Synergistic effect of cholecystokinin octapeptide and angiotensin II in reversal of morphine induced analgesia in rats

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Abstract

The aim of this paper is to study the synergistic anti-analgesic effect of angiotensin II (Ang II) plus cholecystokinin octapeptide (CCK-8). Our previous studies have shown that both CCK-8 and Ang II are potent anti-opioid substances. Intracerebroventricular (i.c.v.) injection of CCK-8 or Ang II dose-dependently antagonizes morphine-induced analgesia (MIA). In the present study, we observed the combined effect of CCK-8 and Ang II in antagonizing MIA. CCK-8 and Ang II were injected intracerebroventricularly to rats in various proportions and doses. The results were analyzed with isobolographic analysis. Combined injection of CCK-8 and Ang II in a ratio of 1 ng: 2.5 μg or 1 ng: 5 μg produced significantly greater effect in antagonizing MIA. The ED₅₀ of the two ratios are only 18.5% and 27.5%, respectively, of the theoretical dose of simple addition. We conclude that CCK-8 and Ang II used in such dose ratios may antagonize MIA synergistically.

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1. Introduction

The research for endogenous substances with anti-opioid activity has provided us several evidences for morphine tolerance and morphine addiction. Among the whole list of anti-opioid substances, cholecystokinin octapeptide (CCK-8) and angiotensin II (Ang II) are probably most attractive in central nervous system (CNS). Both of the small peptides have an abundant and widespread distribution in CNS (Beinfeld, 1983; Muscha-Steeckelings and Unger, 1992). CCK-8 has been shown to be the most potent endogenous anti-opioid substance. It acts selectively as an opiate antagonist in modulating pain and in regulating food intake (Faris et al., 1983). Ang II was first discovered as an anti-opioid substance in CNS in 1985 (Kaneko et al., 1985). We have shown that central Ang II antagonized analgesic effects induced by opiate (Wang and Han, 1989).

Several reports have demonstrated that different opioid peptides produced analgesic synergy, such as the combination of endorphin and dynorphin, enkephalin and dynorphin produce synergistic analgesia (Kimberly et al., 1990; Miaskowski et al., 1992). However, no information is available concerning the interaction between endogenous anti-opioid substances. In the present study, the effect of combination of intracerebroventricular administration of CCK-8 and Ang II on nociceptive thresholds in rats was observed.

The interaction between biologically active substances is a comprehensive event. There are several methodological approaches for analysis of drug interactions. Among them there is a commonly used one, the isobole method, which Gebhart (1992) commented it as the ‘gold standard’ because it is the only model of analysis proven valid. Isobolographic analysis is a mechanism-free model that requires no assumptions about the shapes of the dose-response curves. This method is appropriate for analysis primarily when testing single-dose combinations and when analyzing the interaction between agents when their mechanism of action is unknown or the nature of the dose-response relationship is not known (Berenbaum, 1989). The isobolograph is simple enough to interpret and is convenient for display. Furthermore, using this method, the finding that synergism occurs at only some ratios may aid to uncover its mechanism. In our study, this approach was used as basis of our experimental design and tool of statistical analysis.

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2. Methods

2.1. Animals

Adult male and female Wistar rats weighing 200–300 g (provided by the Animal Center of Beijing Medical University) were used throughout. Rats were housed six to eight per cage with free access to food and water and were maintained on a 12:12 h light/dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Beijing Medical University.

2.2. Intracerebroventricular (i.c.v) injection of drugs

Rats were anesthetized with chlorohydrate (0.4 g/kg, i.p.). The animals were then placed in a stereotaxic headholder. The skull was exposed and a 0.8 mm gauge guide cannula was directed to a lateral ventricle (1.0 mm caudal to bregma, 1.5 mm lateral to the sagittal suture, 3 mm ventral to the skull surface). The guide cannula were cemented in place. The animals were allowed to recover for 4 days before any pharmacological manipulations were made. Intracerebroventricular drug administration was performed by expelling 10 μl of solution through an injection cannula (0.45 mm gauge) inserted through the guide cannula and protruding an additional 2 mm into the ventricular space (Pellegrino et al., 1979).

