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Alphaxalone, a neurosteroid anaesthetic, increases the activity of the glutamate transporter type 3 expressed in *Xenopus* oocytes

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ABSTRACT

Glutamate transporters may be important targets for anaesthetic action in the central nervous system. The authors investigated the effects of alphaxalone, an intravenous neurosteroid anaesthetic, on the activity of glutamate transporter type 3 (EAAT3). EAAT3 was expressed in *Xenopus* oocytes by injecting its mRNA. Two-electrode voltage clamping was used to record membrane currents before, during, and after applying L-glutamate (30 μM) in the presence or absence of alphaxalone. Responses were quantified by integrating current traces and are reported in microCoulombs (μC). Results are presented as means±S.E.M. L-Glutamate induced inward currents in EAAT3 expressing oocytes, and these currents were dose-dependently increased by alphaxalone. Alphaxalone at 0.01 to 3 μM significantly increased the inward currents. In addition, the treatment of oocytes with phorbol-12-myristate-13-acetate (PMA), a protein kinase C (PKC) activator, significantly increased the transporter currents (1.0±0.2 to 1.4±0.2 μC; $P<0.05$). However, treatment with PMA plus alphaxalone did not increase responses further as compared with PMA or alphaxalone alone. Furthermore, pretreatment of oocytes with chelerythrine or staurosporine, two PKC inhibitors, did not affect basal transporter currents, but did significantly reduce alphaxalone-enhanced EAAT3 activity; whereas oocytes pretreated with wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, showed significant reductions in basal and alphaxalone-enhanced EAAT3 activities. The above results suggest that alphaxalone enhances EAAT3 activity and that PKC and PI3K are involved in this effect.

1. Introduction

Neurosteroids are synthesized from cholesterol in the nervous systems (Compagnone and Mellon, 2000; Horishita et al., 2002), and some neurosteroids have anaesthetic activity (Holzbauer, 1976). Alphaxalone (3α-hydroxy-5α-pregnane-11, 20-dione) is a neurosteroid anaesthetic that was available for clinical use (Child et al., 1971), but was withdrawn due to anaphylactoid reactions attributed to the vehicle in the preparation (Cremophor EL) (Winter et al., 2003). Currently, alphaxalone is used as a veterinary anaesthetic with anticonvulsant activity (Hansen et al., 2004; Deutsch et al., 1996). Mechanism underlying the anaesthetic effect of alphaxalone has been studied by a number of investigators. Alphaxalone has been reported to potentiate GABA neurotransmission in rat brain slices (Harrison and Simmonds, 1984), rat hippocampal neurons (Harrison et al., 1987) and adrenal medullary cells (Cottrell et al., 1987), and to inhibit nicotinic acetylcholine receptors (Shiraishi et al., 2002) and norepinephrine transporter function (Horishita et al., 2002) in cultured bovine adrenal chromaffin cells. Shiraishi et al. (2003) suggested that alphaxalone has inhibitory effects on M_1 and M_3 muscarinic receptors expressed in *Xenopus* oocytes, and that these effects underlie its anaesthetic effect. However, to date, the effects of alphaxalone on glutamate transporters have not been investigated.

Glutamate is a major excitatory amino acid neurotransmitter, and glutamate transporters play a critical role in the translocation of glutamate from the extracellular to the intracellular spaces and in maintaining extracellular glutamate concentrations within physiological levels (Danbolt, 2001). Glutamate transporter dysfunction results in extracellular glutamate accumulation and neurotoxicity, which has been implicated in the pathophysiology of ischemic brain damage and neurodegenerative disorders, such as Alzheimer's disease and amyotrophic lateral sclerosis (Tanaka et al., 1997; Rothstein et al., 1995).

