Anesth Pain Med 2008; 3: 264～269

■ 실험연구 ■

Remifentanil increases the activity of the glutamate transporter, EAAC1, expressed in *Xenopus* oocytes

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Background: Remifentanil has gained wide clinical acceptance during anesthesia due to its short context-sensitive half time and organ-independent metabolism. However, its mechanism as an anesthetic remains unclear. Glutamate transporters may be important targets for anesthetic action in the central nervous system, and we tested whether remifentanil affected the activity of the primary neuronal glutamate transporter, EAAC1 (excitatory amino acid carrier 1).

Methods: EAAC1 was expressed in *Xenopus* oocytes by mRNA injection. By using two-electrode voltage clamping, membrane currents were recorded before, during, and after application of L-glutamate (30 μM) in the presence or absence of remifentanil. Oocytes were exposed to a protein kinase C (PKC) activator and inhibitor to study the role of PKC on EAAC1 activity.

Results: L-Glutamate induced an inward current in EAAC1-expressing oocytes. This response increased in a bell-shaped manner in the presence of 0.1 μM to 1 mM remifentanil. Remifentanil significantly increased V_{max} (3.1 ± 0.2 μC for controls vs. 4.9 ± 0.3 μC for remifentanil treatment; n = 12－15; $P < 0.05$). However, remifentanil did not significantly change K_m. Treatment of the oocytes with phorbol-12-myristate-13-acetate (PMA), a PKC activator, caused a significant increase in transporter current (1.00 ± 0.03 to 1.35 ± 0.03 μC; $P < 0.05$). Oocytes pretreated with the PKC inhibitor alone (staurosporine) abolished remifentanil-enhanced EAAC1 activity.

Conclusions: Our data suggests that remifentanil enhances EAAC1 activity and that PKC is involved in mediating this effect. **(Anesth Pain Med 2008; 3: 264～269)**

Key Words: excitatory amino acid carrier 1, glutamate transporter, protein kinase C, remifentanil, *Xenopus* oocyte.

INTRODUCTION

Remifentanil is an ultra short-acting opioid and has gained wide clinical acceptance due to its pharmacologic characteristics. It has a rapid onset with short duration and induces intense analgesia.[1] High doses of remifentanil administered during remifentanil-base anesthesia do not affect early postoperative recovery[2] and allows early return of cognitive functions.[3]

It is well known that intraoperatively administered remifentanil results in acute opioid tolerance or hyperalgesia[4,5] and *N*-methyl-D-aspartate (NMDA) receptors play a critical role in the development of this opioid tolerance and secondary hyperalgesia.[6-8] However, remifentanil effect on glutamate transporters has not been evaluated. Glutamate is the dominating excitatory neurotransmitter that plays an important role in many physiological functions.[9] In addition, modulation of glutamate neurotransmission in the CNS is one of the important mechanisms for anesthetics to induce anesthesia and analgesia.[10,11] The homeostasis of extracellular glutamate concentration is regulated by glutamate transporters, which is critical in neuroprotection against excitotoxicity (neurotoxicity due to high concentration of extracellular glutamate).[12] Five glutamate transporters have been characterized[12] to date: GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5. GLAST (EAAT1) has been observed in both neuron and astroglia. Immunolocalization studies show that GLT1 (EAAT2) is localized in astroglial cells throughout the brain and spinal cord. EAAC1 (EAAT3) is specific for neurons and Purkinje cells, and distributed prominently in cortex, hippocampus, and caudate-putamen and confined to presynaptic and postsynaptic elements. EAAT4 is localized mainly in cerebellar Purkinje cells in rat and human CNS. EAAT5 has only been cloned from human. It is primarily expressed in retina. Among these five transporters, excitatory amino acid carrier 1 (EAAC1) is the dominating isoform of glutamate transporters in nerve terminals[13] and controls termination of the excitatory synaptic transmission by mediating re-uptake of glutamate into the nerve terminal.[14]

논문접수일 : 2008년 9월 15일
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EAAC1 also protects cells from neural toxic effects of high extracellular glutamate[14,15] and therefore reduced EAAC1 function would be expected to increase extracellular glutamate concentration.[16,17]

Previous studies demonstrated that remifentanil has diverse effects on the CNS[18,19] not only as an analgesic (opioid agonist) but also as an anesthetic. In this respect, we hypothesize that remifentanil may have an effect on glutamate transporters. This study was performed to investigate the effect of remifentanil on EAAC1, the major neuronal glutamate transporter in the brain and spinal cord, and to evaluate the role of protein kinase C (PKC) in mediating this effect.

