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Involvement of kainate receptors in the analgesic but not hypnotic effects induced by inhalation anesthetics

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Abstract:
In the present study, the role of kainate (KA) receptors in hypnosis and analgesia induced by emulsified inhalation anesthetics was investigated. A mouse model of hypnosis and analgesia was established by an intraperitoneal injection of emulsified enflurane, isoflurane or sevoflurane. We intracerebroventricularly (*icv*) or intrathecally (*it*) administered KA, a KA receptor agonist to mice. The effects of the KA on the sleep time were observed using a hypnosis test, and the tail-withdrawal latency was analyzed using the tail-withdrawal test. In the hypnosis test, KA (2.5, 5 or 10 ng; *icv* administered) treatment had no distinctive effects on the sleep time of mice treated with emulsified inhalation anesthetics. In the tail-withdrawal test, KA (0.2, 0.4 or 0.8 ng; *it* administered) treatment significantly and dose-dependently decreased the tail-withdrawal latency of mice treated with emulsified anesthetics. These results suggested that KA receptors may modulate the analgesic but not hypnotic effects induced by emulsified enflurane, isoflurane or sevoflurane.

Key words:
hypnosis, analgesia, inhalation anesthetics, KA receptors

Introduction

Inhalation anesthetics have been used for more than 160 years, but the underlying mechanism of their action remains unknown. Recently, ligand-gated ion channels have emerged as the most promising molecular target for inhalation anesthetics [4, 15, 21, 24, 26]. Various *in vitro* studies have suggested that either the enhancement of inhibitory neurotransmitter function or the inhibition of excitatory neurotransmitter function is a plausible method to induce anesthesia [1, 2, 15, 20].

Kainate (KA) receptors, which are ubiquitous in the central nervous system, are members of the ionotropic glutamate receptor family. KA receptors are tetrameric combinations of the following five subunits: GluR5, GluR6, GluR7, KA1 and KA2. Of these subunits, GluR5–7 can form functional homomeric or hetero-

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meric receptors. However, KA1 and KA2 only participate in heteromeric receptors by partnering with any of the GluR5–7 subunits [6, 14, 17]. A considerable amount of data has suggested that this class of receptors is present at both sides of the synapse. Presynaptic and postsynaptic KA receptors are able to regulate both the transmission of information and excitability in a synapse-specific manner. In addition, evidence has implicated that KA receptors are involved in pathophysiological conditions, such as epilepsy, excitotoxicity and pain [17]. Several studies have provided evidence for antinociceptive effects of compounds directed at the GluR5 subunit, which is the key receptor expressed in dorsal root ganglion (DRG) neurons [9, 27]. Inhalation anesthetics can reduce KA-induced membrane currents in cortical neurons *in vitro* [8]. Hence, KA receptors may explain the capacity of inhalation anesthetics to produce the anesthetic state. Inhalation anesthetics supply the following two essential elements of anesthesia: hypnosis and analgesia [23]. However, the involvement of KA receptors in the hypnotic and analgesic effects induced by emulsified inhalation anesthetics has not been determined by an *in vivo* behavioral study.

Therefore, we hypothesized that if KA receptors contribute to the hypnotic and the analgesic effects of anesthetics, application of a KA receptor agonist (KA) should decrease the hypnotic and analgesic effects of emulsified inhalation anesthetics. This hypothesis was investigated using the hypnosis and tail-withdrawal tests.

Materials and Methods

Animals

Kunming mice (22 ± 3 g) were obtained from the Shanghai Laboratory Animal Center. Mice were housed with a 12-h light:dark cycle at room temperature (22 ± 2°C). Food and water were provided *ad libitum*. All experiments were performed at the same time between 8:00 and 12:00 a.m. to avoid diurnal variation during behavioral testing. The experimental protocols were approved by the Animal Care and Use Committee of Jiansu University, and they complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

