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CCK_B receptor antagonist L365,260 potentiates the efficacy to and reverses chronic tolerance to electroacupuncture-induced analgesia in mice

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Abstract

Cholecystokinin octapeptide (CCK-8) is a physiological antagonist of endogenous opioids in the central nervous system (CNS). Our previous work has shown that CCK-8 plays an important role in the development of tolerance to morphine analgesia and electroacupuncture (EA) analgesia in the rat. The present studies were designed to examine whether the CCK_B receptor is involved in the modulation of EA analgesia and the development of EA tolerance in mice. The latency to flick the tail in the radiant heat was used as index to assess the efficacy of EA analgesia. Subcutaneous (s.c.) injection of the CCK_B receptor antagonist L365,260 produced a dose-dependent (0.125–2.0 mg/kg) potentiation of the analgesia induced by 100 Hz EA, with a maximal effect occurred at 0.5 mg/kg. In addition, L365,260 (0.5 mg/kg) significantly reversed chronic tolerance to 100 Hz EA in mice. These results suggest that the CCK_B receptor might play a role in the tonic inhibition of 100 Hz EA-induced analgesia and in the mediation of chronic tolerance to 100 Hz EA in mice. The results opened a way for further investigation of the function of CCK-8 in pain modulation using inbred strains of mice.

Keywords: Cholecystokinin octapeptide; CCK-8; CCK_B receptor; L365,260; Acupuncture; Analgesia; Tolerance

1. Introduction

Acupuncture has been used in China and other Asian countries for more than 2000 years. It is very effective in the treatment of many diseases including pain, and has little aversive side effects [5,11,21]. However, when acupuncture is applied for a prolonged period of time, the analgesic effects will decrease, this phenomenon is called "acupuncture tolerance" [10,12,20,24]. Our previous studies indicated that electroacupuncture (EA) produces analgesic effect through the release of endogenous opioid peptides including endorphins, enkephalins, dynorphin and endomorphin [9,13]. On the other hand, the efficacy of EA-induced analgesia depends on a functional balance between the opioid peptides and the anti-opioid peptides in the central nervous system (CNS) [10,20,26].

Many neurotransmitters and peptides, including cholecystokinin octapeptide (CCK-8), are known to be involved in acupuncture-induced analgesia and tolerance. CCK-8 is widely distributed in the central nervous system [22]. Endogenous CCK-8 has been shown to have a potent antagonistic effect on opioid antinociception [6]. CCK-8 has been shown to exert an anti-opioid effect *via* the activation of CCK_B receptors in the CNS [9,10,19,17,26]. CCK-8 is also involved in opioid tolerance. CCK-8 was markedly increased in rat brain when morphine tolerance or EA tolerance was induced [7,9,26,27]. Both CCK_A and CCK_B receptor mRNA expression in the hypothalamus of rats was significantly increased in the low responders of 100 Hz EA-induced analgesia compared to high responders [16]. This result suggests that the CCK receptor seem to play a key role in determining the effectiveness of EA analgesia. Regarding the involvement of subtypes of CCK receptors in the CCK–opioid interaction, overwhelming evidence, obtained from the administration of highly selective receptor antagonists and antisense oligonucleotides in rodents, have shown that CCK_B receptors mediate the anti-opioid function in the CNS [2,27].

CCK-8 and/or CCK receptors have been reported to be involved in EA analgesia and tolerance in rats and in rabbits [1,3,26]. No information is available whether the same phenomenon applies for mice. We reported in a previous study that different stains of mice with different genetic background

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showed a wide variety in antinociceptive response toward EA stimulation [23]. The aim of the present study was to explore whether CCK_B receptors are involved in EA analgesia and EA tolerance in mice as in the rat. L365,260, a specific CCK_B receptor antagonist, was used as a tool to observe whether it could enhance the efficacy of EA analgesia and reverse the development of EA tolerance in mice.

2. Materials and methods

2.1. Animals and chemicals

Male DBA/2 mice weighing 20–25 g were provided by the Animal Department of Peking University. They were housed four per cage under natural light–dark cycles with food pellets and water *ad libitum* according to the *University Guidelines for Animal Care and Use* adapted from NIH, USA. In all studies, measures were taken to minimize pain and/or discomfort in mice. L365,260 [3*R*(+)-*N*-(2,3-dihydrol-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-*N*-(3-methyl-phenyl)urea] was donated by Drs. Iversen and Freidinger of Merck Sharp and Dohme Laboratory. L365,260 was dissolved in 20% dimethylsulfoxide and 80% propylene glycol. Either different dose of L365,260 (10 mg/kg) or vehicle were injected subcutaneously (s.c.) 20 min prior to EA. All injections were in a volume of 0.1 ml/10 g body weight.

