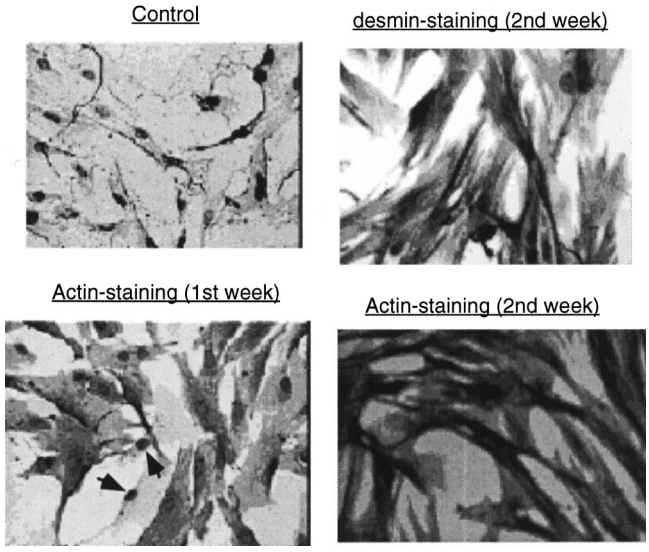

Fig. 2. Immunocytochemical identification of smooth muscle cells cultured from rabbit femoral arteries. Using smooth muscle–specific α-actin and desmin antibodies, the smooth muscle cells cultured from femoral arteries were identified with staining of the antibodies, but not those of fibroblasts or endothelial cells. The nuclei are stained blue with hematoxylin (black circles). Smooth muscle cells were stained with brown precipitation of DAB for desmin and actin (dark areas), and some nonsmooth muscle cells from the first week of migration are not stained (arrows, actin-staining [first week]). This figure shows that the cultured cells from femoral arteries were mostly (> 99%) smooth muscle cells from the second week of migration.

riod to down-regulate PKC,[16] and 3% isoflurane was tested. In the third procedure, VSMCs were treated with the inhibitors of PKC (bisindolylmaleimide and Go6976) or CaMKII (KN-93 and KN-62) for 30 min at room temperature before administration of 3% isoflurane. In the fourth procedure, the localization of PKC isoforms (α and ε) in VSMCs and the effect of 3% isoflurane on the translocation of PKC were examined. For positive control of PKC translocation, VSMCs were treated with the activator phorbol-12-myristate-13-acetate (PMA) for 30 min.

Isoflurane saturated in DMEM (approximately 1.8 ml for a 35 mm-dish and approximately 10 ml for a 100-mm dish) was quickly administered into covered cultured dishes for a specific period of time. The partial pressure of isoflurane in DMEM, expressed as a percentage of 1 atmosphere, was assayed by gas chromatography.[17]

Vascular smooth muscle cells used in the first three experimental procedures were rapidly frozen in 2-propanol–dry ice, suspended in a lysate buffer with 0.2% Triton X-100, and sonicated. The lysate buffer contained 250 mM sucrose, 5 mM EDTA, 10 μg/ml aprotinin, 20 μg/ml soybean tyrosine inhibitor, 100 μg/ml PMSF, 100 μg/ml leupeptin, 10 mM HEPES (pH 7.4), and 5 μM each of phosphoserine, phosphothreonine, phosphotyrosine, β-glycerophosphate, p-nitrophenylphosphate, and sodium orthovanadate.[11]

Homogenates were centrifuged at 14,000*g* for 30 min. The supernatants (samples) from the homogenates were assayed for protein concentrations using bicinchoninic acid (BCA) reagents (Pierce Chemical Co., Rockford, IL) and stored at −70°C.

Immunoblot Analysis of ERK1/2

In each experiment, samples were used at equal concentrations of total protein, which ranged from 20 to 30 μg. Proteins were separated by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were treated with anti-phospho-ERK1/2 antibody (1:1,500) followed by horseradish peroxidase–conjugated anti-mouse antibody (1:5,000). Total ERK1/2 was used to validate changes in phosphorylated ERK1/2 affected by isoflurane and was measured from the same membrane after stripping and staining with anti-ERK1/2 antibody (1:1,500) followed by anti-goat antibody (1:5,000). Immunoreactive bands were visualized using chemiluminescence (ECL Amersham Pharmacia Biotech, Piscataway, NJ, and Santa Cruz Biotechnology, Inc.) and evaluated with image analysis software. The amount of phosphorylated ERK1/2 was expressed as a percentage of the control.

