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Isoflurane Enhances the Expression and Activity of Glutamate Transporter Type 3 in C6 Glioma Cells

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Background: **Glutamate transporters play an important role in maintaining extracellular glutamate homeostasis. Volatile anesthetics have been shown to affect glutamate transporter activity acutely (within minutes after the exposure). It is not known whether volatile anesthetics affect the expression of glutamate transporters.**

Methods: **Rat cultured C6 glioma cells that express excitatory amino acid transporter type 3 (EAAT3) were exposed to isoflurane at various concentrations (0.5–4.0%) or for different periods (1–24 h) at 37°C. EAAT3 mRNA, proteins, and activity were quantified.**

Results: **Isoflurane induced a time- and concentration-dependent increase in the mRNA and protein levels of EAAT3 in C6 cells. The maximal increase was induced by 2% isoflurane, and the cells incubated with 2% isoflurane for 3 and 7 h expressed the highest levels of EAAT3 mRNA and proteins, respectively. Similarly, glutamate uptake was higher in C6 cells exposed to 2% isoflurane for 7 h than in control cells. Actinomycin D and cycloheximide, inhibitors for mRNA and protein synthesis, respectively, did not affect the isoflurane-induced increase in EAAT3 mRNA and protein expression. Phorbol 12-myristate 13-acetate, a protein kinase C activator, also enhanced EAAT3 expression. The combination of 2% isoflurane and phorbol 12-myristate 13-acetate caused a higher level of EAAT3 expression than that induced by 2% isoflurane alone. Neither staurosporine, a protein kinase C inhibitor, nor wortmannin, a phosphatidylinositol 3 kinase inhibitor, inhibited the isoflurane-induced increase in EAAT3 expression.**

Conclusions: **The results of this study suggest that isoflurane increases the expression and activity of EAAT3 by stabilizing EAAT3 mRNA and proteins *via* protein kinase C– and phosphatidylinositol 3 kinase–independent pathways.**

GLUTAMATE is a major excitatory neurotransmitter in the central nervous system (CNS). By means of overexcitation, glutamate also is a potent neurotoxin. Glutamate-mediated neuronal injury has been implicated in the pathophysiology of ischemic brain damage and in the etiology of several major human neurodegenerative disorders, such as Alzheimer and Huntington diseases, as well as in amyotrophic lateral sclerosis.[1,2] Therefore, the extracellular concentrations of glutamate must be kept low for efficient neurotransmission and injury prevention. No extracellular enzyme is known to metabolize glutamate, as is not the case for many other neurotransmitters.[3,4] Thus, glutamate transporters, which remove glutamate from extracellular space into cells, play a critical role in securing a high signal-to-noise ratio in synaptic transmission and in preventing harmful receptor overstimulation.[3] Five glutamate transporters (also called excitatory amino acid transporters [EAATs], EAAT1–5) have been identified to date. They are abundantly and differentially expressed in glial cells and neurons of certain CNS regions.[3]

Volatile anesthetics have been demonstrated to enhance EAAT activity in cultured cells and synaptosomes,[5–7] although no effects or inhibitory effects of volatile anesthetics on EAAT activity have been reported.[8–10] Because native nerve cells usually express more than one type of EAATs, we used oocyte expression system to study anesthetic effects on the activity of single types of EAATs. Our results demonstrated that the activity of EAAT3, a glutamate transporter mainly expressed in neurons, increased within a few minutes after being exposed to volatile anesthetics.[10] Protein kinase C (PKC) may be involved in these anesthetic effects.[10] Anesthetic effects on the expression of glutamate transporters, a potential acting site, have not been investigated yet. Volatile anesthetics have been demonstrated to affect the expression of other proteins, such as nitric oxide synthases.[11] Thus, we designed the present study to test our hypothesis that isoflurane, a commonly used volatile anesthetic in clinical practice, enhances EAAT3 expression in rat C6 glioma cells. We used rat C6 glioma cells in the study because they are the only cells found to express a single type of EAAT (EAAT3).[12] These cells have been used widely as a cell model to study the regulation of EAAT3 activity, trafficking, and expression by various factors, including hypoxia, platelet-derived growth factor, and PKC.[12–14]

Materials and Methods

No human or animal subjects were used for this study. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless specified in the text.

