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Effects of synchronous or asynchronous electroacupuncture stimulation with low versus high frequency on spinal opioid release and tail flick nociception

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Abstract

Electroacupuncture stimulation (EAS) is known to change brain neurotransmitter release. In the present study, we investigated the effects of synchronous or asynchronous electroacupuncture stimulation with low versus high frequency on spinal opioid release and tail flick nociception. Rats were given "2/100 Hz" EAS, which stands for an asynchronous mode of stimulation, in which 2 Hz was alternated with 100 Hz, each lasting for 3 s, or "(2 + 100) Hz" EAS, a mode of stimulation in which 2 Hz stimulation was applied to the left hind leg simultaneously with 100 Hz stimulation on the right hind leg. The rats were subjected to the same total number of electrical stimulations in these two modes. Results were as follows: (1) 2/100 Hz EAS was 40% more potent than (2 + 100) Hz EAS ($P < 0.01$) in producing an anti-nociceptive effect. (2) Intrathecal (i.t.) injection of the μ-opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP) blocked in a dose-dependent manner the anti-nociceptive effect produced by 2/100 Hz EAS but not by (2 + 100) Hz EAS, whereas i.t. injection of the κ-opioid receptor antagonist norbinaltorphimide (Nor-BNI) blocked the anti-nociceptive effect induced by both modes of EAS. (3) I.t. injection of endomorphin-2 antiserum blocked in a dose-dependent manner the anti-nociceptive effect of 2/100 Hz EAS but not that of (2 + 100) Hz EAS, whereas i.t. injection of dynorphin antiserum blocked the anti-nociceptive effect induced by both modes of stimulation. (4) 2/100 Hz EAS increased the release of both endomorphin-2 and dynorphin, whereas (2 + 100) Hz EAS increased the release of dynorphin but not of endmorphin-2. We conclude that the more potent anti-nociceptive effect induced by 2/100 Hz EAS, as compared with that of (2 + 100) Hz EAS, was due, at least partly, to the synergistic interaction of endomorphin-2 and dynorphin in rat spinal cord.

Keywords: Endomorphin-2; Dynorphin; Acupuncture; Electroacupuncture stimulation; Opioids; Synergy

Introduction

Research in the neuroscience field has revealed that the release of neurotransmitters and neuropeptides in the central nervous system (CNS) can be triggered by peripheral electrical stimulation at specific frequencies. Our previous studies have shown that electroacupuncture stimulation of low frequency (2 Hz) increased the release of multiple opioid peptides, including enkephalin (Enk), β-endorphin (End) and endomorphin (EM), which interact with the μ- and δ-opioid receptors in the CNS. High-frequency (100 Hz) stimulation increased the release of dynorphin (Dyn), which interacts with the κ-opioid receptor in the spinal cord. The release of these opioid peptides results in significant anti-nociception (Chen and Han, 1992a,b; Fei et al., 1987; Han, 1986; Han et al., 1991, 1999; Huang et al., 2000).

It was obvious that a sharp demarcation existed between the responses to 2 and 100 Hz stimulation. On a log scale, 15 Hz is midway between 2 and 100 Hz and can partially activate release of both the μ-, δ-receptor-specific opioids and the κ-receptor-specific opioid. In order to maximize the anti-nociceptive effect, EAS with alternating 2 and 100 Hz (dense and disperse, DD) frequencies was tested. The two

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stimulatory electrodes were applied to separate acupoints and connected to a pulse generator that emitted square waves alternating between 2 Hz for 3 s and 100 Hz for 3 s. The anti-nociceptive effect induced by this DD mode of stimulation was found to be significantly more effective than the pure low- or pure high-frequency stimulation (Chen et al., 1994). This pattern of simulation is now accepted by clinicians and is widely used in most EAS devices. A question arises that, if the brain can perceive alternate 2 and 100 Hz (2/100 Hz) electrical stimulation for the differential release of enkephalin and dynorphin, respectively, could we use 2 Hz stimulation on one leg and 100 Hz on the other leg [(2 + 100) Hz] simultaneously, so that enkephalin and dynorphin were both released simultaneously to the full extent? In the present study, we used endomorphin as a representative marker and 2/100 Hz mode EAS as a control to test this hypothesis.