Immunoblot Analysis of PKC in the Cytosol and Membrane

In the fourth experimental procedure, treated VSMCs were frozen in cooled 2-propanol–dry ice, suspended in lysate buffer (as described for ERK1/2 immunoblotting), and sonicated. Homogenates were centrifuged at 1,000*g* for 30 min, the supernatant was collected and centrifuged at 10,000*g* for 30 min, and the cytosolic fraction was separated from the resulting supernatant by further centrifugation at 46,000*g* for 60 min. The resulting supernatant contained the cytosolic fraction. Membrane fractions prepared from the pellets were resuspended in 50 μl lysate buffer containing 1% Triton X-100, incubated on ice for 30 min, and centrifuged at 12,000*g* for 30 min. The resultant supernatants were retained as the membrane fraction. The samples were kept at −70°C until used for immunoblotting of PKC isoforms.

The protein quantification, separation by SDS-PAGE, and immunoblotting of PKC fractions were as described for ERK1/2 immunoblotting, except primary polyclonal antibodies were against specific isoforms of PKC (α and ε at 1:1,000), and secondary antibodies were anti-rabbit (1:5,000).

Statistical Analysis. Test results were expressed as a percentage of the controls in mean and SD. Analysis of variance was used to compare between test results and the control, and $P < 0.05$ was considered statistically significant.[18]

Results

Cultured smooth muscle cells obtained by the cell migration method[15] were examined by immunocytochemical staining of desmin (fig. 2, top right, desmin-staining [second week]) and smooth muscle–specific actin (fig. 2, bottom). During the first 2 weeks of migration, cells migrating from femoral arterial segments contained more than 90% smooth muscle cells (first week, fig. 2). Cells cultured from the second and third week of migration consisted of more than 99% smooth muscle cells, which was confirmed with angiotensin II–induced smooth muscle–specific increases in phosphorylated ERK1/2 (data not shown). This study used the 5th–10th passages of cells from the second week of migration because cells from the third week of migration proliferated more slowly.

Isoflurane Increases ERK1/2 Phosphorylation and the Influence of Inhibitors of MEK, CaMKII, and PKC, or Down-regulated PKC

The increase of ERK1/2 phosphorylation (pERK1/2) by 3% isoflurane reached a plateau at 5–10 min (fig. 3A) and was dose dependent (fig. 3B). Based on immunoblot analysis, total ERK1/2 was relatively constant throughout each experiment (*e.g.*, tERK1/2, fig. 3A) despite the effect of isoflurane on pERK1/2 under various conditions (figs. 3–6, data not shown).

The increase in pERK1/2 by isoflurane was completely inhibited by 10 μM PD98059, a specific inhibitor of MEK (fig. 4) and was also reduced in the presence of 10 μM

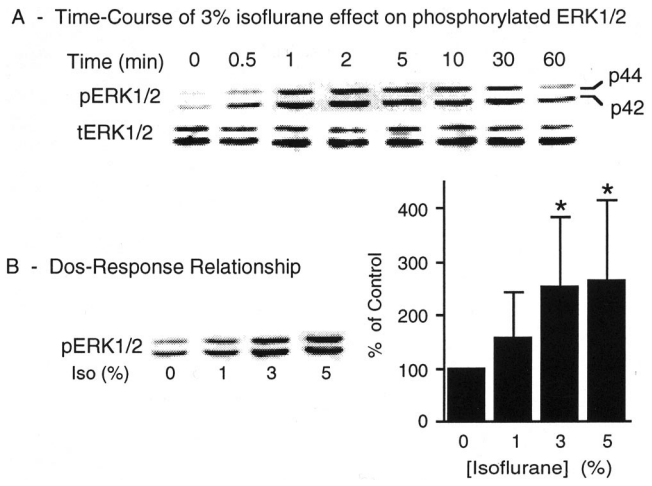

Fig. 3. Time course (*A*) and dose–response relation (*B*) of isoflurane-increased extracellular signal–regulated kinase (ERK1/2) phosphorylation. pERK1/2 = phosphorylated ERK at molecular weight of 44 kDa (p44 or ERK1) and 42 kDa (p42 or ERK2); tERK1/2 = total ERK1/2; Iso (%) = isoflurane, concentrations in percent of partial pressure. Mean ± SD (n = 9). pERK1/2 levels are expressed as a percentage of the control (100%, vehicle without isoflurane). *P < 0.05 compared with the control. Isoflurane increased pERK1/2 (*A* and *B*), which was a direct function of isoflurane concentrations (*B*). However, the tERK1/2 (*A*) values were relatively constant throughout each experiment to confirm the amount of total proteins administered and changes of pERK1/2 by isoflurane.

bisindolylmaleimide, an inhibitor of both cPKC and nPKC (fig. 5A). In contrast, the increase of pERK1/2 by isoflurane was not significantly affected by the inhibitor specific for cPKC, Go6976 (fig. 4B). The PMA-mediated increase in pERK1/2 by PKC was significantly reduced by Go6976 (fig. 5B) and bisindolylmaleimide (data not shown).