2.3. Nociceptive test

The rats were restrained in a plastic holder with the hind legs protruding. The nociceptive threshold was measured by the latency of the tail flick responses elicited by radiant heat applied to the lower 1/3 of the tail. The mean tail flick latency (TFL) of three measurements taken at 5 min intervals at the start of experiments was taken as the basal threshold. Adjust the amplitude of radiant heat so that the basal TFL was within 4–6 s. The tail flick latency taken at 10-min intervals after drug administration was expressed as the percentage change from basal tail flick latency, with a cut-off limit of +150% above baseline, to avoid unnecessary skin damage (Ren and Han, 1979).

2.4. Drugs

Morphine HCl was purchased from Qinhai Drug Company (China). Cholecystokinin octapeptide (CCK-8: Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe) was a gift from Squibb and Sons Inc. (USA). Angiotensin II (Ang II: HAsp-Arg-Var-Tyr-Ile-His-Pro-Phe) was obtained from Sigma Inc. (USA). Chlorohydrate was a product of Beijing Chemical Company (China).

2.5. The rationale of experiment design and statistical analyses

The isobolographic analysis is referred as the ‘gold standard’ because it is the only model of analysis proven valid for analyzing interactions between biologically active agents. The theory about this approach has been already described in Tallarida (1992).

Briefly, the experimental design used to evaluate the interaction between two agents with isobolographic analysis requires determination of the potency of the combination. This potency is then compared with that of the theoretically additive combination. The potency of an agent or of a mixture is measured as a dose that produces a response in 50% of the subjects, i.e. ED50, ED50 of each single agent (Z1* and Z2*) and their variance (V(Z1*) and V(Z2*)) are calculated using the log-probit method of Litchfield and Wilcoxon (1949). The log-probit method uses a quantal or ‘all or none’ approach to analyze data. A quantal dose–response relationship is obtained by specifying an endpoint of agent action and determining the number or percentage of subject that achieve this endpoint at each dose. In our experiment, the endpoint, namely, the reversal of morphine-induced analgesia (MIA), is set anywhere from two standard deviations below the control response, i.e. Mean – 2 SD. Here the MEAN is the mean values of percent change of TFL within the next 20–60 min after the control group was given morphine.

CCK-8 and Ang II were mixed in fixed proportions based on weight (i.e. 1 ng:2.5 μg and 1 ng:5 μg). The mixture was administered in various doses and the responses (quantal) were observed, thereby producing a dose–response relation for the mixture. The ED50 and variance of the mixture, which are denoted Z* mix and V(Z* mix), respectively, were then calculated. This potency of the mixture was then compared with that of a theoretically additive dose, denoted Z*add. The calculation of Z* add and its 95% confidence interval (CI) has been described in detail in Tallarida (1992).

If the 95% CI of Z*add and Z*mix do not overlap, then we conclude that the mixture’s effect depart from simple additivity. If Z*mix < Z*add the mixture is synergistic under this ratio; whereas the relation Z*mix > Z*add means antagonism. Of course, equality means that the mixture is simple additive.

3. Results

3.1. ED50 for CCK-8 and Ang II on antagonizing MIA

The rats were given one s.c. injection of morphine (5 mg/kg) followed by i.c.v. injection of NS or sequentially increasing doses of CCK-8 (0.5, 1, 2, 4 ng) or Ang II (2.5, 5, 10, 20 μg). The mean values of the percent changes of TFL within next 20–60 min were measured. As described in methods, the endpoint for reversal effect of CCK-8 and Ang II on MIA is MEAN − 2 SD, where MEAN is the mean value of TFL within 20–60 min and SD is its standard deviation. As shown in Fig. 1A,B, the ED50 and its variance for each agents were obtained from the dose-response curves.
for CCK-8 and Ang II. ED_{50} for CCK-8 and Ang II was 1.3218 ng and 5.786 μg, respectively.

3.2. The synergistic reversal effect of CCK-8/Ang II in a dose ratio of 1 ng/2.5 μg on MIA

Forty-four rats were divided into six groups and given s.c. injection of 5 mg/kg morphine. Ten minutes later, the rats were given i.c.v. injection of (a) NS 10 μl; (b) sequentially increasing doses of CCK-8/Ang II mixture with the dose ratio fixed as 1 ng/2.5 μg. The mean values of the percent changes of TFL within next 20–60 min were measured. The results were shown in Fig. 2A. TFLs in the CCK-8/Ang II combination groups were significantly decreased compared with that in the morphine + NS control groups, which indicated that the combination of CCK-8 and Ang II effectively antagonize MIA (P < 0.05). As described in methods, dose-response curve for the mixture was obtained and the ED_{50} for the mixture (Z^*_{mix}) was 0.3898, while its 95% CI was (0.1007, 1.5096). Calculation of the theoretical additive ED_{50} yielded Z^*_{add} = 2.1036 and 95% CI was (1.7905, 2.4325). Thus, the two CIs do not overlap. Also there is Z^*_{mix} < Z^*_{add}. That is, the mixture’s ED_{50} is significantly less than expected from single additivity. This means that the mixture in this proportion displayed synergistic effect in antagonizing MIA.

