0026-895X/05/6705-1522–1533$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 5
7443/1198168

Mol Pharmacol 67:1522–1533, 2005
Printed in U.S.A.

Isoflurane Induces a Protein Kinase C α-Dependent Increase in Cell-Surface Protein Level and Activity of Glutamate Transporter Type 3

Yueming Huang and Zhiyi Zuo

Department of Anesthesiology, University of Virginia Health System, Charlottesville, Virginia

Received September 19, 2004; accepted February 11, 2005

ABSTRACT

Glutamate transporters regulate extracellular concentrations of glutamate, an excitatory neurotransmitter in the central nervous system. We have shown that the commonly used anesthetic isoflurane increased the activity of glutamate transporter type 3 (excitatory amino acid transporter 3, EAAT3) possibly via a protein kinase C (PKC)-dependent pathway. In this study, we showed that isoflurane induced a time- and concentration-dependent redistribution of EAAT3 to the cell membrane in C6 glioma cells. This redistribution was inhibited by staurosporine, a pan PKC inhibitor, or by 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole (Gö6976) at a concentration that selectively inhibits conventional PKC isozymes (PKCα, -β, and -γ). This isoflurane-induced EAAT3 redistribution was also blocked when the expression of PKCα but not PKCβ proteins was down-regulated by the respective antisense oligonucleotides. The isoflurane-induced increase of glutamate uptake by EAAT3 was abolished by the down-regulation of PKCα expression. Immunoprecipitation with an anti-EAAT3 antibody pulled down more PKCα in cells exposed to isoflurane than in control cells. Isoflurane also increased the phosphorylated EAAT3 and the redistribution of PKCα to the particulate fraction of cells. Consistent with the results in C6 cells, isoflurane also increased EAAT3 cell-surface expression and enhanced the association of PKCα with EAAT3 in rat hippocampal synaptosomes. Our results suggest that the isoflurane-induced increase in EAAT3 activity requires an increased amount of EAAT3 protein in the plasma membrane. These effects are PKCα-dependent and may rely on the formation of an EAAT3-PKCα complex. Together, these results suggest an important mechanism for the regulation of glutamate transporter functions and expand our understanding of isoflurane pharmacology at cellular and molecular levels.

Glutamate transporters (also called excitatory amino acid transporters, EAAT) are important in regulating extracellular concentrations of glutamate (Danbolt, 2001), a major excitatory neurotransmitter in the mammalian central nervous system (CNS). By transporting glutamate from extracellular to intracellular space under physiological conditions, EAATs prevent extracellular glutamate accumulation and regulate glutamate neurotransmission. Five EAATs have been identified: EAAT1 to EAAT5 (Danbolt, 2001). In rats, EAAT1 and EAAT2 are found in glial cells, and EAAT3 and EAAT4 are mainly expressed in neurons, whereas EAAT5 is located in neurons and glial cells of retina (Rothstein et al., 1994; Lehre et al., 1995; Arriza et al., 1997). The transporting functions of all five EAATs are sodium-dependent. They use the transmembrane gradient of Na^+, K^+, and H^+ as a driving force to uptake glutamate (Billups et al., 1998; Danbolt, 2001).

Studies on regional distribution of EAATs have shown that EAAT3 is widely distributed in forebrain, hippocampus, and cerebellum and that EAAT4 is largely restricted to the molecular cell layer of cerebellum (Rothstein et al., 1994; Danbolt, 2001). Thus, EAAT3 is the major neuronal EAAT in the CNS. We have shown that isoflurane, a commonly used volatile anesthetic in clinical practice, increases EAAT3 activity and that these effects are possibly protein kinase C (PKC)-dependent (Do et al., 2002). It has been shown that PKC activation-induced increase of EAAT3 activity is associated with an increase of EAAT3 proteins in the plasma membrane (Davis et al., 1998) and that PKCα is involved in regulating EAAT3 expression in the plasma membrane (Gonzalez et al., 2002). In this study, we determined whether isoflurane in-

This study was supported by National Institutes of Health grants R01-GM065211 and R01-NS045983 (to Z.Z.).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.007443.

ABBREVIATIONS: EAAT, excitatory amino acid transporter; CNS, central nervous system; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; IPSC, inhibitory postsynaptic current; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; PBS-Ca/Mg, phosphate-buffered saline containing calcium and magnesium.

creases cell-surface expression of EAAT3 proteins and whether the isoflurane-induced increase of EAAT3 activity and cell-surface expression is PKC isozyme-specific. We used C6 glioma cells as one of our study models. C6 cells have been shown to express EAAT3 only and have been frequently used in studies of EAAT3 activity and cell-surface expression (Davis et al., 1998; Gonzalez et al., 2002). We then used rat hippocampal synaptosomes to determine whether the results observed in C6 cells are relevant to the CNS. Our results indicate that isoflurane induced a PKCα-dependent increase of EAAT3 activity and cell-surface expression and that isoflurane also increased the coimmunoprecipitation of PKCα with EAAT3, which may suggest an increased interaction between these two proteins in cells.

Materials and Methods

Materials. Ham's F-10 nutrient mixture was from Invitrogen (Carlsbad, CA). Six-well tissue culture plates and 25- and 75-cm^2 tissue culture flasks were manufactured by Corning Glassworks (Corning, NY). L-[^{3}H]Glutamate (specific activity, 56 Ci/mM) was purchased from Amersham Biosciences Inc. (Piscataway, NJ). Wizard Plus Minipreps were from Promega (Madison, WI). Anti-PKCα, PKCβ I, PKCδ, and PKCϵ antibodies and Protein A/G Plus-Agarose were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Affinity-purified polyclonal rabbit anti-EAAT1 and anti-EAAT3 antibodies were from Alpha Diagnostics International (San Antonio, TX). Sulfo-*N*-hydroxysulfosuccinimidobiotin and immunopure-immobilized monomeric avidin were from Pierce (Rockford, IL). Fluorescent antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA). Oligonucleotides were prepared by the University of Virginia Biomolecular Research Facility (Charlottesville, VA). Nitrocellulose membranes for Western blot were from Bio-Rad (Hercules, CA). Sytox green nucleic acid stain, ProLong Antifade Kit, and Pro-Q Diamond dye were from Molecular Probes (Eugene, OR). Rat C6 glioma cells were from American Type Culture Collection (Manassas, VA). Sprague-Dawley rats were from Hilltop Laboratory Animals, Inc. (Scottsdale, PA). Complete protease inhibitors were from Roche Applied Science (Indianapolis, IN). Gö6976 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Isoflurane Incubation. Rat C6 glioma cells were cultured in flasks (for biotinylation and immunoprecipitation experiments) or on coverslips (for immunocytochemistry experiments, coverslips were placed in six-well plates) in Ham's F-10 nutrient mixture containing 15% horse serum and 2.5% fetal bovine serum at 37°C in a 95% air/5% CO_2 incubator. When cells were ~80% confluent, the culture medium was replaced with a serum-free medium (Ham's F-10 mixture only) for 24 h before isoflurane incubation.

Primary culture of mixed glial cells from rat cerebrum was prepared as described before (Zuo, 2001). They were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. They were exposed to isoflurane when they were ~80% confluent and had been in the serum-free Dulbecco's modified Eagle's medium for 24 h.

C6 cells and primary glial cell culture were incubated with isoflurane in an open system as follows. Fresh serum-free medium (50–200 ml) that had been gassed with 95% air/5% CO_2 through or not through an isoflurane vaporizer at a flow rate of 3 l/min for 20 min was added to the cells for the preset periods at 37°C. Preliminary experiments with gas chromatography showed that isoflurane concentrations in the medium reached equilibrium 5 min after the onset of gassing under the current experimental conditions. During the incubation, the medium was continuously gassed with the carrier gases containing or not containing isoflurane to compensate for isoflurane loss from the solution to air.

Reagent Application before or during Isoflurane Incubation. The PKC inhibitor staurosporine (50 nM) or Gö6976 (10 μM) was applied during the isoflurane incubation. PKCα antisense and random control oligonucleotides in serum-free medium were incubated with the cells for 5 days before the isoflurane incubation. The solution was replaced every 36 h with fresh medium containing the oligonucleotides. The sequences of PKCα antisense and random oligonucleotides were as follows: 5′-GC CGG GTA AAC GTC AGC CAT-3′ (antisense) and 5′-CCTGAGAGCCAATCAGGCGT-3′ (random) (Baltuch et al., 1995). The antisense oligonucleotide was designed to target the translation start site of rat PKCα. The random oligonucleotide has base composition identical with that of antisense oligonucleotide and served as a control. In the case of PKCβ downregulation, cells were incubated with PKCβ antisense and sense oligonucleotides for 3 days with the medium change every 24 h. The sequences of PKCβ antisense and sense oligonucleotides were from a previous publication (Korchak et al., 1998): 5′-AGC CGG GTC AGC CAT CTT G-3′ (antisense) and 5′-C AAG ATG GCT GAC CCG GCT-3′ (sense). The antisense oligonucleotide was designed against the translation start site of rat PKCβ. Because PKCβ I and PKCβ II differ only in the 3′ terminus, this antisense oligonucleotide should bind to the mRNA of both PKCβ I and PKCβ II. All oligonucleotides for PKCα and PKCβ were phosphorothioate derivatives to prolong their half-lives.