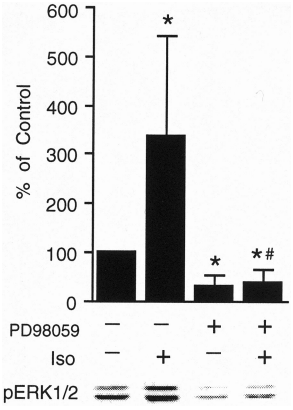

Fig. 4. Effects of the inhibitor of mitogen-activated protein kinase kinase (MEK), PD98059, on isoflurane-increased phosphorylated ERK1/2 (pERK1/2) level. PD98059 = 10 μM; Iso = 3% isoflurane; pERK1/2 = phosphorylated ERK1/2. Mean ± SD (n = 3–8). pERK1/2 levels are expressed as a percentage of the control (vehicle, 100%). *P < 0.05 compared with control containing vehicle. #P < 0.05 compared with 3% isoflurane. The MEK inhibitor, PD98059, significantly decreased pERK1/2 levels, and the isoflurane-increased pERK1/2 levels were completely blocked.

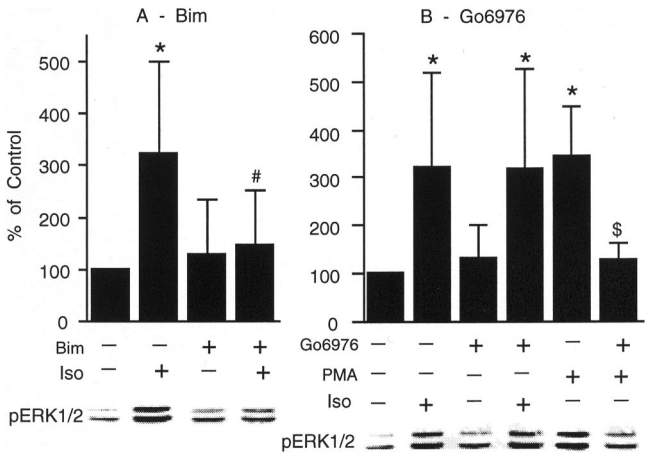

Fig. 5. Effects of protein kinase C inhibitors on isoflurane-increased phosphorylated ERK1/2. Bim = bisindolylmaleidmide, an inhibitor of conventional–novel protein kinase C, at 10 μM; Go6976 = a conventional protein kinase C inhibitor at 10 μM; PMA = phorbol-12-myristate-13-acetate, a protein kinase C activator, at 1 μM; Iso = isoflurane at 3%. Mean ± SD (n = 3–9). Phosphorylated ERK1/2 (pERK1/2) levels are expressed as a percentage of the control (vehicle). *P < 0.05 compared with the control (100%); #P < 0.05 compared with 3% isoflurane; $$P$ < 0.05 compared with PMA. Isoflurane increased pERK1/2 levels, which was completely inhibited by bisindolymaleidmide (*A*). The isoflurane-increased pERK1/2 level was not significantly affected by Go6976, but the PMA-increased pERK1/2 level was completely blocked by Go6976 (*B*).

Another PKC activator, PDBu (1 μM) also increased pERK1/2 when administered for 30 min, but pERK1/2 was reduced when PDBu was used to pretreated cells for 48 h (down-regulation of PKC) (fig. 6). This reduction in pERK1/2 mediated by PDBu-induced down-regulation of PKC also prevented the increase in pERK1/2 by isoflurane (fig. 6). The inhibitors of CaMKII, KN-93 (fig. 7A) or KN-62 (fig. 7B), reduced the increase in pERK1/2 by 3% isoflurane.

Effects of Isoflurane on the Translocation of PKC Isoforms

One of the characteristics of protein kinase signaling is translocation of the kinase (for review, see Cobb[1]). The increase in pERK1/2 by isoflurane was blocked by the inhibitor of c/nPKC (bisindolylmaleimide) but not by the inhibitor of cPKC (Go6976), suggesting that nPKC but not cPKC is activated by isoflurane.