Incubation

Rat C6 glioma cells from American Type Culture Collection (Manassas, VA) were grown in F-10 nutrient mixture (Ham) (GIBCO Invitrogen Corporation, Grand Island, NY) containing 15% horse serum and 2.5% fetal bovine serum at 37°C. Cells were used for experiments when they were 80% confluent. After the indicated agents were added to culture medium that had been

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with C6 cells for 3 days, cells were placed in an airtight chamber (about 5 l in volume) that was passed with 95% air/5% CO_2 through or not through an isoflurane vaporizer at a flow rate of 3 l/min for 20 min. Preliminary experiments with infrared spectroscopy demonstrated that isoflurane concentrations in the outlet gas equaled those in the inlet gas 6 min after the onset of gassing under the current experimental conditions. The inlet and outlet of the chamber were then closed, and the cells were incubated for the preset periods at 37°C. The agents applied during the incubation, in addition to various concentrations of isoflurane, included phorbol 12-myristate 13-acetate (PMA, 100 nM), staurosporine (50 nM), wortmannin (100 nM), cycloheximide (a protein synthesis inhibitor, 50 μg/ml), and actinomycin D (an mRNA synthesis inhibitor, 15 μg/ml) (Biomol Research Laboratories, Plymouth Meeting, PA). PMA is a PKC activator. Staurosporine and wortmannin are a PKC inhibitor and phosphatidylinositol 3-kinase (PI3K) inhibitor, respectively.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA from C6 glioma cells after incubation was purified using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Single-strand cDNA was obtained by using the SuperScript first-strand synthesis system (Invitrogen Life Technologies). Oligonucleotide primers were 22 and 21 base pairs corresponding to positions 548–569 and 1081–1061, respectively, of the published rat cDNA EAAT3 sequence.[15] Twenty-eight cycles of polymerase chain reaction with platinum *Taq*DNA polymerase (Invitrogen Life Technologies) were used to yield amplified polymerase chain reaction products of 534 base pairs. A housekeeper gene, rat β-actin, with polymerase chain reaction products of 764 base pairs (Clontech Laboratories, Palo Alto, CA), was coamplified with the EAAT3 cDNA and used as an internal standard to normalize tube-to-tube variation in amplification efficiencies. The amplified products were separated according to their sizes on 1% agarose gels and stained with ethidium bromide. The intensity of the bands was quantitated by an Alpha Imager 2000 (Alpha Innotech Corporation, San Leandro, CA).

Western Blot Analysis

Cell homogenates were prepared by homogenizing the cells in 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 1 μM pepstatin A. The crude homogenates were centrifuged at 1,000*g* for 10 min at 4°C. Protein content in the supernatants was quantitated by the Lowry assay using a protein assay kit from Sigma. About 100 μg per lane of proteins was subjected to Western blot analysis as previously described.[11] Mouse anti-EAAT3 IgG monoclonal antibody raised against a synthetic peptide corresponding to amino acids 161–177 of the rat EAAT3 was from ZYMED Laboratories (San Francisco, CA). Rabbit anti-β-actin polyclonal antibody was an affinity-purified antibody raised against the C-terminal actin fragment (11 amino acids) attached to multiple antigen peptide backbone from Sigma Chemical. The intensity of the protein bands was quantitated by an ImageQuant 5.0 Molecular Dynamics Densitometer (Molecular Dynamics, Sunnyvale, CA). The EAAT3 results then were normalized to those of β-actin to control for errors in protein sample loading and transferring during the Western blot analysis.

Transporter Activity Assay

The EAAT activity assay was performed as described previously with some modifications.[7,16] Briefly, after being incubated with 2% isoflurane for 7 h, C6 cells growing in six-well culture plates were washed twice with wash buffer (10 mM HEPES, 140 mM NaCl [replaced by 140 mM choline chloride in sodium depletion experiments], 5 mM Tris-base, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, 10 mM dextrose, pH 7.2). They were then incubated with 10 μM L-[^{3}H]-glutamate (specific activity of 56 Ci/mM; Amersham Bioscience, Piscataway, NJ) in the wash buffer for 5 min at 37°C. The incubation was terminated 5 min later by removing the incubation buffer and washing the cells three times with ice-cold wash buffer. The cells were lysed with 0.2 M NaOH, and radioactivity was measured in a liquid scintillation counter.

Cell Survival and Viability Assay

C6 cells were incubated with various concentrations of isoflurane for 7 h at 37°C. They were then used for cell survival and viability assay. Because Trypan blue is impermeable to normal cells, cells stained by this dye are not considered to have survived. Cells were incubated with 0.2% Trypan blue for 4 min at room temperature. At least 100 cells per sample were counted with a hemocytometer to compute the percentage of cell survival (Trypan blue–negatively stained cells in total counted cells).

Cell viability was determined by a colorimetric assay using a kit from Chemicon International (Temecula, CA). This assay is based on cleavage of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium (pale yellow color) to a dark blue formazan product by mitochondrial dehydrogenase. The absorbance was measured with a test wavelength of 540 nm and a reference wavelength of 620 nm. Cell viability in the isoflurane-treated samples was expressed as percentage of the absorbance of the controls.

