

# Effects of Volatile Anesthetics on Glutamate Transporter, Excitatory Amino Acid Transporter Type 3

## The Role of Protein Kinase C

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**Background:** Glutamate transporters play an important role in maintaining extracellular glutamate homeostasis. The authors studied the effects of volatile anesthetics on one type of glutamate transporters, excitatory amino acid transporter type 3 (EAAT3), and the role of protein kinase C in mediating these effects.

**Methods:** Excitatory amino acid transporter type 3 was expressed in *Xenopus* oocytes by injection of EAAT3 mRNA. Using two-electrode voltage clamp, membrane currents were recorded before, during, and after application of L-glutamate. Responses were quantified by integrating the current trace and are reported as microcoulombs. Data are mean  $\pm$  SEM.

**Results:** L-Glutamate-induced responses were increased gradually with the increased concentrations of isoflurane, a volatile anesthetic. At 0.52 and 0.70 mM isoflurane, the inward current was significantly increased compared with control. Isoflurane (0.70 mM) significantly increased Vmax (maximum velocity) ( $3.6 \pm 0.4$  to  $5.1 \pm 0.4 \mu\text{C}$ ;  $P < 0.05$ ) but not Km (Michaelis-Menten Constant) ( $55.4 \pm 17.0$  vs.  $61.7 \pm 13.6 \mu\text{M}$ ;  $P > 0.05$ ) of EAAT3 for glutamate compared with control. Treatment of the oocytes with phorbol-12-myristate-13-acetate, a protein kinase C activator, caused a significant increase in transporter current ( $1.7 \pm 0.2$  to  $2.5 \pm 0.2 \mu\text{C}$ ;  $P < 0.05$ ). Responses in the presence of the combination of phorbol-12-myristate-13-acetate and volatile anesthetics (isoflurane, halothane, or sevoflurane) were not greater than those when volatile anesthetic was present alone. Oocytes pretreated with any of the three protein kinase C inhibitors alone (chelerythrine, staurosporine, or calphostin C) did not affect basal transporter current. Although chelerythrine did not change the anesthetic effects on the activity of EAAT3, staurosporine or calphostin C abolished the anesthetic-induced increase of EAAT3 activity.

**Conclusions:** These data suggest that volatile anesthetics enhance EAAT3 activity and that protein kinase C is involved in mediating these anesthetic effects.

GLUTAMATE is a major excitatory neurotransmitter. It is also neurotoxic when extracellular concentration is high. Glutamate transporters play an important role in removing glutamate from extracellular space into cells.

Dysfunction of glutamate transporters causes glutamate accumulation that results in glutamate-mediated neuronal injury, which has been implicated in the pathophysiology of ischemic brain damage and other neurodegenerative disorders, such as amyotrophic lateral sclerosis.<sup>1,2</sup> Five glutamate transporters have been characterized to date: excitatory amino acid transporters 1-5 (EAAT1-5). EAAT1-2 are glial, EAAT3-4 are neuronal, and the mRNA of EAAT5 is distributed in the neurons and glia of retina. Volatile anesthetics (VAs) have been demonstrated to enhance the uptake of glutamate in *in vitro* systems.<sup>3,4</sup> Such effects have been highlighted as a mechanism of neuroprotective effects of VAs. However, previous studies used either cultured neuroglial cells or synaptosomes, which contain more than one type of EAATs. To date, type-selective inhibitors on EAATs are not available. Thus, the effects of VAs on a single type of glutamate transporters have not yet been reported.

Protein kinase C (PKC) is implicated in a variety of physiologic and pathophysiologic functions, including neuronal signaling.<sup>5</sup> Activation of PKC causes diverse effects on glutamate transporters. A recent study demonstrated that phorbol 12-myristate 13-acetate (PMA), a PKC activator, increased the activity of EAAT3.<sup>6</sup> PKC also may mediate the action of VAs on various components of neurotransmission, such as the signaling pathways through the activation of muscarinic, serotonin, or metabotropic glutamate receptors.<sup>7-9</sup> In addition, a number of studies suggest that VAs may activate PKC in certain study models.<sup>10</sup> Thus, we designed experiments to address the following questions: Do VAs enhance the activity of EAAT3 and are the effects of VAs on EAAT3 PKC-mediated?