3.3. The synergistic reversal effect of CCK-8/Ang II in a dose ratio of 1 ng/5 μg on MIA

Forty-six rats were assigned to 6 groups and given s.c. injection of 5 mg/kg morphine followed by i.c.v. injection of (a) NS 10 μl; (b) sequentially increasing doses of CCK-8/Ang II mixture with the dose ratio fixed as 1 ng/5 μg. The mean values of the percent changes of TFL within next 20–60 min were measured. As shown in Fig. 2B, TFLs in the CCK-8/Ang II combination groups, except the lowest dose group, were significantly decreased compared with that in the morphine + NS control groups, indicating that the combination of CCK-8 and Ang II effectively antagonize MIA (P < 0.05). With the dose-response curve for this mixture, the ED_{50} (Z^*_{mix}) was 0.8489, while its 95% CI was (0.6057, 1.1899). Theoretical calculation again yielded Z^*_{add} = 3.0857 and 95% CI was (2.6553, 3.002). The two CIs do not overlap. Also there is Z^*_{mix} < Z^*_{add}. Thus, the mixture’s ED_{50} is significantly less than expected from single additivity. So the mixture in this proportion is also synergistic.

3.4. Summary

As shown in Fig. 3, an isobologram for the evaluation of interaction between CCK-8 and Ang II was constructed. Doses of Ang II were arranged on the X axis and doses of

![Fig. 1](image1.png)

![Fig. 2](image2.png)

![Fig. 3](image3.png)
CCK-8 were on the Y axis. The x-y coordinates of a single point represent doses of Ang II and CCK-8 of a certain combination. Points that produce equal effect on MIA were connected as the isobolograph. The additive relationship between CCK-8 and Ang II was described by the equation of a straight line joining the \( Z_{i}^{*} \) and \( Z_{2}^{*} \). All points on this isobole of additivity represent dose pairs that are additive, where as the points lie below or above this line represent synergism or antagonism, respectively. In this study, \( Z_{i}^{*} \) for the two investigated ratios of CCK-8 and Ang II were shown in figure 5. Here \( P_{1} \) is the position for \( Z_{i}^{*} \) when CCK-8/Ang II equals 1 ng/2.5 \( \mu g \), and \( P_{2} \) is the position for \( Z_{i}^{*} \) when CCK-8/Ang II equals 1 ng/5 \( \mu g \). Both \( P_{1} \) and \( P_{2} \) lie below the line of additivity, indicating that the combination of CCK-8 and Ang II at these two ratios display synergistic effect on reversal of MIA.

4. Discussion

It has been demonstrated that the combinations of various opiates and opioid peptides with different receptor specificity produce synergistic or antagonistic interaction in antinociceptive effects in the rat CNS. For example, co-administration of low-antinociceptive dose of a selective \( \delta \) agonist DPDPE with sequentially increasing doses of a selective \( \mu \) agonist DAMGO produced synergistic antinociceptive effect. Co-administration of low-antinociceptive dose of a selective \( \kappa \) agonist U50,488H with sequentially increasing doses of DAMGO also produced synergistic antinociceptive effect (Kimberly et al., 1990). On the other hand, a hypothesis has been developed for a descending antianalgesia system mediated in the spinal cord by dynorphine A 1-17 (Fujimoto et al., 1990), indicating that antagonistic interactions exist between endogenous opioids in nociceptive effects. However, there are no data to verify whether various endogenous anti-opioid substances could produce similar interaction. Our present study was designed to address this question.

Isobolographic interpretation of drug interaction can be done with both graded and probit dose-response curves. Although the former is usually preferred, it is not proper for the data collected in the present study. Since we were investigating the antagonism of MIA, a moderate dose of morphine (5 mg/kg) was selected. The analgesic effect of this dose showed certain individual variation. With the addition of CCK-8 and Ang II, or both agents, the remaining analgesia became even more fluctuating. It does not make much sense to average the reversal of MIA from originally different levels. So we decided to employ the probit analysis, using \( \text{MEAN} - 2 \text{ SD} \) as the artificial endpoint indicating the successful reversal of MIA.