2.2. Nociceptive testing

Experiments were performed in a temperature-controlled room (22 ± 1 °C). Mice were kept in a specially designed polyethylene holder, with the hind legs and tail exposed. Nociception was assessed using the radiant heat-induced tail flick latency (TFL) as described previously [14,23]. Focused light from a projection bulb was applied to the tail 2–3 cm from the tip and the TFL was measured. The intensity of thermal stimulus was adjusted by changing the voltage of electricity to obtain a basal latency within the range of 3.5–5.5 s. To avoid tissue damage, a cut-off limit of 10 s was used. Mice were placed in the holder and inserted with acupuncture needles, and remaining *in situ* for 30 min prior to the assessment of the basal TFL. The mean of three consecutive measures at 5-min interval was taken as the basal TFL.

2.3. Electroacupuncture stimulation

EA was applied as reported previously [14]. The apparatus is shown in Fig. 1. The bent "L" shaped distal ends of the stainless-steel needles (0.15 mm diameter) were inserted in each hind leg, 3 mm in depth at the Zusanli acupoint (ST 36) near the knee joint and just penetrable at the Sanyinjiao acupoint (SP 6) near the ankle joint [23]. The proximal ends of the needles were connected to a Han's Acupoint Nerve Stimulator (HANS) (LH series, manufactured in Peking University). After insertion, the needles were fixed *in situ* with adhesive tape. Square waves generated from the HANS were applied to acupoints simultaneously. The frequency was 100 Hz and the pulse width was 0.2 ms. To prevent mice from stressful response, the stimulation intensity used in the present study was not higher than 1.5 mA as described previously [14]. During the experiment, mice kept quite and did not show struggling and other stressful responses. The intensity of stimulation (0.5–1.0–1.5 mA) was increased in a stepwise manner (0.5 mA increment), for 10 min at each intensity, lasting for a total of 30 min. TFL was measured every 10 min, with the electrical stimulation paused temporarily during the TFL assessment. The percent increase of TFL for the three assessments was averaged as a measure of EA-induced analgesia and was calculated as follows: TFL (%) = (latency after EA − basal latency)/basal latency) × 100%.

2.4. Electroacupuncture tolerance

Chronic tolerance to 100 Hz EA was developed according to our previous report [12]. EA was applied to mice once daily, 30 min/session, for 6 days consecutively. The basal TFL was examined before EA administration in each day. The thermal latency was examined at the end of EA each day to evaluate the EA-induced analgesic effect. The analgesic effect decreased along with the increase of number of EA applications, indicating the development of chronic tolerance to EA [20].

Fig. 1. Apparatus of electroacupuncture in mice. Mice were kept in a specially designed polyethylene holder, with the hind legs and tail exposed. The stainless-steel needles were inserted in each hind leg at the acupoints of Zusanli (ST 36) and Sanyinjiao (SP 6). After insertion, the needles were fixed *in situ* with adhesive tape. The proximal ends of the needles were connected to a Han's Acupoint Nerve Stimulator (HANS).

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. Group difference was analyzed by one-way analyses of variance (ANOVA) followed by Newman-Keuls *post hoc* test. $P < 0.05$ was used to determine a significant difference.

3. Results

One week before the experiment with L365,260, 160 mice were divided into three groups according to the analgesic effect induced by EA. The 100 Hz EA was delivered to mice for 30 min. Those mice with an over 50% increase of TFL were classified as high responders ($n = 45$); those lower than 20% as low responders ($n = 58$), and those intercalated between 20% and 50% were discarded ($n = 57$).

3.1. Dose–response curve of L365,260 on 100 Hz EA-induced analgesia in low responder mice

To observe the effect of L365,260 on basal thermal latency, TFL was measured at 10-min interval for a total of 60 min after injection. L365,260, ranged from 0.125, 0.25, 0.5, 1.0 to 2.0 mg/kg was used. The TFL fluctuated within the range of 0–4 ± 2.4% in all groups (9–10 per group), suggesting that s.c. L365,260 produced neither antinociception nor hyperalgesia in mice (data not shown).

