Sevoflurane Stimulates MAP Kinase Signal transduction through the Activation of PKC α and βII in Fetal Rat Cerebral Cortex Cultured Neuron

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I. Introduction

The protein kinase C (PKC) family consists of more than 11 phospholipid-dependent serine/threonine kinases which take part in cellular responses to various agonists, including hormones, some growth factors and neurotransmitters [1, 2, 16, 17, 25]. PKC is abundant in the central nervous system and plays an important role in the regulation of neuronal functions [22, 26]. Many studies have demonstrated that PKC may participate as a potential target molecule in general anesthetic actions [8, 15]. Halothane and propofol stimulate the activation of rat partial purified PKC in the presence of physiologically relevant bilayer vesicles in vitro [4]. Halothane also stimulates PKC activity in rat
cerebrocortical synaptosomes [6]. Although these reports suggest that anesthetics may function in the brain through PKC signal transduction, little is known about the detailed effects of anesthetics on PKC signaling in neuronal cells.

Multiple discrete isoforms of PKC have been identified. These isoforms show subtly different enzymological properties, differential tissue expression, and specific intracellular localization [16, 18]. The PKC isoforms are divided into the following three groups according to their sensitivity toward the activators: conventional PKCs (cPKC: α, β1, β2, γ) are calcium-dependent, and stimulated by DAG; novel PKCs (nPKC: δ, ε, η, θ) are calcium-independent, but are diacylglycerol (DAG)-stimulatable; atypical PKCs (aPKC: ζ, μ) require neither calcium nor DAG for optimal activity. Various reports have demonstrated that individual PKCs mediate different biological processes in the cell. In the physiological activation of cPKC isoforms by DAG, binding of DAG increases the affinity of cPKC for Ca2+ and phosphatidylserine, facilitates cPKC translocation and binding to cell membranes, and increases cPKC catalytic activity [30]. In cultured vascular smooth muscle cells, isoflurane, a volatile anesthetic, evokes the translocation of PKC ε, but not PKC α from the cytosol to the membrane fraction [29]. This result suggests that anesthetics activate isotype-specific PKC in vascular smooth muscle cells. Isoflurane also stimulates the phosphorylation of extracellular signal-regulated kinase (ERK) in smooth muscle cells [29]. ERK is a component of the mitogen-activated protein (MAP) kinase signaling pathway together with small G proteins such as Raf and MAP kinase kinase (MEK). However it is not clear whether isoflurane-induced ERK phosphorylation is mediated by the Raf-MEK cascade. PKC is an important initiator of the MAP kinase signaling pathway in the central nervous system [20]. However, it remains unclear which of the PKC isoforms are specifically activated by volatile anesthetics in neuronal cells.

Sevoflurane is most frequently used as a volatile anesthetic because it has a low blood-gas partition coefficient and the lowest pungency of commercially available inhaled anesthetics. In spite of the many investigations aimed at elucidating sevoflurane function in recent years there are still numerous unanswered questions concerning intercellular signaling transduction in neuronal cells. The purpose of this study was to examine the effects of sevoflurane on the PKC-MAP kinase signaling pathway in rat cerebral cortex cultured neurons using biochemical and morphological procedures. Our results indicated that treatment with sevoflurane led specifically to activation of PKC α and βI followed by the stimulation of MAP kinase signaling, accompanied by the phosphorylation of ERK in the cell body and neurites of neurons.

II. Materials and Methods

Materials

Sevoflurane was purchased from Abbott Japan (Tokyo, Japan). DNase-I (D-4527), polyethyleneimine, Poly-L-lysine (0.01% Solution); PD98059 and staurosporine from Sigma Chemical Co. (St. Louis, MO); phosphate buffered saline (PBS) from Dainippon Seiyaku (Osaka, Japan); α-Minimum Essential medium (α-MEM) and horse serum from Invitrogen Japan K.K. (Tokyo, Japan); neurobasal medium and B27 supplement from Life Technologies (Gaithersburg, MD); Lab-Tek Chamber Slides from Nalgen Nune International (Naperville, IL). Anti-Raf antibody and anti-PKC antibody (α, β, βII, γ, δ, ε, η, ζ, μ) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphorylated-Raf (p-Raf), MEK, p-MEK, p-ERK and ERK antibodies were purchased from New England Biolabs, Inc. (Beverly, MA). Anti-mouse and anti-rabbit antibodies labeled with horseradish peroxidase were purchased from Amersham Biosciences (Piscataway, NJ).