To examine this possibility, we determined whether nPKC but not cPKC would be translocated from the cytosol to the membrane on activation. The PKC activator PMA (1 nM–0.1 μM) increased the membrane fraction and decreased the cytosolic fraction of both PKCα and PKCε (fig. 8) in a dose-dependent manner. Isoflurane increased PKCε in the membrane fraction (fig. 9A) throughout the time course (0.5–60 min), but not PKCα (fig. 9B). This isoflurane-increased PKCε in the membrane fraction was dose dependent (membrane, fig. 10). However, PKCε in the cytosol fraction was not significantly decreased (cytosol, fig. 10).

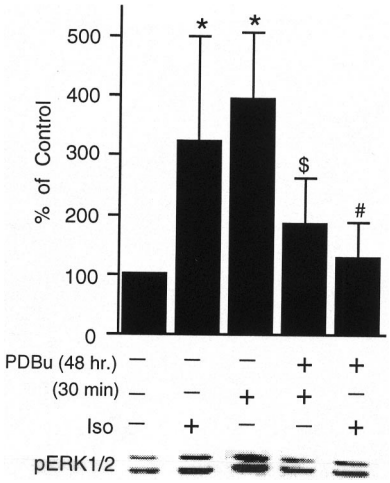

Fig. 6. Influence of protein kinase C (PKC) down-regulation on isoflurane-increased phosphorylated ERK1/2 level. PDBu = phorbol-12,13-dibutyrate, a PKC activator, at 1 μM; PDBu (48 h) = the cultured cells were treated with PDBu for 48 h to down-regulate PKC; PDBu (30 min) = the cultured cells were treated with PDBu for 30 min to activate PKC; Iso = isoflurane at 3%; pERK1/2 = phosphorylated ERK1/2. Mean ± SD (n = 3–9). pERK1/2 levels are expressed as a percentage of the control (100%). *P < 0.05 compared with control containing vehicle; #P < 0.05 compared with 3% isoflurane; $$P$ < 0.05 compared with PDBu (48 h). PDBu increased the pERK1/2 level (*, PDBu [30 min]), which was greatly reduced in vascular smooth muscle cells with down-regulated PKC ($, PDBu [48 h]). The isoflurane-increased pERK1/2 level (*, Iso) was completely blocked in vascular smooth muscle cells with down-regulated PKC (#, PDBu [48 h] and Iso).

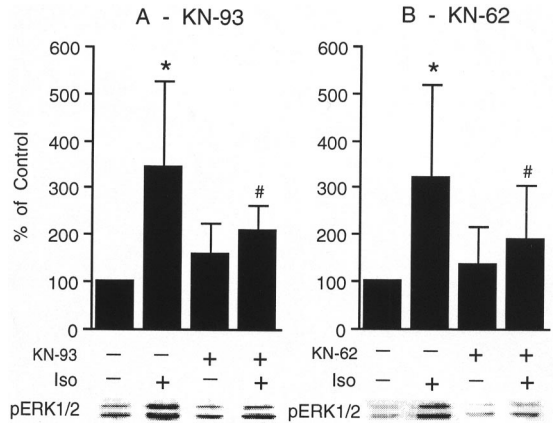

Fig. 7. Effects of Ca^{2+}–calmodulin-dependent protein kinase (CaMKII) inhibitors on isoflurane-increased phosphorylated ERK1/2 level. KN-93, KN-62 = CaMKII inhibitors by competing with calmodulin binding sites; Iso = isoflurane at 3%; pERK1/2 = phosphorylated ERK1/2. Mean ± SD (n = 4–10). pERK1/2 levels are expressed as a percentage of the control (100%). *P < 0.05 compared with the control; #P < 0.05 compared with 3% isoflurane. The isoflurane-increased pERK1/2 level (*, *A* and *B*) was significantly reduced by the CaMKII inhibitors, 10 μM KN-93 (#, *A*) and 10 μM KN-62 (#, *B*).

Discussion

This study showed that isoflurane increased phosphorylated extracellular signal–regulated kinases (pERK1/2) in cultured smooth muscle cells from rabbit femoral arteries and that the increase was dose dependent and sustained. Isoflurane-increased pERK1/2 was initiated upstream by activation of CaMKII and Ca^{2+}-independent protein kinase C (nPKC), which was followed by translocation of the nPKCε from the cytosol to the membrane.