## Materials and Methods

### Oocyte Preparation and Expression of Excitatory Amino Acid Transporter Type 3

The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Virginia (Charlottesville, VA). Mature female *Xenopus laevis* frogs were purchased from *Xenopus* I (Ann Arbor, MI) and fed regular frog brittle twice weekly. For removal of oocytes, frogs were anesthetized in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO) in water until unresponsive to painful stimuli (toe pinching) and underwent surgery on ice. A 5-mm incision was made in the lower lateral abdominal quadrant,

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and a lobule of ovarian tissue, containing approximately 200 oocytes, was removed and placed immediately in modified Barth solution (containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 0.41 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 0.3 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.1 mM gentamicin, 15 mM HEPES, pH adjusted to 7.6). The oocytes were defolliculated with gentle shaking for approximately 2 h in calcium-free OR-2 solution (containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, 0.1% collagenase type Ia, pH adjusted to 7.5) and then incubated in modified Barth's solution at 16°C.

Excitatory amino acid transporter type 3 cDNA was provided by Mattias A. Hediger, Ph.D. (Associate Professor of Medicine, Laboratory of Molecular and Cellular Physiology, Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA). The cDNA was subcloned in a commercial vector (BluescriptSKm). The plasmid DNA was linearized with restriction enzyme (Not I), and mRNA was synthesized *in vitro* using a commercially available kit (Ambion, Austin, TX). The resulting mRNA was quantified spectrophotometrically and diluted in sterile RNase-free water. This mRNA was used for the cytoplasmic injection of oocytes in a concentration of 40 ng/30 nl using an automated microinjector (Drummond Nanoject; Drummond Scientific Co., Broomall, PA). This was followed by the incubation of the oocytes at 16°C for 3 or 4 days before the current recording.

#### *Electrophysiologic Recording*

Experiments were performed at room temperature (approximately 21–23°C). A single defolliculated oocyte was placed in a recording chamber (0.5 ml volume) and perfused with 3 ml/min Tyrode solution (containing 150 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 10 mM dextrose, and 10 mM HEPES, pH adjusted to 7.5). Microelectrodes were pulled in one stage from 10- $\mu$ l capillary glass (Drummond Scientific Co., Broomall, PA) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA). Tips were broken to a diameter of approximately 10  $\mu$ m. These microelectrodes provided resistance of 1–3 M $\Omega$  when they were filled with 3 M KCl. The oocytes were voltage clamped using a two-microelectrode oocyte voltage clamp amplifier (OC725-A; Warner Corporation, New Haven, CT) connected to a data acquisition and analysis system running on an IBM-compatible personal computer. The acquisition system consisted of a DAS-8A/D conversion board (Keithley-Metrabyte, Taunton, MA), and analyses were performed with OoClamp software.<sup>11</sup> All measurements were performed at a holding potential of –70 mV. Oocytes that did not show a stable holding current less than 1  $\mu$ A were excluded from analysis. L-Glutamate was diluted in Tyrode solution and superfused over the oocyte for 20 s (3 ml/min). L-Glutamate-induced inward currents were sampled at 125 Hz for 1 min: 5 s of baseline,

20 s of agonist application, and 35 s of washing with Tyrode solution. Responses were quantified by integrating the current trace and reported as microcoulombs. Because the transport of one negatively charged glutamate molecule cotransport 2–3 Na<sup>+</sup> into the cell, the glutamate transport is an electrogenic process. Thus, the size of the glutamate-induced current reflects the amount of transported glutamate. Each experiment was performed with oocytes from at least three different frogs.

