

**Isoflurane induces a protein kinase C alpha-dependent increase in cell surface
protein level and activity of glutamate transporter type 3**

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Abbreviations: EAAT, excitatory amino acid transporter; PKC, protein kinase C; CNS, central nervous system; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; EPSC, excitatory post-synaptic current; IPSC, inhibitory post-synaptic current; GABA, γ -aminobutyric acid; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

Abstract

Glutamate transporters regulate extracellular concentrations of glutamate, an excitatory neurotransmitter in the CNS. We have shown that the commonly used anesthetic isoflurane increased the activity of glutamate transporter type 3 (EAAT3) possibly via a protein kinase C (PKC)-dependent pathway. Here we show that isoflurane induced a time- and concentration-dependent redistribution of EAAT3 to cell membrane in C6 glioma cells. This redistribution was inhibited by staurosporine, a pan PKC inhibitor, or by Gö6976 at a concentration that selectively inhibits conventional PKC isoforms (PKC α , β and γ). This isoflurane-induced EAAT3 redistribution was also blocked when the expression of PKC α but not PKC β 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 PKC α expression. Immunoprecipitation with an anti-EAAT3 antibody pulled down more PKC α in cells exposed to isoflurane than in control cells. Isoflurane also increased the phosphorylated EAAT3 and the redistribution of PKC α 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 PKC α 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 PKC α -dependent and may rely on the formation of an EAAT3-PKC α 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.

Introduction

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 - 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 (Arriza et al., 1997; Lehre et al., 1995; Rothstein et al., 1994). The transporting functions of all five EAATs are Na^+ -dependent. They use the transmembrane gradient of Na^+ , K^+ and H^+ as 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 (Danbolt, 2001; Rothstein et al., 1994). 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 $\text{PKC}\alpha$ is involved in regulating EAAT3 expression in the plasma membrane (Gonzalez et al., 2002). In this study, we determined whether isoflurane increases 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 PKC α -dependent increase of EAAT3 activity and cell surface expression and that isoflurane also increased the co-immunoprecipitation of PKC α with EAAT3, which may suggest an increased interaction between these two proteins in cells.

Materials and Methods

Materials. F-10 nutrient mixture (Ham) was from GIBCO™ Invitrogen Corporation (Grand Island, NY). Six-well tissue culture plates, 25 cm² and 75 cm² tissue culture flasks were manufactured by Corning (Corning, NY). L-[³H]-glutamate (specific activity of 56 Ci/mM) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Wizard plus minipreps was from Promega (Madison, WI). Anti-PKCα, PKCβI, PKCδ and PKCε antibodies and Protein A/G plus-agarose were purchased from Santa Cruz Biotechnology (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 (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 (Scottsdale, PA). Complete protease inhibitors (catalog number 1697498) were from Roche Diagnostics Corporation Roche Applied Science (Indianapolis, IN). Gö6976 was from BioMol (Plymouth Meeting, PA). Other reagents were purchased from Sigma Chemical (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 F-10 nutrient mixture (Ham) containing 15% horse serum and 2.5% fetal bovine serum at 37°C in a 95% air-5% CO₂ incubator. When cells were ~ 80% confluent, the culture medium was replaced by a serum-free medium (F-10 mixture only) for 24 hours 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 at ~80% confluent and had been in the serum-free Dulbecco's modified Eagle's medium for 24 hours.

C6 cells and primary glial cell culture were incubated with isoflurane in an open system as follows. Fresh serum-free medium (50 to 200 ml) that had been gassed with 95% air-5% CO₂ through or not through an isoflurane vaporizer at a flow rate 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 inhibitors staurosporine (50 nM) or Gö6976 (10 µM) were 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 hr with fresh medium containing the oligonucleotides. The sequences of PKC α antisense and random oligonucleotides were: 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 PKC α . The random oligonucleotide has base composition identical to that of antisense oligonucleotide and served as a control. In the case of PKC β down regulation, cells were incubated with PKC β antisense and sense oligonucleotides for 3 days with the medium change every 24 hr. The sequences of PKC β 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 PKC β . Since PKC β I and PKC β II differ only in the 3' terminus, this antisense oligonucleotide should bind to the mRNA of both PKC β I and PKC β II. All oligonucleotides for PKC α and PKC β were phosphorothioate derivatives to prolong their half-life.

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 cm² 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% sodium dodecyl sulfate (SDS), 1 µg/ml leupeptin, 250 µM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 mg/ml trypsin inhibitor, and 1 mM iodoacetamide for 1 hr at 4°C with vigorous shaking. The total lysates were centrifuged at $20,000 \times g$ 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 figure 1) were incubated with equal volumes of suspension of avidin-conjugated beads (600 µl of bead suspension to 600 µl of lysates) for 1 hr at room temperature with occasional stirring. The mixture was then centrifuged at $16,500 \times g$ for 15 min at 4°C. This second supernatant was also collected for Western analysis and is labeled as intracellular fraction in figure 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 $16,500 \times g$ for 10 min at 4°C and this third supernatant was kept for Western blot as the biotinylated fraction in figure 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, 5 µg/ml leupeptin. The homogenate was centrifuged at $45,000 \times g$ for 60 min at 4°C. The supernatant was harvested as the soluble fraction. The pellet that was the

particulate/membrane 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 quantitated by the Lowry assay using a protein assay kit, 25 μ g of protein per lane were subjected to Western analysis as described before (Huang and Zuo, 2003; Zuo and Johns, 1997). Briefly, 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 μ g/ml; polyclonal rabbit anti-EAAT1 at 1:500 dilution; polyclonal rabbit anti- β -actin at 1:3000 dilution; monoclonal mouse anti-PKC α and monoclonal mouse anti-PKC β I at 1:500 dilution; polyclonal rabbit anti-PKC δ and rabbit anti-PKC ϵ 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 due to the limited linear range of intensity produced by this method. Quantitative analysis of the protein bands was performed using an ImageQuant 5.0 Molecular Dynamics Densitometer (Molecular Dynamics, Sunnyvale, CA). The relationship between the protein band signal and exposure time of the heaviest band on the films was established. Protein bands on a film where the intensity of the heaviest band was still within the linear range were measured to generate the data reported here.

Immunoprecipitation. As described previously (Gonzalez et al., 2003), C6 cells cultured in 75 cm² dishes were lysed in 2 ml of buffer containing 50 mM Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mM NaCl and complete protease inhibitors for 1

hr 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 hr 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 blot. 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 – 95°C for 5 min. The samples were also used for Western blot. 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 Bioscience, Piscataway, NJ) at an excitation wavelength 546 nm and an emission wavelength 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. Briefly, after decapitation of adult male Sprague-Dawley rats under halothane anesthesia, hippocampi were immediately dissected on ice and weighed

(usually about 100 mg, the volumes described in the following steps were based on 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, New York, NY). The solution was then centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged again at $20,000 \times g$ for 20 min. The pellet was resuspended in 40 volumes of sucrose and repelleted by centrifugation at $20,000 \times g$ 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 $20,000 \times g$ for 20 min at 4°C . The biotinylated proteins in the pellet were separated from the non-biotinylated proteins as described in the section Biotinylation. The immunoprecipitation with the anti-EAAT3 antibody was performed on synaptosomes incubated with or without 2% isoflurane as described in the section 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 X100 in PBS for 2 min on ice, and then with 3% bovine serum albumin solution for 1 hour at 37°C . Anti-EAAT3 antibody (1.25 $\mu\text{g}/\text{ml}$) was added for 1 hour at 37°C . The cells were subsequently incubated with Donkey anti-rabbit IgG-Texas red at 1:500 dilution for 1 hr 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 2 - 3 times and finally were washed with water before the coverslips were mounted with the use of prolong antifade kit. Serial sections of 0.5 μm of the labeled cells were obtained using a Zeiss 410 Axiovert 100 microsystems LSM confocal microscope (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 (Huang and Zuo, 2003; Zuo, 2001), 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 , 10 mM dextrose, pH 7.2. They were then incubated with 10 μM L-[^3H]-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 \pm SD of the fold changes over the

controls, with controls being set as 1. Results of glutamate uptake assay are means \pm SD 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 $P < 0.05$ was considered significant.