In order to explore whether the low responders can be changed into high responders, we observed the effect of s.c. L365,260 on 100 Hz EA-induced analgesia. Fifty-eight low responder mice were randomly divided into six groups (9–10 per group) and given s.c. injection of vehicle or L365,260 at

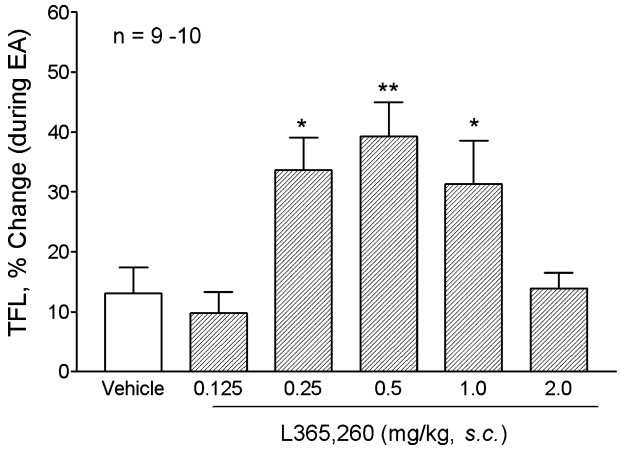

Fig. 2. Effect of the CCK_B antagonist L365,260 on 100 Hz EA analgesia in low responder mice. Mice were injected with either L365,260 (0.125, 0.25, 0.5, 1.0 and 2.0 mg/kg s.c.) (hatched columns) or vehicle (blank columns) 20 min prior to 100 Hz EA. The TFL was measured every 10 min during EA for 30 min. Data are expressed as means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ compared with the vehicle group by one-way ANOVA followed by Newman-Keuls *post hoc* test; $n=9$–10.

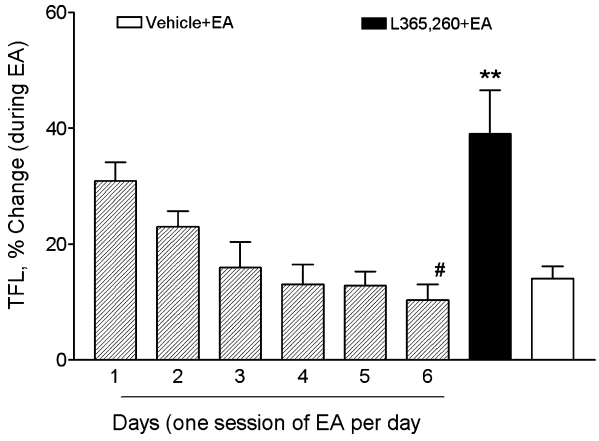

Fig. 3. Reversal effect of CCK_B receptor antagonist L365,260 on chronic tolerance of 100 Hz EA-induced analgesia in mice. Chronic tolerance developed as 100 Hz EA (1.0, 1.5, or 2.0 mA, 10 min) was given once daily for 6 days (hatched columns). The mice were randomly and evenly divided into two groups. At day 7, mice were injected with either L365,260 at a dose of 0.5 mg/kg (black columns) or vehicle (blank columns) 20 min prior to the administration of another session of 100 Hz EA. Data are expressed as means ± S.E.M. $^{\#}P<0.05$ compared to the first session of 100 Hz EA treatment, $^{**}P<0.01$ compared with the EA plus vehicle group by one-way ANOVA followed by Newman-Keuls *post hoc* test; $n=10$.

five different doses (0.125, 0.25, 0.5, 1.0 and 2.0 mg/kg) 20 min prior to EA. The results are shown in Fig. 2. L365,260 produced a dose-dependent potentiation of EA-induced analgesia with a maximal effect at 0.5 mg/kg ($P<0.01$). L365,260 at dose of 0.5 mg/kg was used in the next experiment.

3.2. Reversal effect of L365,260 on chronic 100 Hz EA tolerance

In order to explore whether the EA tolerance can be reversed by the CCK_B antagonist L365,260, mice were made tolerant to EA following the procedure stated in the methodology. As shown in Fig. 3, the analgesic effect induced by 100 Hz EA decreased gradually from day 1 to 6 ($P<0.05$), indicating the development of chronic tolerance to EA. The mice were then randomly divided into 2 groups of 10 each. On day 7, another session of 100 Hz EA was applied 20 min after s.c. injection of either L365,260 (0.5 mg/kg) or vehicle. The results are shown in Fig. 2. The EA analgesic effects in the L365,260 group (39.4 ± 7.0%) were significantly higher than those in the vehicle group (13.7 ± 1.8%) ($P<0.01$).