#### *Anesthetic Administration and Protein Kinase C Manipulation*

A reservoir filled with 40 ml Tyrode solution was bubbled by output from a calibrated anesthetic-specific vaporizer. Air at a flow rate of 500 ml/min was used as a carrier gas, and 10 min was allowed for equilibration. In the control group, oocytes were perfused with Tyrode solution for 4 min before the responses were measured. In the VA group, oocytes were perfused with Tyrode solution for the first 1 min, followed by Tyrode solution equilibrated with VA for the next 3 min before the response measurement. The concentrations of VA in the solution of the recording chamber were periodically verified by the following method. After an aqueous sample was drawn from the chamber into an airtight glass syringe, a fourfold greater quantity of atmospheric air was drawn up as well. After agitation to equilibrate the anesthetic between the air-water phases, the anesthetic concentration in air was analyzed in a gas chromatograph (Aerograph 940; Varian Analytical Instruments, Walnut Creek, CA) calibrated with standards for each VA. The anesthetic concentrations in the original chamber solution were then calculated by applying partition coefficients, as previously described.<sup>12</sup>

Isoflurane, halothane, and sevoflurane, three commonly used VAs, were tested in this study. To study isoflurane dose response of EAAT3 activity, oocytes were exposed to 0 (control), 0.17, 0.35, 0.52, and 0.70 mM, respectively. The concentration of VAs used in other experiments was 0.70 mM for isoflurane, 0.59 mM for halothane, and 0.78 mM for sevoflurane. At 22°C, the aqueous anesthetic concentrations equilibrated with the minimum alveolar concentrations (in adult rats)<sup>13</sup> of isoflurane (1.12%), halothane (0.88%), and sevoflurane (1.97%) were 0.5, 0.48, and 0.33 mM, respectively. However, because of the decreased water:gas partitioning at 37°C (*vs.* 22°C), the corresponding equilibrated aqueous concentrations at 37°C were reduced by 40–50%.<sup>12</sup> To determine the effects of isoflurane on Km and Vmax of EAAT3, serial concentrations of L-glutamate (3, 10, 30, 100, and 300  $\mu$ M) were used. In other experiments, 30  $\mu$ M L-glutamate was used to induce the glutamate transporter currents.

To study the effect of PKC activation on EAAT3, oocytes were preincubated with PMA (100 nM) for 10 min before recording. To investigate whether there was in-

teraction between PMA and VAs, PMA-treated oocytes were exposed to VAs as described above. To study the effect of PKC inhibition on EAAT3 activity, oocytes were preincubated with one of three PKC inhibitors: staurosporine (1  $\mu\text{M}$  for 1 h), chelerythrine (50  $\mu\text{M}$  for 1 h), or calphostin-C (3  $\mu\text{M}$  for 2 h).

### Materials

Isoflurane and sevoflurane were purchased from Abbott Laboratories (North Chicago, IL), and halothane was purchased from Halocarbon Laboratories (River Edge, NJ). Other chemicals were obtained from Sigma (St. Louis, MO).

### Data Analysis

Responses are reported as mean  $\pm$  SEM. As variability in responses among batches of oocytes is common, responses were at times normalized to the same-day controls of each batch. Differences among groups were analyzed using either the student *t* test or analysis of variance, followed by Bonferroni or Student-Newman-Keuls correction as appropriate.  $P < 0.05$  was considered significant.

## Results

### Functional Expression of Excitatory Amino Acid Transporter Type 3 in Xenopus Oocytes

Whereas uninjected oocytes were unresponsive to L-glutamate (data not shown), oocytes injected with EAAT3 mRNA showed inward currents after application of L-glutamate (fig. 1). The response was concentration-dependent, and the  $\text{EC}_{50}$  for L-glutamate was determined to be 27.2  $\mu\text{M}$ , similar to that reported in the literature.<sup>14</sup> Thus, 30  $\mu\text{M}$  L-glutamate was used for other studies. No response was observed in the presence or absence of VAs when sodium chloride was replaced with choline chloride in the perfusion solution (data not shown), a characteristic of EAATs, because of the fact that EAATs are sodium cotransporters. This observation is in accordance with previous reports.<sup>14</sup>