Results

Isoflurane induces EAAT3 redistribution to cell surface of C6 cells

Since previous studies have shown that isoflurane increased EAAT3 activity and that EAAT3 redistribution to plasma member is one of the mechanisms to increase 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. Similarly, 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 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 reached maximal 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 significantly increased EAAT3 proteins at cell surface ($240 \pm 25\%$ of the control, $n = 3$) and 2% isoflurane induced a maximal effect (Fig. 3).

Isoflurane-induced EAAT3 redistribution in C6 cells is PKC α -dependent

Since our previous study suggests that the isoflurane-induced increase in EAAT3 activity may be PKC-mediated (Do et al., 2002), we tested whether 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 μ M 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-PKC α antibody detected a protein band at ~80 kDa and that the anti-PKC β I antibody detected a protein band at ~70 kDa in C6 cells (Fig. 5). These protein sizes are similar to those that reported for PKC α and PKC β I in the literature (Dean and McKay, 1994; Korchak et al., 1998). These proteins were selectively down regulated by PKC α and PKC β antisense oligonucleotides, respectively, but not by their corresponding sense or random control oligonucleotides (Fig. 5). PKC α antisense oligonucleotides at 5 μ M for 5 days and PKC β antisense oligonucleotides at 1 μ M for 3 days reduced the expression of the corresponding PKC isozymes to less than 20% of the control levels (Fig. 5). The expression of PKC α was not affected by the PKC β antisense and sense oligonucleotides. The expression of PKC β I was not affected by the PKC α antisense and random oligonucleotides (Fig. 5). In the cells in which PKC α was down regulated, isoflurane (2% for 5 min) no longer induced an EAAT3 redistribution to the plasma membrane (Fig. 6), whereas PKC α down regulation did not affect the surface expression of EAAT3 proteins under basal conditions (EAAT3 protein in the cell surface in the presence of PKC α

antisense and random oligonucleotides was 94 ± 14 and $103 \pm 6\%$ of the control, respectively, $n = 5$). In contrast, the level of isoflurane-induced increase in EAAT3 cell surface expression in cells with PKC β I down regulation was similar to the level of increase in control cells and in cells without PKC β I down regulation (PKC β sense oligonucleotide-treated cells) (Fig. 6). These results suggest that PKC α is important for the isoflurane-induced increase of EAAT3 cell surface expression.

Isoflurane-increased EAAT3 activity in C6 cells is PKC α -dependent

Since 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). Similarly, isoflurane also increased EAAT3 activity in C6 cells treated with PKC α random control oligonucleotides. However, isoflurane did not change the EAAT3 activity in cells that had decreased PKC α expression (Fig. 7). These results suggest that PKC α 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 PKC α with EAAT3 in C6 cells

Since PKC activation has been shown to increase the formation of PKC α and EAAT3 complex (Gonzalez et al., 2003), we tested whether isoflurane increased the association of PKC α 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 PKC α in the immunoprecipitates produced by the anti-EAAT3 antibody ($358 \pm 49\%$ of the control, $n = 4$, Fig. 8). These results are specific because neither PKC α 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 PKC δ and PKC ϵ (almost no band in the case of PKC β I) were detected in the immunoprecipitates, isoflurane did not change the amount of PKC β I, PKC δ and PKC ϵ in the immunoprecipitates (Fig. 8). These results suggest that isoflurane specifically increases the formation of PKC α 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 PKC α 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 PKC α in the immunoprecipitates produced by the anti-EAAT3 antibody ($128 \pm 37\%$ and $94 \pm 26\%$, respectively, of the control, $n = 3$, Fig. 8).

Isoflurane increases EAAT3 phosphorylation and PKC α 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 ($460 \pm 79\%$ 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 PKC α redistribution to the particulate/membrane fraction of C6 cells (Fig. 9). Since PKC activation is often associated with redistribution from the soluble fraction to the particulate/membrane fraction (Dorn et al., 1999; Kraft and Anderson, 1983), these results are evidence of PKC α activation after isoflurane exposure.

Isoflurane increases EAAT3 redistribution to cell surface and enhances the association of PKC α 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 PKC α 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 ($418 \pm 63\%$ of the control, $n = 3$) and a decrease of intracellular EAAT3 protein ($26 \pm 2\%$ of the 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 PKC α in the immunoprecipitates produced with the anti-

EAAT3 antibody ($293 \pm 56\%$ of the control, $n = 3$, Fig. 10), suggesting that isoflurane increases the association of PKC α with EAAT3 in rat hippocampal synaptosomes.

Discussion

Our study has the following major findings: 1) isoflurane induced a time- and concentration-dependent increase in EAAT3 cell surface expression, 2) the isoflurane-induced increase in EAAT3 activity and cell surface expression is PKC α -dependent, and 3) isoflurane increased the co-immunoprecipitation of PKC α 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 of PKC α in the increase of both EAAT3 activity and cell surface expression after PKC activation by PMA (Gonzalez et al., 2003; Gonzalez et al., 2002). PKC activation may also increase EAAT3 activity without changing its cell surface expression possibly through another PKC isozyme, PKC ϵ (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 PKC α -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 PKC α in the EAAT3 complex and the phosphorylation of EAAT3, which are additional evidence to support the role of PKC α 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 (Dempsey et al., 2000; Goekjian and Jirousek, 1999). 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 PKC α abolished the isoflurane-induced increase in EAAT3 activity and cell surface expression. These results suggest that PKC ϵ , 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 PKC α to mediate its effects on EAAT3. Consistent with this idea, our results showed that isoflurane induced a redistribution of PKC α 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 PKC α . 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. suggested that PKC contains a hydrophobic binding site for alcohols and anesthetics in the regulatory region of PKC (Slater et al., 1993) and observed that the activity of purified PKC α was increased by long chain n-alkanols (Slater et al., 1997). However, a more recent study suggests that alcohols may activate PKC α 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 cell surface returned to the basal level at longer incubations and higher concentrations of isoflurane. However, the recovery phenomenon may not be due to isoflurane toxicity to the cells because incubation of cells with isoflurane at concentrations up to 4% for 7 hr 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 PKC α with a diminished EAAT3 expression at cell surface. In support of this explanation, our results showed that the co-immunoprecipitation of PKC α 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 to a slowed excitatory post-synaptic current (EPSC) decay at some synapses (Barbour et al., 1994; Diamond and Jahr, 1997; Mennerick and Zorumski, 1994). In addition, a recent study showed that inhibition of neuronal EAATs in hippocampal slices decreased the inhibitory neurotransmitter γ -aminobutyric acid (GABA)-mediated inhibitory post-synaptic 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 1 - 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 one 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) (Orliaguet et al., 2001; Stevens et al., 1975); and isoflurane concentrations higher than one minimum alveolar concentration are frequently used in clinical practice. Interestingly, 2% isoflurane did not affect EAAT1 cell surface expression in rat glial cells, suggesting the effects of isoflurane on EAAT3 cell surface expression are specific.