METHODS AND MATERIALS

Oocyte Harvesting and Preparation

The study protocol was approved by the Institutional Animal Care and Use Committee at the Seoul National University College of Medicine. Procedure for isolation and microinjecion of *Xenopus* oocytes was published previously by Do et al.[20] Mature female *Xenopus laevis* frogs were purchased from Kato S Science (Chiba, Japan), and fed with regular frog brittle twice weekly. For removal of oocytes, frogs were anesthetized by immersion in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO, USA) until they were unresponsive to a painful stimulus (toe pinching), and operated on ice. Approximately 150 oocytes were surgically removed and placed immediately in the calcium-free OR-2 solution (containing in mM: NaCl 82.5, KCl2, $MgCl_2$ 1, HEPES 5, collagenase type Ia 0.1%, pH 7.5) in order to remove additional vitelline membrane surrounding the oocytes. The oocytes were defolliculated with gentle shaking for approximately 2 hr and then incubated at 18°C in the 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).

Glutamate Transporter (EAAC1) Expression

The rat EAAC1 complementary DNA (cDNA) construct was provided by Dr. M. A. Hediger (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 a restriction enzyme (Not I), and messenger RNA (mRNA) was synthesized *in vitro* with a commercially available kit (Ambion, Austin, TX). The resulting mRNA was quantified spectrophotometrically and diluted in sterile water. Oocytes were injected with this mRNA in a concentration of 30 ng/30 nl using an automated microinjector (Nanoject; Drummond Scientific Co., Broomall, PA) via a glass micropipette whose tip diameter was about 17−20 μm. Oocytes were then incubated for 3 days in modified Barth solution at 18°C before the current recording.

Electrophysiology

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 vertical micropipette puller. Electrode tips were broken to a diameter of approximately 10 μm. A single oocyte was positioned in a recording chamber (0.5-ml volume) and superfused with the Tyrode's solution containing (in mM) NaCl 150, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, dextrose 10, and HEPES 10, pH 7.5 at the flow rate of 3 ml/min for 4 min before measuring currents. Recording electrodes (a resistance of 1−5 MΩ) filled with 3 M KCl were inserted into the oocyte. A Warner Oocyte-clamp OC 725-C (Warner, Hamden, CT) was used to voltage clamp each oocyte at −70 mV. Data acquisition and analysis system was run on a personal computer and analyses were performed with OoClamp software. Oocytes that did not show a stable holding current less than 1 μA were excluded from analysis. L-Glutamate was diluted in Tyrode's solution and superfused over the oocyte for 20 sec at 3 ml/min. L-Glutamate-induced inward currents were sampled at 125 Hz for 1 min: 5 sec of baseline, 20 sec of agonist application, and 35 sec of washing with Tyrode's solution. Responses were quantified by integrating the current trace and reported as microcoulombs (μC), which reflected the amount of transported glutamate. Each experiment was performed with oocytes from at least four different frogs.

In the control group, oocytes were perfused with Tyrode's solution for 1 min before the responses were measured. In this investigation, remifentanil was initially dissolved as concentrated stock solutions in distilled water and subsequently diluted in modified Barth solution to appropriate final concentrations (1 mM, 100, 10, 1, and 0.1 μM). In the remifentanil-treated group, oocytes were preincubated with remifentanil for the 10 min, followed by perfusion with Tyrode's solution for one minute before the response to L-glutamate was measured.

To study remifentanil dose response relationship on EAAC1 activity, oocytes were exposed to 0 (control), 0.1, 1, 10, 100 μM, and 1 mM, respectively. To determine the effects of remifentanil on the K_m and V_{max} of EAAC1 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 the glutamate transporter currents. To study the role of PKC on EAAC1 activity, oocytes were preincubated with 50 nM phorbol-12-myristate-13-acetate (PMA) for 10 min before recording. Some of the PMA-treated oocytes were exposed to remifentanil in the same way as described previously. To study the effect of PKC inhibition on EAAC1 activity, oocytes were exposed to the PKC inhibitor (2 μM staurosporine) for 1 h before current recording.

Statistical Analysis

Results are reported as means ± SEM. Because variability in response between batches of oocytes is common due to different expression levels of EAAC1 proteins, responses were at times normalized to the same-day controls for each batch and at least 4 frogs were studied for each data. Differences between treatment groups were analyzed using ANOCOVA (responses vs. remifentanil concentrations), nonlinear regression analysis (responses vs. L-glutamate concentrations) and two way ANOVA (PKC activator or inhibitor), followed by the Bonferroni for post hoc comparison. A P value < 0.05 was considered significant.