Statistical Analysis

The intensity of all EAAT3 mRNA and protein bands was first normalized to that of β-actin as described in the

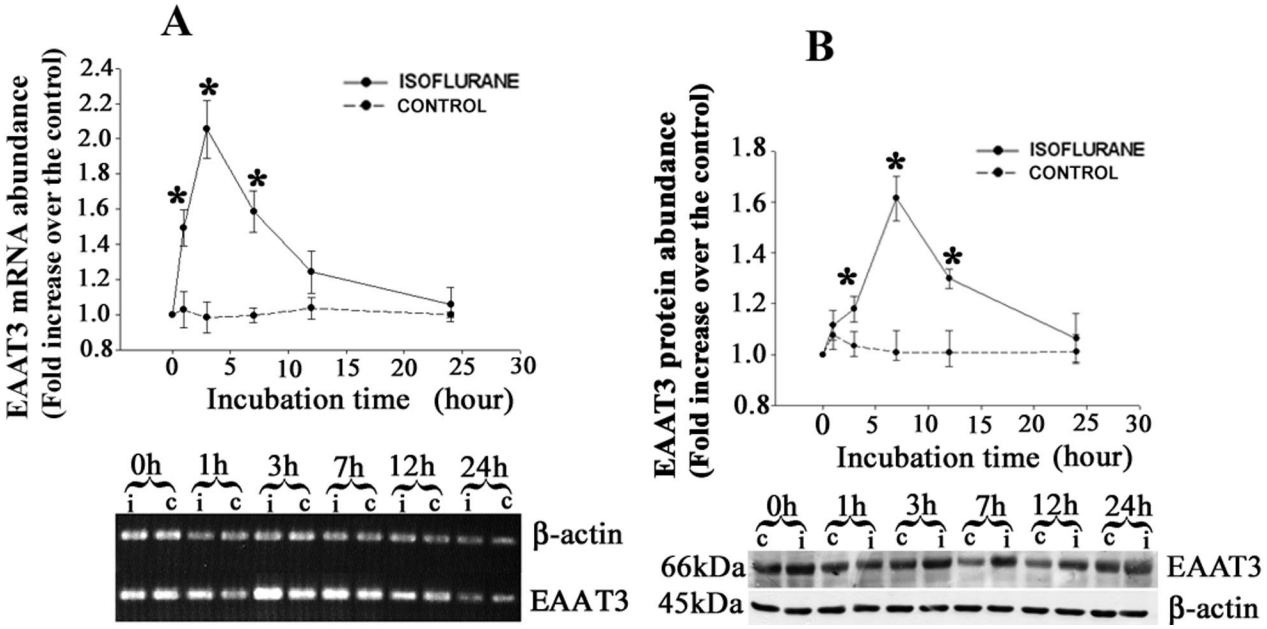

Fig. 1. Time course of 2% isoflurane-induced increase of excitatory amino acid transporter type 3 (EAAT3) mRNA (*A*) and proteins (*B*) in C6 glioma cells. The *graphs* present the EAAT3 mRNA or protein abundance quantified by integrating the volume of bands from four separate experiments, and a representative film image of the bands is shown. Values depicted as *solid lines* in the *graphs* are the mean ± SD of the fold change over the corresponding time controls, with the controls being set as 1. The *dashed lines* show the values of time controls as the mean ± SD of the fold change over time 0 control, with time 0 control being set as 1. *$P < 0.05$ compared with values at time 0 by unpaired *t* test. c = control; i = 2% isoflurane.

section on Western blot analysis. The results in the treatment groups were then normalized to those of the corresponding time controls. Specific activity of EAATs was calculated by subtracting the Na^+-independent glutamate uptake from the total glutamate uptake, because EAATs are Na^+ cotransporters and extracellular Na^+ is required for EAATs to perform their normal glutamate transport activity.[17] Results are presented as means ± SD of the fold change over the controls, with controls being set as 1. Statistical analysis was performed by unpaired *t* test (expression, activity, and viability data) or by the Mann–Whitney rank sum test (survival data). $P < 0.05$ was considered significant.

Results

Isoflurane Dose and Time Dependently Increased the Expression of EAAT3 mRNA and Proteins

Only one product with a size of ~500 base pairs was detected after reverse transcriptase–polymerase chain reaction with primers for EAAT3. Similarly, one product with a size of ~760 base pairs was detected with the use of β-actin primers. The sizes of these products correspond to the predicted sizes from the sequences.[15,18] There was no significant difference in EAAT3 mRNA expression among the time controls (fig. 1A). Isoflurane (2%) caused a time-dependent increase of EAAT3 mRNA in C6 cells, however, with the highest increase seen after the incubation with isoflurane for 3 h (fig. 1A).