Extracellular signal–regulated kinases (ERK1/2) belong to a superfamily of MAP kinases (for review, see Cobb[1]) that have an important role in cellular functions, including proliferation, apoptosis, cell migration, and possibly smooth muscle contraction. The increase in pERK1/2 by isoflurane *via* PKC and CaMKII suggests that isoflurane activates ERK1/2. The ability of the inhibitor (PD98059) of MAP kinase kinase (MEK, the ERK1/2 upstream kinase) in blocking the isoflurane-increased pERK1/2 suggests that it is mediated through MEK–ERK signaling pathway.

This study also shows that cultured smooth muscle cells from femoral arteries retain components of ERK1/2 signaling pathway affected by isoflurane. This conclusion is based on the similarities in response to inhibitors of PKC and CaMKII of isoflurane-increased pERK1/2 in cultured smooth muscle cells of this study, as well as the isoflurane-increased force in skinned strips observed in femoral arteries.[6] The isoflurane-increased pERK1/2 and force are both blocked by the inhibitor of PKC (bisindolylmaleimide, which inhibits both c/nPKC) but not by a specific inhibitor of cPKC (Go6976), suggesting that isoflurane-increased pERK1/2 and force are mediated by activation of nPKC. The activation of nPKC is further confirmed by the ability of isoflurane to cause an increase in nPKCε in the membrane fraction. The evidence of isoflurane-increased nPKCε in the membrane fraction suggests the translocation of nPKCε from the cytosolic fraction to the membrane fraction. No statistically significant decrease in the cytosolic nPKCε could be attributed to that the change by isoflurane is too small to be detectable compared to the large cytosolic nPKCε. The fact that isoflurane-induced nPKCε translocation and isoflurane-increased pERK1/2 are long-lasting shown in this study is consistent with the sustained force in-

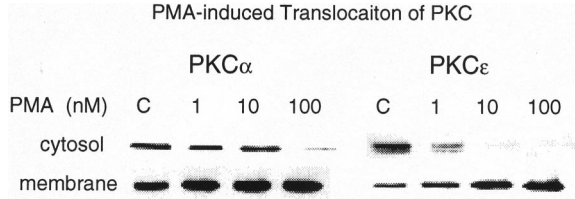

Fig. 8. Phorbol-12-myristate-13-acetate (PMA)-induced protein kinase C ([PKC] PKCε and PKCα) translocation. PMA (nM) = an activator of PKC from 1 to 100 nM; cytosol = cytosolic fraction; membrane = membrane fraction; C = control, 0 μM PMA; PKCα = an isoform of conventional Ca^{2+}-dependent PKC (cPKC); PKCε = an isoform of novel Ca^{2+}-independent PKC. PMA decreased dose-dependently the amount of PKCα and PKCε in the cytosolic fraction, which were associated with increases in the membrane fraction. The immunoblots are representative of three separate experiments.

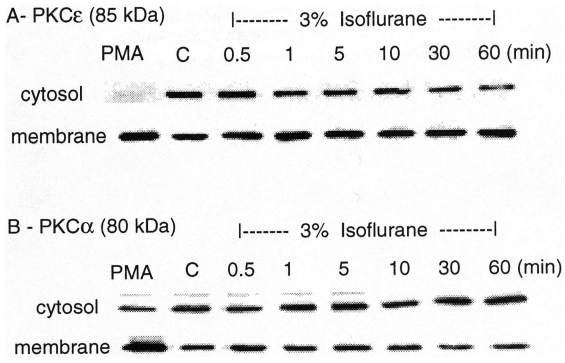

Fig. 9. Time course of the effect of isoflurane on translocation of protein kinase C (PKC) isoforms. PMA = phorbol-12-myristate-13-acetate, an activator of PKC at 0.1 μM; C = control; (min) = 3% isoflurane was administered at various time period (0.5–60 min); cytosol = cytosolic fraction; membrane = membrane fraction. PMA again increased the membrane fraction but decreased the cytosolic fraction of both isoforms of PKC (PMA; *A*, PKCε; *B*, PKCα). Isoflurane increased in the membrane fraction of PKCε at 30 s and sustained up to 60 min (membrane, *A*) but not that of PKCα (membrane, *B*). The immunoblots are representative of three separate experiments.

creased by isoflurane observed in skinned strips,[6] suggesting that the increased force is by ERK1/2 signaling. These findings show the ability to use cultured cells of arterial origin to elucidate the molecular mechanisms underlying anesthetic effects on force in skinned strips.