Activation of PKC α 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 (GluR2) 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 PKC α 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 PKC α , 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 PKC α -dependent. Isoflurane also increased the amount of PKC α 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.

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 U S A* **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. *Journal of Neuro-Oncology* **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. *New 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* **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 epsilon protein kinase C translocation.
Proc Natl Acad Sci U S A **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. *Current Medicinal
Chemistry* **6**:877-903.
- Gonzalez M, Bannerman P and Robinson M (2003) Phorbol myristate acetate-dependent
interaction of protein kinase C alpha 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 Ca²⁺, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **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. *Brit 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* **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 Kuncel 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, JV., Ho C, Kelly M, Rubin E and Stubbs C (1993) Inhibition of protein kinase C by alcohols and anesthetics. *Nature* **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 alpha. *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.

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Figure Legends

Fig. 1. *Isoflurane-induced EAAT3 and EAAT1 redistribution.* C6 glioma cells (panel **A**) or primary glial cell culture of rat (panel **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 in the top panel and the graphic presentation of the EAAT3 or EAAT1 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in graphs are expressed as fold changes over the control and are presented as the means \pm S.D. * $P < 0.05$ compared with the corresponding control. C: control; iso: 2% isoflurane.

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 in the top panel and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in 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 in each panel, C6 cells were immunostained with an anti-EAAT3 antibody (red). Nuclei were stained with sytox green nucleic acid stain (green). Serial sections of 0.5 μ m 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.

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 in the top panel and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in 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. 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, panel **A**), a pan inhibitor of protein kinase C, or Gö6976 (10 μ M, panel **B**), a relatively selective inhibitor of conventional protein kinase C isozymes, were applied during isoflurane incubation. The biotinylated fractions of the cells were analyzed by Western blot. A representative Western blot is shown in the top panel and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in 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. # $P < 0.05$ compared with 2% isoflurane alone. C: control; iso: 2% isoflurane; st: 50 nM staurosporine.

Fig. 5. *Selective PKC isozyme down regulation by antisense oligonucleotides.* C6 glioma cells were incubated with various concentrations of PKC α antisense or random control oligonucleotides for 5 days (panel **A**) or with various concentrations of PKC β antisense or sense oligonucleotides for 3 days (panel **B**). A representative Western blot

for antisense oligonucleotides (**a**) and for random or sense control oligonucleotides (**b**) is shown. The graphic presentation of the PKC α or PKC β 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 \pm S.D. * $P < 0.05$ compared with the control. The expression of PKC β I in C6 cells incubated with 5 μ M of PKC α antisense or random control oligonucleotides for 5 days is shown in panel **C**. The expression of PKC α in C6 cells incubated with 1 μ M of PKC β sense or antisense oligonucleotides for 3 days is shown in panel **D**. C: control.

Fig. 6. *The effects of PKC isozyme down regulation on isoflurane-induced EAAT3 redistribution.* C6 glioma cells were pretreated with or without PKC α antisense or random control oligonucleotides (5 μ M) for 5 days or pretreated with or without PKC β antisense or sense oligonucleotides (1 μ M) for 3 days. They were then incubated with or without 2% isoflurane for 5 min. Panels **A** and **C**: The biotinylated fractions of the cells were analyzed by Western blot. A representative Western blot is shown in the top panel and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in 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. # $P < 0.05$ compared with isoflurane alone. \$ $P < 0.05$ compared with isoflurane plus PKC α random control oligonucleotides. Panel **B**: The cells were immunostained with an anti-EAAT3 antibody (red). Nuclei were stained with sytox green nucleic acid stain (green). Serial sections of 0.5 μ m 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: PKC α antisense oligonucleotides; α -random: PKC α random control oligonucleotides.

Fig. 7. *The effects of PKC α down regulation on isoflurane-induced increase of glutamate uptake.* C6 glioma cells were pretreated with or without PKC α antisense or random control oligonucleotides 5 μ M for 5 days. Glutamate uptake was performed by incubating the cells with 10 μ M of L-[3 H] glutamate in the presence or absence of 2% isoflurane for 5 min at 37 $^{\circ}$ C. Data are means \pm 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 PKC α random control oligonucleotides. Na $^{+}$ free: L-[3 H] glutamate associated with C6 cells in a Na $^{+}$ -free incubation solution under control conditions.

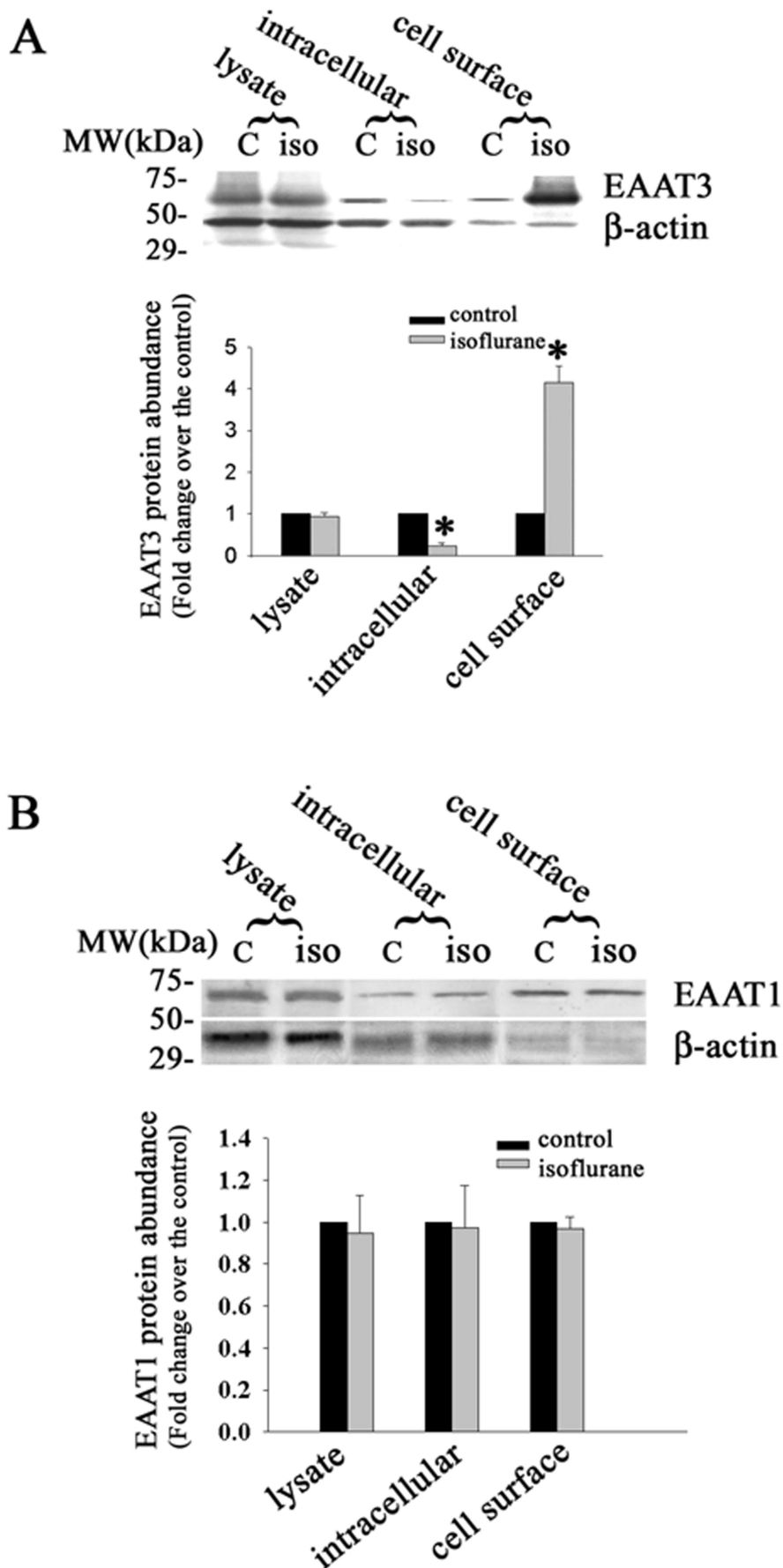
Fig. 8. *Isoflurane-increased co-immunoprecipitation of PKC with EAAT3.* Panel **A:** C6 glioma cells were incubated with 2% isoflurane for 5 min and the total lysates were immunoprecipitated with an anti-EAAT3 antibody. The precipitates were analyzed by Western blot with anti-PKC antibodies and an anti-EAAT3 antibody. A representative Western blot of the precipitates is shown in the top panel and the graphic presentation of the PKC protein abundance quantified by integrating the volume of autoradiograms from four separate experiments is shown in the bottom panel. Values in 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. Panel **B:** C6 glioma cells were incubated with 2% isoflurane for 5 or 10 min or 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-PKC α antibody and an anti-EAAT3 antibody. A representative Western blot of the precipitates is shown in the top panel and the graphic presentation of the PKC protein

abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in 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. C: control; iso: 2% (unless specified) isoflurane.

Fig. 9. *Isoflurane-induced EAAT3 phosphorylation and PKC α redistribution.*

Panel 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% sodium dodecyl sulfate-polyacrylamide gel that is stained with Pro-Q Diamond dye for phosphoproteins (top right panel). The same gel was used for Western analysis with an anti-EAAT3 antibody and an anti-PKC α antibody (top left panel). 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 in the bottom panel. Values in 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. **Panel 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-PKC α antibody. Top panel is a representative Western blot. Bottom panel is a graphical presentation of the PKC α protein abundance quantified by integrating the volume of autoradiograms from three separate experiments. Values in 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. C: control; iso: 2% isoflurane.

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. Panel **A**: The total lysates were then separated into intracellular and cell surface fractions by a biotinylation method. A representative Western blot is shown in the top panel and the graphic presentation of the EAAT3 protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in 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. Panel **B**: The total lysates were immunoprecipitated with an anti-EAAT3 antibody. The precipitates were analyzed by Western blot with an anti-PKC α antibody and an anti-EAAT3 antibody. A representative Western blot is shown in the top panel and the graphic presentation of the PKC protein abundance quantified by integrating the volume of autoradiograms from three separate experiments is shown in the bottom panel. Values in 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. C: control; iso: 2% isoflurane.