A protein band with a mobility corresponding to ~70 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis was detected by Western blot analysis with the anti-EAAT3 antibody and a protein band at ~45 kDa when anti-β-actin antibody was used. The sizes of these detected proteins are consistent with the sizes reported in the literature.[12,13] There was no significant difference in EAAT3 protein expression among the time controls (fig. 1B). Similar to the results of mRNA, isoflurane (2%) also caused a time-dependent increase in EAAT3 proteins with the peak level at 7 h of incubation (fig. 1B), a time point after the peak increase of EAAT3 mRNA. Isoflurane also caused a concentration-dependent increase of both mRNA and proteins of EAAT3, with the highest level of expression at 2% isoflurane (fig. 2). In contrast, various concentrations (0.5– 4%) of isoflurane did not affect the survival and viability of C6 glioma cells (fig. 3).

Isoflurane Increased the Glutamate Uptake via *EAAT3*

Consistent with the expression data, C6 cells also had increased EAAT activity after being incubated with 2% isoflurane for 7 h (fig. 4). This increased activity was from EAAT3, because 300 μM dihydrokainate did not inhibit the isoflurane-induced increase in EAAT activity in C6 cells (fig. 4). Dihydrokainate is a relatively selective inhibitor for EAAT2 (the Ki for inhibition of EAAT2 is in the low-micromolar range, whereas about 10 mM is required to block transport by other EAATs)[19] and is the

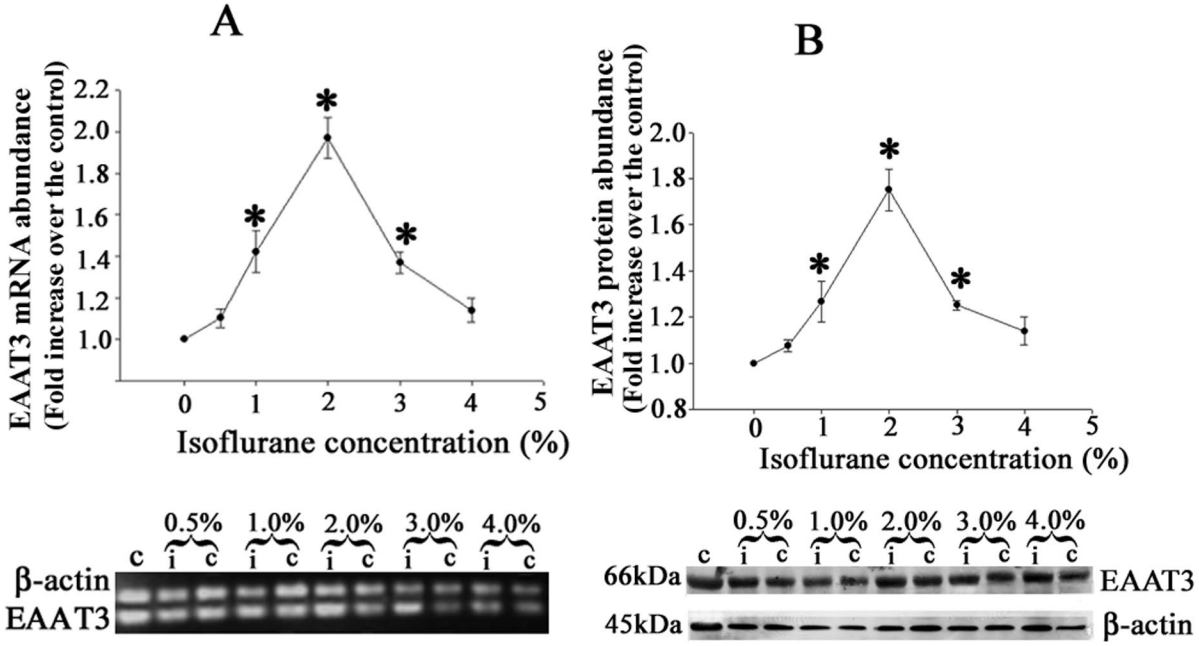

Fig. 2. Dose responses of isoflurane-induced increase of excitatory amino acid transporter type 3 (EAAT3) mRNA (*A*) and proteins (*B*) in C6 glioma cells. Cells were incubated with various concentrations of isoflurane for 3 h at 37°C for mRNA quantification and for 7 h at 37°C for protein quantification. The *graphs* present the EAAT3 mRNA or protein abundance quantified by integrating the volume of bands from three separate experiments, and a representative film image of the bands is shown. Values in the *graphs* are the mean ± SD of the fold change over the corresponding controls, with the controls being set as 1. **P* < 0.05 compared with values at isoflurane concentration 0 by unpaired *t* test. c = control; i = 2% isoflurane.

only available type-selective inhibitor for EAATs. Because EAAT2 is the major form of EAATs in normal glial cells,[20] we used dihydrokainate to inhibit the activity of EAAT2 that could be expressed in small quantity in our C6 glioma cells.