Cell culture

The Tokai University Institutional Animal Care and Use Committee approved all animal studies. Pregnant Wistar-Imamichi rats were purchased from Imamichi Institute for Animal Reproduction, Tsukuba, Japan. Primary rat cerebral cortex cultured neurons were established from embryonic day 18 fetuses. Brains were aseptically excised and septal tissue areas were dissected out with a pair of scalpels. Dissociation was done under a stereomicroscope according to the description of fetal rat brain anatomy. After dissociation using 10 units/ml papain (Worthington Biochemical Corp., Lakewood, NJ) and 0.01% DNase I, tissue fragments were sedimented following the addition of a few milliliters of horse serum. The pellet was resuspended in a medium containing 5% (v/v) heat-inactivated horse serum (HS, Gibco BRL, Grand Island, NY), 5% (v/v) precolostrum newborn calf serum (Nakashibetsu Serum Center, Mitsubishi Chemical Industry, Tokyo), α-MEM containing 1.9 mg/ml sodium bicarbonate, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate. The cell suspension was passed through two different sizes of plastic tips. Cells were seeded onto polyethyleneimine-coated 60-mm plates at 10⁴ cells/cm². Surface coating was done by using a solution of 1 mg/ml polyethyleneimine in 150 mM sodium borate buffer, pH 8.4, for 3 hr at room temperature. The plates were washed twice with sterile distilled water and then twice with serum-containing medium. They were cultured in a humidified 10% CO2-90% air atmosphere at 37°C, and the medium was changed to neurobasal medium containing B-27 supplement. Sevoflurane was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in each medium was 0.1%. PD98059 and staurosporine was dissolved in DMSO at 10 mM and 1 mM stock solution which, when needed, were further diluted to reach final concentrations.

Assay of cell viability

Cell viability was determined by using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). At the end of the treatment period, 10 µl of 5 mM WST-8, 0.2 mM 1-methoxy PMS, 150 mM NaCl was added to each well. The plates were placed at 37°C for 4 hr in the dark and the absor-
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Immunoblot analysis

Cells were lysed in 2 ml of ice-cold hypotonic lysis buffer (10 mM HEPES-NaOH buffer at pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 µg/ml leupeptin) by homogenization with a glass/Teflon homogenizer with five up-and-down strokes. The homogenate was centrifuged at 200,000 g for 15 min at 4°C to yield supernatant (“cytosol”) and pellet (“membrane”) fractions, which were collected separately. The pellet fraction was resuspended and homogenized in 1 ml of lysis buffer containing 0.5% (v/v) Nonidet P-40 and incubated on ice for 30 min to extract PKC activity. After an additional 1 ml of lysis buffer was added to decrease the detergent concentration, the pellet was centrifuged at 200,000 g for 15 min at 4°C, and the supernatant fraction was removed. Total protein content of the supernatant and pellet solution was determined using the Bio-Rad protein assay kit. In each experiment, samples were tested at equal concentrations of total protein, which ranged from 20 to 30 µg. Samples were boiled in sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% 2-mercaptoethanol) and separated by SDS-PAGE (10% polyacrylamide gels) and then electro-transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% non-fat dry milk, 0.1% Tween 20 in 0.01 M Tris-buffered saline (TBS) for 1 hr at room temperature. After three washes with 0.05% Tween 20 in 0.01 M phosphate buffered saline (T-PBS), the membranes were incubated with antibodies against specific isoforms of PKC (PKC α, βI, βII, γ, δ, ε and ζ at 1:400) for 1 hr at room temperature. Membranes were washed five times with T-PBS and incubated with horseradish peroxidase-conjugated anti-rabbit Ig antibody diluted 1:5000 with T-PBS for 1 hr at room temperature. After five washes with T-PBS, immuno-reactive bands were detected using the enhanced chemiluminescence method (ECLplus Amersham Biosciences). Quantitative analysis was performed using image analysis software (NIH Image).