### Enhancement of Excitatory Amino Acid Transporter Type 3 Activity by Isoflurane in a Concentration-dependent Manner

When oocytes without injection of EAAT3 mRNA were exposed to isoflurane, no inward or outward currents were recorded (data not shown). Oocytes injected with EAAT3 mRNA showed increased responses to L-glutamate in an isoflurane concentration-dependent manner (isoflurane concentration, 0.18–0.70 mM). At 0.52 and 0.70 mM isoflurane, the responses were significantly increased compared with control values (fig. 2). In addition to enhancing the responses induced by 30  $\mu\text{M}$  L-glutamate, 0.70 mM isoflurane also significantly increased

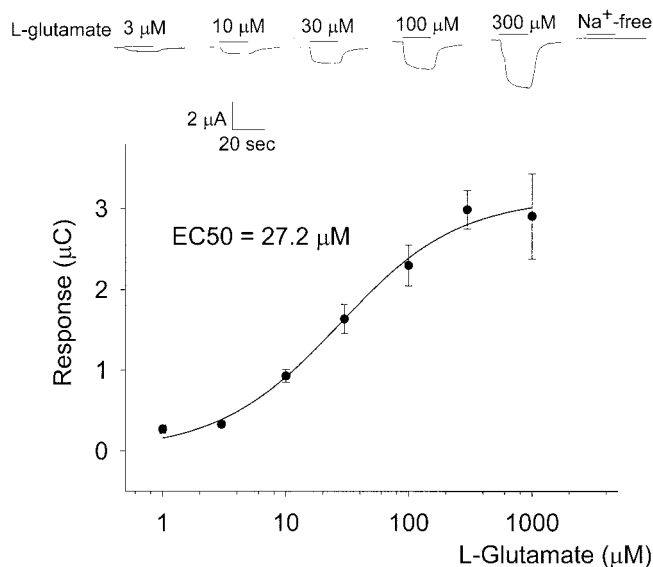


Fig. 1. Dose–response curve of glutamate transporter, excitatory amino acid transporter type 3. Oocytes were injected with excitatory amino acid transporter type 3 mRNA and incubated at 16°C for 3 or 4 days before the current recording. (Top) Typical current traces induced by the application of serial concentrations of L-glutamate are shown. Responses were quantified by integrating the current trace by quadrature. Data are mean  $\pm$  SEM (n = 15–27). The minor ticks on the abscissa indicate double concentrations of the previous ticks on the log scale.

the responses induced by 100 or 300  $\mu\text{M}$  L-glutamate (fig. 3). Further analyzing the data (Prism ver 2.0; GraphPad, San Diego, CA) demonstrated that isoflurane did not cause a significant change in  $K_m$  ( $55.4 \pm 17.0 \mu\text{M}$  for control *vs.*  $61.7 \pm 13.6 \mu\text{M}$  for the isoflurane group; n = 18;  $P > 0.05$ ). However, isoflurane significantly increased  $V_{max}$  from  $3.6 \pm 0.4$  to  $5.1 \pm 0.4 \mu\text{C}$  (n = 18;  $P < 0.05$ ), corresponding to a 42% increase.