Biotinylation. Biotinylation of cell-surface proteins was performed as described previously (Davis et al., 1998), with some modifications. After incubation with or without isoflurane, cells that were grown in 75-cm^2 tissue culture flasks were rinsed twice with warm phosphate-buffered saline (PBS) containing 0.1 mM calcium and 1.0 mM magnesium (PBS-Ca/Mg). The cells were then incubated with 2 ml of biotin solution (sulfo-*N*-hydroxysulfosuccinimidobiotin, 1 mg/ml in PBS-Ca/Mg) for 20 min at 4°C with gentle shaking. The biotinylation reaction was terminated by washing the cells three times with ice-cold PBS-Ca/Mg containing 100 mM glycine. After the cells were incubated in this wash solution for 45 min at 4°C with gentle agitation, they were then lysed in 2 ml of lysis buffer containing 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 250 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mg/ml trypsin inhibitor, and 1 mM iodoacetamide for 1 h at 4°C with vigorous shaking. The total lysates were centrifuged at 20,000*g* for 20 min at 4°C to remove nuclei and debris. The resulting supernatants (aliquots of the supernatants were kept for Western analysis and are labeled as lysates in Fig. 1) were incubated with equal volumes of suspension of avidin-conjugated beads (600 μl of bead suspension to 600 μl of lysates) for 1 h at room temperature with occasional stirring. The mixture was then centrifuged at 16,500*g* for 15 min at 4°C. This second supernatant was also collected for Western analysis and is labeled as intracellular fraction in Fig. 1. After being washed four times, each time with 1 ml of lysis buffer, the pellet that contained the biotinylated cell-surface proteins was resuspended in 500 μl of Laemmli buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, and 5% 2-mercaptoethanol for 30 min to dissolve the biotinylated proteins. The mixture was centrifuged at 16,500*g* for 10 min at 4°C, and this third supernatant was kept for Western blot as the biotinylated fraction in Fig. 1.

Soluble and Particulate Fractionation. The soluble and the particulate fractions of C6 cells were prepared by centrifugation as described before (Zuo and Johns, 1995). Cells were homogenized in ice-cold 20 mM Tris-HCl, pH 7.5, containing 1 mM DL-dithiothreitol, 5 mM EGTA, 2 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin. The homogenate was centrifuged at 45,000*g* for 60 min at 4°C. The supernatant was harvested as the soluble fraction. The pellet that was the particulate-mem-

brane fraction was washed three times with ice-cold homogenization buffer and then resuspended in the buffer by sonication.

Western Blotting. After protein content in samples was quantified by the Lowry assay using a protein assay kit, 25 μg of protein per lane was subjected to Western analysis as described before (Zuo and Johns, 1997; Huang and Zuo, 2003). In brief, proteins were separated with 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then were electrotransferred to nitrocellulose membranes. The protein bands were probed with primary antibodies (polyclonal rabbit anti-EAAT3 at 0.5 μg/ml; polyclonal rabbit anti-EAAT1 at 1:500 dilution; polyclonal rabbit anti-β-actin at 1:3000 dilution; monoclonal mouse anti-PKCα and monoclonal mouse anti-PKCβ I at 1:500 dilution; and polyclonal rabbit anti-PKCδ and rabbit anti-PKCϵ at 1:1000 dilution) and then a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibody (1:1000) and finally visualized by the enhanced chemiluminescence method with multiple exposures of films because of the limited linear range of intensity produced by this method. Quantitative analysis of the protein bands was performed using an ImageQuant 5.0 densitometer (Amersham Biosciences). The relationship between the protein band signal and exposure time of the heaviest band on the films was established. Protein bands on a film in which the intensity of the heaviest band was still within the linear range were measured to generate the data reported here.

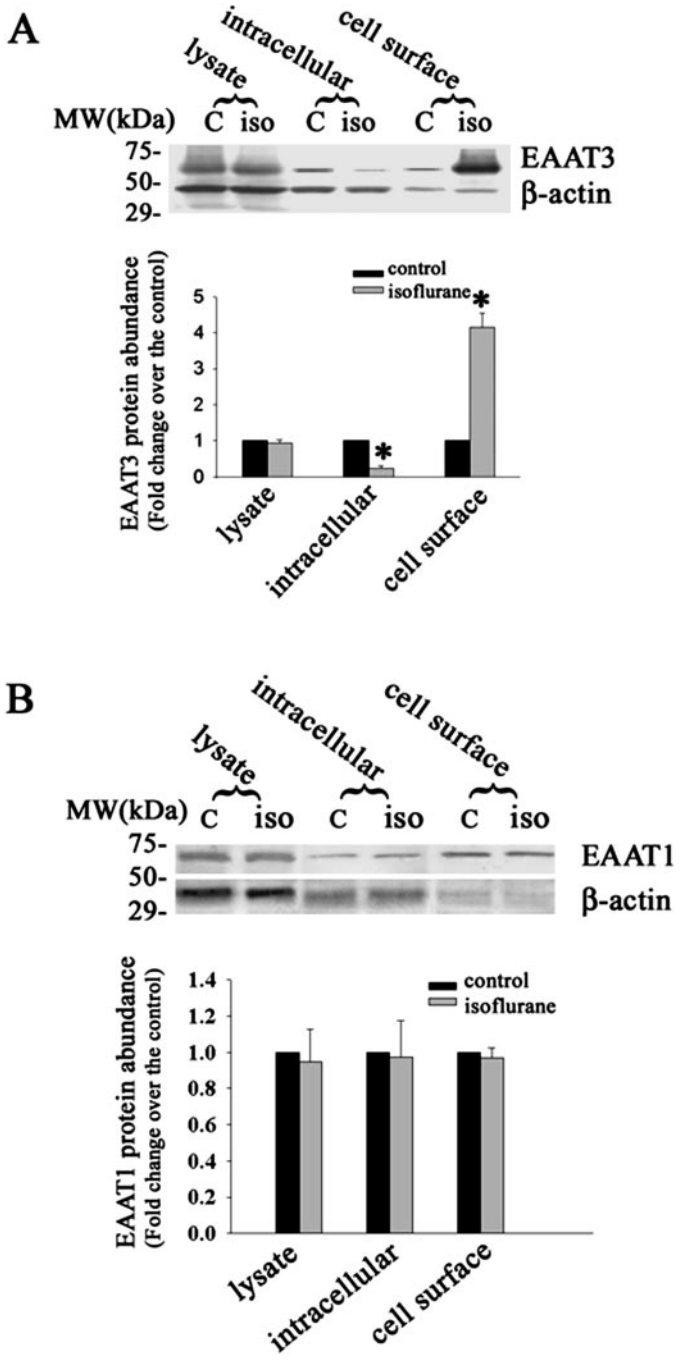

Fig. 1. Isoflurane-induced EAAT3 and EAAT1 redistribution. C6 glioma cells (A) or primary glial cell culture of rat (B) were incubated with 2% isoflurane for 5 min at 37°C. Total cell lysates were then separated into intracellular and cell-surface fractions by biotinylation method. A representative Western blot is shown at the top, and the graphic presentation of the EAAT3 or EAAT1 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in the graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the corresponding control. C, control; iso, 2% isoflurane.

Immunoprecipitation. As described previously (Gonzalez et al., 2003), C6 cells cultured in 75-cm^2 dishes were lysed in 2 ml of buffer containing 50 mM Tris-HCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM NaCl, and complete protease inhibitors for 1 h at 4°C. The lysates were centrifuged at 14,000*g* for 15 min to remove cell debris. The resulting supernatants were incubated overnight with 2 μg of affinity-purified polyclonal rabbit anti-EAAT3 antibody at 4°C. The mixture was then incubated with 40 μl of Protein A/G Plus-Agarose beads for 1 h at 4°C with gentle shaking. The sample was then centrifuged at 500*g* for 2 min at 4°C. An aliquot of supernatant was saved for Western blotting. The pellet containing bead-bound immune complexes was washed four times with the lysis buffer, and the immune complexes were then eluted by incubation with 100 μl of Laemmli buffer at 90 to 95°C for 5 min. The samples were also used for Western blotting. Control experiments using beads alone or rabbit IgG to replace the rabbit anti-EAAT3 antibody were performed to show the specificity of the anti-EAAT3 antibody.

Phosphoprotein Staining. Immunoprecipitates produced from C6 cell protein samples with an anti-EAAT3 antibody were separated with 10% SDS-PAGE. The gel was then stained with Pro-Q Diamond dye. The staining was visualized with a Typhoon 9400 Variable Mode Imager (Amersham Biosciences) at an excitation wavelength of 546 nm and an emission wavelength of 580 nm. This dye technology is very sensitive to stain phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins.

Preparation of Crude Synaptosomes and Use of Synaptosomes for Biotinylation and Immunoprecipitation Studies. The preparation of crude synaptosomes was performed as described before (Robinson, 1998). All steps during the preparation were done at 4°C. In brief, after decapitation of adult male Sprague-Dawley rats under halothane anesthesia, hippocampi were immediately dissected on ice and weighed (usually approximately 100 mg; the volumes described in the following steps were derived from this wet weight of the hippocampi). The hippocampi were homogenized in 20 volumes of 0.32 M sucrose at 400 rpm for 8 strokes with a homogenizer (Brinkmann Instruments, Westbury, NY). The solution was then centrifuged at 800*g* for 10 min. The supernatant was centrifuged again at 20,000*g* for 20 min. The pellet was resuspended in 40 volumes of sucrose and repelleted by centrifugation at 20,000*g* for 20 min. This washed P2 pellet was crude synaptosomes and was resuspended in 4 ml of PBS-Ca/Mg containing 10 mM dextrose that had been gassed with or without 2% isoflurane at 37°C for 20 min. The incubation was for 5 min at 37°C, and sulfo-*N*-hydroxysulfosuccinimidobiotin was added to reach a concentration of 1 mg/ml. After incubation for 20 min at 4°C, the solution was centrifuged at 20,000*g* for 20 min at 4°C. The biotinylated proteins in the pellet were separated from the nonbiotinylated proteins as described under *Biotinylation*. The immunoprecipitation with the anti-EAAT3 antibody was performed on synaptosomes incubated with or without 2% isoflurane as described under Immunoprecipitation.