4. Discussion

The main finding of the present study is that CCK_B receptor antagonist L365,260 can potentiate 100 Hz EA-induced analgesia and reverse chronic tolerance to 100 Hz EA in mice. This suggests that endogenous CCK_B receptors might be involved in the modulation of EA analgesia and EA chronic tolerance in mice.

The interaction between CCK-8 and opioids was first reported by Ito et al. [15], and CCK-8 has been shown to possess potent anti-opioid activities in the CNS [6]. Faris et al. observed that intracerebroventricilar (i.c.v.) injection of CCK-8 suppressed antinociception induced by β-endorphin, and that CCK given i.p. prior to systemic morphine application significantly reduced the analgesic effect of morphine [7]. The antagonism of CCK-8 to morphine induced-antinociception was dose-dependent [25]. Proglumide, a nonselective CCK antagonist, was also reported to potentiate morphine induced antinociception without causing antinociception by itself [18,25]. These results suggest that endogenous CCK-8 attenuates antinociception induced by the administration of opioids, thus acting as a negative feedback modulator. Regarding the involvement of subtypes of CCK-8 receptors in the CCK–opioid interaction, overwhelming evidences obtained from the administration of highly selective receptor antagonists and antisense oligonucleotides in rodents have shown that CCK_B receptors mediate the anti-opioid function of CCK-8 in the CNS [2,27]. Reports also show that CCK-8 might be involved in the EA-induced analgesia. Han et al. [9] measured the CCK-8 release in the rat spinal perfusate and found that those rats, showing a significant CCK-8 increase during 100 Hz EA, were low responders (*i.e.* exhibiting weaker EA analgesia), whereas rats, showing little CCK-8 increase, were high responders (*i.e.* exhibiting stronger EA analgesia) [8]. Tang et al. found that the CCK-8 release was significantly higher in the low responder rats compared to high responders in the midbrain periaqueductal gray (PAG), and i.c.v. injection of antisense oligonucleotides to CCK-8 mRNA enhanced 100 Hz EA induced analgesia and convert low responder rats into high responder for EA analgesia [19]. Chen et al. demonstrated that i.c.v. injection of L365,260 into the PAG significantly increased the analgesic effect induced by 100 Hz EA in rats [3]. In the present study, we used L365,260, a highly specific CCK_B receptor antagonist, to study the anti-opioid effect of CCK against 100 Hz EA in mice. As shown in Fig. 2, L365,265 can potentiate the analgesic effect induced by 100 Hz EA in low responder mice, suggesting that low responders have been changed to

higher responders. This finding has shown that CCK_B receptors in mice is involved in the anti-opioid effect in antinociception. The bell shape of the dose–response curve (Fig. 2) is very similar to that observed in the experiment showing the potentiation of 100 Hz EA-induced analgesia in mice by nocistatin, another endogenous antiopioid peptide [12]. Given that the opioid receptor and CCK receptor are existing in one and the same neuron [17], the kinetics of the physiological interaction between the opioid receptor and the receptors of CCK/nocistatin (regarded as physiological opioid antagonists) deserve further investigation.

As shown in Fig. 3, mice, given daily 100 Hz EA for 6 consecutive days, developed chronic tolerance to 100 Hz EA, and that L365,260 can reverse the chronic 100 Hz EA tolerance. As mentioned above, EA can release opioid peptides in the CNS to produce analgesia [9]. A high level of CCK-8 in the CNS may exert a suppressive effect on EA-induced analgesia by antagonizing the endogenous opioids [15]. A number of studies support this viewpoint. For example, Zhou et al. [26,27] measured the content of CCK-8-immunoreactivity (CCK-*ir*) in rat spinal perfusate, and found that CCK-8 increased significantly in rats made tolerant to EA. Our previous works have shown that L365,260 can delay the development of EA tolerance in rats, and antisense oligonucleotides to CCK mRNA can also delay the development of tolerance elicited by prolonged 100 Hz EA stimulation [4,19]. The present study further suggests that the endogenous release of CCK-8 is also involved in 100 Hz EA tolerance in mice, as in rats [10,16,19,26].

When EA is applied for a prolonged period of time such as in the treatment of chronic pain, the analgesic effects will decrease. The current results may help in finding a useful method to improve the therapeutic effects of EA in the treatment of chronic diseases when "EA tolerance" occurs [9,12,20]. In addition, this study will redound to further study on EA using inbred strains of mice.

Conflict of interest

There is no financial or other conflict of interest with any person or institution regarding publication of this manuscript.

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