Materials

Primary product of remifentanil-hydrochloride was obtained from GlaxoSmithKline (Brentfoed, UK) and this substance was devoid of glycine. Molecular biology reagents were obtained from Ambion (Austin, TX), and other chemicals were obtained from Sigma (St. Louis, MO) unless specified in the text.

RESULTS

L-glutamate did not induce any current in uninjected oocytes (data not shown). However, oocytes injected with mRNA of EAAC1 showed inward currents after application of L-glutamate (Fig. 1). The responses were concentration-dependent (Fig. 2). The EC_{50} of EAAC1 for L-glutamate was determined to be 27 μM in the previous investigation of the same system[20] and therefore 30 μM L-glutamate was used for other studies.

Five concentrations of remifentanil were tested (0.1 μM, 1 μM, 10 μM, 100 μM, and 1 mM). Application of remifentanil to uninjected oocytes induced no inward currents (data not shown). In contrast, oocytes injected with EAAC1 mRNA showed significantly increased transporter current at 0.1 μM, 1 μM, 10 μM, and 100 μM remifentanil in a bell-shaped manner (Fig. 1) and 0.1 μM remifentanil (the lowest concentration) was chosen for further experiments.

In addition to enhancing the responses induced by 30 μM L-glutamate, 0.1 μM remifentanil also significantly increased the responses induced by 100 or 300 μM L-glutamate (Fig. 2). Further data analysis (Prism version 2.0, GraphPad, San Diego, CA) showed that remifentanil significantly increased V_{max} from 3.1 ± 0.2 μC for controls to 4.9 ± 0.3 μC for the remifentanil-treated group (n = 12−15; $P < 0.05$), corresponding to a 58% increase. However, remifentanil did not cause a significant change in K_m (27.1 ± 3.2 μM for controls vs. 28.6 ± 4.3 μM for the remifentanil group; n = 12−15; $P > 0.05$).

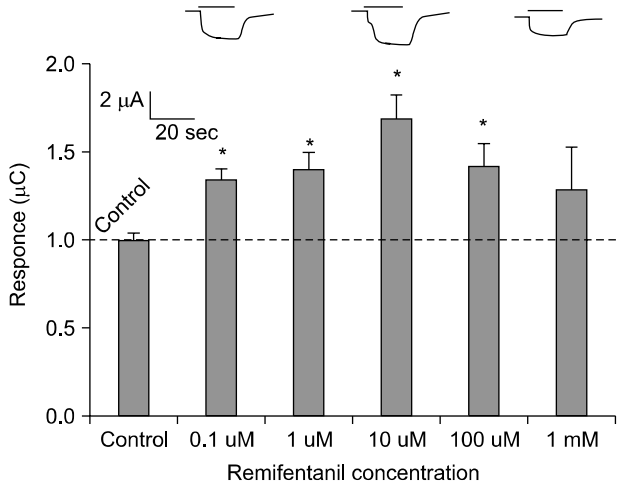

Fig. 1. Concentration-response of remifentanil on the activity of excitatory amino acid transporter type 3. Oocytes were exposed to various concentrations of remifentanil for 10 min. The EAAC1 response was then induced by 30 μM L-glutamate. Inset graphs are representative current traces. Data are means ± SEM (n = 12−15 in each group). *P < 0.05 compared with control.

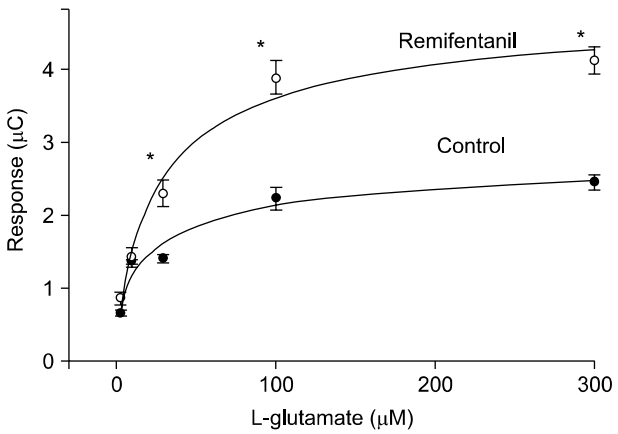

Fig. 2. Concentration-response curve of EAAC1 to glutamate in the presence or absence of 0.1 μM remifentanil for 10 min. Data are means ± SEM (n = 12−15 in each group). *P < 0.05 compared to the corresponding control.