The mechanism basis for the synergism between CCK-8 and Ang II is unknown. Both CCK-8 and Ang II are potent endogenous anti-opioid substances and both of them antagonize MIA in rat CNS. The distribution of CCK-8 neurons and their receptors in rat CNS was observed overlapping with that of Ang II neurons and receptors, especially in some regions involved in the pain modulation, such as periaque ductal gray (PAG), nucleus accumbens and nucleus amygdal (Beinfeld, 1983; Muscha-Steekelings and Unger, 1992). This might be the morphological basis for the interaction between CCK-8 and Ang II. Further more, systemic morphine produced a marked increase of both CCK-8 and Ang II immunoreactivity in the rat spinal cord (Wang and Han, 1990a; Zhou et al., 1993), suggesting that the two peptides may share common functions in pain modulation.

There are also several lines of evidence supporting the possibilities of interaction between spinal receptors on a single cell. The presence of both \( \mu \)- and \( \delta \)-opioid receptors on primary afferent fibers and on dorsal horn neurons in the rat have been reported (Fields et al., 1980). On a single dorsal root ganglion cell, \( \mu \), \( \delta \) and \( \kappa \)-receptors have been characterized (Werz et al., 1987). Meanwhile, our previous studies have demonstrated that i.c.v. administration of CCK-8 or Ang II alone efficiently antagonizes analgesia mediated by different opioid receptors (Han et al., 1985; Wang and Han, 1989). This may be due to that opioid receptor and CCK-8 or Ang II receptor reside at the same regions and that binding of one receptor affect the other. Our laboratory also reported that CCK-8 increased the \( K_{d} \) of \( \kappa \)-opioid binding sites but decreased the \( B_{\text{max}} \) of \( \mu \)-opioid sites (Wang and Han, 1990b). Although Ang II did not suppress the binding of \([^{3}H]\)etorphine to rat brain membrane opioid receptors, it abolished the suppressive effect of DPDPE on the mobilization of intracellular calcium storage. This abolishment was mediated by Ang II receptor (Wang et al., 1992b). Similarly, our recent results have shown that CCK-8 antagonizes the depressant effect on voltage-gated calcium current mediated by \( \kappa \)-opioid receptor. This antagonizing effect appears to be mediated via CCK receptor (Wang et al., 1992a; Xu et al., 1996).

In addition, the post-receptor mechanisms of CCK-8 and Ang II have some common features. It has been clearly established that both CCK-8 and Ang II receptors are G-protein coupled receptor (Zhang et al., 1992; Raizada et al., 1993). Activation of G protein-coupled receptor stimulates PLC to hydrolyze the lipid precursor phosphatidylinositol 4,5-biphosphate (PIP2), liberating inositol (1,4,5)-triphosphate (IP3) and diacylglycerol (DAG). The intracellular release of IP3 and DAG has many consequences. IP3 can stimulate the mobilization of calcium from intracellular stores, while DAG can activate PKC. Further studies are needed to clarify the possible post-receptor mechanisms underlying the interaction between CCK-8 and Ang II, such as receptor phosphorylation, impaired coupling of receptor to G-protein or G-protein to effector.

Thus, we speculated that the activation of different opioid receptors may result in the changes of the interaction between another neurotransmitter and its receptor, such as CCK-8 and Ang II, therefore the synergy, addition or antagonism between the receptors occur.
There is also a possibility that the interaction occurs on the pain-modulatory circuit levels. Several lines of evidence has raised the possibility that certain nuclei in the brain may form a complex network to modulate the analgesic action of morphine (Zhou et al., 1984; Ma et al., 1992). It is possible that both CCK-8 and Ang II attend this network. This mechanism needs to be verified experimentally.

In conclusion, using isobolographic analysis, we studied the interaction between CCK-8 and Ang II at different combination. Our present results, for the first time, revealed that i.c.v. co-administration of these two endogenous anti-opioid substances produced synergistic interaction on reversal of MIA. The magnitude of the synergy depends highly upon the ratio of the combination of CCK-8 and Ang II, indicating that the synergy occur only under certain functional circumstances, such as the certain ratio of the neurotransmitters releases. This study provides a useful evidence to further explore the central mechanism of pain modulation.

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