Cells were scraped in 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM PMSF, 1 mg/ml aprotinin and 10 mg/ml leupeptin. Cell homogenates were vortexed for 1 hr at 4°C and centrifuged at 12,000 g for 15 min. Protein quantification, separation by SDS-PAGE, and immunoblotting were performed as described above using primary antibody (anti-Raf, MEK, ERK1/2, pRaf, pMEK and pERK1/2, diluted 1:400) and secondary antibody (horseradish peroxidase-conjugated anti-mouse Ig and antirabbit Ig antibodies, diluted 1:5000 with T-PBS).

Immunohistochemistry

Cells were cultured on Poly-L-lysine coated Lab-Tek Chamber Slides. The slides were prefixed in 4% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, in a microwave oven for 45 sec at 35°C. They were then fixed in 4% paraformaldehyde, 0.03% Triton X-100 in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 1 hr. After ten washes with 0.01 M PBS, the slides were incubated with anti-phospho-ERK antibody diluted 1:400 with 3% BSA-PBS containing 0.03% Triton-X-100 at 4°C for 12 hr. After ten washes with 0.01 M PBS, the slides were examined with a confocal laser scanning microscope (LSM-410, Carl Zeiss, Jena, Germany)

Statistical analysis

Test results are expressed as a percentage of the control as mean and SD. Differences in results were evaluated for significance using Student’s t-test. Statistical significances of differences were indicated as * P<0.01, ** P<0.001, *** P<0.0001.

III. Results

Effects of sevoflurane on cell viability and morphological characteristics of primary cultured neurons

The viability of sevoflurane-treated primary cultured neurons was examined. Neuronal cells were treated with 0.5 mM sevoflurane for various times (0, 3, 30, 60, 120 min) as illustrated in Figure 1A, and with 120 min sevoflurane various concentrations (0, 0.25, 0.5 mM) as illustrated in Figure 1B. Sevoflurane did not affect the cell viability. In addition, at the light microscopic level, the cells appeared healthy and without vacuolation or other evidence of damage (Fig. 1C and D). These results demonstrated that sevoflurane has no detectable cytotoxicity and does not significantly stimulate primary cultured neurons.

Immunoblot analysis for PKC isoforms in the cytosol and membrane fractions

One of the characteristics of PKC signaling is the translocation of the PKC from the cytosol to the membrane fraction. The effects of sevoflurane on the translocation of PKC isoforms in cultured fetal rat cerebral cortex neurons were examined by immunoblotting using PKC isoform-specific antibodies. PKC isoforms (α, βI, βII, γ, δ, ε and ζ) were enriched in the cytosol fraction compared with the membrane fraction under the control conditions (Fig. 2). Treatment with sevoflurane induced the translocation of PKC α and βII species from the cytosol to the membrane fraction which indicated the activation of these isoforms. However there were no clear changes in the distribution of PKC βI, γ, δ, ε or ζ in sevoflurane-treated neuronal cells. The quantitative analysis of PKC isoforms in the cytosol and membrane fractions are shown in Figure 3. When neuronal cells were
treated with sevoflurane for various times (2.5, 5, 10, 15, 30, 60, 120 min), significant increases of the relative amount of PKC α and βII isoforms in membrane fraction and decreases in the cytosol fraction occurred in a time-dependent manner (Fig. 3A, C).