Five glutamate transporters have been characterized to date (Danbolt, 2001), i.e., the excitatory amino acid transporters 1–5 (EAAT1–5). EAAT1 and EAAT2 are glial, EAAT3 and EAAT4 are neuronal, and EAAT5 is mainly localized to the retina. EAAT3 is the major neuronal transporter in the brain and spinal cord and plays a neuroprotective role by maintaining

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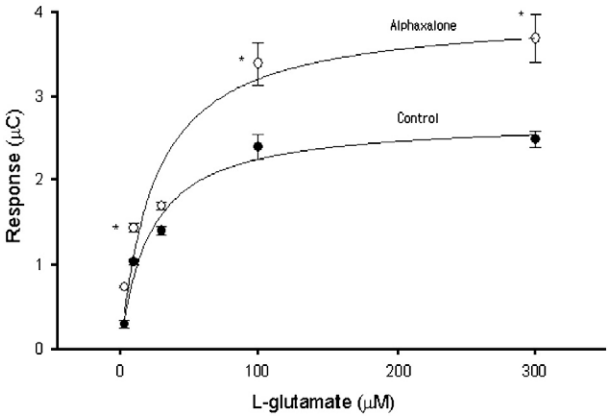

Fig. 1. Concentration-response curve of EAAT3 to glutamate in the presence or absence of 0.1 μM alphaxalone for 3 min. In addition to enhancing the responses induced by 30 μM L-glutamate, 0.1 μM alphaxalone also significantly increased the responses induced by 100 or 300 μM L-glutamate. Data are means±S.E.M. (n=19 in each group). *P<0.05 compared to the corresponding control.

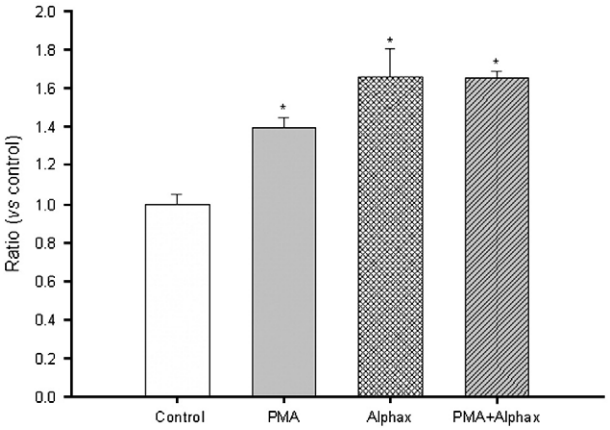

Fig. 3. Effects of protein kinase C (PKC) activation on EAAT3 activity in the presence or absence of 0.1 μM alphaxalone for 3 min. Oocytes exposed to phorbol-12-myristate-13-acetate (PMA), alphaxalone, or both showed a significant increase in EAAT3 activity compared with control; whereas there was no statistical difference among the PMA, alphaxalone, or PMA plus alphaxalone groups. PMA; phorbol-12-myristate-13-acetate. Alphax; alphaxalone. Data are means±S.E.M. (n=19–22 in each group). *P<0.05 compared with control.

extracellular glutamate homeostasis in the cortex and hippocampus (Danbolt, 2001; Sepkuty et al., 2002) that are vulnerable to excitotoxic damage after brain ischemia, e.g., stroke and head trauma (Greene and Greenamyre, 1996; Nilsson et al., 1996; Palmer et al., 1993). In addition, EAAT3 dysfunction may be linked to seizure propensity, e.g., animals with reduced EAAT3 levels are prone to epileptiform fits (Danbolt et al., 1998), and Gorter et al. (2002) suggested that EAAT3 dysfunction underlies epilepsy development. Furthermore, the down-regulation of EAAT3, rather than EAAT2, in rat hippocampal regions was found to contribute to the early phase of seizure (Simantov et al., 1999).

In this study, we evaluated the effects of alphaxalone on EAAT3 expressed in *Xenopus* oocytes by using two-electrode voltage clamping, and the involvements of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K), two signaling molecules that regulate EAAT3 activity (Do et al., 2002a,b, 2003), in the alphaxalone effects.