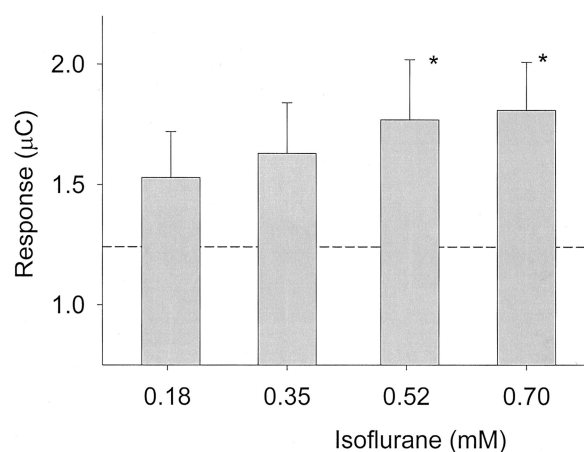
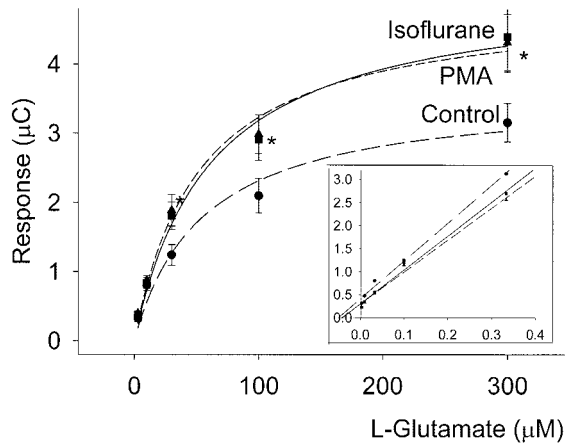


Fig. 2. Effects of isoflurane on the activity of excitatory amino acid transporter type 3. The concentration of L-glutamate was 30  $\mu\text{M}$ . Dashed line shows the mean value of the control group ( $1.2 \pm 0.2 \mu\text{C}$ ). Oocytes exposed to isoflurane (3 min) showed increased responses to L-glutamate in a concentration-dependent manner. Isoflurane at 0.52 and 0.70 mM significantly increased the responses compared with control. Data are mean  $\pm$  SEM (n = 18 in each group). \* $P < 0.05$  compared with control.





**Fig. 3.** Dose–response curve of excitatory amino acid transporter type 3 in the presence or absence of isoflurane or phorbol 12-myristate 13-acetate (PMA). Oocytes were exposed to isoflurane (0.70 mM for 3 min) or PMA (100 nM for 10 min). Inset graph is a Lineweaver-Burk plot using the mean value of the respective groups with abscissa for reciprocal of L-glutamate concentration (micromolars) and ordinate for reciprocal of response (microcoulombs).  $r^2 = 0.99$  for the control, isoflurane, or PMA group. \* $P < 0.05$  compared with control.

#### Effects of Protein Kinase C Activation on Excitatory Amino Acid Transporter Type 3 Activity in the Presence or Absence of Volatile Anesthetics

Preincubation of the oocytes with PMA (100 nM) for 10 min significantly increased EAAT3 activity ( $1.7 \pm 0.2$  vs.  $2.5 \pm 0.2$   $\mu\text{C}$ ;  $n = 20$ ;  $P < 0.05$ ). To determine whether PMA interacts with VAs, PMA-treated oocytes were exposed to VAs, and the responses were compared with controls. Oocytes treated with PMA or VAs showed greater responses than those in the control group. There was no statistical difference among the PMA, isoflurane, or PMA plus isoflurane groups (table 1). Thus, there seems to be no additive or synergistic interaction between PMA and isoflurane effects on EAAT3 activity, suggesting that these two agents might increase the EAAT3 activity through the same pathway. To support

**Table 1.** Effects of PKC Activation on EAAT3 in the Presence or Absence of VAs

	Control	PMA	Anesthetic	PMA and Anesthetic
Isoflurane	$1.0 \pm 0.1$	$1.5 \pm 0.1^*$	$1.6 \pm 0.2^*$	$1.6 \pm 0.2^*$
Halothane	$1.0 \pm 0.1$	$1.8 \pm 0.2^*$	$2.2 \pm 0.4^*$	$2.0 \pm 0.3^*$
Sevoflurane	$1.0 \pm 0.1$	$1.8 \pm 0.3^*$	$1.8 \pm 0.1^*$	$1.7 \pm 0.2^*$

Oocytes either preincubated with PMA (100 nM for 10 min) or exposed to VA (0.70 mM for isoflurane, 0.59 mM for halothane, and 0.78 mM for sevoflurane) for 3 min showed greater responses than the control. Each set of data has been normalized by using the mean value of the control group from the same batch of oocytes ( $n = 3$  or 4). Units are folds of controls, with controls being 1. Data are mean  $\pm$  SEM ( $n = 12$ –18).