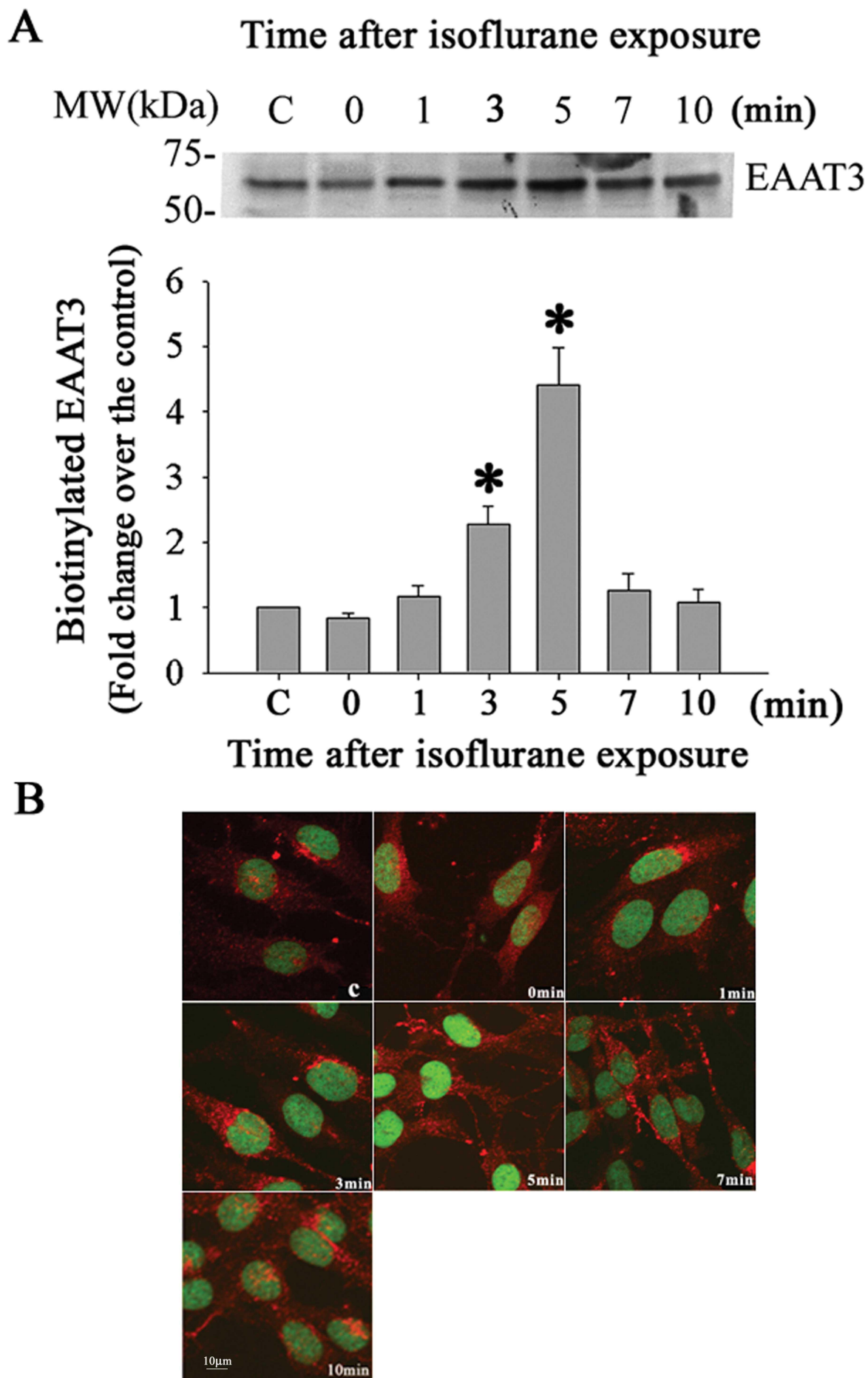


Fig. 2

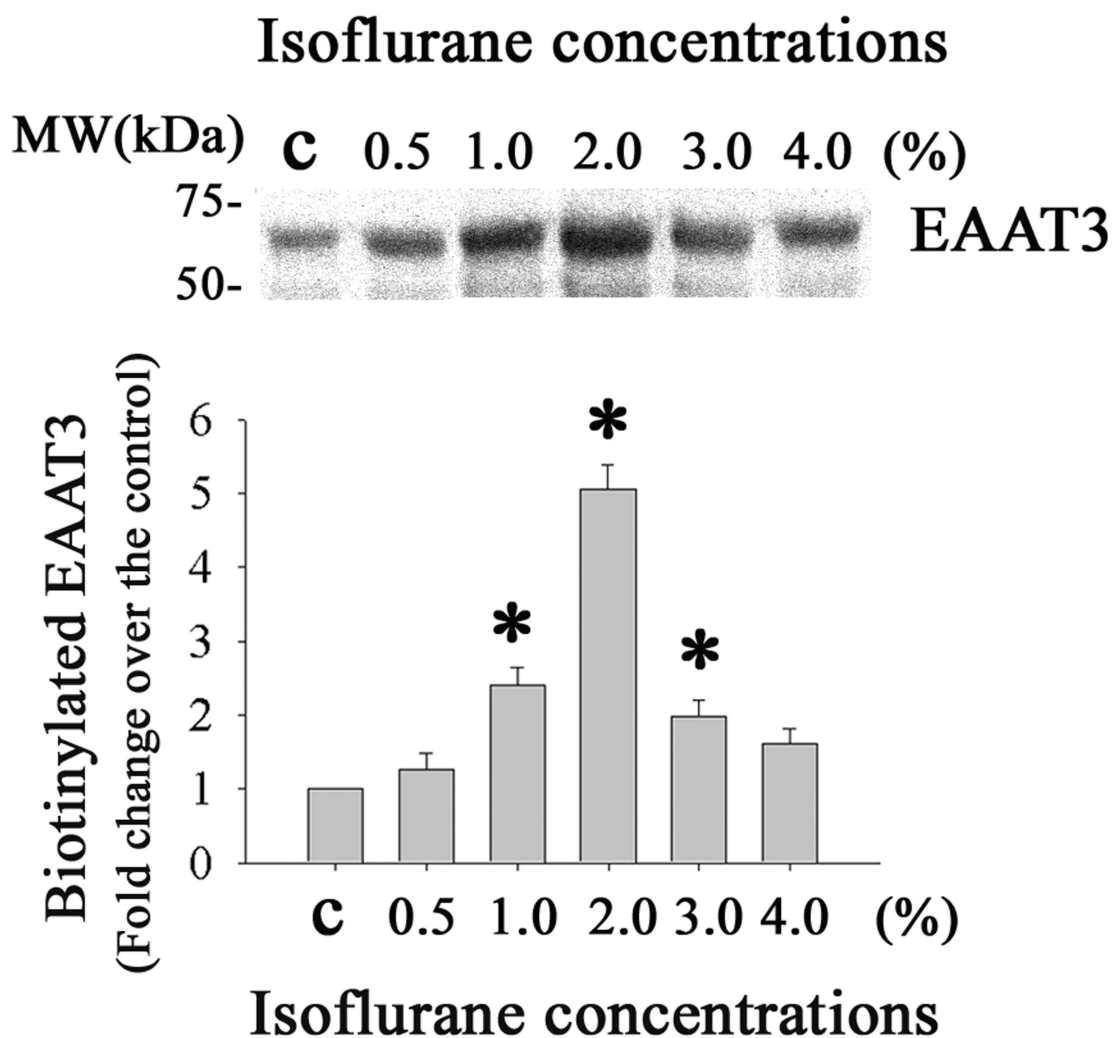


Fig. 3

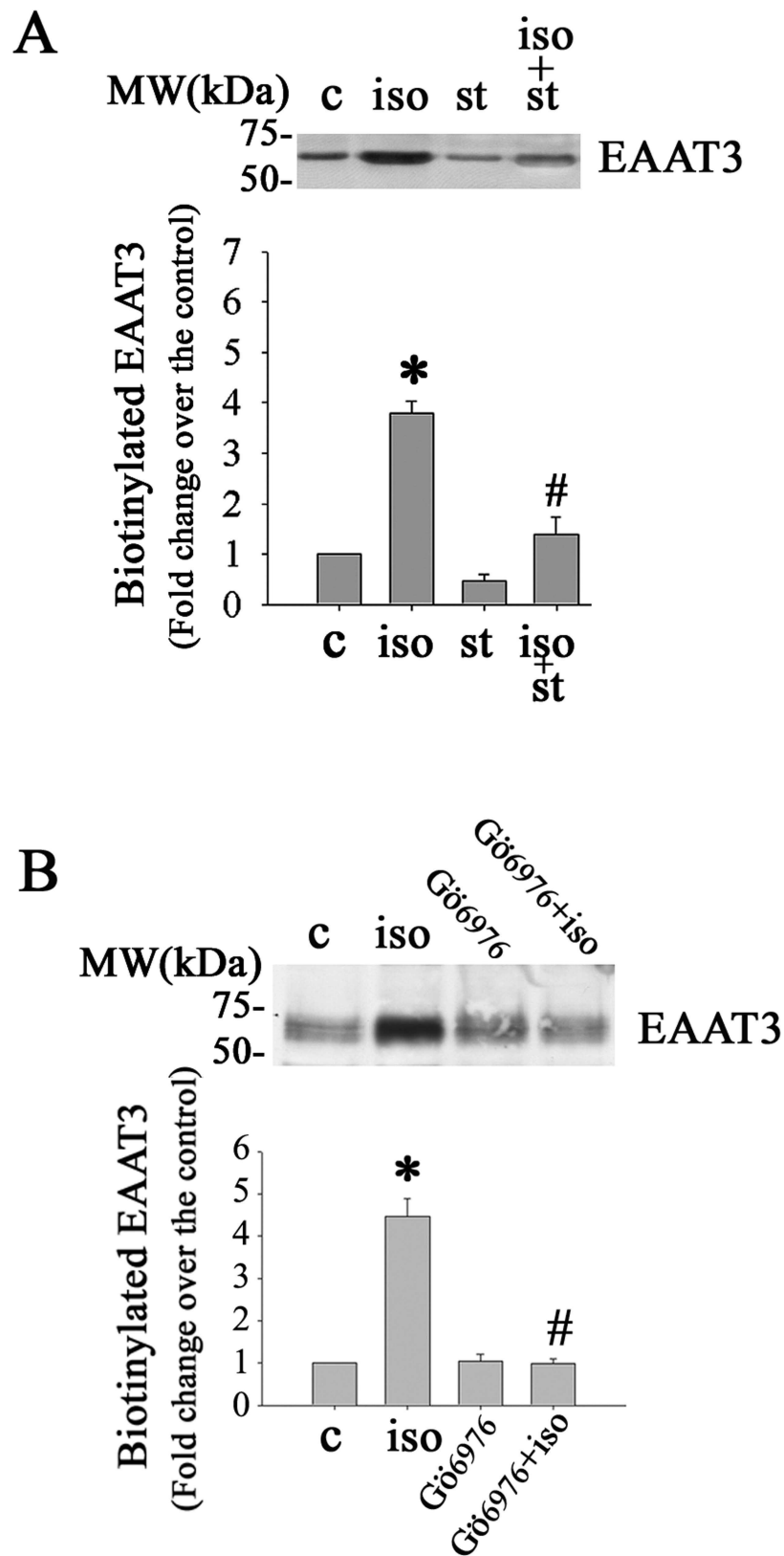


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