2. Materials and methods

The study protocol adopted was approved by the Institutional Animal Care and Use Committee at Seoul National University College of Medicine. *Xenopus* oocytes were isolated and microinjected as described by Do et al. (2002a). Mature female *Xenopus laevis* frogs were purchased from Kato S Science (Chiba, Japan), and fed with regular frog brittle twice weekly. To remove 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). Operations were performed on ice. Briefly, a 5-mm long incision was made in the lower lateral abdominal quadrant, and then a lobule of ovarian tissue (containing approximately 150 oocytes) was removed and placed immediately in calcium-free OR-2 solution

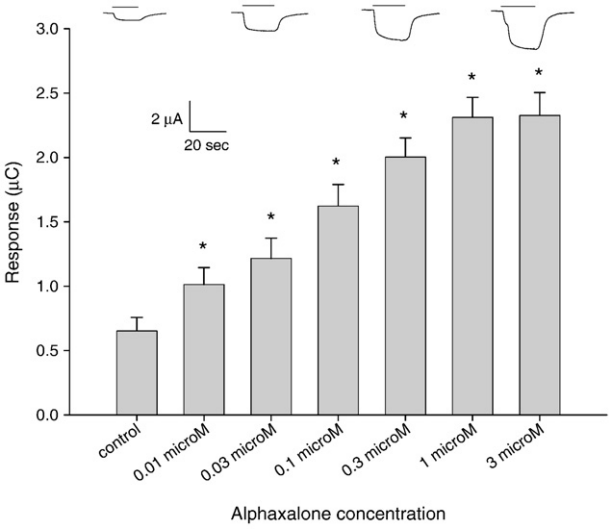

Fig. 2. Concentration-response of alphaxalone on the activity of excitatory amino acid transporter type 3. Oocytes were exposed to various concentrations (0.01 μM, 0.03 μM, 0.1 μM, 0.3 μM, 1 μM, 3 μM) of alphaxalone for 3 min. The EAAT3 response was then induced by 30 μM L-glutamate. Oocytes injected with EAAT3 mRNA showed increased responses to L-glutamate in an alphaxalone concentration-dependent manner. Inset graphs are representative current traces. Data are means±S.E.M. (n=17–19 in each group). *P<0.05 compared with control.

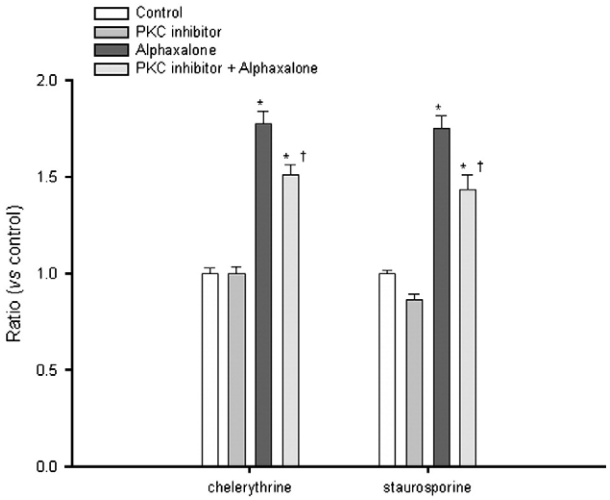

Fig. 4. Effects of protein kinase C (PKC) inhibition on EAAT3 activity in the presence or absence of 0.1 μM alphaxalone for 3 min. These two PKC inhibitors (staurosporine or chelerythrine) decreased but did not abolish the 0.1-μM alphaxalone-enhanced EAAT3 activity. PKC inhibitor: protein kinase C inhibitor. Data are means±S.E.M. (n=15–22 in each group). *P<0.05 compared with control. †P<0.05 compared with alphaxalone alone.

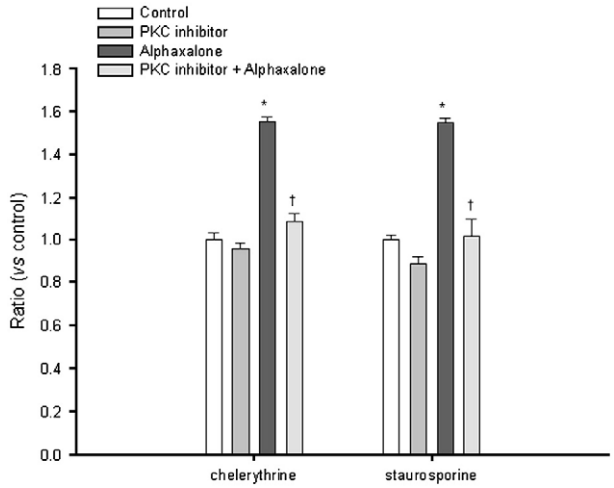

Fig. 5. Effects of protein kinase C (PKC) inhibition on EAAT3 activity in the presence or absence of 0.04 μM alphaxalone for 3 min. These two PKC inhibitors (staurosporine or chelerythrine) abolished the 0.04-μM alphaxalone-enhanced EAAT3 activity. PKC inhibitor: protein kinase C inhibitor. Data are means±S.E.M. (n=13–18 in each group). *P<0.05 compared with control. †P<0.05 compared with alphaxalone alone.