**Sevoflurane enhances the phosphorylation of Raf, MEK and ERK1/2 in neuronal cells**

We next examined whether the specific activation of PKC α and βII by sevoflurane stimulated the MAP kinase signaling pathway in cultured fetal rat cerebral cortex neurons (Figs. 4 and 5). An increase of Raf phosphorylation was observed within 2.5 ml of the administration of 0.25 mM sevoflurane for various times (2.5, 5, 10, 15, 30, 60, 120 min), significant increases of the relative amount of PKC α and βII isoforms in membrane fraction and decreases in the cytosol fraction occurred in a time-dependent manner (Fig. 3A, C).
Fig. 3. Quantitative time-course analysis of subcellular distribution of PKC isoforms in rat cerebral cortex cultured neurons exposed to 0.25 mM sevoflurane for 0, 15, 60 or 120 min (A: PKC α, B: PKC βI, C: PKC βII, D: PKC γ, E: PKC δ, F: PKC ε, G: PKC ζ). Band intensities were measured by densitometry. Values are expressed as the mean±SD of five determinations (n=5). Significance of differences was determined by Student’s t-test; * P<0.01, ** P<0.001.
sevoflurane (Fig. 5A). Subsequently, the level of phosphorylated MEK protein was increased at 10–15 min after sevoflurane treatment (Fig. 5C). In addition, the phosphorylation of ERK1/2 protein reached a maximum at 15–90 min (Fig. 5E). In contrast, the contents of total Raf, MEK and ERK1/2 proteins were relatively constant at all times examined (Fig. 5B, D, F). These data demonstrate that the activation of the MAP kinase cascade occurred after sevoflurane administration in neuronal cells.

Fig. 5. Time course of Raf, MEK and ERK activation by sevoflurane. Cultured neurons were incubated at 37°C for 0, 2.5, 5, 10, 15, 30, 60, 90 or 120 min in the presence of 0.25 mM sevoflurane. Band intensities were measured by densitometry. Each value is expressed as the mean±SD of five determinations (n=5). The significance of differences was determined by Student’s t-test; * P<0.01, ** P<0.001, *** P<0.0001.
**Effects of staurosporine and PD98059 on sevoflurane-induced ERK phosphorylation**

The PKC inhibitor (staurosporine: 100 nM) and MEK inhibitor (PD98059: 20 µM) significantly decreased the phosphorylation of ERK1/2 induced by sevoflurane treatment (Fig. 6). This result strongly demonstrated that the sevoflurane-induced ERK1/2 phosphorylation mediated by the activation of PKC and MEK in fetal rat cerebral cortex cultured neuron.

**Immunohistochemistry**

In order to examine the localization of phosphorylated-ERK1/2 (pERK1/2) protein, immunohistochemical studies of sevoflurane-treated cultured neuronal cells were performed. In control neurons at 0 min after sevoflurane treatment, the cell bodies were weakly stained with antibody specific for the pERK1/2 protein (Fig. 7). pERK1/2 proteins were remarkably increased in both the cell body and the neurites after 0.25 mM sevoflurane-treatment.

**IV. Discussion**

Volatile anesthetics, including halothane, isoflurane and sevoflurane, produce behavioral effects associated with altered neuronal functions [14]. Despite the widespread use of these anesthetics, their mechanism of action via their effects on intracellular signaling pathways remains obscure. The PKC family comprises signal transduction enzymes consisting of several isoforms which are highly concentrated in the nervous system and have been implicated in the modulation of neuronal synaptic transmission [9, 19, 21]. Furthermore, many studies have implicated PKC as a potential target for general anesthetic effects in various tissues. In addition, it is well documented that PKC is an important initiator of the MAP kinase signaling, which is considered to mediate numerous cellular functions such as proliferation, differentiation and also volatile anesthetic-induced pharmacological preconditioning [27]. However, the effects of volatile anesthetics on PKC and MAP kinase signaling, especially which PKC molecules are responsible, in neuronal cells remain unclear. The results of this study clearly demonstrated that in cultured neurons from the rat cerebral cortex, a volatile anesthetic, sevoflurane, induced the phosphorylation of a series of proteins related to MAP kinase signaling, including Raf, MEK and ERK1/2. This sevoflurane-induced activation of the MAP kinase pathway was initiated by the isoform-specific activation of PKC α and βII, which are Ca2+-dependent PKC molecules.