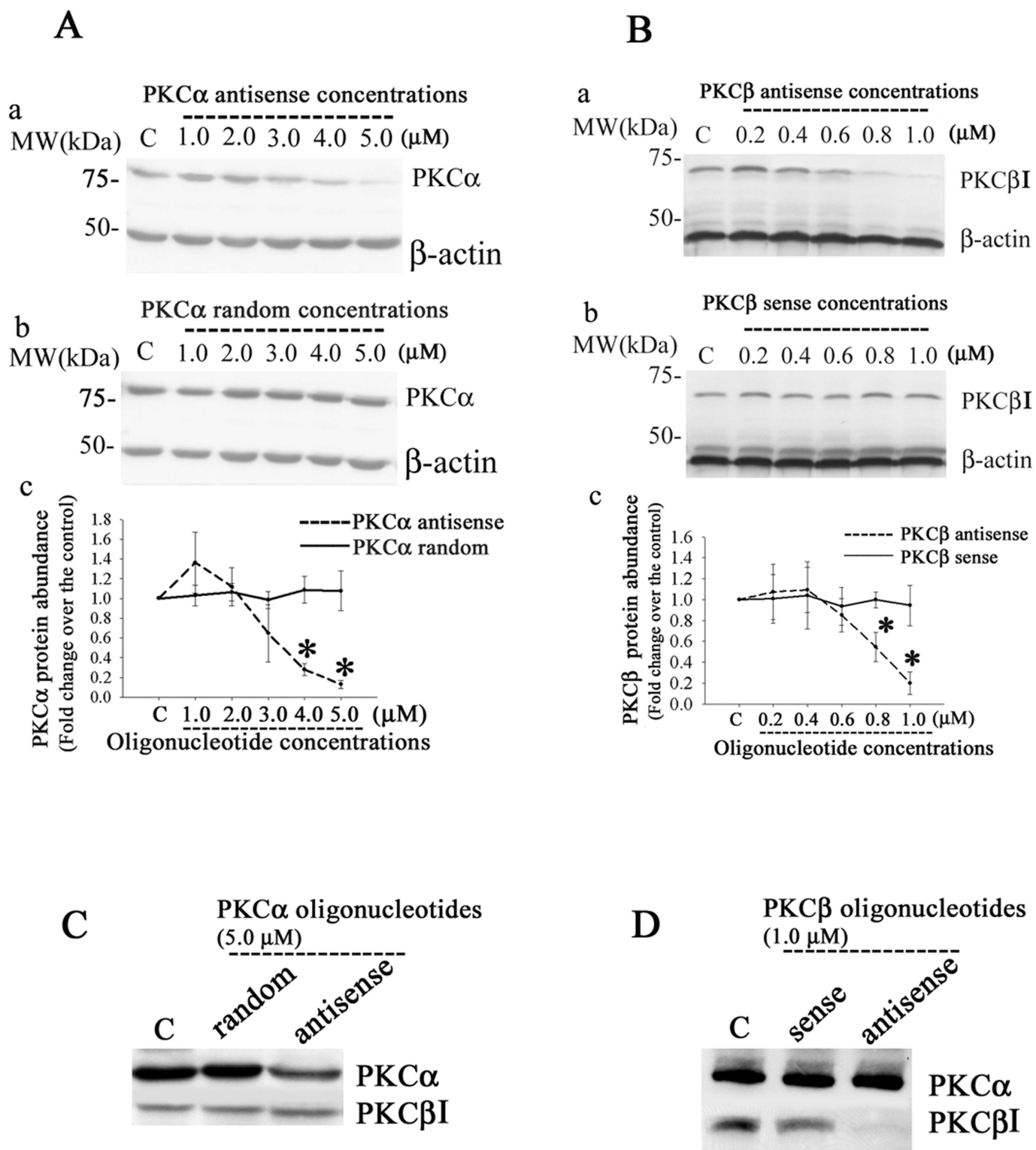


Fig. 5

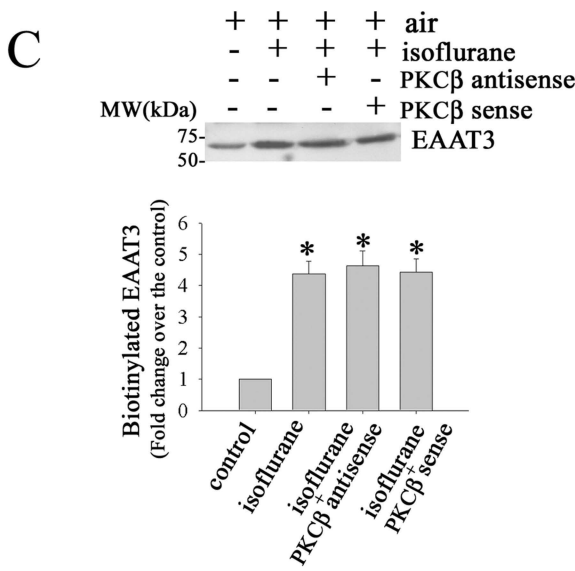
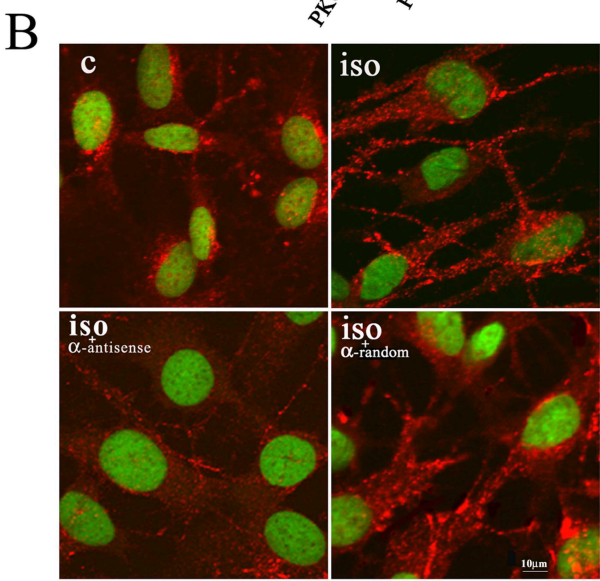
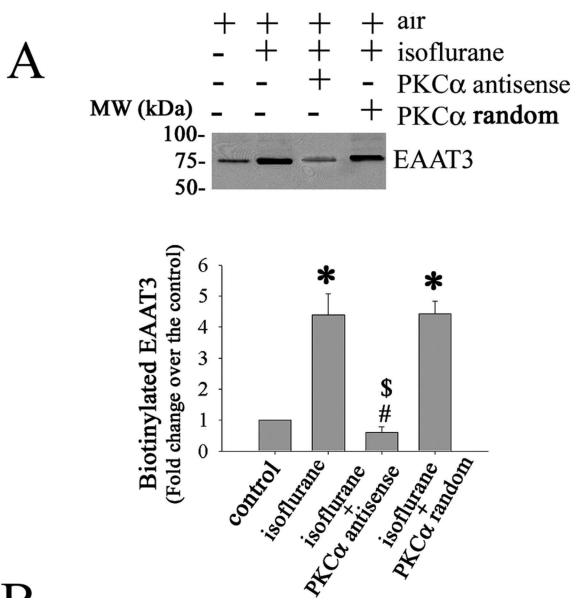