Immunocytochemistry and Observation under Confocal Microscopy. As described before (Davis et al., 1998; Gonzalez et al., 2003), after incubation with or without isoflurane, C6 glioma cells on glass coverslips were fixed with 2% paraformaldehyde for 20 min. They were then incubated with 50 mM ammonium chloride in PBS for 10 min at room temperature, with 0.2% Triton X-100 in PBS for 2 min on ice, and then with 3% bovine serum albumin solution for 1 h at 37°C. Anti-EAAT3 antibody (1.25 μg/ml) was added for 1 h at 37°C. The cells were subsequently incubated with Donkey anti-rabbit IgG-Texas red at 1:500 dilution for 1 h at 37°C and with 5 mM sytox green nucleic acid stain at 1:1000 dilution for 20 min at room temperature. Cells were washed after each step with PBS two to three times and finally were washed with water before the coverslips were mounted with the use of the ProLong Antifade Kit. Serial sections (0.5 μm) of the labeled cells were obtained using a Zeiss 410 Axiovert 100 microsystems LSM confocal microscope (Carl Zeiss GmbH, Jena, Germany). Attenuation, contrast, brightness, and pinhole aperture remained constant during the observation of control cells and cells subjected to various treatments.

Glutamate Uptake Assay. As described before (Zuo, 2001; Huang and Zuo, 2003), C6 cells grown in 25-cm^2 flasks were washed twice with wash buffer containing 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, and 10 mM dextrose, pH 7.2. They were then incubated with 10 μM L-[^{3}H]glutamate in the wash buffer in the presence or absence of 2% isoflurane for 5 min at 37°C. The incubation was terminated 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.

Statistical Analysis. The intensity of EAAT1, EAAT3, and PKC protein bands was normalized to that of β-actin to control for errors in protein-sample loading and transferring during the Western blot analysis when total cell lysates or cytosolic fractions were used for the analysis. The intensity of PKC protein bands was normalized to that of EAAT3 bands when immunoprecipitates were analyzed by Western blotting. The band intensity of phosphoproteins corresponding to EAAT3 was normalized to that of total EAAT3 bands on Western blot. Results are means ± S.D. of the -fold changes over the controls, with controls being set as 1. Results of glutamate uptake assay are means ± S.D. of the measured numbers in each sample. Statistical analysis was performed by unpaired t test or one-way analysis of variance followed by the Student-Newman-Keuls test for post hoc comparison as appropriate. A P value <0.05 was considered significant.

Results

Isoflurane Induces EAAT3 Redistribution to Cell Surface of C6 Cells. Because previous studies have shown that isoflurane increased EAAT3 activity and that EAAT3 redistribution to the plasma membrane is one of the mechanisms that increases EAAT3 activity (Davis et al., 1998; Do et al., 2002; Gonzalez et al., 2002), we tested whether isoflurane induced an EAAT3 redistribution to the plasma membrane in this study. The anti-EAAT3 antibody detected a protein band with a mobility corresponding to ~60 kDa on SDS-PAGE. Likewise, a protein band at ~45 kDa was detected by the anti-β-actin antibody (Fig. 1). The sizes of these detected proteins are consistent with the sizes reported for these proteins in the literature (Gonzalez et al., 2002; Huang and Zuo, 2003). The total amount of EAAT3 protein in C6 cells after exposure to 2% isoflurane for 5 min was similar to that in control cells. However, EAAT3 protein at the cell surface (the biotinylated EAAT3) was significantly increased after the isoflurane exposure (415 ± 39% of the control, n = 3). This increase of EAAT3 protein at the cell surface was associated with a decrease of cytosolic EAAT3 protein (22 ± 9% of the control, n = 3) (Fig. 1). These results suggest that a short exposure of cells to isoflurane induced a redistribution of EAAT3 to the plasma membrane without changing the total amount of EAAT3 protein in C6 glioma cells. However, isoflurane exposure did not change the distribution of EAAT1 in the plasma membrane and intracellular compartments of the primary culture of rat glial cells (Fig. 1). These results suggest that isoflurane-induced EAAT3 redistribution is specific.

Isoflurane-Induced EAAT3 Redistribution in C6 Cells Is Time- and Concentration-Dependent. The isoflurane (2%)-induced EAAT3 redistribution to the plasma membrane was rapid and its reached maximum when the cells had been incubated with isoflurane for 5 min (441 ± 56% of the control, n = 3) (Fig. 2). The EAAT3 protein at the cell surface in cells with longer isoflurane exposures gradually returned to the control levels (Fig. 2). Immunocytochemistry studies showed punctate immunostaining of EAAT3 throughout the cytoplasm under control conditions, a pattern reported previously (Davis et al., 1998). Isoflurane caused significant EAAT3 clustering, especially at the periphery of the cells (Fig. 2). Consistent with the biotinylation results, these isoflurane-induced changes detected by immunocytochemistry were also time-dependent (Fig. 2).

The isoflurane-induced increase in EAAT3 cell-surface expression was also concentration-dependent. Isoflurane at 1% for 5 min had already increased significantly EAAT3 proteins at the cell surface (240 ± 25% of the control, n = 3), and 2% isoflurane induced a maximal effect (Fig. 3).

Isoflurane-Induced EAAT3 Redistribution in C6 Cells Is PKCα-Dependent. Because our previous study suggested that the isoflurane-induced increase in EAAT3 activity may be PKC-mediated (Do et al., 2002), we tested whether an isoflurane-induced increase of EAAT3 cell-surface expression was PKC-dependent. Our results showed that the isoflurane (2% for 5 min)-induced increase in EAAT3 cell-surface expression was significantly decreased by staurosporine, a pan PKC inhibitor (Fig. 4). These results suggest that the isoflurane-induced increase in EAAT3 cell-surface expression may be PKC-dependent. We then used Gö6976, a more selective inhibitor of conventional PKC isozymes, at low micromolar concentrations (Davis et al., 1998; Gonzalez et al., 2003). Gö6976 at 10 μM alone did not affect the cell-surface expression of EAAT3 proteins. However, it significantly inhibited the isoflurane-induced increase of EAAT3 cell-surface expression (Fig. 4), suggesting the involvement of conventional PKC isozymes in the isoflurane effect.

To identify the PKC isozymes that are involved in the isoflurane effects on EAAT3 cell-surface expression, we used antisense oligonucleotides to selectively down-regulate the expression of PKC isozymes. Our results showed that the anti-PKCα antibody detected a protein band at ~80 kDa and that the anti-PKCβ I antibody detected a protein band at ~70 kDa in C6 cells (Fig. 5). These protein sizes are similar to those that are reported for PKCα and PKCβ I in the literature (Dean and McKay, 1994; Korchak et al., 1998). These proteins were selectively down-regulated by PKCα and PKCβ antisense oligonucleotides, respectively, but not by their corresponding sense or random control oligonucleotides

(Fig. 5). PKCα antisense oligonucleotides at 5 μM for 5 days and PKCβ antisense oligonucleotides at 1 μM for 3 days reduced the expression of the corresponding PKC isozymes to less than 20% of the control levels (Fig. 5). The expression of PKCα was not affected by the PKCβ antisense and sense oligonucleotides. The expression of PKCβ I was not affected by the PKCα antisense and random oligonucleotides (Fig. 5). In the cells in which PKCα was down-regulated, isoflurane (2% for 5 min) no longer induced an EAAT3 redistribution to the plasma membrane (Fig. 6), whereas PKCα down-regulation did not affect the surface expression of EAAT3 proteins under basal conditions (EAAT3 protein in the cell surface in the presence of PKCα antisense and random oligonucleotides was 94 $\pm$ 14 and 103 $\pm$ 6% of the control, respectively; $n = 5$).