Formulation of emulsified inhalation anesthetics

Inhalation anesthetics were emulsified using the method of Chiari et al. [5]. Enflurane, isoflurane and sevoflurane were dissolved in soy bean oil containing dispersed egg lecithin, and these solutions comprised the oil phase of the emulsion. The dissolution of egg lecithin in soy bean oil was facilitated with heat. After dissolution of lecithin, the oil phase was cooled to approximately 10°C before the addition of the inhalation anesthetic. The aqueous phase of the emulsion contained glycerin dissolved in water. The aqueous phase was cooled to a temperature similar to that of the oil phase (10°C). The oil phase was then added to the aqueous phase while it was vigorously stirred to form the primary emulsion. The primary emulsion was homogenized at high pressure to form the final emulsion. After homogenization, the inhalation anesthetic emulsion was stored in glass vials, which were capped and refrigerated (2–5°C) until use. Vials containing the emulsion were warmed to 37°C for 2 h before administration. The target concentrations of the anesthetics in emulsion were approximately 8%. After warming the emulsion for 2 h at 37°C, the actual concentrations of enflurane, isoflurane and sevoflurane in the emulsion were approximately 7.5, 8.1 and 7.0%, respectively, as determined by gas chromatography.

Intracerebroventricular (*icv*) injection

Icv administration was performed following the method of Laursen and Belknap [13]. Briefly, animals were injected at the bregma with a 50-µl Hamilton syringe fitted with a 26-ga needle, which had a tip that was adjusted for 2.4-mm penetration. The injection volume was 5 µl, and injection sites were verified by injecting an equal volume of 1% methylene blue. Dye distribution into the ventricular space was observed. The dye seeped into the ventricular space, ventral surface of the brain and upper cervical portion of the spinal cord.

Intrathecal (*it*) injection

Conscious mice were *it* injected using the method of Hylden and Wilcox [11]. At the 5th lumbar vertebra, a 26-ga needle fitted with a microsyringe was inserted into the spinal canal at a 30° angle. Transient tail extension indicated a successful injection. The solution was injected in a volume of 5 µl over a period of 5 s. Lidocaine (2%) was *it* injected into 10 mice, and these

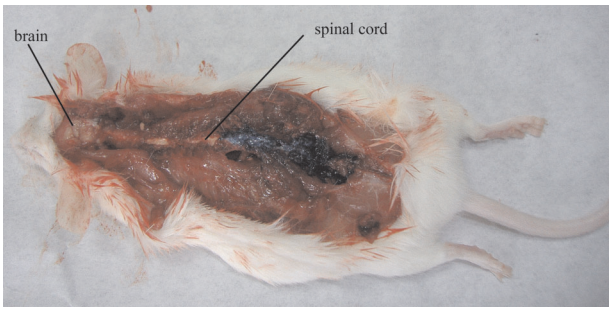

Fig. 1. A picture (spinal cord and brain) of a mouse *it* injected with 5 μl of methylene blue dye. Thirty minutes after the injection, the methylene blue dye did not diffuse beyond the rostral thoracic segments

mice immediately exhibited hind limb paralysis, which lasted for approximately 10 min in the preliminary experiments. A picture (spinal cord and brain) of a mouse *it* injected with methylene blue dye is shown in Figure 1.

Hypnosis test

The hypnotic doses of emulsified enflurane (16 ml/kg), isoflurane (9 ml/kg) and sevoflurane (20 ml/kg) were determined in preliminary experiments [10].

One hundred and twenty Kunming mice (male or female) were randomly divided into 12 groups (n = 10) as follows: groups treated with emulsified enflurane, isoflurane and sevoflurane combined with artificial cerebral spinal fluid (aCSF) and groups treated with emulsified enflurane, isoflurane and sevoflurane combined with various concentrations of KA (2.5, 5 or 10 ng).

Mice were kept on a heating pad (38°C) during the test. Every group was *ip* injected with a hypnotic dose of emulsified inhalation anesthetics. One minute after the loss of the righting reflex (animals remaining on their back for at least 30 s), the mice were *icv* injected with aCSF or KA. Sleep time (duration of the loss of righting reflex) was then observed [16]. Moreover, blood pressure (BP), heart rate (HR) and respiratory rate (RR) were measured for each mouse.

Tail-withdrawal test

All experiments were started at 10:00 a.m. and performed according to the method of Dableh et al. [7]. Briefly, each mouse was placed in a plastic tube (50-ml polypropylene conical tube) such that the tail protruded from an opening in the bottom of the tube. The distal half of the tail was dipped into a bath of circulating water thermostatically controlled at 48.0 ± 0.5°C. The tail-withdrawal latency (TWL) time was measured by an experienced observer in a single-blinded manner. A cutoff time of 20 s was used to avoid tissue damage.

The analgesic doses of emulsified enflurane (8 ml/kg), isoflurane (5 ml/kg) and sevoflurane (10 ml/kg) were determined in preliminary experiments [10].