Isoflurane Increased EAAT3 Expression Probably via Stabilization of EAAT3 mRNA and Proteins

Actinomycin D significantly decreased EAAT3 mRNA expression level in both control and isoflurane-treated cells (fig. 5A). Nevertheless, 2% isoflurane caused a similar degree of increase in EAAT3 mRNA expression compared with the control levels no matter whether actinomycin D was present or not (EAAT3 mRNA levels in the isoflurane groups were 194 ± 8% and 206 ± 20% of the corresponding controls, respectively, in the presence or absence of actinomycin D, n = 3, $P > 0.05$) (fig. 5A). Similarly, cycloheximide significantly decreased EAAT3 protein expression level in both control and isoflurane-treated cells (fig. 5B). However, cycloheximide did not change the degree of 2% isoflurane–induced increase in EAAT3 protein expression (EAAT3 protein levels in the isoflurane groups were 255 ± 51% and 198 ± 17% of the corresponding controls, respectively, in the presence or absence of cycloheximide, n = 3, $P > 0.05$) (fig. 5B).

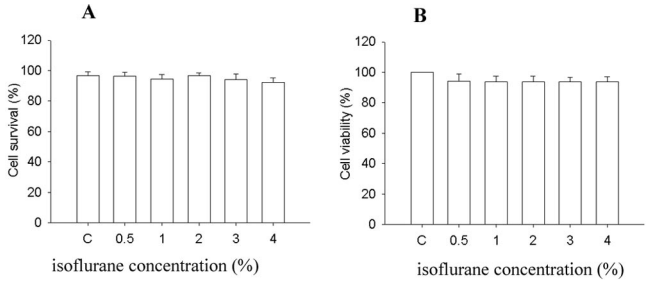

Fig. 3. The effects of isoflurane on C6 cell survival (*A*) and viability (*B*). Cells were incubated with isoflurane for 7 h at 37°C. Data are mean ± SD (n = 6 for survival data and n = 5 for viability data). There is no statistically significant difference between controls and samples treated with isoflurane at any tested concentrations. C = control.

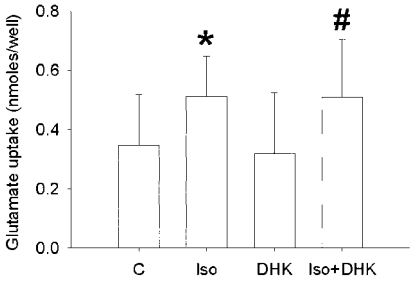

Fig. 4. Isoflurane induced an increase in excitatory amino acid transporter type 3 activity. C6 cells were exposed to 2% isoflurane for 7 h at 37°C and then incubated with 10 μM L-[^{3}H] glutamate in the absence of isoflurane for 5 min at 37°C. Data are mean ± SD after subtraction of background (Na^+-independent uptake, n = 18). **P* < 0.05 compared with control. #*P* < 0.05 compared with dihydrokainate alone. C = control; Iso = 2% isoflurane; DHK = 300 μM dihydrokainate.