Materials and methods

Animals

Adult female Wistar rats weighing 150–200 g were used throughout the experiment. The protocols for animal experiments were approved by the animal use committee of the Peking University Health Science Center.

Nociceptive test

The nociceptive threshold of the animal was measured by the tail flick latency response (TFL) elicited by radiant heat. Briefly, the rats were kept in special holders, with the tail and the hind legs protruding naturally. Light from a 12-W projection bulb was applied to the lower 1/3 of the tail through an aperture of 5-mm diameter and the tail flick latency was recorded by an electronic timer to an accuracy of 0.1 s. Room temperature was maintained at 21 ± 1°C and the tail temperature was more than 52°C during radiant heat stimulation. At the beginning of the experiment, the tail flick latency was assessed three times at 5-min intervals. The mean value from the first three assessments was taken as the basal nociceptive threshold and was usually within the range of 4–6 s. The values of the subsequent measurements, after EAS administration, were expressed as percent changes from the basal TFL. An elevation over 150% of the basal level was taken as the cutoff limit to avoid skin damage. The cutoff limit was determined based on our previous extensive experience testing the effect of morphine on TFL (Ren and Han, 1979). The results indicated that, when a moderate or large dose of morphine was given, rats with basal TFL of 5 s presented no response at all to radiant heat, even if the period of irradiation was prolonged to 15, 20 or 30 s, which results in blistering of the skin. When we used 150% of the basal TFL as the upper limit, the average effect of morphine was directly proportional to the logarithm of the dosage, yielding a typical dose–response relationship. This determination of cutoff limit is also supported by the literature (Bars et al., 2001). After EAS, about 30% of the rats reached the cutoff level of 150% of the basal TFL, which artificially lowered the average TFL and thus somewhat underestimated the effect of the EAS. However, the statistical analysis was not affected. Details of the method have been described elsewhere (Ren and Han, 1979).

Electroacupuncture stimulation (EAS)

Two stainless-steel needles, 0.25 mm in diameter, were inserted into each hind leg, one in the Zusanli point (S36, 5 mm lateral to the anterior tubercle of the tibia) and the other in the Sanyingjiao point (Sp6, 3 mm proximal to the medial malleolus, at the posterior border of the tibia). Biphasic square waves generated from a Han's Acupoint Nerve Stimulator (HANS, manufactured at Peking University) were applied to the needles inserted in each leg. The pulse width was 0.6 ms at 2 Hz, and 0.2 ms at 100 Hz. For 2/100 Hz EAS, the two electrodes for stimulation were applied at the two acupoints and connected to a pulse generator that emitted square waves alternating between 2 Hz for 3 s and 100 Hz for 3 s. For (2 + 100) Hz EAS, 2 Hz stimulation was given to one leg, whereas 100 Hz stimulation was given to the other leg simultaneously. The intensity was initially set at 1 mA and then increased stepwise to 2 mA and then to 3 mA, with each period of stimulation lasting for 10 min. The reason for the stepwise increments in intensity was to allow the animal to gradually adapt to the EAS and to minimize the possible stress that might be induced by the higher intensity. This intensity activated Aβ and some of the Aδ-fibers but not the C-fibers (Xing et al., submitted for publication). No signs of distress such as vocalization or struggling, but only minor muscle tremors, were observed during the stimulation. The total stimulation period was 30 min. The tail flick latency was measured every 10 min during the period of electrical stimulation, followed by another three tests after the termination of the stimulation to assessment of the after effect.