\* $P < 0.05$  compared with control.

EAAT3 = excitatory amino acid transporter type 3; PKC = protein kinase C; PMA = phorbol 12-myristate 13-acetate; VA = volatile anesthetic.

**Table 2.** Effects of PKC Inhibition on EAAT3 in the Presence or Absence of VAs

	Control	Staurosporine (1 $\mu\text{M}$ for 1 h)	Anesthetic	Staurosporine and Anesthetic
Isoflurane	$1.0 \pm 0.1$	$0.9 \pm 0.1$	$1.7 \pm 0.2^*$	$1.1 \pm 0.2$
Halothane	$1.0 \pm 0.1$	$0.8 \pm 0.1$	$1.6 \pm 0.2^*$	$1.3 \pm 0.2$
Sevoflurane	$1.0 \pm 0.1$	$0.7 \pm 0.1$	$1.6 \pm 0.2^*$	$0.9 \pm 0.1$
	Control	Calphostin C (3 $\mu\text{M}$ for 2 h)	Anesthetic	Calphostin C and Anesthetic
Isoflurane	$1.0 \pm 0.1$	$1.0 \pm 0.2$	$1.7 \pm 0.1^*$	$1.4 \pm 0.3$
Halothane	$1.0 \pm 0.1$	$1.1 \pm 0.2$	$1.7 \pm 0.1^*$	$1.2 \pm 0.2$
Sevoflurane	$1.0 \pm 0.1$	$1.1 \pm 0.2$	$1.7 \pm 0.2^*$	$1.0 \pm 0.1$
	Control	Chelerythrine (50 $\mu\text{M}$ for 1 h)	Anesthetic	Chelerythrine and Anesthetic
Isoflurane	$1.0 \pm 0.1$	$1.4 \pm 0.2$	$1.9 \pm 0.3^*$	$1.7 \pm 0.2^*$
Halothane	$1.0 \pm 0.1$	$1.0 \pm 0.2$	$1.7 \pm 0.2^*$	$2.1 \pm 0.3^*$
Sevoflurane	$1.0 \pm 0.1$	$1.0 \pm 0.2$	$1.9 \pm 0.2^*$	$1.9 \pm 0.4^*$

The concentration of VAs is 0.70 mM for isoflurane, 0.59 mM for halothane, and 0.78 mM for sevoflurane. Each set of data has been normalized by using the mean value of the control group from the same batch of oocytes ( $n = 3$  or 4). Units are folds of controls, with controls being 1. Data are mean  $\pm$  SEM ( $n = 10$ –18).

\* $P < 0.05$  compared with control; EAAT3 = excitatory amino acid transporter type 3; PKC = protein kinase C; VA = volatile anesthetic.

this, kinetic study of PMA-exposed oocytes (100 nM for 10 min) demonstrated that PMA increased  $V_{\text{max}}$  significantly compared with control ( $3.6 \pm 0.4$   $\mu\text{C}$  for control vs.  $4.9 \pm 0.3$   $\mu\text{C}$  for the PMA group;  $n = 18$ ;  $P < 0.05$ ) but caused no changes in  $K_m$  ( $55.4 \pm 17.0$   $\mu\text{M}$  for control vs.  $52.4 \pm 9.9$   $\mu\text{M}$  for the PMA group;  $n = 18$ ;  $P > 0.05$ ; fig. 3), a similar pattern to isoflurane effect.