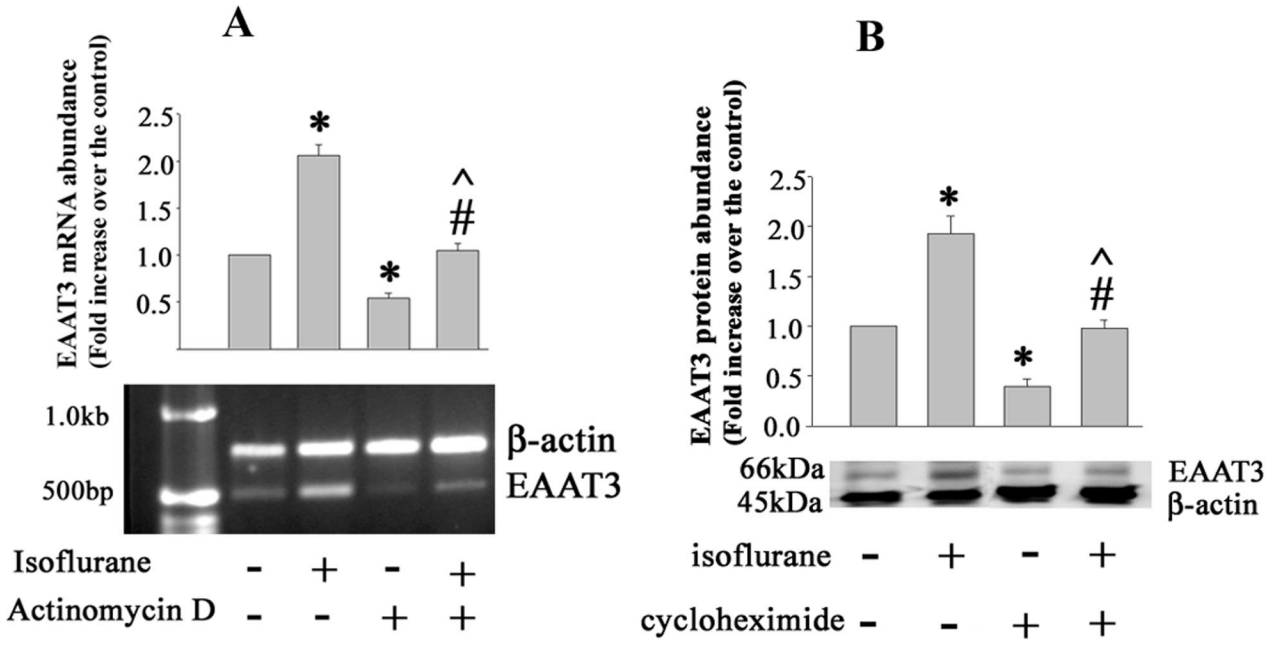

Fig. 5. The effects of actinomycin D (15 μg/ml) and cycloheximide (50 μg/ml) on 2% isoflurane–induced increase of excitatory amino acid transporter type 3 (EAAT3) mRNA (*A*) and protein (*B*) expression in C6 glioma cells. Cells were incubated with isoflurane in the presence or absence of indicated agents for 3 h at 37°C for mRNA quantification and 7 h at 37°C for protein quantification. The *graphs* present the EAAT3 mRNA or protein abundance quantified by integrating the volume of bands from three separate experiments, and a representative film image of the bands is shown. Values in the *graphs* are the mean ± SD of the fold change over controls, with controls being set as 1. **P* < 0.05 compared with control. #*P* < 0.05 compared with actinomycin D or cycloheximide alone; ^*P* < 0.05 compared with isoflurane alone.

Isoflurane-induced Increase of EAAT3 Expression Was Independent of Protein Kinase C or Phosphatidylinositol 3-kinase

Similar to isoflurane, PMA also increased EAAT3 mRNA and protein expression (fig. 6). Although the maximal increase of EAAT3 mRNA and proteins induced by isoflurane was at 2%, the combination of 2% isoflurane and 100 nM PMA caused an even greater increase in EAAT3 mRNA and protein expression than that in the presence of 2% isoflurane alone (fig. 6). Staurosporine decreased the expression of EAAT3 mRNA and proteins in control cells. Although cells incubated with the combination of staurosporine and isoflurane had decreased EAAT3 mRNA expression compared with cells incubated with isoflurane alone, this decrease mainly resulted from the decrease in control level (fig. 6). Staurosporine did not decrease the EAAT3 protein expression level in isoflurane-treated cells (fig. 6). Wortmannin did not affect the expression of EAAT3 mRNA and proteins in either control or isoflurane-treated cells (fig. 6).

β-actin Expression Was Not Changed under Any Experimental Conditions Used in This Study

β-actin, an abundant cellular protein, has often been used as an internal standard to normalize expression data of other proteins. As shown in table 1, various concentrations of isoflurane did not change the expression of β-actin mRNA and proteins. Similarly, all other treatment conditions used in this study did not alter the expression of β-actin compared with the corresponding controls (data not shown).

Discussion

Glutamate transporters play an important role in extracellular glutamate homeostasis.[3] Because inhibition of glutamate-mediated excitatory neurotransmission seems to be one of the mechanisms by which general anesthetics induce anesthesia,[21] multiple studies have been performed to investigate anesthetic effects on EAAT activity.[5–9] Some early studies have suggested no effects or inhibitory effects of volatile anesthetics on EAAT activity.[8,9] Recently, it has been reported that isoflurane at clinical concentrations did not affect glutamate uptake by synaptosomes prepared from rat cerebral cortex.[22] However, multiple other studies have demonstrated that acute exposure to volatile anesthetics enhances the activity of EAATs.[5–7] These reported anesthetic effects on EAAT activity may represent one mechanism by which volatile anesthetics affect glutamate-mediated neurotransmission in the CNS. We now show that isoflurane also enhances the expression of EAAT3, a novel site of interaction for volatile anesthetics to affect glutamate transporters. This interaction is important, because the isoflurane-induced increase in EAAT3 expression results in increased specific activity of EAAT3.