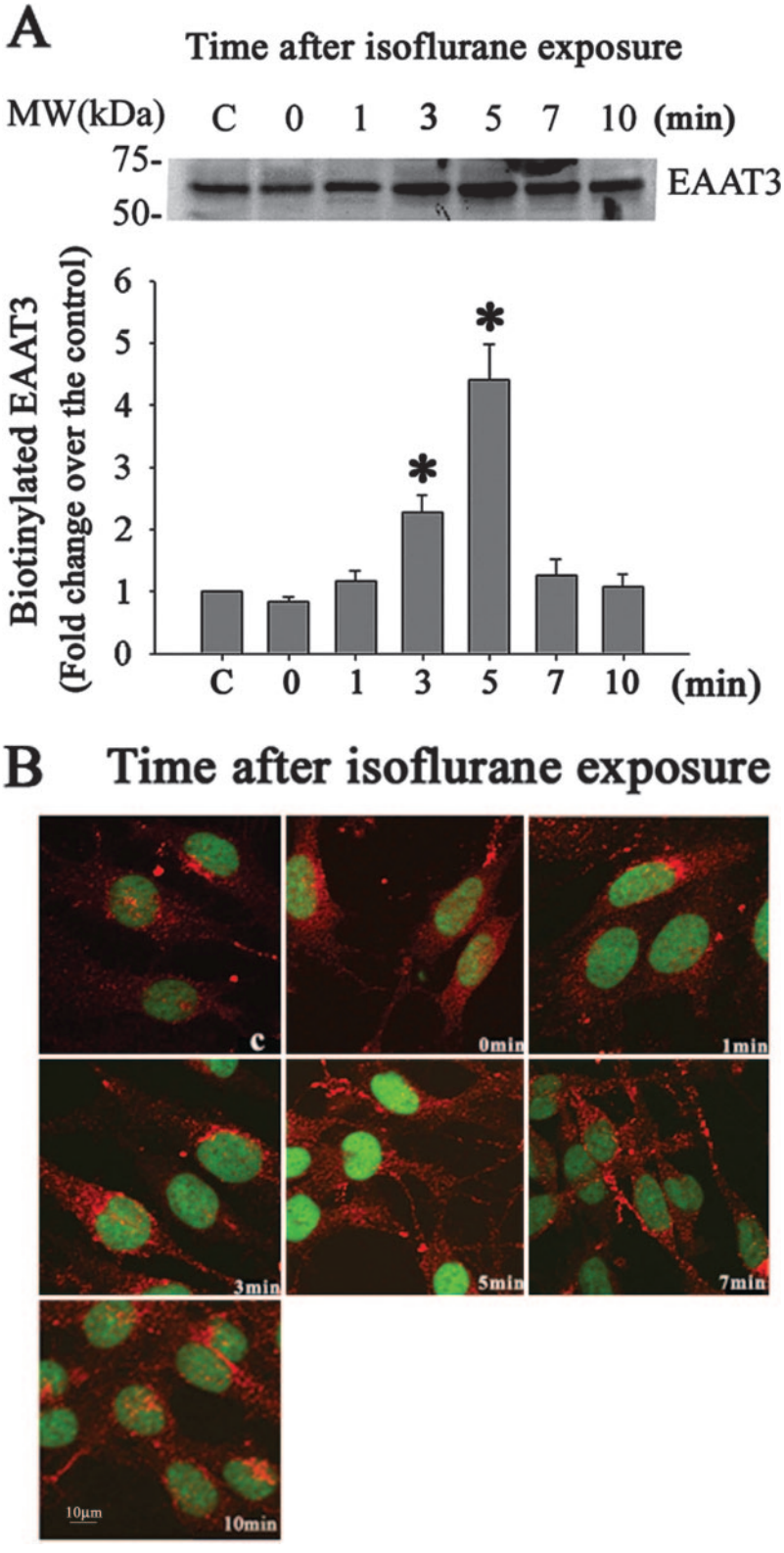

Fig. 2. Time course of isoflurane-induced EAAT3 redistribution. C6 glioma cells were incubated with 2% isoflurane for various times at 37°C. A, the biotinylated fractions of the cells were analyzed by Western blot. A representative Western blot is shown at the top, and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in the graphs are expressed as -fold changes over the control and are presented as the means $\pm$ S.D. *, $P < 0.05$ compared with the control. B, after incubation with 2% isoflurane for various times as indicated, C6 cells were immunostained with an anti-EAAT3 antibody (red). Nuclei were stained with sytox green nucleic acid stain (green). Serial sections (0.5 μm) were observed under confocal microscopy, and the sections through the center of the cell corresponding to the largest cross-sectional nuclear area are shown. C, control.

In contrast, the level of isoflurane-induced increase in EAAT3 cell-surface expression in cells with PKCβ I down-regulation was similar to the level of increase in control cells and in cells without PKCβ I down-regulation (PKCβ sense oligonucleotide-treated cells) (Fig. 6). These results suggest that PKCα is important for the isoflurane-induced increase of EAAT3 cell-surface expression.

Isoflurane-Increased EAAT3 Activity in C6 Cells Is PKCα-Dependent. Because C6 cells have been concluded to express EAAT3 only (Davis et al., 1998) and the glutamate uptake in C6 cells was not inhibited by dihydrokainate (Huang and Zuo, 2003), a relatively selective inhibitor for EAAT2, the sodium-dependent glutamate uptake in C6 cells is considered to be mediated through EAAT3. Consistent with our previous study (Do et al., 2002), isoflurane (2% for 5 min) significantly increased EAAT3 activity in control C6 glioma cells (Fig. 7). Likewise, isoflurane also increased EAAT3 activity in C6 cells treated with PKCα random control oligonucleotides. However, isoflurane did not change the EAAT3 activity in cells that had decreased PKCα expression (Fig. 7). These results suggest that PKCα is important for the isoflurane-induced increase of EAAT3 activity and that isoflurane-induced increase of EAAT3 activity is associated with an isoflurane-induced increase of EAAT3 cell-surface expression.

Isoflurane Increases the Association of PKCα with EAAT3 in C6 Cells. Because PKC activation has been shown to increase the formation of PKCα and EAAT3 complex (Gonzalez et al., 2003), we tested whether isoflurane increased the association of PKCα with EAAT3. When an anti-EAAT3 antibody was used to immunoprecipitate EAAT3 from the lysates of C6 cells, there were no EAAT3 proteins detected by Western analysis in the supernatant ($n = 3$; data not shown), suggesting that the majority of EAAT3 in the samples were immunoprecipitated by this antibody. Our results showed that isoflurane significantly increased the amount of PKCα in the immunoprecipitates produced by the anti-EAAT3 antibody (358 ± 49% of the control, $n = 4$) (Fig. 8). These results are specific because neither PKCα nor EAAT3 protein bands were detected by Western analysis in the precipitates produced by rabbit IgG or beads alone ($n = 3$; data not shown). In addition, although very weak bands of PKCδ and PKCε (almost no band in the case of PKCβ I) were detected in the immunoprecipitates, isoflurane did not change the amount of PKCβ I, PKCδ, and PKCε in the immunoprecipitates (Fig. 8). These results suggest that isoflurane specifically increases the formation of PKCα and EAAT3 complex, which may increase the interaction between these two proteins.

Similar to the results of isoflurane-induced EAAT3 redistribution, the effects of isoflurane on the association of PKCα and EAAT3 are time- and concentration-dependent, because a longer incubation (2% isoflurane for 10 min) or a higher concentration (4% isoflurane for 5 min) did not significantly increase the amount of PKCα in the immunoprecipitates

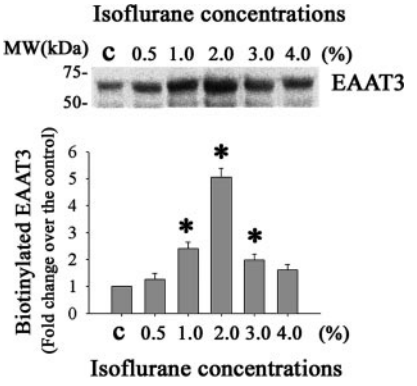

Fig. 3. Concentration-responses of isoflurane-induced EAAT3 redistribution. C6 glioma cells were incubated with various concentrations of isoflurane for 5 min at 37°C. The biotinylated fractions of the cells were analyzed by Western blot. A representative Western blot is shown at the top, and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in the graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. C, control.

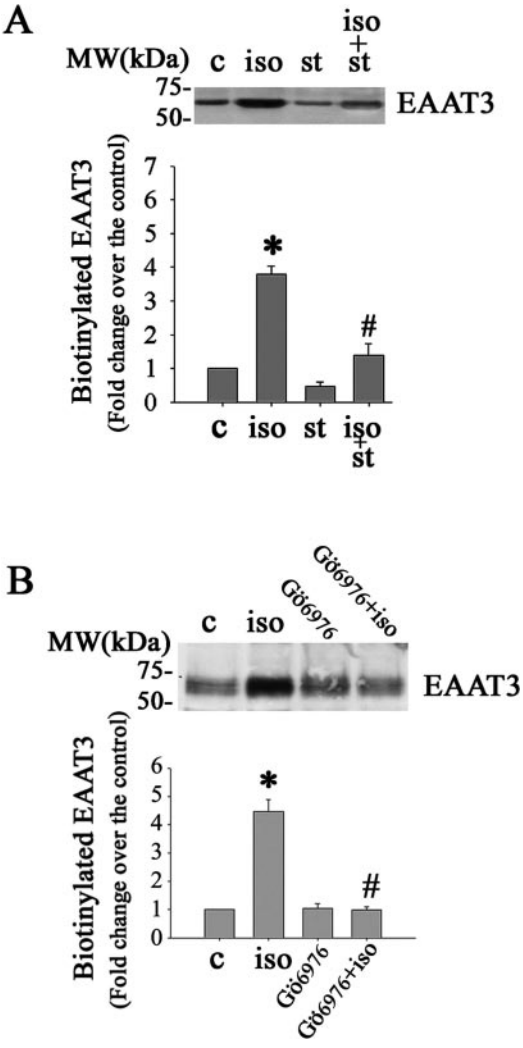

Fig. 4. The effects of PKC inhibition on isoflurane-induced EAAT3 redistribution. C6 glioma cells were incubated with 2% isoflurane for 5 min at 37°C. Staurosporine (50 nM, A), a pan inhibitor of protein kinase C, or Gö6976 (10 μM, B), a relatively selective inhibitor of conventional protein kinase C isozymes, was applied during isoflurane incubation. The biotinylated fractions of the cells were analyzed by Western blot. A representative Western blot is shown at the top, and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control; #, $P < 0.05$ compared with 2% isoflurane alone; C, control; iso, 2% isoflurane; st, 50 nM staurosporine.

produced by the anti-EAAT3 antibody (128 ± 37 and 94 ± 26%, respectively, of the control; $n = 3$) (Fig. 8).