Collection and preparation of the spinal perfusate

Rats were anesthetized with chlorohydrate, and a PE-10 tube was inserted 7.5 cm down the cisterna magna into the subarachnoid space. Another piece of polyethylene tubing (PE-50) was inserted 1 cm into the cisterna. Artificial cerebrospinal fluid containing aminopeptidase inhibitors captopril (1 μM) and bestatin (1 μM) was perfused at a rate of 1.0 ml/30 min via the PE-10 tube with a push–pull pump and the perfusate was collected via the PE-50 tube (Fei et al., 1987). Aliquots of the spinal perfusate (1.0 ml) were collected in polyethylene tubes that were immersed in an ice-water bath. The tubes were then heated for 10 min in a boiling water bath. After cooling, the perfusate was centrifuged at 12,000 rpm for 10 min. The supernatants were dried under vacuum and kept at −20°C.

Radioimmunoassay of dynorphin A and endomorphin-2

For the radioimmunoassay of Dyn A (1–13) and EM-2, the residue of the lyophilized spinal perfusate was reconstituted with 250 μl H_2O. Dyn A (1–13) (RK-021-21) and EM-2 RIA (RK-011-11) kits were generous gifts from Phoenix Pharmaceutical Int., USA. The procedure for the RIA was performed according to the manufacturer's instructions. The sensitivity and specificity of these two kits were as follows: For the Dyn-A (1–13) kit, the sensitivity was IC_{50} ~ 6 pg/tube, with a detection range of 1–128 pg/tube. The cross reactivity was 100% for Dyn A 1–13 (porcine) and Dyn-A 1–12 (porcine), 25% for Big Dynorphin (209–240) (porcine), and 1.5% for Dyn-A (1–10) amide and Dyn (1–11) (porcine), and 0% for Dyn (1–6, 1–7, 1–8 and 1–9). For the EM-2 kit, the sensitivity was IC_{50} ~ 39 pg/tube, with a detection range of 10–1280 pg/tube, and a lower limit of ~10.4 pg/tube. The cross reactivity was 100% for endomorphin-2, 0.47% for EM-1, and 0% for neuropeptide FF (human), CGRP (human), CGRP (rat), orphanin FQ (nociceptin), leucine-enkephalin, dynorphin A, β-endorphin (human), and methionine–enkephalin.

Intrathecal (i.t.) injection

Rats were anesthetized with chlorohydrate (300 mg/kg, i.p.) and prepared with lumbar catheterization of the spinal subarachnoid space as described (Storkson et al., 1996). Three days later, the viability of the catheterized animals was examined, and the rats with signs of neurological impairment were removed from the study. I.t. injection of pharmacological agents dissolved in normal saline was performed via the PE-10 catheter using a volume of 10 μl, followed by 10 μl of normal saline for flushing. Injections were completed within 30 s. For the control, 20 μl normal saline alone was injected.

Data processing and statistical analyses

The results were expressed as mean ± SEM. The data were evaluated by analysis of variance (ANOVA) followed by Duncan's test. A P value of less than 0.05 was considered statistically significant.

Results

A much more potent anti-nociceptive effect was induced by 2/100 Hz EAS compared to that of (2 + 100) Hz EAS

Fifty rats were randomly and evenly divided into two groups, individually assessed for their baseline nociceptive sensitivity and then given 2/100 Hz or (2 + 100) Hz EAS for 30 min. The TFL was determined every 10 min, and the percent change was calculated to represent the extent of EAS-induced anti-nociception. The TFL was then assessed for an additional 30 min following the cessation of EAS to represent the post-treatment effect. The average of the 30-min EAS stimulation period and the 30-min post-treatment period was designated as the total anti-nociceptive effect. The results shown in Fig. 1 indicated that 2/100 Hz EAS produced a much greater anti-nociceptive effect than did (2 + 100) Hz EAS ($P < 0.01$).