One hundred and sixty Kunming mice (male or female) were randomly divided into 16 groups (n = 10) as follows: a group treated only with aCSF; groups treated only with KA (0.2, 0.4 or 0.8 ng); groups treated with emulsified enflurane, isoflurane and sevoflurane combined with aCSF; and groups treated with emulsified enflurane, isoflurane and sevoflurane combined with various concentrations of KA (0.25, 0.5 or 1 ng). The aCSF and KA groups were *it* injected with aCSF and KA, respectively. Groups receiving emulsified enflurane, isoflurane and sevoflurane were *ip* injected with an analgesic dose. After 5 min, each group was *it* injected with aCSF or KA. Baseline TWL and TWL at 5, 10, 15, 20 and 25 min after injection were observed.

Statistics

The results are expressed as the mean ± SD. The data were statistically analyzed using SPSS16.0 software (SPSS Inc., Chicago, IL, USA). Comparisons were performed by one-way or two-way (treatment × time) ANOVA followed by Dunnett's *post-hoc* test; p-values less than 0.05 were considered statistically significant.

Results

Hypnosis test

There were no significant differences in the BP, HR and RR values among the four groups. Compared with the aCSF groups, the KA (2.5, 5 and 10 ng; *icv* administered) groups exhibited no effects on the sleep time of mice treated with emulsified inhalation anesthetics ($p > 0.05$) (Fig. 2).

Tail-withdrawal test

The TWL of conscious mice was not affected by *it* injected KA (0.2, 0.4 or 0.8 ng) when compared to the

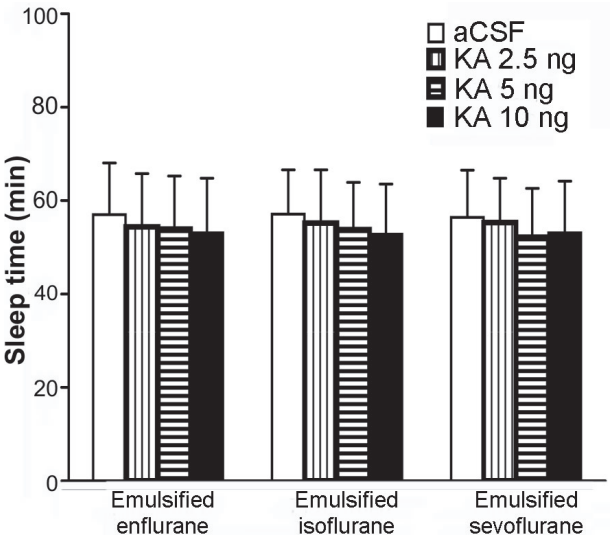

Fig. 2. Effects of KA treatment on the sleep time of mice treated with emulsified inhalation anesthetics as analyzed by the hypnosis test. KA (2.5, 5 or 10 ng) was *icv* injected into mice. The data represent the mean ± SD. Ten mice were used in each group (statistically insignificant; one-way ANOVA followed by Dunnett's *post-hoc* test)

aCSF group ($p > 0.05$) (Fig. 3A). In the groups receiving *ip* injected emulsified inhalation anesthetics, KA (0.2, 0.4 or 0.8 ng; *it* administered) significantly and dose-dependently decreased the TWL ($p < 0.05$, $p < 0.05$ and $p < 0.01$) when compared to the aCSF groups.

Ten minutes after the drugs were *it* injected, KA (0.2, 0.4 or 0.8 ng; *it* administered) decreased the TWL as follows: by 9.8 ($p > 0.05$), 25.9 ($p < 0.01$) and 36.6% ($p < 0.01$), respectively, in mice treated with emulsified enflurane; by 17.2 ($p < 0.05$), 33.8 ($p < 0.01$) and 50.3% ($p < 0.01$), respectively, in mice treated with emulsified isoflurane; and by 17.1 ($p < 0.05$), 34.9 ($p < 0.01$) and 43.4% ($p < 0.01$), respectively, in mice treated with emulsified sevoflurane. Compared to the baseline of TWL, KA (0.8 ng; *it* administered) almost completely antagonized the analgesic effects of the emulsified inhalation anesthetics ($p < 0.01$) (Figs. 3B–3D).