Phosphorylation of specific proteins is a key biochemical reaction in the regulation of many aspects of neuronal function. Various lines of evidence have implicated PKC-mediated protein phosphorylation as a target for general anesthetic effects in the central nervous system [4, 23]. *In vitro* biochemical experiments have demonstrated that general anesthetics directly affect PKC activity [4, 5, 23, 28]. Those studies demonstrated the ability of volatile anesthetics and intravenous anesthetics to induce significant increases in the sensitivity of purified brain PKC to its endogenous activators phosphatidylerine, diacylglycerol, and Ca2+ *in vitro*. Eleven isoforms of PKC have been identified in mammalian tissues. Individual PKCs mediate different biological processes in the cell, and accordingly these isoforms differ in their tissue distribution, regional distribution in the brain, subcellular localization, and sensitivity to various activators [2, 24]. Previous studies using isoform-selective antibodies to PKC α, PKC β or PKC γ demonstrated that halothane, a volatile anesthetic, promotes the translocation of the three conventional PKC isoforms from the cytosol to the membrane fraction of synaptosomes [7]. On the other hand, Zhong and Su reported the activation of novel PKC ε but not of conventional PKC α by isoflurane in vascular smooth muscle cells [29]. They also showed that isoflurane-activated ERK1/2 in cultured smooth muscle cells is initiated by novel PKC. Furthermore, in rat heart, PKC δ and PKC ε mediate isoflurane-induced preconditioning [12]. These reports indicated that volatile anesthetics activate specific PKC isoforms in various tissues under certain conditions. In the present study, one of our objectives was to identify the PKC isoforms activated by sevoflurane in cultured neurons derived from the rat cerebral cortex. We also assessed the intracellular localization of individual PKC isoforms before and after treatment with sevoflurane. The major finding of the current study was that sevoflurane specifically activates PKC α and PKC βII, resulting in their translocation to distinct intracellular sites in rat cerebral cortex cultured neurons, as demonstrated using isoform-selective antibodies to PKC α, PKC βI, PKC βII, PKC γ, PKC δ, PKC ε and PKC
This result suggests that PKCα and PKCβII molecules have an important role in the function of volatile anesthetics in neuronal cells. Hemmings et al. demonstrated that halothane had no significant effects on PKCγ translocation, while significant effects of halothane were observed on the distributions of PKCα and β in cytosol and membrane [7]. This report suggested that volatile anesthetics stimulated the translocation process itself, perhaps by increasing the affinity of activated PKC for its specific membrane receptor in particular cells.

It has been reported that high levels of activation of PKC can cause neurodegeneration and are consistent with the possibility that altered cellular signaling contributed to pathological neuronal degeneration in cultured human cortical neurons [13]. Moreover, it has been reported that PKCδ is involved in nitric oxide-induced dopaminergic cell death [11]. These reports clearly suggested that altered intracellular signaling, such as over-activation of PKCs, elicit neuronal cell degeneration. In this study, it became apparent that the treatment of sevoflurane induced the activation of PKCα and βII specifically. Nevertheless, data on cell viability after sevoflurane treatment further demonstrated that there is no detectable cytotoxicity of fetal rat cerebral neurons in response to sevoflurane in the range of 0.25–0.5 mM for 2 hr.

Fig. 7. Immunohistochemical localization of phosphorylated-ERK in sevoflurane-treated fetal rat cerebral cultured neurons. After treatment with 0.25 mM sevoflurane, cultured neurons were fixed and immunohistochemistry was performed. Immunohistochemical localization of green reaction for pERK. Blue color indicates the nucleus. Bar=25 μM.
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VI. References

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