I.t. injection of the μ-opioid receptor antagonist (CTOP) attenuated the anti-nociceptive effect induced by 2/100 Hz EAS but not (2 + 100) Hz EAS

Forty-eight rats were randomly divided into four groups, individually assessed for their baseline nociceptive sensitivity and then given i.t. injections of CTOP (0.25, 0.5, 1 μg) or normal saline. They were then immediately given 2/100 Hz or (2 + 100) Hz EAS for 30 min, and the TFL was

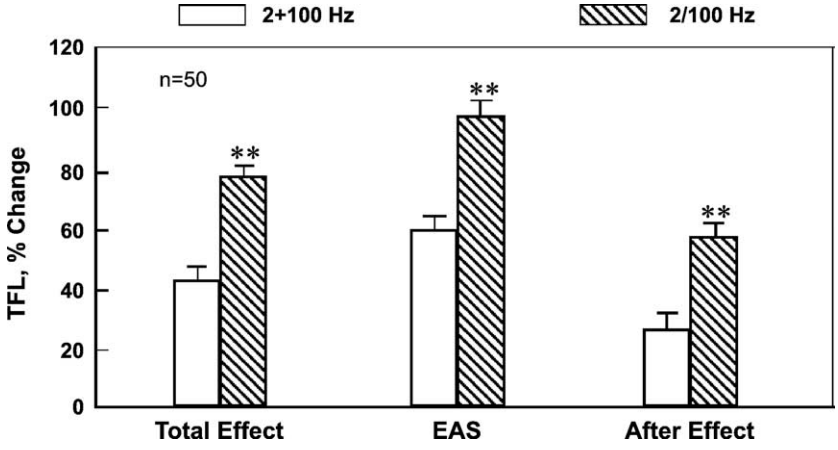

Fig. 1. Anti-nociceptive effect produced by electroacupuncture stimulation (EAS) of different frequencies. EAS: Indicates mean percent increase over basal tail flick latency (TFL) of three measurements during the 30-min stimulation period. After-effect: Indicates mean percent increase over TFL of three measurements in the 30 min immediately after the cessation of EAS. The average of the two periods during the 60-min observation period was designated as the total anti-nociceptive effect. **$P < 0.01$ as compared with the corresponding data in the (2 + 100) Hz group (ANOVA followed by Duncan test).

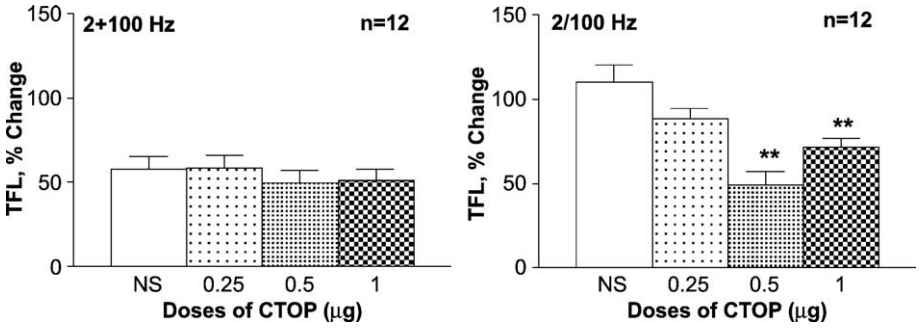

Fig. 2. Intrathecal (i.t.) injection of the μ-opioid receptor antagonist CTOP attenuated the anti-nociceptive effect induced by 2/100 Hz but not by (2 + 100) Hz EAS. Values are expressed as the mean percent increase over basal tail flick latency (TFL) of three measurements during the 30-min stimulation period. NS: normal saline. $^{**}P < 0.01$ as compared with the corresponding data in normal saline group (ANOVA followed by Duncan test).

measured as indicated in the previous experiment. The results are shown in Fig. 2. CTOP attenuated the 2/100 Hz EAS induced anti-nociception with the maximal effect occurring at 0.5 μg. The anti-nociceptive effect produced by (2 + 100) Hz EAS was not affected by CTOP at this dose range.