Isoflurane Increases EAAT3 Phosphorylation and PKCα Redistribution to the Particulate Fraction of Cell. More phosphoproteins were presented in the anti-EAAT3 antibody-induced immunoprecipitates prepared from cells exposed to isoflurane than from control C6 cells (460 ± 79% of the control, $n = 3$) (Fig. 9). These phosphoproteins had the same mobility as EAAT3 on SDS-PAGE (Fig. 9). These results suggest that the proteins with increased phosphorylation after isoflurane exposure are EAAT3. Isoflurane exposure also induced a time-dependent increase of PKCα redistribution to the particulate/membrane fraction of C6 cells (Fig. 9). Because PKC activation is often associated with redistribution from the soluble fraction to the particulate/membrane fraction (Kraft and Anderson, 1983; Dorn et al., 1999), these results are evidence of PKCα activation after isoflurane exposure.

Isoflurane Increases EAAT3 Redistribution to the Cell Surface and Enhances the Association of PKCα with EAAT3 in Rat Brain Tissue. To determine whether the results obtained with C6 cells are relevant for the CNS, the effects of isoflurane on EAAT3 redistribution and association with PKCα in rat hippocampal synaptosomes were studied. Consistent with the results in C6 cells, synaptosomes exposed to 2% isoflurane for 5 min had an increase in EAAT3 at their surface (418 ± 63% of the control, $n = 3$) and a decrease of intracellular EAAT3 protein (26 ± 2% of the

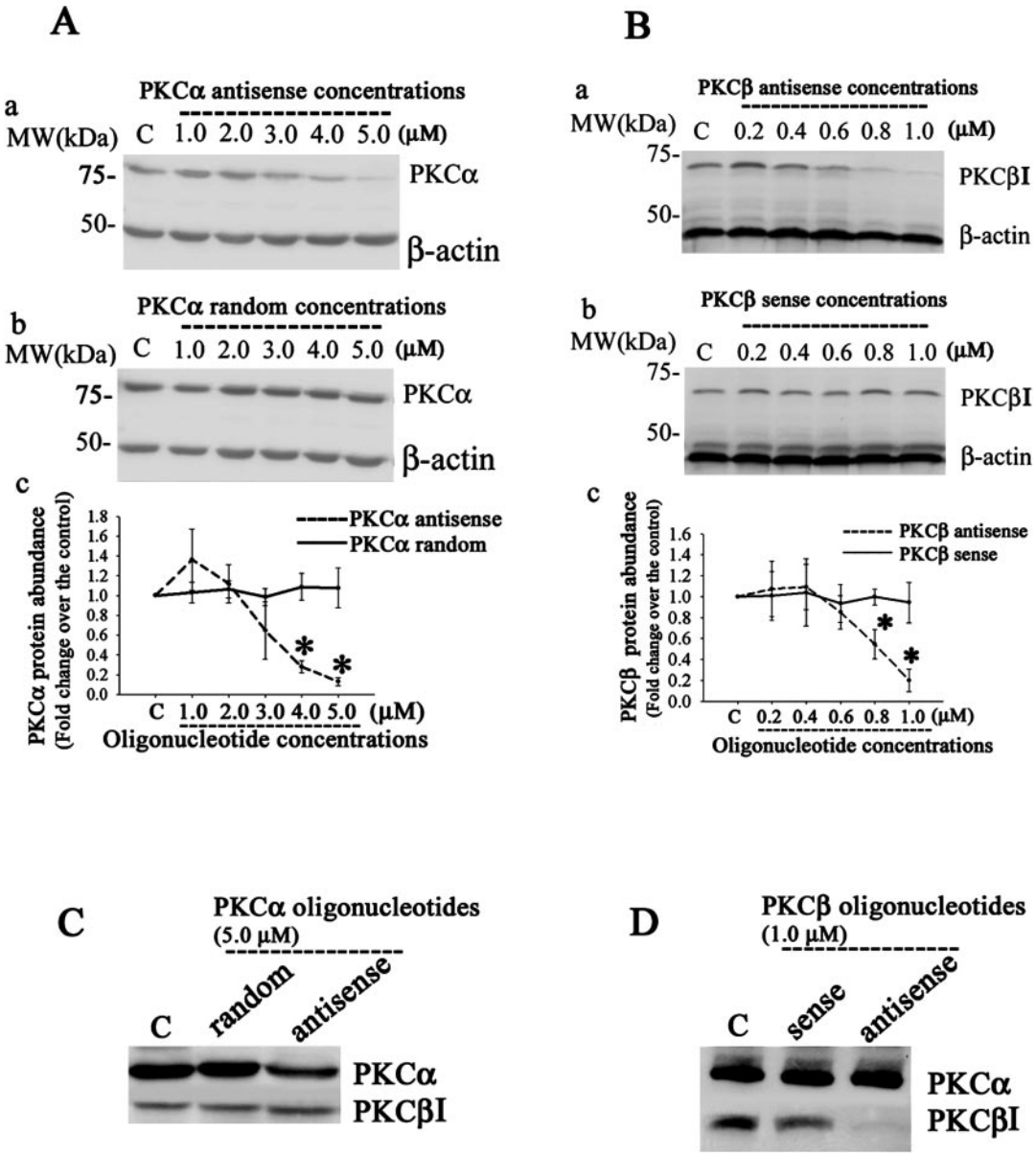

Fig. 5. Selective PKC isozyme down-regulation by antisense oligonucleotides. C6 glioma cells were incubated with various concentrations of PKCα antisense or random control oligonucleotides for 5 days (A) or with various concentrations of PKCβ antisense or sense oligonucleotides for 3 days (B). A representative Western blot for antisense oligonucleotides (a) and for random or sense control oligonucleotides (b) is shown. The graphic presentation of the PKCα or PKCβ I protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in c. Values in graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. C, the expression of PKCβ I in C6 cells incubated with 5 μM PKCα antisense or random control oligonucleotides for 5 days is shown. D, the expression of PKCα in C6 cells incubated with 1 μM PKCβ sense or antisense oligonucleotides for 3 days is shown. C, control.

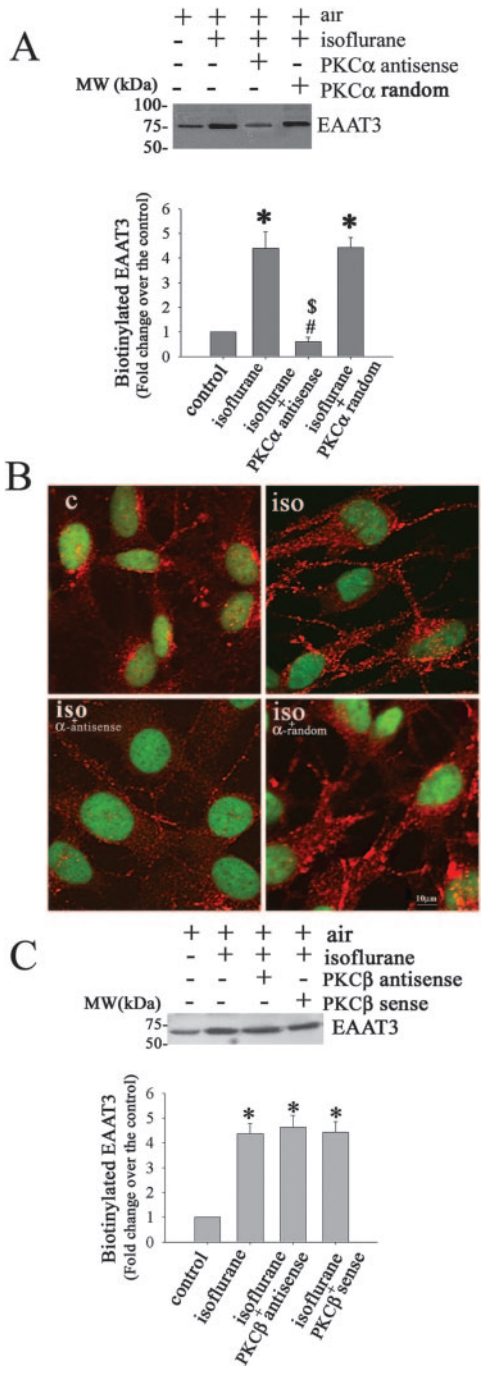

Fig. 6. The effects of PKC isozyme down-regulation on isoflurane-induced EAAT3 redistribution. C6 glioma cells were pretreated with or without PKCα antisense or random control oligonucleotides (5 μM) for 5 days or pretreated with or without PKCβ antisense or sense oligonucleotides (1 μM) for 3 days. They were then incubated with or without 2% isoflurane for 5 min. A and C, the biotinylated fractions of the cells were analyzed by Western blot. A representative Western blot is shown at the top, and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in the graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control; #, $P < 0.05$ compared with isoflurane alone; $, $P < 0.05$ compared with isoflurane plus PKCα random control oligonucleotides. B, the cells were immunostained with an anti-EAAT3 antibody (red). Nuclei were stained with sytox green nucleic acid stain (green). Serial sections (0.5 μm) were observed under confocal microscopy, and the sections through the center of the cell corresponding to the largest cross-sectional nuclear area are shown. C, control; iso, 2% isoflurane; α-antisense, PKCα antisense oligonucleotides; α-random, PKCα random control oligonucleotides.

control, $n = 3$) (Fig. 10), suggesting that isoflurane induces EAAT3 redistribution to the surface of the synaptosomes. Synaptosomes exposed to isoflurane also had an increased amount of PKCα in the immunoprecipitates produced with the anti-EAAT3 antibody (293 ± 56% of the control, $n = 3$) (Fig. 10), suggesting that isoflurane increases the association of PKCα with EAAT3 in rat hippocampal synaptosomes.