(containing in mM: NaCl 82.5, KCl 2, $MgCl_2$ 1, HEPES 5, collagenase type Ia 0.1%, pH 7.5), in order to remove vitelline membrane surrounding oocytes. They were then defolliculated by gentle shaking for approximately 2 h and kept at 18 °C in modified Barth's solution (containing in mM: NaCl 88, KCl 1, $NaHCO_3$ 2.4, $CaCl_2$ 0.41, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.3, gentamicin 0.1, HEPES 15, pH adjusted to 7.6).

The rat EAAT3 complementary DNA (cDNA) construct used was provided by Dr. M. A. Hediger (Brigham and Women's Hospital, Harvard Institute of Medicine, Boston, MA), and contained cDNA subcloned in a commercial vector (BluescriptSKm). Plasmid DNA was linearized using a restriction enzyme (*Not* I), and messenger RNA (mRNA) was synthesized *in vitro* using a commercially available kit (Ambion, Austin, TX). The resulting mRNA was quantified spectrophotometrically and diluted in sterile water. This mRNA was subsequently injected directly into oocyte cytoplasm at 30 ng/30 nl using an automated microinjector (Nanoject; Drummond Scientific Co., Broomall, PA) via a glass micropipette of tip diameter at 17–20 μm. Oocytes were then incubated for 3 days at 18 °C before current recording.

Electrophysiological recordings were performed at room temperature (approximately 21–23 °C). Microelectrodes were pulled in one stage from 10-μl capillary glass (Drummond Scientific Co.) on a micropipette puller. Tips were broken at a diameter of approximately 10 μm. A single defolliculated oocyte was placed in a recording chamber (0.5-ml volume) and perfused with Tyrode's solution containing (in mM) NaCl 150, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, dextrose 10, and HEPES 10 at pH 7.5 at a flow rate of 3 ml/min for 4 min before measuring currents. Two recording electrodes (1–5 MΩ) filled with 3 M KCl were inserted into individual oocytes. A Warner Oocyte-clamp OC 725-C (Warner, Hamden, CT) was used to voltage clamp each oocyte at −70 mV. Data acquisition and the analysis were performed by using a personal computer running OoClamp software. Oocytes that did not show a stable holding current of less than 1 μA were excluded from the analysis. L-Glutamate was diluted in Tyrode's solution and superfused over clamped oocytes for 20 s at 3 ml/min. L-Glutamate-induced inward currents were sampled at 125 Hz for 1 min, i.e., 5 s at baseline, 20 s of agonist application, and 35 s of washing period (conducted with Tyrode's solution). Responses were quantified by integrating current traces and are reported as microCoulombs (μC), which reflect the total amount of glutamate transported. Each experiment was performed using oocytes from at least four different frogs.

In the control group, oocytes were perfused with Tyrode's solution for 4 min before responses were measured. In the alphaxalone-treated group, oocytes were perfused with Tyrode's solution for the first minute, and this was followed by Tyrode's solution with alphaxalone for 3 min before response to L-glutamate was measured. During this investigation, alphaxalone was initially dissolved in dimethylsulphoxide (DMSO) and this concentrated stock solution was subsequently diluted with Tyrode's solution to concentrations of 3, 1, 0.3, 0.1, 0.03 and 0.01 μM.