Fig. 6

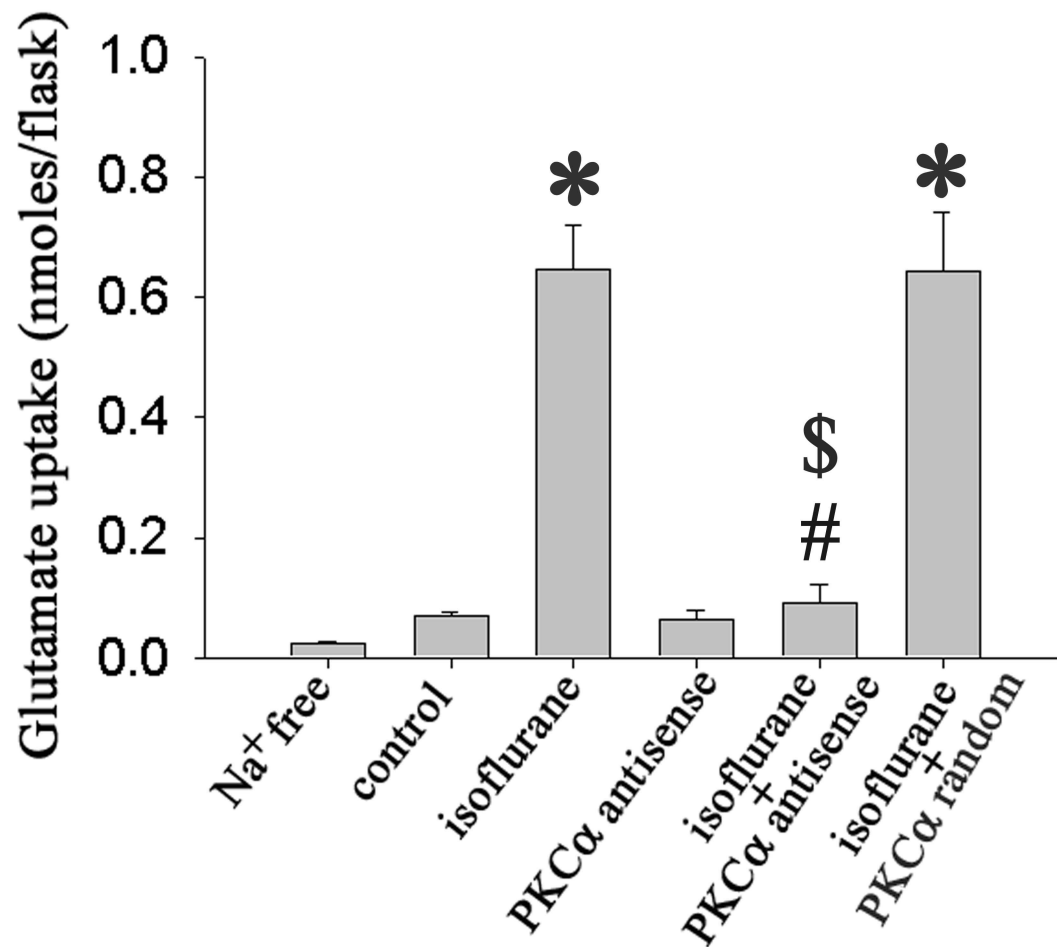


Fig. 7

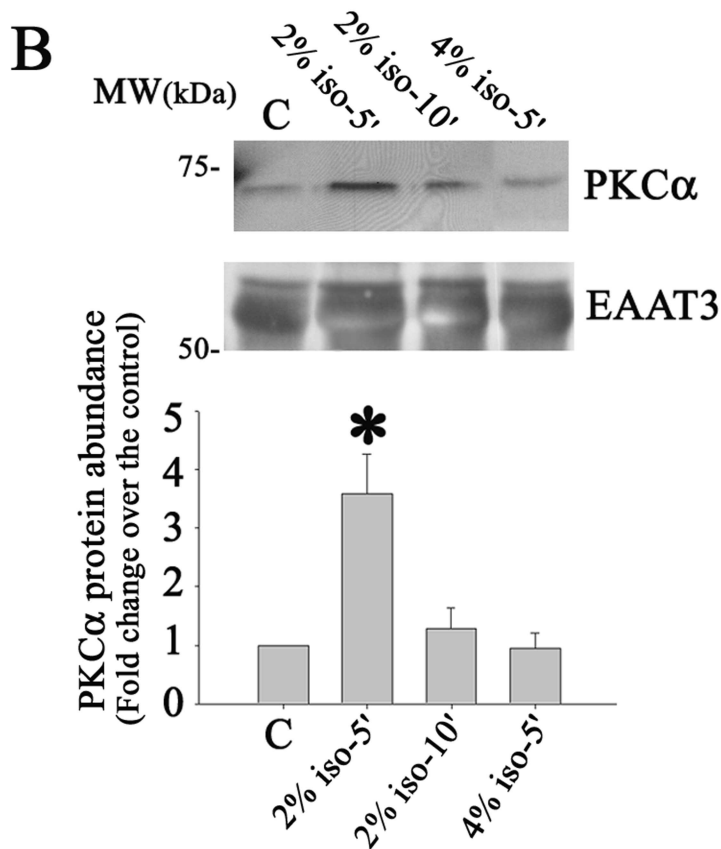
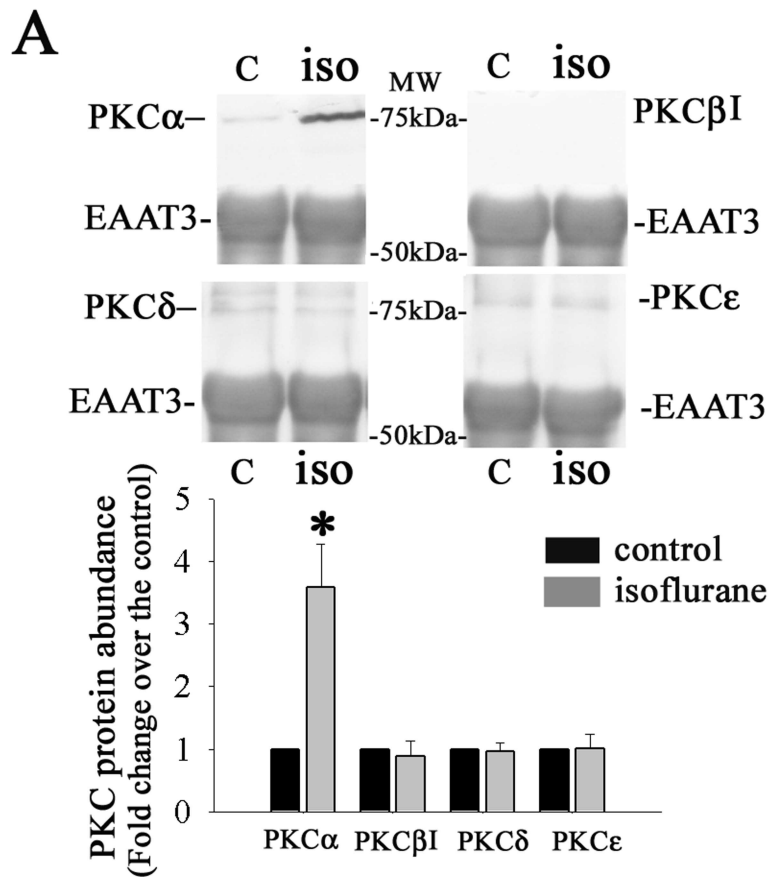