Discussion

Our study produced the following major findings: 1) isoflurane induces a time- and concentration-dependent increase in EAAT3 cell surface expression; 2) the isoflurane-induced increase in EAAT3 activity and cell surface expression is PKCα-dependent; and 3) isoflurane increases the coimmunoprecipitation of PKCα with EAAT3.

The effects of volatile anesthetics on EAAT activity have been investigated in several studies. Larsen et al. (1997) showed that isoflurane increased glutamate uptake in synaptosomes prepared from rat cerebral cortex. However, using a similar experimental model, Nicol et al. (1995) failed to demonstrate any increases in glutamate uptake by isoflurane. Although the reasons for this discrepancy are not known, synaptosomes have more than one type of EAAT. Thus, the reported isoflurane effects on glutamate uptake may represent sum of the effects of isoflurane on a mixed population of EAATs expressed in the synaptosomes. To study anesthetic effects on a single type of EAAT, we expressed glutamate transporters in oocytes. Our results indicated that isoflurane increased EAAT3 activity and that PKC may be involved in this effect (Do et al., 2002).

PKC activation by phorbol 12-myristate 13-acetate (PMA) has been shown to increase EAAT3 activity in C6 glioma cells (Davis et al., 1998; Gonzalez et al., 2002). PMA also increased the cell-surface expression of EAAT3 (Davis et al., 1998; Gonzalez et al., 2002). Recent studies suggest an important role for PKCα in the increase of both EAAT3 activity and cell-surface expression after PKC activation by PMA (Gonzalez et al., 2002, 2003). PKC activation may also increase EAAT3 activity without changing its cell-surface expression,

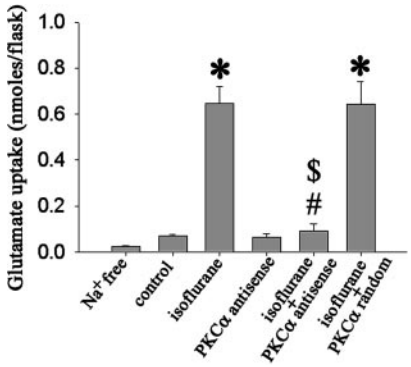

Fig. 7. The effects of PKCα down-regulation on isoflurane-induced increase of glutamate uptake. C6 glioma cells were pretreated with or without PKCα antisense or random control oligonucleotides at a concentration of 5 μM for 5 days. Glutamate uptake was performed by incubating the cells with 10 μM L-[^{3}H]glutamate in the presence or absence of 2% isoflurane for 5 min at 37°C. Data given are means ± S.D. ($n = 6$). *, $P < 0.05$ compared with the control. #, $P < 0.05$ compared with isoflurane alone. $, $P < 0.05$ compared with isoflurane plus PKCα random control oligonucleotides. Na^+ free, L-[^{3}H]glutamate associated with C6 cells in a Na^+-free incubation solution under control conditions.

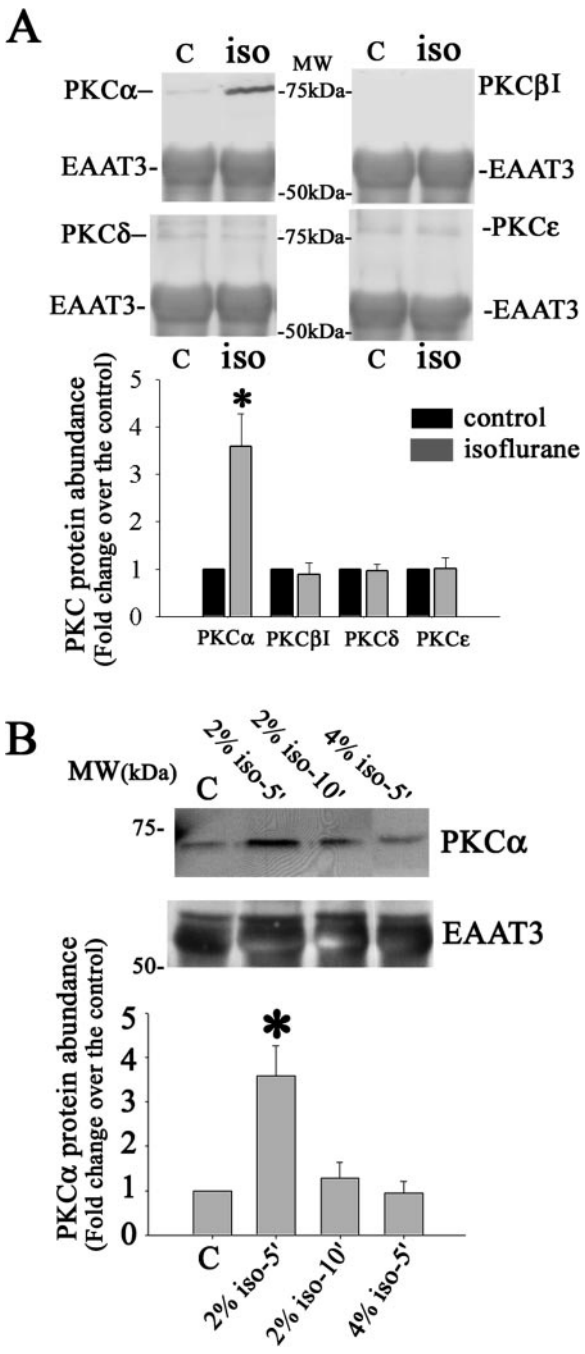

Fig. 8. Isoflurane-increased coimmunoprecipitation of PKC with EAAT3. A, C6 glioma cells were incubated with 2% isoflurane for 5 min, and the total lysates were immunoprecipitated with an anti-EAAT3 antibody. The precipitates were analyzed by Western blot with anti-PKC antibodies and an anti-EAAT3 antibody. A representative Western blot of the precipitates is shown at the top, and the graphic presentation of the PKC protein abundance quantified by integrating the volume of autoradiograms from four separate experiments is shown at the bottom. Values in the graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. B, C6 glioma cells were incubated with 2% isoflurane for 5 or 10 min or with 4% isoflurane for 5 min. The total lysates were immunoprecipitated with an anti-EAAT3 antibody. The precipitates were analyzed by Western blot with an anti-PKCα antibody and an anti-EAAT3 antibody. A representative Western blot of the precipitates is shown at the top, and the graphic presentation of the PKC protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. C, control; iso, 2% (unless specified) isoflurane.

possibly through another PKC isozyme, PKCϵ (Gonzalez et al., 2002). In this study, we showed that isoflurane-increased EAAT3 activity was associated with an increase of EAAT3 cell-surface expression and that both effects are PKCα-dependent. These results suggest that the isoflurane-induced increase of EAAT3 activity is caused mainly by redistribution of EAAT3 proteins to their functional location, the plasma membrane. Our study also showed that isoflurane increased the amount of PKCα in the EAAT3 complex and the phosphorylation of EAAT3, which is additional evidence to support the role of PKCα in regulating EAAT3 functions.

At least 11 isozymes of PKC have been identified. They are classified into three groups: conventional PKCs (α, β I, β II, and γ), novel PKCs (δ, ϵ, η, and θ), and atypical PKCs (ζ and ι/λ) (Way et al., 2000). Each isozyme has distinct subcellular distribution and may mediate unique functions (Goekjian and Jirousek, 1999; Dempsey et al., 2000). Our results showed that Gö6976, a selective inhibitor for conventional PKC isozymes, inhibited isoflurane-induced EAAT3 redistribution in C6 cells and that down-regulation of PKCα abolished the isoflurane-induced increase in EAAT3 activity and cell-surface expression. These results suggest that PKCϵ, the other PKC isozyme that has been shown to increase EAAT3 activity but without an effect on EAAT3 cell-surface expression in C6 glioma cells (Gonzalez et al., 2002), may not be important in mediating the isoflurane effects on EAAT3. Thus, our results support that isoflurane selectively affects PKCα to mediate its effects on EAAT3. Consistent with this idea, our results showed that isoflurane induced a redistribution of PKCα to the membrane/particulate fraction of C6 cells after isoflurane exposure. It is not known from our study whether isoflurane directly or indirectly via other mediators affects PKCα. However, halothane, another volatile anesthetic, has been shown to stimulate the activity of purified brain PKC in the presence of physiologically relevant lipid bilayer vesicles (Hemmings et al., 1995). In addition, Slater et al. (1993) suggested that PKC contains a hydrophobic binding site for alcohols and anesthetics in the regulatory region of PKC, and they observed that the activity of purified PKCα was increased by long-chain *n*-alkanols (Slater et al., 1997). However, a more recent study suggests that alcohols may activate PKCα not by direct binding to PKC but by altering lipid structure and by enhancing PKC-lipid bilayer binding (Shen et al., 1999).

Our time-course and dose-response experiments showed bell-shaped responses of EAAT3 redistribution to isoflurane. It is not known why the amount of EAAT3 at the cell surface returned to the basal level at longer incubations and higher concentrations of isoflurane. However, the recovery phenomenon may not be caused by isoflurane toxicity to the cells, because incubation of cells with isoflurane at concentrations up to 4% for 7 h did not affect the survival and viability of C6 glioma cells in our previous study (Huang and Zuo, 2003). It has been shown that PKC activation by alcohols and cell-membrane lipids is also bell-shaped (Sando et al., 1998; Shen et al., 1999). It has been suggested that this bell-shaped PKC activation may occur via an increase in the area of PKC-activating lipid domains in the presence of high concentrations of PKC activators, such that enzyme-substrate aggregates and/or enzyme-enzyme aggregates on the activating lipid domains are diluted out, thus decreasing PKC activity (PKC phosphorylation of its substrates) (Sando et al., 1998).