To study the dose–response effect of alphaxalone on EAAT3 activity, oocytes were exposed to 0 (control), 0.01, 0.03, 0.1, 0.3, 1, and 3 μM, respectively. To determine the effects of alphaxalone on the K_m and V_{max} values of EAAT3 for glutamate, serial concentrations of L-glutamate (3, 10, 30, 100, and 300 μM) were used. In other experiments, 30 μM L-glutamate was used to induce glutamate transporter currents. To study the role of PKC on EAAT3 activity, oocytes were preincubated with 100 nM phorbol-12-myristate-13-acetate (PMA) for 10 min before recording. Some of the PMA-treated oocytes were exposed to alphaxalone in the same manner as described above. To study the effects of PKC inhibitors on EAAT3 activity, oocytes were exposed to the PKC inhibitors staurosporine (2 μM for 1 h) or chelerythrine (100 μM for 1 h). To investigate the role of PI3K on EAAT3 activity regulation, oocytes were incubated with wortmannin (a PI3K inhibitor; 10 μM for 1 h). The incubation with PMA, staurosporine, chelerythrine and wortmannin occurred in a dish that was not perfused and contained the chemicals at the corresponding concentrations.

Molecular biology reagents were obtained from Ambion (Austin, TX), and other chemicals from Sigma (St. Louis, MO), unless otherwise specified. Data are provided as means±S.E.M. Because batch-to-batch variability in oocyte response is common due to different expression levels of EAAT3, responses were normalized by the results of the same-day controls for each oocyte batch. Statistical analysis was performed using the Student's t-test or by ANOVA followed by Bonferroni's post hoc comparison as appropriate. P values of <0.05 were considered significant.

3. Results

Although non-injected oocytes were not responsive to L-glutamate (data not shown), oocytes injected with EAAT3 mRNA showed inward currents after L-glutamate was applied (Fig. 1). In addition, we found that this response was concentration-dependent and that the EC_{50} of L-glutamate for inducing EAAT3 responses was 27.2 μM (Do et al., 2002a). Thus, L-glutamate was used at 30 μM for other studies.

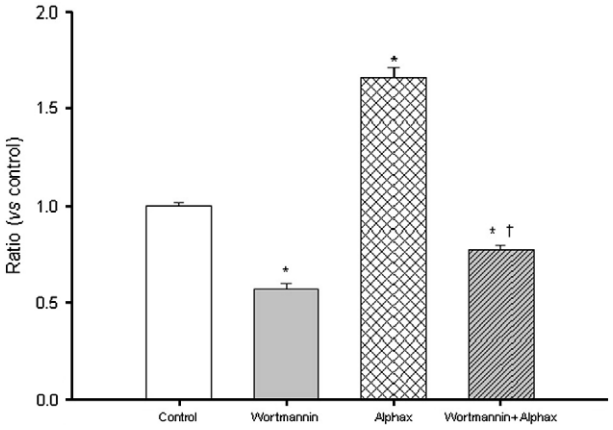

Fig. 6. Effects of phosphatidylinositol 3-kinase (PI3K) inhibition on EAAT3 activity in the presence or absence of alphaxalone. Oocytes were exposed to wortmannin (a PI3K inhibitor; 10 μM for 1 h), alphaxalone (0.1 μM for 3 min), or both. Pretreatment of the oocytes with wortmannin significantly decreased the basal and alphaxalone-enhanced EAAT3 activity. Alphax; alphaxalone. Data are means±S.E.M. (n=19–22 in each group). *P<0.05 compared with control. †P<0.05 compared with alphaxalone alone.

When oocytes without EAAT3 mRNA injection were exposed to alphaxalone, no inward or outward currents were recorded (data not shown). In vehicle control experiments, 0.06% of DMSO (the highest final concentration in alphaxalone solution) had no effect upon current responses to glutamate (1.02±0.15 μC for control group *vs.* 0.98±0.12 μC for DMSO-treated group, $n=10–11$; $P>0.05$). The exposure of oocytes injected with EAAT3 mRNA to serial concentrations of alphaxalone (0.01 μM, 0.03 μM, 0.1 μM, 0.3 μM, 1 μM, 3 μM) concentration-dependently increased their responses to 30 μM L-glutamate (Fig. 2). The EC_{50} of alphaxalone for increasing EAAT3 response was 0.04 μM, and thus, 0.1 μM alphaxalone was chosen for further studies.