The blockade of isoflurane effect by down-regulation of PKC substantiates a role for PKC in pERK1/2[16] increased by isoflurane. Whether small G proteins, such as ras–raf, act upstream of MEK/ERK1/2 signaling (for review, see Cobb[1]) remains to be examined. This isoflurane–PKCε–MEK–ERK1/2 pathway may account for the isoflurane-increased force observed in skinned femoral arterial strips.[6]

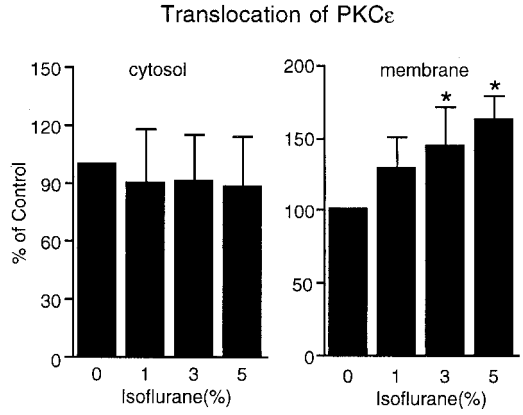

Fig. 10. Dose–response relation of isoflurane effect on protein kinase C-ε (PKCε) translocation. cytosol = PKCε in the cytosolic fraction; membrane = PKCε in the membrane fraction. Mean ± SD (n = 5). The amount of PKCε was measured at 5 min after administration of isoflurane and expressed as a percentage of that of the control (100%). *$P < 0.05$ compared with the control. Isoflurane dose-dependently increased the membrane fraction of PKCε (membrane).

The evidence that the inhibitors of CaMKII block the isoflurane-increased pERK1/2 suggests an activation of CaMKII. This CaMKII–ERK1/2 signaling, affected by isoflurane, has also been shown in cultured smooth muscle cells from rat aorta[12] and rabbit aorta.[11] Whether this CaMKII–ERK1/2 signaling pathway contributes to isoflurane-increased force in skinned femoral arterial strips,[6] as observed in agonist-induced force generation,[13,14] remains to be examined. However, the activation of CaMKII by isoflurane has been implicated in skinned femoral arterial strips[6] but by a different mechanism, *i.e.*, phosphorylation of MLCK resulting in desensitization of the contractile proteins.[9,19] Thus, CaMKII may have at least two downstream targets in vascular smooth muscle, namely, MLCK and ERK1/2. Whether these two mechanisms contribute to muscle contraction and relaxation remains to be examined.

The downstream targets of isoflurane-activated PKC could be by ERK1/2–MLCK–MLC (fig. 1). A ras–ERK–MLCK pathway has been associated with cell migration shown in urokinase-type plasminogen activator–stimulated cells.[20] Thus, activation of CaMKII by isoflurane may also be *via* ERK1/2–MLCK, shown to contribute partially to force generation in skinned femoral arterial strips.[6] The persistence of activation of ERK1/2 by isoflurane shown in this study could account for the sustained force development by isoflurane in skinned femoral arterial strips.[6] This speculation is based on the characteristics of CaMKII (for review[21]), *i.e.*, CaMKII is initially activated by Ca^{2+}–calmodulin followed by Ca^{2+}-independent autophosphorylation.

The cascade of isoflurane effect in cultured vascular smooth muscle cells leading to increased ERK1/2 phosphorylation can therefore be hypothesized as follows (fig. 1): (1) isoflurane causes Ca^{2+} release from the sarcoplasmic reticulum, which then activates CaMKII, leading by one pathway to phosphorylation of MLCK as shown in skinned arterial strips,[6] and by another pathway to ERK1/2 signaling, as shown in this study; (2) isoflurane activates PKCε, leading to its translocation from the cytosol to the membrane, transducing the signal by the MEK–ERK1/2 pathway. This activation of CaMKII and PKC *via* ERK1/2 signaling in cultured cells could lead to phosphorylation of MLCK, resulting in increased MLCK activity as shown in other cell types[20,22] and in chicken gizzard.[23] This CaMKII or PKC–MEK–ERK1/2–MLCK signaling in cultured cells could be responsible for isoflurane-increased force shown in skinned arterial strips.[6] It is known that ERK1/2 signaling may result in cellular proliferation or transcription (for review, see Cobb[1]). Whether this ERK1/2 signaling increased by isoflurane in cultured cells results in cellular proliferation and transcription remains to be determined. In summary, this study shows that isoflurane-activated ERK1/2 in cultured smooth muscle cells is initiated by nPKC and CaMKII.

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