I.t. injection of the κ-opioid receptor antagonist nor-BNI attenuated the anti-nociceptive effect induced by both 2/100 Hz EAS and (2 + 100) Hz EAS

Forty-eight rats were randomly divided into four groups. After the assessment of the baseline TFL, they were given i.t. injections of nor-BNI (3.125, 6.25 or 12.5 μg) or normal saline, and then immediately administered 2/100 Hz EAS or (2 + 100) Hz EAS for 30 min. The TFL was again measured as described in the above studies. The results shown in Fig. 3 indicated that nor-BNI attenuated the anti-nociceptive effects induced by EAS of both 2/100 Hz and (2 + 100) Hz stimulation in a dose-dependent manner.

I.t. injection of endomorphin-2 antiserum attenuated the anti-nociceptive effect induced by 2/100 Hz EAS but not (2 + 100) Hz EAS

Forty-eight rats were randomly and evenly divided into four groups. After the assessment of baseline TFL, they were given i.t. injections of antiserum against EM-2 (H-044-11 from Phoenix Pharmaceuticals) at dilutions of 1:100, 1:10, or the original concentration without dilution, or normal rabbit serum. They were then immediately given 2/100 Hz EAS or (2 + 100) Hz EAS for 30 min. The TFL was measured as described in the above studies. The results are shown in Fig. 4. The anti-nociceptive effect induced by 2/100 Hz EAS was attenuated by the EM-2 antiserum in a dose-dependent manner, showing a maximal suppression of 60%. In contrast, the anti-nociceptive effect produced by (2 + 100) Hz EAS was not affected by the EM-2 antiserum, even without dilution.

I.t. injection of dynorphin antiserum attenuated the anti-nociception induced by both 2/100 Hz EAS and (2 + 100) Hz EAS

The design of the present experiment was the same as the previous one, except that Dyn A antiserum (H-021-21 from Phoenix Pharmaceuticals) was used instead of EM-2 antiserum. The results are shown in Fig. 5. I.t. injection of difsfferent dilutions of Dyn A antiserum (1:100, 1:10 or original concentration without dilution) attenuated the anti-nociceptive effect induced by 2/100 Hz EAS in a dose-dependent manner with a maximal inhibition of about 60% ($P < 0.01$). A similar experiment was performed with (2 + 100) Hz EAS. An inhibition of 30–40% ($P < 0.05$) of the anti-nociceptive effect of the EAS was observed, but no clear dose–response relationship was seen.

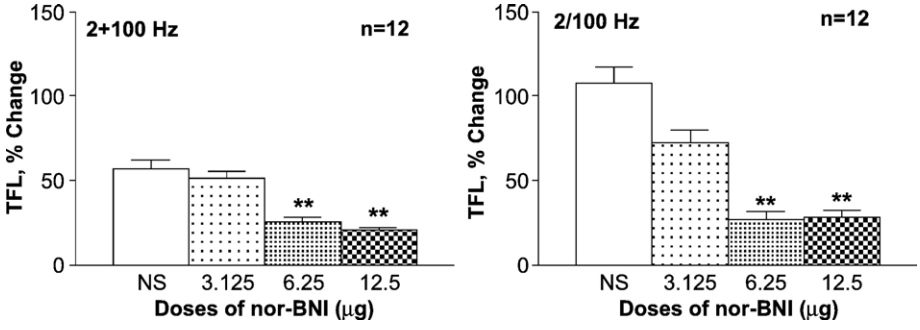

Fig. 3. Intrathecal (i.t.) injection of the κ-opioid receptor antagonist nor-BNI attenuated the anti-nociceptive effect induced by both 2/100 Hz and (2 + 100) Hz EAS. Values are expressed as the mean percent increase over basal tail flick latency (TFL) of three measurements during the 30-min stimulation period. NS: normal saline. $^{**}P < 0.01$ as compared with the corresponding data in normal saline group (ANOVA followed by Duncan test).