Fig. 8

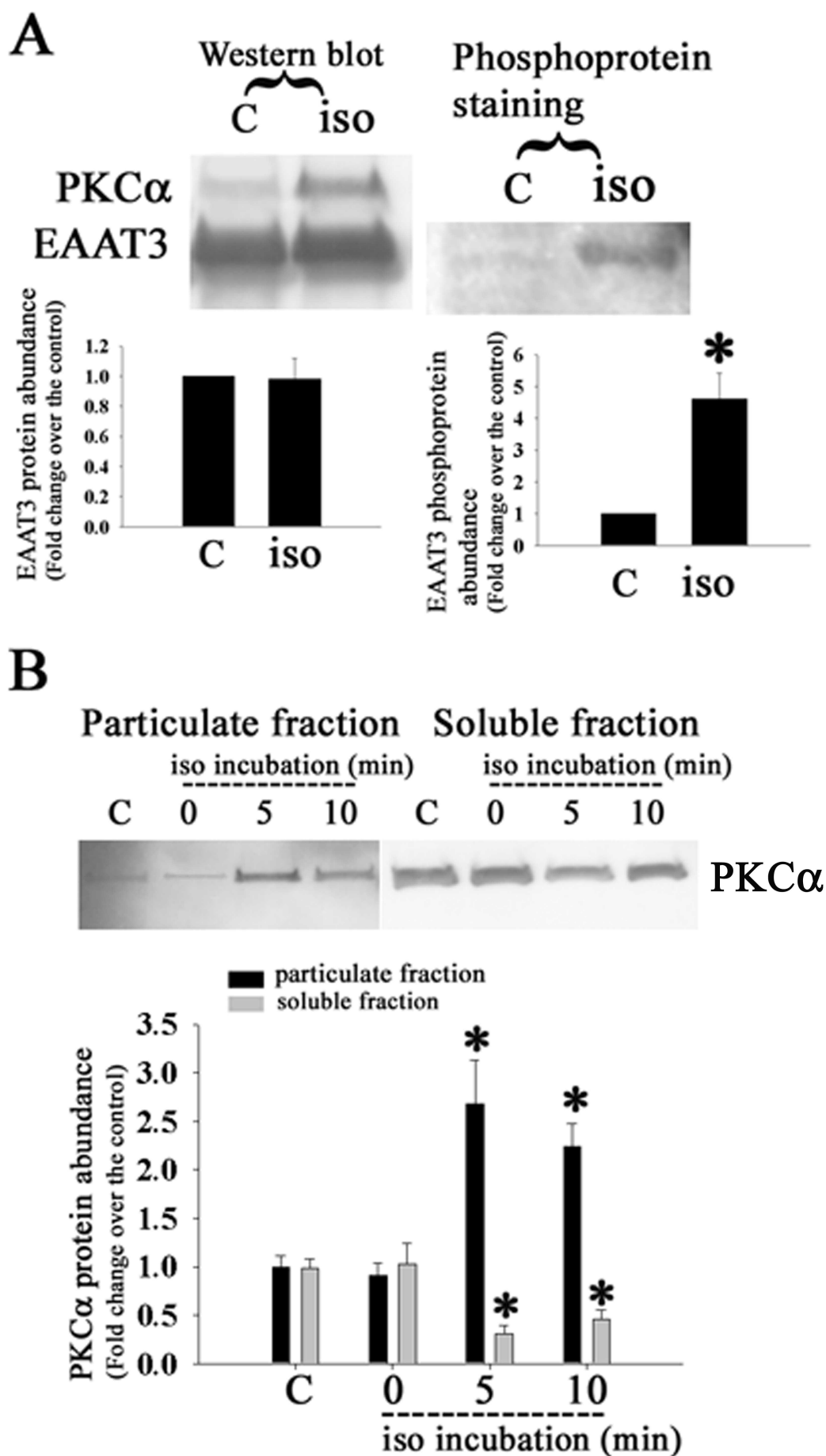


Fig. 9

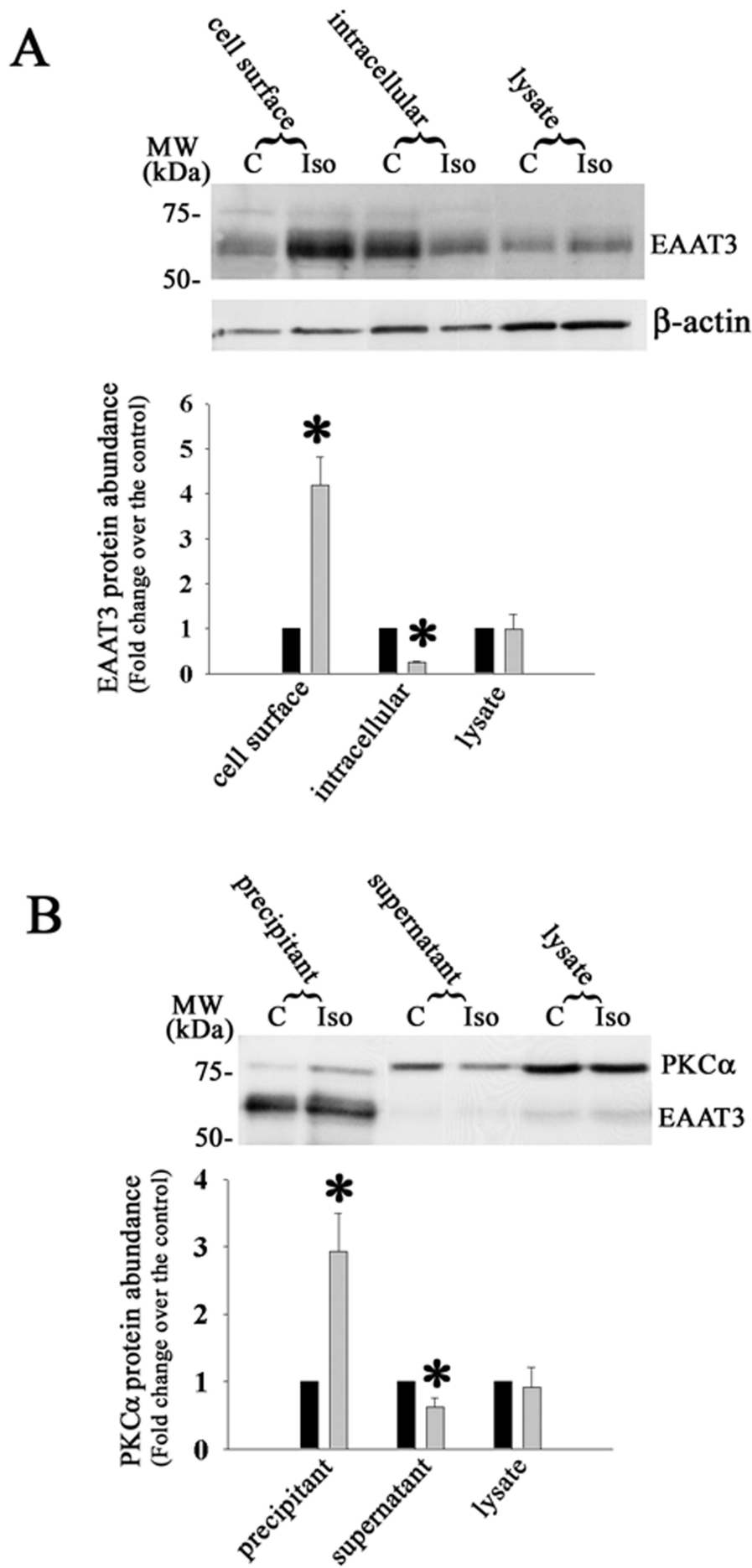


Fig. 10