Discussion

Inhalation anesthetics are often administrated through the airway, and this administration requires specific types of equipment. Therefore, some inhalation experiments cannot be performed. Novel formulations of inhalation anesthetics have been developed to facilitate drug administration *in vivo*. These formulations are composed of a drug emulsification in a lipid vehicle, and this type of preparation may be clinically useful to produce anesthesia [5]. Previous studies have supported the efficacy and safety of *iv* administrated emulsified inhalation anesthetics in animals [28, 29]. In the present study, hypnotic doses of emulsified inhalation anesthetics induced the loss of the mouse righting reflex as observed in the hypnosis test. The mouse sleep time was approximately 60 min, and drug treatment minimally affected breathing and blood circulation. In the tail-withdrawal test, an *ip* administered injection was used to produce analgesia. Mice not only showed the effects of analgesia but also had essentially normal behavior, and the righting reflex was not lost. There are some differences between inhalation and *ip* administered injections. Onset velocity measures the absorption velocity of *ip* administered injection. Emulsified sevoflurane may be absorbed more slowly than other anesthetics, which may explain why the dose of emulsified sevoflurane applied to mice was approximately twice that of emulsified isoflurane, even though the ratio of the minimum alveolar concentration (MAC) between isoflurane (1.15% in humans) and sevoflurane (1.71% in humans) is approximately 1.5.

General anesthesia induces immobility, amnesia, hypnosis and analgesia, and it suppresses stress responses to noxious stimuli. To simplify the experiments in the present study, individual components of general anesthesia were observed [25]. However, there was a caveat to this approach because the effects of anesthesia are not independent. Pain increases awareness. Noxious stimuli during anesthesia increases arousal, which is indicated by increased neuronal activity in the reticular formation and thalamus [3]. Moreover, the spinal cord (responsible for analgesia-induced and anesthesia-induced immobility) interacts with the brain cortex (responsible for awareness and hypnosis).

Inhalation anesthetics supply two essential elements of anesthesia: hypnosis and analgesia. Hypnosis is defined as the lack of 'perceptive awareness', which is assessed by the response to non-noxious stimuli [15]. The righting reflex test determines the ability of an animal to regain an upright posture within 30 s of being placed in a supine position. In the absence of central nervous system depression or impairment, maintaining an upright posture is a natural