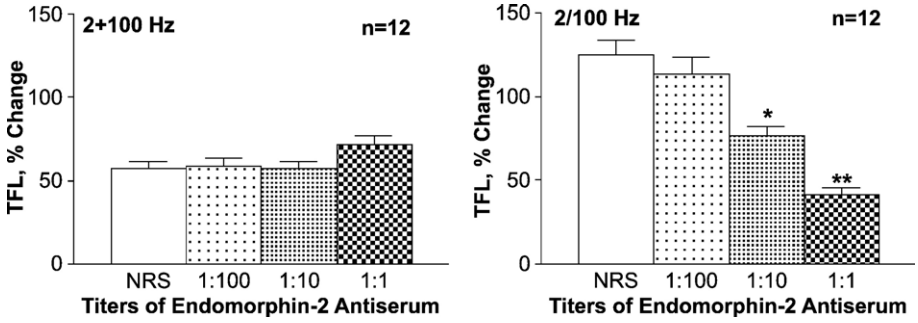

Fig. 4. Intrathecal (i.t.) injection of endomorphin-2 antiserum attenuated EAS anti-nociception induced by 2/100 Hz but not by (2 + 100) Hz EAS. Values are expressed as the mean percent increase over basal tail flick latency (TFL) of three measurements during the 30-min stimulation period. NRS: normal rabbit serum. *$P < 0.05$, **$P < 0.01$ as compared with the corresponding data in the normal rabbit serum group (ANOVA followed by Duncan test).

Comparison of the contents of EM-2-ir and Dyn A-ir in the spinal perfusate obtained from rats receiving 2/100 Hz EAS or (2 + 100) Hz EAS

Thirty rats were randomly and evenly divided into two groups, receiving 2/100 Hz EAS and (2 + 100) Hz EAS, respectively. Spinal perfusate was collected in three periods before, during and after EAS; each collection lasting for 30 min. The results are shown in Figs. 6 and 7. Both 2/100 Hz and (2 + 100) Hz EAS increased the release of Dyn A-ir in the spinal cord. However, an increase of EM-2-ir was observed only in rats receiving 2/100 Hz EAS but not that of (2 + 100) Hz EAS.

Discussion

The scientific questions dealt with in the present study can be analyzed at two levels: scientific and practical. The practical goal was to find the optimal electrical parameters for EAS to produce a maximal anti-nociceptive effect. The scientific objective was characterization of the neurochemical response of the brain toward EAS of specific frequencies, and, more specifically, to characterize the response of the CNS toward signals of two concurrent EAS of different frequencies arriving synchronously.

It is already known that 2 Hz EAS can increase the release of Enk/End/EM from brain and spinal cord, whereas 100 Hz EAS increases the release of Dyn in the spinal cord (Han et al., 1991). How can the release of both Enk/End/EM and Dyn be increased? An already accepted approach is to use low- and high-frequency stimulations at alternating 3 s intervals, so that the chemicals produced in the first 3 s, e.g., by 2 Hz EAS, will have a chance to interact with the chemicals produced in the second 3 s, i.e., by 100 Hz EAS. A problem with this approach is that the 2 or 100 Hz stimulation is only for one half of the 30-min duration of total stimulation. Why not make use of the whole period of stimulation if 2- and 100-Hz stimulations are working synchronously at two different sites of the body, so that the signals can reach the brain separately but synchronously. The best estimate is that the two endogenous opioid systems would be fully activated at the same time to produce a maximal synergistic interaction, resulting in a maximal anti-nociceptive effect. However, one can have an alternative hypothesis that the two separate signals (2 and 100 Hz) will converge in the CNS, at least in the reticular formation of the brain stem. In this case, the 2 Hz signal will merge into the 100-Hz signal and hence its own effect will be lost. Another possibility may be effects of habituation over the long 30-min continuous (2 + 100) Hz EAS stimulation in contrast to the constantly changing and dynamic 2/100 Hz stimulation. Our studies were conducted to test these hypotheses.