It has been concluded in the literature that C6 glioma

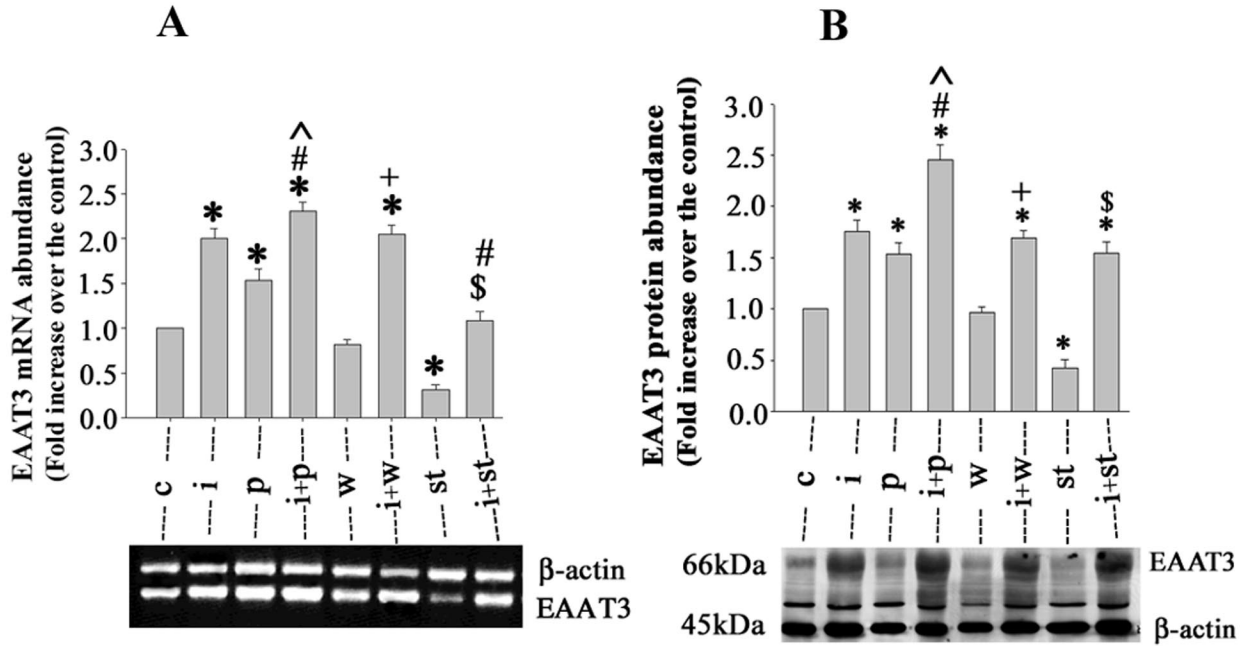

Fig. 6. The effects of PMA (100 nM), staurosporine (50 nM), and wortmannin (100 nM) on 2% isoflurane–induced increase of excitatory amino acid transporter type 3 (EAAT3) mRNA (*A*) and protein (*B*) expression in C6 glioma cells. Cells were incubated with isoflurane in the presence or absence of indicated agents for 3 h at 37°C for mRNA quantification and 7 h at 37°C for protein quantification. The *graphs* present the EAAT3 mRNA or protein abundance quantified by integrating the volume of bands from three separate experiments, and a representative film image of the bands is shown. Values in the *graphs* are the mean ± SD of the fold change over controls, with controls being set as 1. **P* < 0.05 compared with control. #*P* < 0.05 compared with isoflurane alone. ^*P* < 0.05 compared with PMA alone. +*P* < 0.05 compared with wortmannin alone. $*P* < 0.05 compared with staurosporine alone. c = control; i = 2% isoflurane; p = phorbol 12-myristate 13-acetate; st = staurosporine; w = wortmannin.

cells express EAAT3 only.[12] Through its effects on transporting glutamate into neurons, EAAT3 may be important in regulating extracellular glutamate concentrations and preventing glutamate-induced neurotoxicity.[3,23] In addition, EAAT3, the widely distributed neuronal EAAT in the CNS (EAAT4, the other neuronal EAAT, is mainly expressed in the cerebellum), has been implicated to contribute to neurotransmitter γ-aminobutyric acid synthesis and inhibitory synaptic strength (*via* its function to uptake glutamate, which is a substrate for γ-aminobutyric acid synthesis).[24,25] Dysfunction of EAAT3 has also been linked to the development of epilepsy.[24,26] Thus, our results may have significant implications in biology and clinical medicine. Although exposure for 3 h or longer to isoflurane was needed to increase EAAT3 expression significantly in our study and 3 h may be a long exposure time for many surgeries, anesthetics are frequently administrated in excess of 3 h for numerous surgical procedures, including major vascular surgery, major neurosurgery, and organ transplantation. In our study, isoflurane at concentrations of 1–3% significantly increased EAAT3 expression. These isoflurane concentrations are clinically relevant, because 1 minimum alveolar concentration (MAC) that inhibits 50% of human subjects in response to surgical stimuli is 1.15%[27] and isoflurane concentrations higher than 1 MAC are frequently used during anesthesia. Therefore, our current clinical practice indeed provides the conditions for isoflurane to enhance EAAT3 expression in some patients. Although it is not appropriate to extrapolate our results directly to *in vivo* conditions or clinical practice, our data suggest a potential site for volatile anesthetics to act in the CNS.