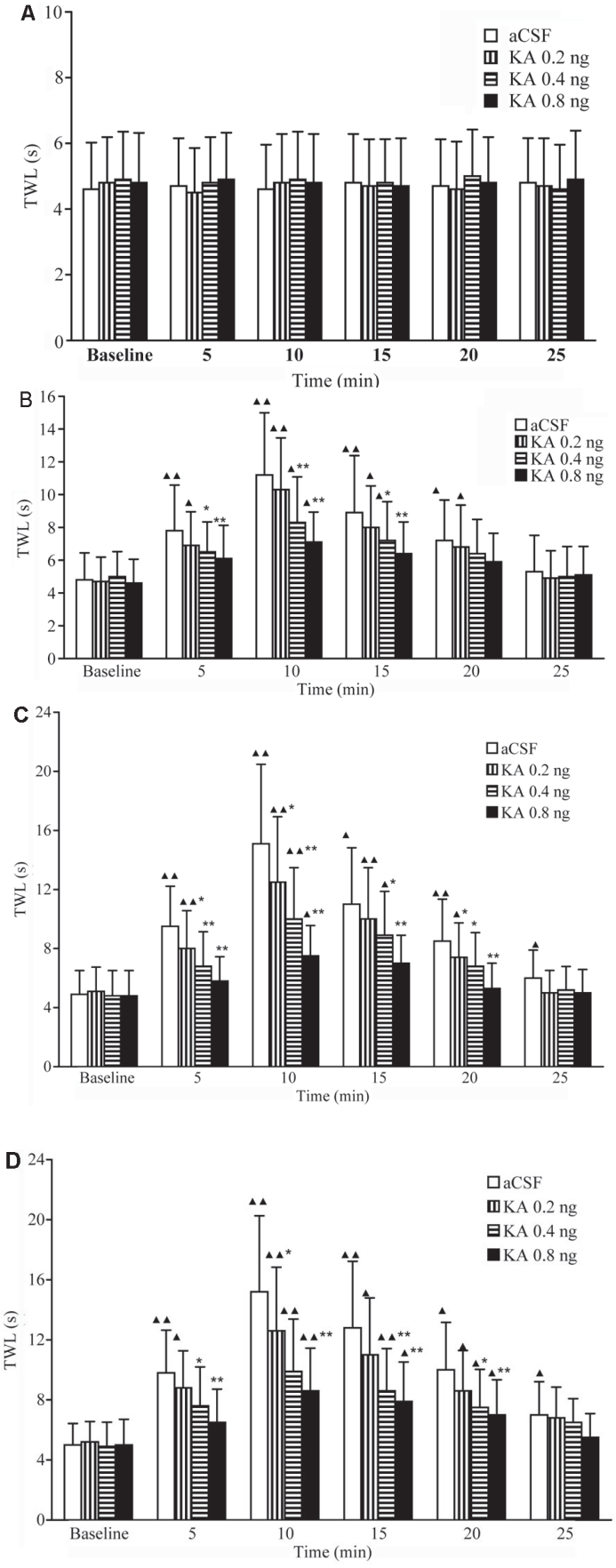

Fig. 3. Effects of KA treatment on the tail withdrawal latency (TWL) in conscious mice (**A**), mice treated with emulsified enflurane (**B**), mice treated with emulsified isoflurane (**C**), and mice treated with emulsified sevoflurane (**D**). KA (0.2, 0.4 or 0.8 ng) was *it* injected into mice. The data represent the mean ± SD. Ten mice were used in each group (* represents $p < 0.05$ and ** represents $p < 0.01$ when compared to the aCSF group using the two-way ANOVA followed by Dunnett's *post-hoc* test; and ▲ represents $p < 0.05$ and ▲▲ represents $p < 0.01$ when compared to each baseline using two-way ANOVA followed by Dunnett's *post-hoc* test)

reflex. Therefore, this assay is used to test drugs that cause significant central nervous system depression [16]. The tail withdrawal-test is a method used to measure analgesia. Analgesia is defined as the loss of pain sensation without loss of consciousness or mobility.

KA is a specific agonist of the KA receptors. Treatment with KA agitates animals and may even produce convulsions. In the preliminary experiments, KA (10 ng; *icv* administered) treatment induced scratching and biting behaviors, suggesting that the dose used in the hypnosis test was sufficient. In this study, however, *icv* administered doses of KA were unable to significantly attenuate the hypnotic effects of emulsified inhalation anesthetics. Hence, we concluded that KA receptors may not have a key role in the hypnotic effects of emulsified inhalation anesthetics. Hypnotic effects of inhalation anesthetics may refer to various levels, sites and mechanisms of the central nervous system. In addition to the function of receptors or ion channels, hypnotic effects may be regulated by many factors including nerves, humors and internal secretion.

Studies in rats have demonstrated that the spinal cord mediates the analgesic and immobility effects of inhalation anesthetics [18, 19]. An appropriate method to study spinally mediated drug effects in mice is *it* administered injections. Hylden and Wilcox [11] injected 5 μl of methylene blue dye in mice, and they reported that the dye does not diffuse beyond the rostral thoracic segments. Moreover, they also found that *it* injected [^{3}H]-morphine does not exist in significant quantities in either the midbrain or forebrain after injection. Therefore, we determined the behavioral effects of drugs at the spinal level. In preliminary experiments, KA (> 1.5 ng; *it* administered) treatment increased the scratching behavior of mice. In the present study, however, the doses used had no effect on the normal behavior of mice. The present study showed that KA (*it*) treatment significantly and dose-dependently decreased the TWL of mice treated with emulsified enflurane, isoflurane and sevoflurane. A dose of 0.8 ng (*it*) almost completely antagonized the analgesic effects of the three emulsified inhalation anesthetics. As predicted by our hypotheses, we conclude that spinal KA receptors may be important targets for the analgesic effects of emulsified enflurane, isoflurane and sevoflurane. These findings were inconsistent with the observation of Sonner et al., who found that GluR6 knockout mice have normal desflurane, halothane and isoflurane minimum alveolar concentration (MAC) values. GluR6 editing mutant mice also do not demonstrate consistent changes in MAC values for these anesthetics [22]. However, the findings for GluR6 mutations do not conclusively eliminate KA receptors as mediators of immobility because KA receptors can be assembled from other subunits even in the absence of the GluR6 subunit. Ko et al. demonstrated that noxious responses to capsaicin and formalin are strongly reduced in GluR5$^{-/-}$ mice but not GluR6$^{-/-}$ mice [12].

The results of the present *in vivo* study indicate that KA receptors may modulate the analgesic but not hypnotic effects induced by emulsified enflurane, isoflurane or sevoflurane. For the elucidation of post-receptor mechanisms, further research is essential.

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