First, we determined the extent of the anti-nociceptive effect that served as the endpoint of the physiological response. The result indicated that the anti-nociceptive

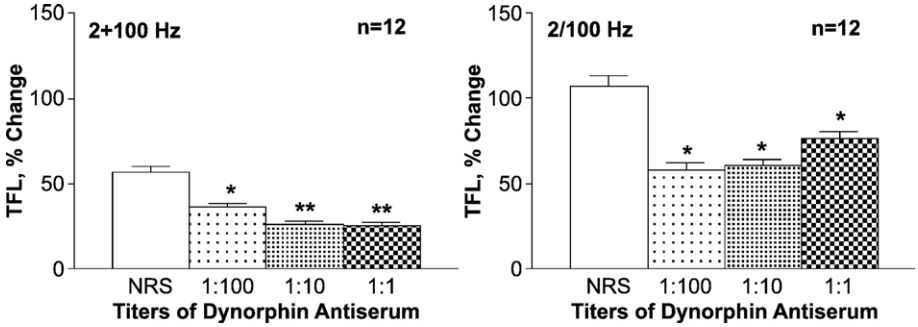

Fig. 5. Intrathecal (i.t.) injection of dynorphin antiserum attenuated EAS anti-nociception induced both by 2/100 Hz and (2 + 100) Hz EAS. Values are expressed as the mean percent increase over basal tail flick latency (TFL) of three measurements during the 30-min stimulation period. NRS: normal rabbit serum. *$P < 0.05$, **$P < 0.01$ as compared with the corresponding data in the normal rabbit serum group (ANOVA followed by Duncan test).

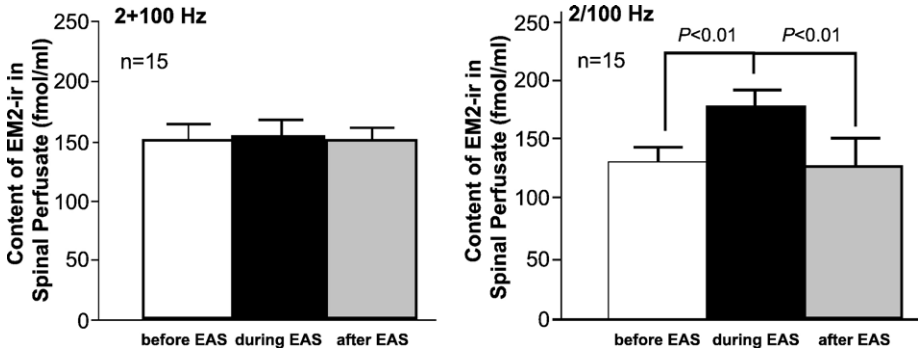

Fig. 6. Comparison of the content of endomorphin-2-ir (EM-2-ir) in spinal perfusate between 2/100 Hz and (2 + 100) Hz EAS. EAS was administered for 30 min with increasing intensity of 1–2–3 mA. Data are shown as the mean ± S.E.M of the level of EM2-ir in the spinal perfusate (fmol/ml). Samples of 1.0 ml in volume were collected every 30 min.

effect of (2/100) Hz was 40% weaker than that of 2/100 Hz. As the introduction mentioned, 2/100 Hz EAS gave a more effective anti-nociceptive effect than did pure 2 or 100 Hz EAS, which was partly due to the synergy by the opioid peptides. Instead of measuring all three kinds of μ and δ opioid receptor selective peptides (Enk, End and EM), we measured only EM-2, which is recognized as the one of the endogenously produced peptides with a pure μ-opioid receptor agonist property (Zadina et al., 1997). The results can be analyzed as follows:

(1) Blockade of the anti-nociceptive effect by type-specific opioid receptor antagonists. Since EM-2 is considered as a μ-selective agonist (Zadina et al., 1997) and Dyn as a κ-selective agonist (Goldstein and Naidu, 1989), it would be ideal to use type-specific antagonists as tools to elucidate the receptor mechanisms. Results shown in Figs. 2 and 3 indicated that the μ-specific antagonist CTOP blocked the effect of 2/100 Hz EAS but not that of (2 + 100) Hz EAS, whereas the κ-specific antagonist nor-BNI attenuated the effects induced by both EAS in a dose dependent manner. It seems as if the μ-opioid receptor was not involved in the anti-nociceptive effect of the (2 + 100) Hz EAS.