In addition to enhancing the responses induced by 30 μM L-glutamate, 0.1 μM alphaxalone also significantly increased the responses induced by 100 or 300 μM L-glutamate (Fig. 1). Further data analysis (Prism version 2.0, GraphPad, San Diego, CA) showed that alphaxalone significantly increased V_{max} from 2.8±0.2 μC for controls to 4.9±0.3 μC for the alphaxalone-treated group ($n=19$; $P<0.05$), which corresponded to a 75% increase. However, alphaxalone did not cause a significant change in K_m (52.9±15.2 μM for controls *vs.* 63. 2±16.3 μM for the alphaxalone group; $n=19$; $P>0.05$).

Oocytes preincubated with PMA (100 nM) for 10 min showed an increase in EAAT3 activity (1.0±0.1 vs. 1.5±0.1 μC; $n=22$; $P<0.05$), which is in accordance with our previous findings (Do et al., 2002a,b). PMA (100 nM)-treated oocytes were exposed to alphaxalone to determine whether an interaction exists between PMA and alphaxalone (0.1 μM) to affect EAAT3 activity. Oocytes exposed to PMA, alphaxalone, or both showed a significant increase in EAAT3 activity compared with non-treated controls. However, no statistical difference was observed between the PMA, alphaxalone, or PMA plus alphaxalone groups (Fig. 3). Furthermore, basal EAAT3 activity was not affected by preincubation with staurosporine (2 μM) and chelerythrine (100 μM) for 1 h (Fig. 4). To determine whether an interaction exits between PKC inhibitors and alphaxalone in terms of EAAT3 activity, oocytes preincubated with PKC inhibitors (staurosporine or chelerythrine) were superfused with alphaxalone (0.1 μM for 3 min). These two PKC inhibitors at the selected concentrations significantly decreased but did not abolish alphaxalone-enhanced EAAT3 activity (Fig. 4). This incomplete inhibition of the alphaxalone effect may be a dose-dependent phenomenon because staurosporine and chelerythrine at the same concentrations used in the above experiments abolished the EAAT3 activity enhancement caused by 0.04 μM alphaxalone (Fig. 5), a lower concentration than that in the above experiments.

In addition, pretreatment of oocytes with wortmannin (1 μM for 1 h; a PI3K inhibitor), significantly decreased basal and alphaxalone-enhanced EAAT3 activities (Fig. 6).

4. Discussion

Our results demonstrate that alphaxalone produces a concentration-dependent (0.01–3 μM) increase in the activity of EAAT3, a major neuronal glutamate transporter, and suggest that PKC and PI3K mediate this effect. In addition, our kinetic study showed that 0.1 μM alphaxalone increased the V_{max}, but not the K_m, of EAAT3 for glutamate. An increased V_{max} and no change in K_m are a commonly observed kinetic feature of the effects of various anaesthetics on EAAT3 (Do et al., 2002a,b, 2003). This pattern of changes suggests that alphaxalone exposure does not change the affinity of EAAT3 for glutamate, but rather increases available EAAT3 numbers in the plasma membrane, which could be the result of EAAT3 redistribution from intracellular compartments to the plasma membrane, as have been shown by previous studies (Davis et al., 1998; Huang and Zuo, 2005).

We also studied the mechanism whereby alphaxalone enhances EAAT3-mediated currents. PKC is a family of intracellular signaling enzymes that have been implicated in the regulation of EAAT3 activity (Do et al., 2002b; Casado et al., 1993), and several investigations have shown that PKC is involved in the mechanisms whereby volatile anaesthetics affect various proteins, including EAAT3 (Do et al., 2002b; Minami et al., 1997). Our results show that PMA, a PKC activator, and alphaxalone do not act additively or synergistically to increase EAAT3 activity, suggesting that these two agents increase EAAT3 activity via the same pathway. Moreover, chelerythrine or staurosporine, two PKC inhibitors (Herbert et al., 1990; Matsumoto and Sasaki, 1989), at concentrations that did not affect the basal EAAT3 activity, decreased EAAT3 activity enhancement by alphaxalone. Although staurosporine and chelerythrine at the concentrations used in this study may affect other protein kinases, these two inhibitors are structurally different and the inhibition of alphaxalone-enhanced EAAT3 activity by both of them increased the likelihood for PKC to be involved in the alphaxalone effect on EAAT3. Thus, our PKC activator and inhibitor results consistently suggest that PKC participates in the enhancement of EAAT3 activity by alphaxalone.