#### Effects of Protein Kinase C Inhibition on Excitatory Amino Acid Transporter Type 3 in the Presence or Absence of Volatile Anesthetics

Preincubation of the oocytes with staurosporine (1  $\mu\text{M}$ ) for 1 h did not decrease EAAT3 activity compared with controls ( $1.5 \pm 0.3$  to  $1.2 \pm 0.2$   $\mu\text{C}$ ;  $n = 17$ ;  $P > 0.05$ ). However, staurosporine abolished the isoflurane-enhanced EAAT3 activity. Similarly, staurosporine also abolished the enhancement of EAAT3 activity caused by halothane or sevoflurane (table 2). To further confirm the involvement of PKC in the anesthetic effects on EAAT3, other PKC inhibitors were used. Although oocytes treated with calphostin C (3  $\mu\text{M}$  for 2 h) alone had similar responses to L-glutamate as controls, they did not show enhanced responses to L-glutamate as controls did in the presence of VAs (table 2). Likewise, chelerythrine (50  $\mu\text{M}$  for 1 h) alone had no effects on EAAT3 activity. However, chelerythrine failed to block the VA effects on EAAT3 activity (table 2). Similar results were obtained even with higher concentration of chelerythrine and longer preincubation time (100  $\mu\text{M}$  for 2 h,

$1.2 \pm 0.2 \mu\text{C}$  for control,  $1.1 \pm 0.2 \mu\text{C}$  for chelerythrine group;  $n = 13$ ;  $P > 0.05$ ).

## Discussion

### *Effects of Isoflurane on the Activity of Excitatory Amino Acid Transporter Type 3*

Several studies have investigated the effects of VAs on glutamate uptake. Larsen *et al.*<sup>3</sup> reported that isoflurane increased glutamate uptake in synaptosomes. In contrast, using a similar experimental model, Nicol *et al.*<sup>15</sup> failed to demonstrate any increases in glutamate uptake by anesthetic agents including isoflurane. Miyazaki *et al.*<sup>4</sup> reported that several VAs increased glutamate uptake in cultured astrocytes. Recently, Liachenko *et al.*<sup>16</sup> concluded that isoflurane inhibited glutamate uptake with an  $\text{EC}_{50}$  at approximately 0.8 mM in mouse cerebrocortical slices. This conclusion was based on their findings that high concentrations ( $> 0.4$  mM) of isoflurane produced higher concentrations of glutamate in the effluent solution from brain slices depolarized by 40 mM KCl than that in the presence of low concentrations ( $< 0.4$  mM) of isoflurane, and *L-trans*-pyrrolidine-2,4-dicarboxylic acid, a specific EAAT inhibitor, significantly decreased the  $\text{EC}_{50}$  for this isoflurane effect. Although the reasons for the different conclusions between the previous studies and the study by Liachenko *et al.* are not clear, the latter study did not directly measure glutamate uptake by cells. In addition, the previous studies used brain slices, synaptosomes, or cultured astrocytes, which have more than one type and different population of EAATs. Thus, the reported anesthetic effects on glutamate uptake in the previous studies may represent summary effects of anesthetics on a mixture population of EAATs expressed in the study models. To further characterize the effects of VAs on the EAAT system, we used oocyte expression system to investigate the anesthetic effects on individual EAATs. Although the oocyte expression system provides an artificial environment for EAATs, oocytes have components of all major intracellular signaling pathways of mammalian cells and have been used for studies of EAAT activity.<sup>7,9,14,17</sup>

Our study clearly demonstrates that VAs enhance the activity of EAAT3, a major neuronal glutamate transporter. This study also showed that 0.70 mM isoflurane increased the  $V_{\text{max}}$ , but not  $K_{\text{m}}$ , of glutamate uptake, suggesting that isoflurane increases the available number or turnover rate of EAAT3 rather than affecting the affinity of EAAT3 to glutamate. These results are consistent with a previously published report.<sup>3</sup> We previously reported that isoflurane affected both  $V_{\text{max}}$  and  $K_{\text{m}}$  of EAATs in cultured glial cells, which mainly express EAAT1 and EAAT2.<sup>18</sup> Differences in the studied EAATs and experimental conditions may explain the discrepancy between the current and our previous studies.