Thus, in our study, higher concentrations of isoflurane and longer incubation times may have resulted in less activation of PKCα, with a diminished EAAT3 expression at the cell surface. In support of this explanation, our results showed that the coimmunoprecipitation of PKCα with EAAT3 was no longer increased in C6 cells exposed to a high concentration (4%) of isoflurane or a long incubation time (10 min).

Our study may have significant implications for the anti-epilepsy and anesthesia properties of isoflurane. EAATs, by uptaking glutamate under physiological conditions, maintain extracellular glutamate homeostasis (Danbolt, 2001). The inhibition of EAAT activity in brain slices or cell cultures increased the peak glutamate concentration in the synaptic cleft and prolonged the glutamate-induced current, leading

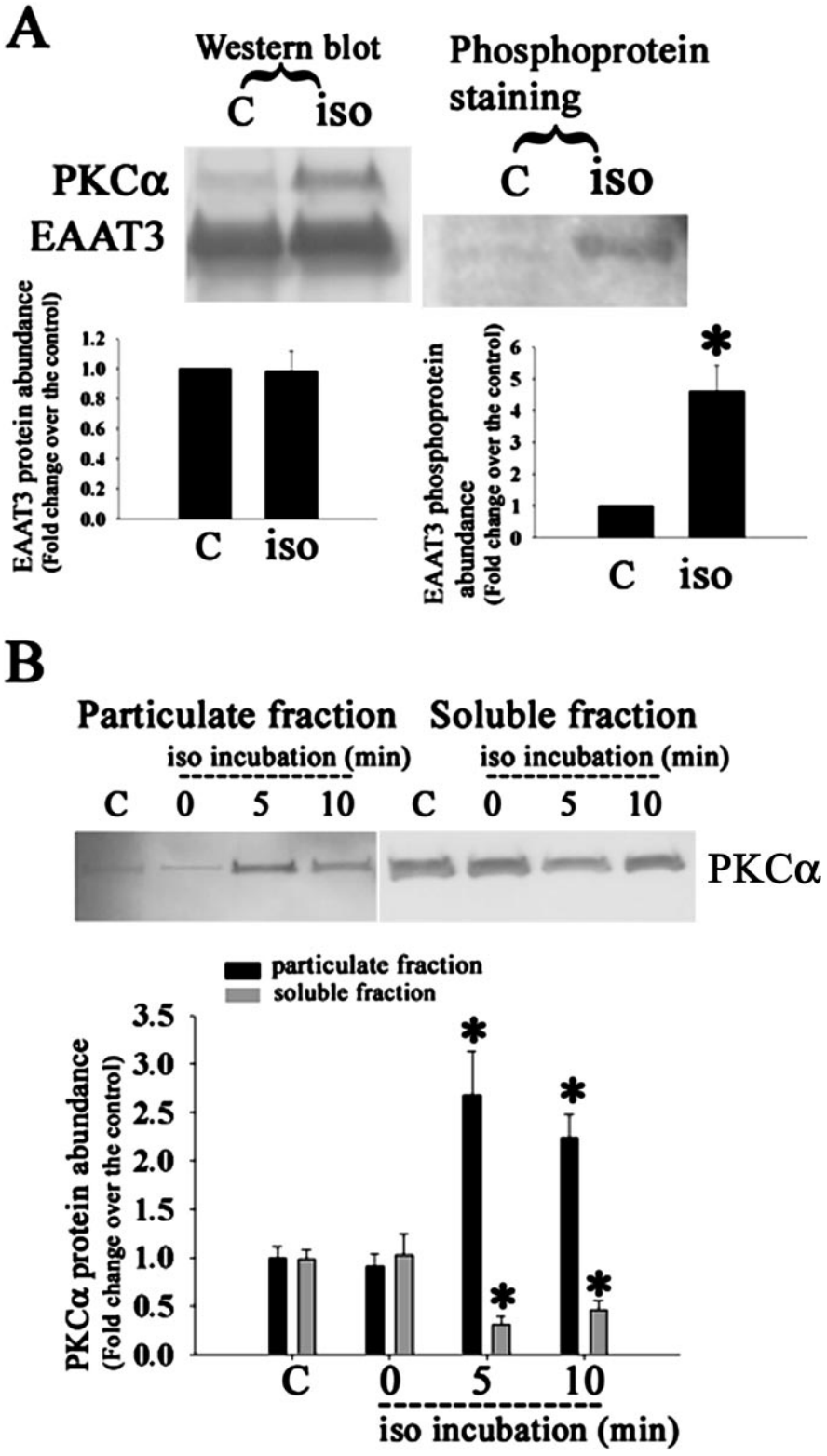

Fig. 9. Isoflurane-induced EAAT3 phosphorylation and PKCα redistribution. A, C6 glioma cells were incubated with or without 2% isoflurane for 5 min. The total lysates were immunoprecipitated with an anti-EAAT3 antibody. The immunoprecipitates were separated by electrophoresis in a 10% SDS-polyacrylamide gel that was stained with Pro-Q Diamond dye for phosphoproteins (top, right). The same gel was used for Western analysis with an anti-EAAT3 antibody and an anti-PKCα antibody (top, left). The graphic presentation of the abundance of the phosphoproteins corresponding to EAAT3 and the total EAAT3 proteins as quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in the graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. B, C6 glioma cells were incubated with or without 2% isoflurane for various times. The cells were homogenized, and the homogenates were fractionated into the soluble and the particulate fractions by centrifugation. These fractions were then used for Western analysis with an anti-PKCα antibody. Top, a representative Western blot. Bottom, a graphical presentation of the PKCα protein abundance quantified by integrating the volume of autoradiograms from three separate experiments. Values in the graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. C, control; iso, 2% isoflurane.

to a slowed excitatory postsynaptic current decay at some synapses (Barbour et al., 1994; Mennerick and Zorumski, 1994; Diamond and Jahr, 1997). In addition, a recent study showed that inhibition of neuronal EAATs in hippocampal slices decreased the inhibitory neurotransmitter GABA-mediated inhibitory postsynaptic current (IPSC) and miniature IPSC via reduced GABA synthesis (because glutamate uptaken by EAATs is a substrate for GABA synthesis) (Matthews and Diamond, 2003). These results are consistent with the data from an early study showing that antisense knockdown of EAAT3 induced epilepsy in rats and that the hippocampal slices from these rats had decreased GABA synthesis, total GABA levels, and miniature IPSC (Sepkuty et al., 2002). Thus, the isoflurane-induced increase in EAAT3 activity and cell-surface expression may be a mechanism for the antiepilepsy property of isoflurane and for isoflurane-induced anesthesia (anesthesia status is hypothesized to occur by inhibiting excitatory neurotransmission or enhancing inhibitory neurotransmission or the combination of these effects) (Campagna et al., 2003). In our study, isoflurane at concentrations of 1 to 3% induced a rapid (within minutes of the exposure) and significant increase in EAAT3 expression in the plasma membrane. These isoflurane concentrations are clinically relevant because 1 minimum alveolar concentration that inhibits a response to surgical stimuli in 50% of human subjects is 1.15% (in rats, it is ~1.4%; rat C6 cells and rat hippocampal synaptosomes were used in the study) (Stevens et al., 1975; Orliaguet et al., 2001); isoflurane concentrations higher than 1 minimum alveolar concentration are frequently used in clinical practice. It is interesting that 2% isoflurane did not affect EAAT1 cell-surface expression in rat glial cells, suggesting that the effects of isoflurane on EAAT3 cell-surface expression are specific.

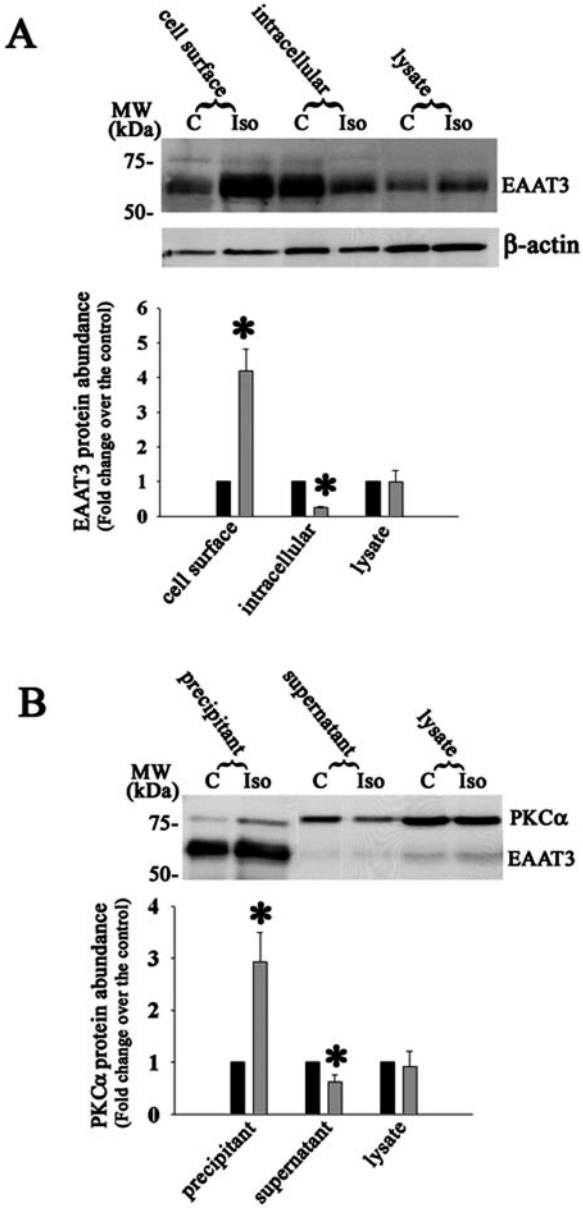

Fig. 10. Isoflurane-induced EAAT3 redistribution and association of EAAT3 with PKC in rat hippocampal synaptosomes. Synaptosomes were incubated with 2% isoflurane for 5 min at 37°C. A, the total lysates were then separated into intracellular and cell-surface fractions by a biotinylation method. A representative Western blot is shown at the top, and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. B, the total lysates were immunoprecipitated with an anti-EAAT3 antibody. The precipitates were analyzed by Western blot with an anti-PKCα antibody and an anti-EAAT3 antibody. A representative Western blot is shown at the top, and the graphic presentation of the PKC protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown at the bottom. Values in the graphs are expressed as -fold changes over the control and are presented as the means ± S.D. *, $P < 0.05$ compared with the control. C, control; iso, 2% isoflurane.