(2) Determination of whether neuropeptide release contributed to the lack of function of the μ-opioid receptor upon (2 + 100) Hz EAS. We evaluated this question by intrathecal injection of specific neuropeptide antibodies (see Han, 1987). Results shown in Figs. 4 and 5 indicated that a dose-dependent suppression of the anti-nociceptive effect by EM2 antiserum occurred only in 2/100 Hz EAS but not in (2 + 100) Hz EAS, whereas antiserum against Dyn attenuated the anti-nociceptive effect produced by both 2/100 Hz EAS and (2 + 100) Hz EAS. This latter response was expected since Dyn was released in either case. This result was also consistent with our previous results that both EM-1 and EM-2 are involved in mediating low-frequency EAS anti-nociception (Han et al., 1999; Huang et al., 2000). Moreover, the results from radioimmunoassay (Fig. 6) indicated that an increase in the content of EM-2 IR was observed only in 2/100 Hz but not in (2 + 100) Hz EAS. Taken together, the results suggested that the effect of pure 2 Hz EAS was absent in the (2 + 100) Hz EAS mode.

(3) One can also find that the (2 + 100) Hz EAS produced a marked after effect that was not seen in 2/100 Hz EAS, suggesting a more profound release of Dyn following (2 + 100) Hz EAS (Fig. 7). This is reasonable since the

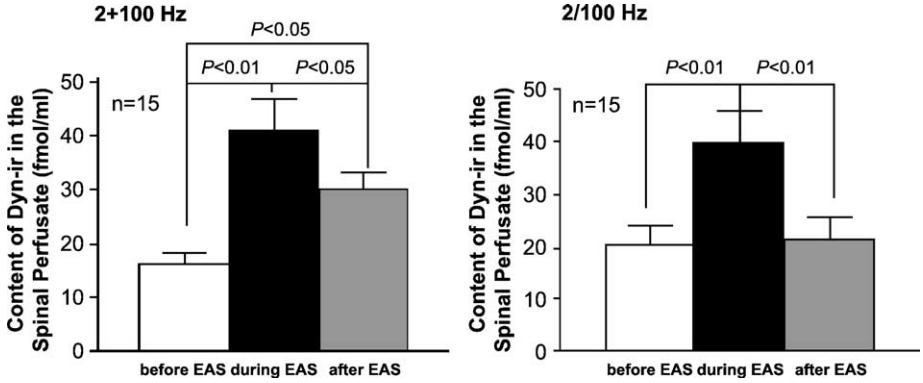

Fig. 7. Comparison of the content of dynorphin-ir (dyn-ir) in spinal perfusate between 2/100 Hz and (2 + 100) Hz EAS. EAS was administered for 30 min with increasing intensity of 1–2–3 mA. Data are shown as the mean ± SEM of the level of dyn-ir in the spinal perfusate (fmol/ml). Samples of 1.0 ml in volume were collected every 30 min.

high-frequency EAS lasted for the whole 30-min stimulation period of (2 + 100) Hz EAS, as compared with a total of only 15 min (1/2 of 30 min) with the 2/100 Hz EAS regime. Moreover, the result also excluded the possibility of habituation in long lasting stimulation of pure 2 or 100 Hz in the (2 + 100) Hz EAS mode.

In this report, we have shown that 2/100 Hz EAS increased the release of both dynorphin and endomorphin, whereas (2 + 100) Hz EAS increased the release of only dynorphin, but not endomorphin, and this may contribute to the more potent anti-nociceptive effect of 2/100 Hz EAS than of (2 + 100) Hz EAS. The present work also suggested that (2 + 100) Hz EAS does not convey the pure 2-Hz information, although further studies are needed.

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