### *Involvement of Protein Kinase C in the Effects of Volatile Anesthetics on Excitatory Amino Acid Transporter Type 3 Activity*

In this study, PKC activation by PMA caused increased activity of EAAT3, consistent with a previous report.<sup>6</sup> However, there was no additive or synergistic interaction between PMA and VAs on the activity of EAAT3, suggesting that VAs act on EAAT3 *via* PKC signaling. In addition, PMA induced a similar kinetic change in EAAT3 activity as that induced by isoflurane (increase in  $V_{\text{max}}$  but no change in  $K_{\text{m}}$ ). Therefore, VAs may enhance EAAT3 activity *via* PKC activation. This observation is further supported by the findings with PKC inhibitors.

Of three PKC inhibitors tested, staurosporine and calphostin C abolished the effects of VAs on EAAT3 activity, whereas chelerythrine did not. These three PKC inhibitors have preferable working sites on PKC: staurosporine acts on the catalytic domain of PKC, calphostin C interacts with lipid site (regulatory domain) of PKC, and chelerythrine acts on the peptide site of PKC.<sup>19,20</sup> Thus, the aforementioned results would suggest that peptide site of PKC might not be so important as other sites in the regulation of VA effects on EAAT3. However, the specificity of these inhibitors to their respective sites is imperfect. In addition, these inhibitors may have different inhibitory potency among PKC isozymes.<sup>21</sup> Therefore, these results also suggest that a subset of PKC isozymes may be involved in these anesthetic effects on EAAT3. It is known that at least several PKC isozymes (PKC  $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ,  $\delta$ ,  $\zeta$ ) are present in *Xenopus* oocytes.<sup>22</sup> Because PKC  $\zeta$ , an atypical PKC isozyme, is not activated by phorbol ester,<sup>23</sup> this PKC isozyme may not play an important role in mediating the PMA or VA effects on EAAT3 activity.

### *Implication of the Anesthetic Effects on Excitatory Amino Acid Transporters*

Volatile anesthetics have been shown to decrease EAA release<sup>24</sup> and signaling.<sup>25</sup> This and other studies indicate that VAs increase glutamate uptake.<sup>3,4</sup> These effects (on glutamate release, uptake, and signaling) may all be important components contributing to the final presentation of VA effects on the central nervous system. In addition, accumulation of extracellular glutamate has been implicated in some brain pathophysiology. Glutamate itself is toxic to neuron at high concentrations.<sup>26</sup> Hence, enhanced glutamate uptake by VAs could at least partly prevent or ameliorate the accumulation of glutamate to a toxic level, thus serving as a neuroprotective mechanism for VAs. There are some debates on the long-term outcome of neuroprotection by VAs. However, studies have consistently demonstrated that VAs improved short-term outcome after an ischemic insult.<sup>27,28</sup> Moreover, although physiologic functions of each type of glutamate transporters are not known completely, there is some emerging evidence that dysfunc-

tion of EAAT3 can be linked to epilepsy. Researchers, using antisense oligonucleotides, demonstrated that animals with decreased concentration of EAAT3 had epileptiform fits.<sup>29</sup> Thus, our results may have suggested a mechanism for the antiseizure property of VAs.

Astroglial glutamate transporters (EAAT1, EAAT2) behave differently after PKC activation. The activity of EAAT1 was inhibited by PMA,<sup>30</sup> and that of EAAT2 was reported to be inhibited,<sup>31</sup> enhanced,<sup>32</sup> or unaffected<sup>33</sup> by PKC activation. The functional diversity of each type of glutamate transporter on PKC activation is not yet understood. Because VAs appear to have direct effects on PKC,<sup>10,34,35</sup> it is probable that VAs might alter the activity of individual EAATs differently. Therefore, further study to investigate the anesthetic effects on other types of EAATs will provide a more complete picture of the anesthetic effects on glutamate uptake *via* EAATs.

In this report, we studied the effects of VAs on a single type of glutamate transporter and investigated the mechanism of these effects. In conclusion, our data suggest that VAs increase EAAT3 activity *via* PKC activation. This may be an important mechanism for the anesthetic, neuroprotective, and antiepileptic effects of VAs.

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