Activation of PKCα has been shown to reduce cell-surface levels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors that contain a glutamate receptor 2 subunit (Perez et al., 2001). It has been suggested that a reduction in the surface level of AMPA receptors is important for the development of long-term depression of synaptic efficacy (Malinow and Malenka, 2002). Although the role of EAAT3 in long-term depression is not known yet, the PKCα activation-induced reduction in cell-surface levels of AMPA receptors and increase in cell-surface levels of EAAT3 may have a complementary effect on excitatory neurotransmission. Thus, isoflurane, via its effects on PKCα, may regulate the development of long-term depression and synaptic plasticity.

In summary, we have shown that the isoflurane-induced increase of EAAT3 activity is associated with a redistribution of EAAT3 to the plasma membrane. The changes in activity and in subcellular localization of EAAT3 are PKCα-dependent. Isoflurane also increased the amount of PKCα in the EAAT3 immunocomplex. These results suggest an important mechanism for the regulation of glutamate transporter functions. These results also reveal cellular and molecular mechanisms for the isoflurane effects on EAAT3, which may be important for the commonly seen CNS inhibition after the application of volatile anesthetics, including isoflurane.

Acknowledgments

We thank Dr. Julianne J. Sando for reviewing this manuscript.

References

Arriza JL, Eliasof S, Kavanaugh MP, and Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc Natl Acad Sci USA* **94:**4155–4160.

Baltuch G, Dooley N, Rostworowski K, Villemure J, and Young V (1995) Protein

kinase C isoform α overexpression in C6 glioma cells and its role in cell proliferation. *J Neurooncol* **24:**241–250.
Barbour B, Keller BU, Llano I, and Marty A (1994) Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells. *Neuron* **12:** 1331–1343.
Billups B, Rossi D, Oshima T, Warr O, Takahashi M, Sarantis M, Szatkowski M, and Attwell D (1998) Physiological and pathological operation of glutamate transporters. *Prog Brain Res* **116:**45–57.
Campagna J, Miller K, and Forman S (2003) Mechanisms of action of inhaled anesthetics. *N Engl J Med* **348:**2110–2124.
Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* **65:**1–105.
Davis KE, Straff DJ, Weinstein EA, Bannerman PG, Correale DM, Rothstein JD, and Robinson MB (1998) Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. *J Neurosci* **18:**2475–2485.
Dean NM and McKay R (1994) Inhibition of protein kinase C-α expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. *Proc Natl Acad Sci USA* **91:**11762–11766.
Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, and Messing RO (2000) Protein kinase C isozymes and the regulation of diverse cell responses. *Am J Physiol* **279:**L429–L438.
Diamond JS and Jahr CE (1997) Transporters buffer synaptically released glutamate on a submillisecond time scale. *J Neurosci* **17:**4672–4687.
Do S-H, Kamatchi GL, Washington JM, and Zuo Z (2002) Effects of volatile anesthetics on glutamate transporter, excitatory amino acid transporter type 3. *Anesthesiology* **96:**1492–1497.
Dorn GW 2nd, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ, Csukai M, Wu G, Lorenz JN, and Mochly-Rosen D (1999) Sustained in vivo cardiac protection by a rationally designed peptide that causes ϵ protein kinase C translocation. *Proc Natl Acad Sci USA* **96:**12798–12803.
Goekjian PG and Jirousek MR (1999) Protein kinase C in the treatment of disease: signal transduction pathways, inhibitors and agents in development. *Curr Med Chem* **6:**877–903.
Gonzalez M, Bannerman P, and Robinson M (2003) Phorbol myristate acetate-dependent interaction of protein kinase C α and the neuronal glutamate transporter EAAC1. *J Neurosci* **23:**5589–5593.
Gonzalez MI, Kazanietz MG, and Robinson MB (2002) Regulation of the neuronal glutamate transporter excitatory amino acid carrier-1 (EAAC1) by different protein kinase C subtypes. *Mol Pharmacol* **62:**901–910.
Hemmings HJ, Adamo A, and Hoffman M (1995) Biochemical characterization of the stimulatory effects of halothane and propofol on purified brain protein kinase C. *Anesth Analg* **81:**1216–1222.
Huang Y and Zuo Z (2003) Isoflurane enhances the expression and activity of glutamate transporter type 3 in C6 glioma cells. *Anesthesiology* **99:**1346–1353.
Korchak HM, Rossi MW, and Kilpatrick LE (1998) Selective role for beta-protein kinase C in signaling for O-2 generation but not degranulation or adherence in differentiated HL60 cells. *J Biol Chem* **273:**27292–27299.
Kraft A and Anderson W (1983) Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature (Lond)* **301:**621–623.
Larsen M, Hegstad E, Berg-Johnsen J, and Langmoen IA (1997) Isoflurane increases the uptake of glutamate in synaptosomes from rat cerebral cortex. *Br J Anaesth* **78:**55–59.
Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, and Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J Neurosci* **15:**1835–1853.
Malinow R and Malenka R (2002) AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* **25:**103–126.
Matthews G and Diamond JS (2003) Neuronal glutamate uptake contributes to GABA synthesis and inhibitory synaptic strength. *J Neurosci* **23:**2040–2048.
Mennerick S and Zorumski CF (1994) Glial contributions to excitatory neurotransmission in cultured hippocampal cells. *Nature (Lond)* **368:**59–62.
Nicol B, Rowbotham DJ, and Lambert DG (1995) Glutamate uptake is not a major target site for anaesthetic agents. *Br J Anaesth* **75:**61–65.
Orliaguet G, Vivien B, Langeron O, Bouhemad B, Coriat P, and Riou B (2001) Minimum alveolar concentration of volatile anesthetics in rats during postnatal maturation. *Anesthesiology* **95:**734–739.
Perez J, Khatri L, Chang C, Srivastava S, Osten P, and Ziff E (2001) PICK1 targets activated protein kinase C alpha to AMPA receptor clusters in spines of hippocampal neurons and reduces surface levels of the AMPA-type glutamate receptor subunit 2. *J Neurosci* **21:**5417–5428.
Robinson MB (1998) Examination of glutamate transporter heterogeneity using synaptosomal preparations. *Method Enzymol* **296:**189–202.
Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, and Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. *Neuron* **13:**713–725.
Sando JJ, Chertihin OI, Owens JM, and Kretsinger RH (1998) Contributions to maxima in protein kinase C activation. *J Biol Chem* **273:**34022–34027.
Sepkuty JP, Cohen AS, Eccles C, Rafiq A, Behar K, Ganel R, Coulter DA, and Rothstein JD (2002) A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. *J Neurosci* **22:**6372–6379.
Shen YM, Chertihin OI, Biltonen RL, and Sando JJ (1999) Lipid-dependent activation of protein kinase C-alpha by normal alcohols. *J Biol Chem* **274:**34036–34044.
Slater S, Cox KL, Lombardi JV, Ho C, Kelly M, Rubin E, and Stubbs C (1993) Inhibition of protein kinase C by alcohols and anesthetics. *Nature (Lond)* **364:**82–84.
Slater S, Kelly M, Larkin J, Ho C, Mazurek A, Taddeo F, Yeager M, and Stubbs C (1997) Interaction of alcohols and anesthetics with protein kinase C α. *J Biol Chem* **272:**6167–6173.
Stevens WC, Dolan WM, Gibbons RT, White A, Eger EI 2nd, Miller RD, De Jong R, and Elashoff RM (1975) Minimum alveolar concentrations (MAC) of isoflurane with and without nitrous oxide in patients of various ages. *Anesthesiology* **42:**197–200.
Way KJ, Chou E, and King GL (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* **21:**181–187.
Zuo Z (2001) Isoflurane enhances glutamate uptake via glutamate transporters in rat glial cells. *Neuroreport* **12:**1077–1080.
Zuo Z and Johns RA (1995) Halothane, enflurane and isoflurane do not affect the basal or agonist-stimulated activity of partially isolated soluble and particulate guanylyl cyclases of rat brain. *Anesthesiology* **83:**395–404.
Zuo Z and Johns RA (1997) Inhalational anesthetics up-regulate constitutive and lipopolysaccharide-induced inducible nitric oxide synthase expression and activity. *Mol Pharmacol* **52:**606–612.

Address correspondence to: Dr. Zhiyi Zuo, Department of Anesthesiology, University of Virginia Health System, P.O. Box 800710, One Hospital Drive, Charlottesville, VA 22908-0710. E-mail: zz3c@virginia.edu