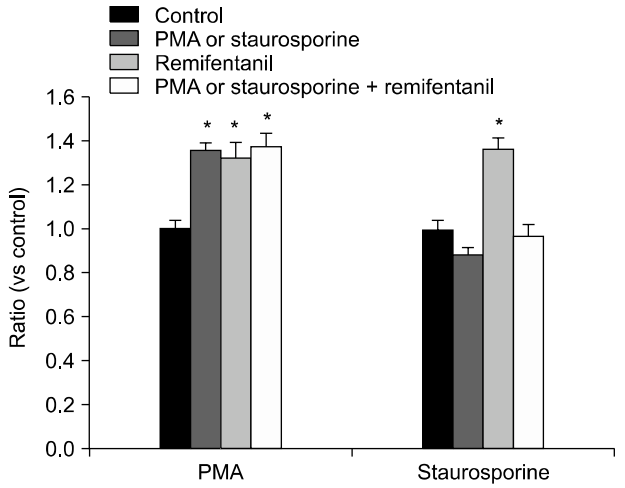

Fig. 3. Effects of protein kinase C (PKC) activator or PKC inhibitor on EAAC1 activity in the presence or absence of 0.1 μM remifentanil for 10 min. PMA; phorbol-12-myristate-13-acetate. Data are means ± SEM (n = 19−22 in each group). *P < 0.05 compared with control.

Oocytes preincubated with PMA (50 nM) for 10 min showed increased EAAC1 activity (1.0 ± 0.1 vs. 1.4 ± 0.1 μC; n = 14−16; P > 0.05). This was in accordance with our previous study (18, 19). PMA (50 nM)-treated oocytes were exposed to remifentanil to determine whether there is an interaction between the effects of PMA and remifentanil (0.1 μM) on EAAC1 activity. Oocytes exposed to PMA, remifentanil, or both showed a significant increase in EAAC1 activity compared with control whereas there was no statistical difference among the PMA, remifentanil, or PMA plus remifentanil groups (Fig. 3).

Preincubation with staurosporine (2 μM) for 1h (Fig. 3) did not significantly affect the EAAC1 activity (1.0 ± 0.2 vs. 0.9 ± 0.1 μC; n = 14 in each group; P > 0.05). To determine whether there is an interaction between the effects of PKC inhibitor and remifentanil on EAAC1, oocytes preincubated with staurosporine were immersed with remifentanil (0.1 μM) for 10 min. Staurosporine at the selected concentration abolished the remifentanil-enhanced EAAC1 activity (Fig. 3).

DISCUSSION

The uptake of glutamate by EAATs is electrogenic and the size of the glutamate-induced current reflects the amount of transported glutamate.[21,22] *Xenopus* oocyte expression system was used to investigate the anesthetic effects on a single subtype of EAAT as oocytes have components of all major intracellular signaling pathways of mammalian cells and have been widely used for studies on EAAC1 activity[20,22-24] through measuring the glutamate-induced currents to quantify EAAC1 activity.

Our results demonstrated that remifentanil produced a bell-shaped dose-response curve in the activity of EAAC1 and that PKC seemed to mediate this effect. This study differs from the previous investigation[8] in that raw material of remifentanil was used to exclude the effect of glycine in Ultiva®.

Remifentanil at concentrations of 0.1, 1, 10 and 100 μM enhanced EAAC1 activity, whereas the higher concentration (1 mM) tested did not have statistically significant increase on EAAC1 activity. In addition, the kinetic study showed that 0.1 μM remifentanil increased the V_{max}, but not the K_m of EAAC1 for glutamate. An increased V_{max} and no change in K_m could be explained as the major regulatory mechanism of EAAC1 activity.[20,23,24] EAAC1 moves between intracellular compartments and plasma membrane of cell surface[25] and our result suggests that remifentanil exposure increases the surface expression of EAAC1 in the plasma membrane rather than changing the affinity of EAAC1 for glutamate.

We then studied the mechanisms for remifentanil effects on EAAC1 activity. PKC is included in the intracellular signaling enzyme system that has been implicated in the regulation of EAAC1 activity.[20,22] Our results showed that there were no additive or synergistic interactions between PMA and remifentanil effects on EAAC1 activity, suggesting that these two agents might increase the EAAC1 activity through the same pathway. PKC inhibition by staurosporine at the concentrations that did not affect the basal EAAC1 activity abolished the enhancement of EAAC1 activity by remifentanil. Thus, the results employing PKC activator and inhibitor suggest the involvement of PKC in the remifentanil-enhanced EAAC1 activity.