Although EAAT3 is widely distributed in the CNS, the data on the regulation of expression of this glutamate transporter are scarce in the literature. Transection of

Table 1. The Effects of Isoflurane on the Expression of β-actin mRNA and Proteins

	Isoflurane (%)					
	0	0.5	1	2	3	4
mRNA	1.0 ± 0.0	0.94 ± 0.05	1.01 ± 0.06	0.96 ± 0.06	0.94 ± 0.07	0.86 ± 0.09
Proteins	1.0 ± 0.0	0.97 ± 0.05	0.99 ± 0.05	0.95 ± 0.07	0.91 ± 0.02	0.87 ± 0.10

β-actin data that were used to normalize excitatory amino acid transporter type 3 data in fig. 2 are presented here. Results are mean ± SD of the fold change over the corresponding controls, with controls being set as 1. There is no statistically significant difference between controls and samples treated with isoflurane at any tested concentrations.

nervus hypoglossus led to an increase in EAAT3 mRNA in nucleus nervi hypoglossi; however, the study was not followed up to see if EAAT3 proteins were increased.[28] Hypoxia also induced a time-dependent increase in EAAT3 mRNA and protein expression in cultured C6 cells (this increased expression was considered to be a compensatory mechanism for the dysfunction of EAATs during hypoxia in the study).[13] However, neither study included mechanistic investigation of the increase in EAAT3 expression. Volatile anesthetics have been demonstrated to affect the expression of some genes, such as nitric oxide synthases[11]; however, the mechanisms for these anesthetic actions are generally unknown. Our results demonstrated that isoflurane increased the expression of both mRNA and proteins of EAAT3 and that the degree of the isoflurane-induced increase in mRNA and proteins was not affected by either actinomycin D (an mRNA synthesis inhibitor) or cycloheximide (a protein synthesis inhibitor). These results suggest that the effects of isoflurane on EAAT3 expression are not *via* mRNA or protein synthesis and thus may be *via* processes such as prolongation of EAAT3 mRNA and protein half-life.

We then investigated if PKC or PI3K mediated the effects of isoflurane on EAAT3 expression. Both PKC and PI3K have been shown to regulate EAAT activity and trafficking.[12,29] PKC also may mediate the anesthetic effects on EAAT3 activity.[10] In addition, hypoxia-induced increase in EAAT2 expression was shown to be PKC-dependent (the study did not determine if hypoxia-induced increase in EAAT3 expression also was PKC-dependent).[13] However, our results did not suggest the involvement of PKC in the isoflurane effects on EAAT3 expression. PMA, a PKC activator, increased EAAT3 expression. The combination of PMA and 2% isoflurane produced an increase in EAAT expression higher than that caused by 2% isoflurane alone. Our dose–response experiments showed that 2% isoflurane induced a maximal effect on EAAT3 expression. These results suggest that isoflurane may act through mechanisms that are different from those for PMA to increase EAAT3 expression. Consistent with this idea, staurosporine did not affect the isoflurane-induced enhancement of EAAT3 expression. Although staurosporine is considered to be a general PKC inhibitor that can also inhibit other kinases such as Ca^{2+}/calmodulin kinase,[30] the failure of staurosporine to inhibit isoflurane-induced increase in EAAT3 expression suggests that PKC is not involved in these isoflurane effects. In the case of PI3K, the activation of PI3K has been implicated in the mechanisms of upregulation of EAAT2 expression in cultured glial cells by dibutyryl cyclic adenosine monophosphate, epidermal growth factor, and conditioned media from neuronal cultures.[31] Wortmannin, a PI3K inhibitor, did not affect the isoflurane-induced increase in EAAT3 expression in our study, however, suggesting that PI3K is not involved in these isoflurane effects.

Interestingly, both dose–response and time course experiments demonstrated bell-shaped responses of EAAT3 expression to isoflurane. The mechanisms for the recovery of EAAT3 expression to basal level at high isoflurane concentrations and with long incubations are not clear. Because there were no changes of EAAT3 expression over time in control cells, the effects of the cultured cells at different time points of the cell cycle on EAAT3 expression may not contribute to the phenomenon: recovery of EAAT3 expression at high isoflurane concentrations and with long duration of isoflurane incubation. In addition, these bell-shaped responses of EAAT3 expression to isoflurane may not be due to isoflurane toxicity, because the survival and viability of C6 cells were not affected by isoflurane at the concentrations used in this study.

In summary, we demonstrated that isoflurane at clinically relevant concentrations enhanced EAAT3 expression and activity. This represents a novel site of action for anesthetic effects on glutamate transporters, a group of proteins that are important to regulate the extracellular glutamate concentrations. The mechanisms of these anesthetic effects seem to be through processes such as stabilization of EAAT3 mRNA and proteins. PI3K and PKC, two kinases that have been demonstrated to be important in regulating EAAT activity and expression, may not be involved in the isoflurane effects on EAAT3 expression.

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