In addition to PKC, PI3K has also been implicated in the regulation of EAAT3 activity (Davis et al., 1998). In this study, we found that wortmannin, a PI3K inhibitor, decreased alphaxalone-enhanced EAAT3 activity, suggesting that PI3K is involved in EAAT3 activity enhancement by alphaxalone. Different from PKC inhibition, PI3K inhibition by wortmannin also reduced the basal EAAT3 activity. In addition to activating PKC, PI3K activation also regulates other signaling molecules (Toker and Cantley, 1997; Cantrell, 2001). This difference may explain the different effects of PKC inhibitors and PI3K inhibitor on the basal EAAT3 activity.

The regulation of glutamate neurotransmission in the central nervous system is an important mechanism whereby anesthetics induce anaesthesia (Larsen et al., 1997; Miyazaki et al., 1997). Glutamate at high concentrations is toxic to neurons (Choi et al., 1987), and therefore, cellular glutamate uptake and the prevention of glutamate accumulation in the extracellular space via EAAT3 activity enhancement may serve as a neuroprotective mechanism. Moreover, a previous study demonstrated that animals with low levels of EAAT3 had epileptiform fits (Danbolt et al., 1998). Thus, the EAAT3 activity enhancement shown in the present study, and the potentiation of GABA neurotransmission (Harrison and Simmonds, 1984; Harrison et al., 1987; Cottrell et al., 1987) shown by previous studies may explain, in part, why alphaxalone acts as an anticonvulsant and anaesthetic.

In a previous study, the free drug plasma concentration of alphaxalone was estimated to be 3.5–7.5 μM during clinical anaesthesia (Shiraishi et al., 2003), and others have shown that the IC_{50} values of alphaxalone for muscarinic and nicotinic receptors are 1.8–5.3 μM and 0.17 μM, respectively (Shiraishi et al., 2003; Sumikawa et al., 1983). Our present study showed that the EC_{50} of alphaxalone to increase EAAT3 activity was 0.04 μM, which is much lower than the plasma concentration required for the induction of anaesthesia.

The *Xenopus* oocyte expression system has been used to investigate anaesthetic effects on individual EAATs (Do et al., 2002a,b, 2003; Minami et al., 1997) because oocytes posses the components of all major mammalian intracellular signaling pathways. The effects of several commonly used intravenous anaesthetics on EAAT3 activity have been investigated (Do et al., 2002a, 2003; Yun et al., 2006). However, the effects of alphaxalone differ from those of previously studied intravenous anaesthetics: alphaxalone enhances EAAT3 activity in a concentration-dependent manner, propofol and lidocaine have bell-shaped dose–response curves (Do et al., 2002a, 2003), etomidate decreases EAAT3 activity in a concentration-dependent manner, and thiopental and ketamine have no significant effect at any concentration tested (Yun et al., 2006). Similar to the pattern of alphaxalone, the volatile anesthetic isoflurane also dose-dependently increases the EAAT3 activity (Do et al., 2002b) and this effect is PKCα-dependent (Huang and Zuo, 2005).

The intravenous anaesthetic Althesin® contains two active steroids, alphaxalone and alphadolone, in an aqueous vehicle (Cremophor EL, a polyethoxylated castor oil surfactant). The anaesthetic effect of

Althesin® is attributed mainly to alphaxalone; alphadolone was added to the formulation to improve solubility (Stock, 1973). However, Cremophor EL (the vehicle) has been associated with major anaphylactoid reactions, and thus, Althesin® was withdrawn from clinical use (Winte et al., 2003).

Propofol, the most widely used intravenous anaesthetic in current practice, was also withdrawn from the market soon after its introduction, and it was also initially formulated on Cremophor EL for human use. Eventually, it was reintroduced as 1% solution in 10% soybean oil emulsion after improvements had been made to emulsion quality (Baker and Naguib, 2005). Thus, we believe that alphaxalone is likely to be re-released for clinical applications as an anaesthetic and anticonvulsant if a satisfactory emulsion can be developed.

In conclusion, our data suggest that alphaxalone increases EAAT3 activity and that PKC and PI3K mediate this effect. This alphaxalone effect on EAAT3 activity may contribute to the anaesthetic and anticonvulsant effects of alphaxalone.

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