Because of the bell-shaped dose-response curve, we tested the lowest concentration (0.1 μM) to see whether significant activation could be achieved. A free plasma concentration of remifentanil was estimated to be 5−21 nM at clinical anesthesia, considering a continuous infusion of 0.1−0.5 μg/kg/min and a plasma protein binding capacity of 70%.[8] The concentration of remifentanil tested in this study (0.1 μM) is much higher than the plasma concentrations during anesthesia. In previous investigations,[8] the EC_{50} of Ultiva® was 1.4 ng/ml (3.5 nM) for the NMDA receptor (NR1A/2A combination) expressed in *Xenopus* oocytes. Considering the large discrepancy in the concentration range of remifentanil between current and previous study,[8] EAAC1 appears out of the primary target of anesthetic effect of remifentanil.

Bell-shaped dose-response relationship of remifentanil is

similar to those of lidocaine and propofol on EAAC1.[23,24] The enhancement of EAAC1 activity may be translated such that remifentanil is neuroprotective. In contrast, previous study has hypothesized a neurotoxic effect of remifentanil in the fact that remifentanil activated NMDA human receptors, a receptor implicated in playing an important role in ischemic brain injury.[8] However, Guntz et al.[26] showed that the NMDA current recorded after application of Ultiva® is related to the presence of glycine and that NMDA-induced current is potentiated by application of remifentanil hydrochloride through a pathway involving μ-opioid receptor. More extensive researches on remifentanil in the field of neuroprotection are required.

Inhibition of glutamate neurotransmission in the CNS is also one of the important mechanisms for anesthetics to induce anesthesia and neurotransmitter uptake systems are considered as a possible target for the presynaptic action of anesthetic agents.[10,11] Activated glutamate transporters uptake glutamates and subsequently decrease the concentration of glutamate in the synaptic cleft. Previous study showed that inhibition of glutamate transporters increased the minimum alveolar concentration for isoflurane in rats.[27] In contrast, remifentanil has been shown to decrease the inhalation anesthetic requirement[28,29] and modulation of EAAC1 activity by remifentanil may be related to these clinical findings.

In addition to the well-documented role of the glutamate transporter in the mechanism of anesthesia and neuroprotection, the enhancement of EAAC1 activity may contribute to the anticonvulsant action since animals with decreased EAAC1 expression had epileptiform fits.[30] Interestingly, several investigators reported that seizures in patients with no history of epilepsy have been associated with administration of remifentanil[31,32] and that remifentanil prolonged the duration of seizure during electoconvulsive therapy.[33,34] In the dose-response study, the change of EAAC1 activity by remifentnil showed a bell-shaped curve. We could not study with higher concentration of remifentanil because the resting currents of the oocytes became unstable when the concentration went beyond 1 mM. Higher concentration of remifentail might inhibit EAAC1 activity as the effects of lidocaine and benzodiazepines on the EAAC1 activity.[23,35]

This is the first study to investigate the direct effect of remifentanil on EAAC1. In the recent studies[36,37] glutamate transporters have been suggested to be involved in the modulation of the glutamatergic system by opioids. After chronic morphine administration, expression of EAAC1 and GLAST was reduced in spinal cord[36] and glutamate uptake and surface expression of GLT1 at hippocampal synapses were increased after morphine withdrawal.[37] In addition, opioid receptors are found to modulate the glutamatergic system in brain. Among opioid recptors, δ-opioid receptor (DOR) played a crucial role in neuroprotection against glutmate-induced injury[38] and activation of DOR lead to an immediate increase in EAAC1 activity and subsequent decrease in extracellular glutamate concentration.[39] Remifentanil binds to DOR with much lower affinity (EC_{50} = 66 nM) than to μ-opioid receptor (EC_{50} = 2.6 nM).[40] The EC_{50} of remifentanil for DOR (66 nM) is close to the concentration of this study (100 nM) but whether remifentanil increases EAAC1 activity via DOR remains to be investigated.

In conclusion, we demonstrated the activation of the glutamate transporter, EAAC1, expressed in *Xenopus* oocytes by remifentanil. The role of such enhancement may have implications in anesthetic, analgesic, and neuroprotective actions of remifentanil. Interpretation of the result from clinical perspectives may need correlations with *in vivo* studies that best replicate the clinical scenario and other mechanisms of action of remifentanil remain to be clarified.

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