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☐ Original Article ☐

Antinociceptive Effects of Intrathecal Metabotropic Glutamate Receptor Compounds and Morphine in Rats

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= **Abstract** =

Background: Spinal metabotropic glutamate receptors (mGluRs) and opioid receptors are involved in the modulation of nociception. Although opioid receptors agonists are active for pain, the effects of the compounds for the mGluRs have not been definitely investigated at the spinal level. We examined the effects of the intrathecal mGluR compounds and morphine in the nociceptive test, and then we further clarified the role of the spinal mGluRs. In addition, the nature of the pharmacological interaction after the coadministration of mGluRs compounds with morphine was determined.

Methods: Catheters were inserted into the intrathecal space of male SD rats. For the induction of pain, 50 μl of 5% formalin solution or a thermal stimulus was applied to the hindpaw. An isobolographic analysis was used for the evaluation of the drug interaction.

Results: Neither group I mGluR compounds nor group III mGluR compounds produced any antinociceptive effect in the formalin test. The group II mGluR agonist (APDC) had little effect on the formalin-induced nociception. The group II mGluR antagonist (LY 341495) caused a dose-dependent suppression of the phase 2 flinching response on the formalin test, but it did not reduce the phase 1 response of the formalin test nor did it increase the withdrawal latency of the thermal stimulus. Isobolographic analysis revealed a synergistic interaction after the intrathecal delivery of a LY 341495-morphine mixture.

Conclusions: These results suggest that group II mGluRs are involved in the facilitated processing at the spinal level, and the combination of LY 341495 with morphine may be useful to manage the facilitated pain state. **(Korean J Pain 2005; 18: 1-9)**

Key Words: antinociception, formalin test, interaction, metabotropic glutamate receptors, rat, spinal cord.

INTRODUCTION

Noxious stimuli to the periphery release glutamate, being recognized to play a principal role in the transmission of pain in the nervous system including in the spinal cord.[1,2] Glutamate exerts its action on the dorsal horn neurons via activation of two major classes of receptors: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs).[3] The iGluRs mediate fast synaptic transmission through ligand-gated ion channels, and the mGluRs are coupled to various intracellular second messenger systems through G proteins, and these receptors are responsible for slower synaptic events. The mGluRs have been traditionally divided into groups I (mGluR1 and mGluR5), II (mGluR2 and mGluR3), and III (mGluR4, mGluR6-mGluR8) according to their sequence similarities, signal transduction mechanism, and selectivity of drug.[4] While a role of iGluRs in nociception is well established, studies of the modulation of nociception by mGluRs have yielded differing results. It has been shown that intrathecally administered group I mGluRs antagonist had no effect or reduced nociceptive behaviors during phase 1 and phase 2 in the formalin test.[5,6]

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Intrathecal group I mGluRs agonist and antagonoist enhanced or decreased phase 2 response, respectively, without affecting phase 1 response.[7] Intrathecal group I mGluRs antagonist attenuated carrageenan-induced hyperalgesia.[8] Systemic and intrathecal mGluR5 antagonist was effective or against for Freund's complete adjuvant-induced hyperalgesia.[9,10] On the other hand, systemic and intrathecal group II mGluRs agonist decreased or increased phase 2 response in the formalin test with lack of effect on phase 1 response.[7,11] And also intrathecal group II mGluRs agonist attenuated carrageenan-induced hyperalgesia.[8] Furthermore, mGluR5 antagonist and group II mGluRs agonist had no significant effects on acute nociceptive stimuli.[9,11,12] Additionally, intrathecal group III mGluRs agonist slightly reduced just phase 2 response in the formalin test, inhibited the responses to cutaneous mechanical stimuli and suppressed synaptic transmission.[7,12-14] The effects of group II and III mGluRs antagonists have not been determined in pain model.

Morphine acts via a number of central nervous system sites, including the spinal cord, where presynaptic and, to a lesser extent, postsynaptic μ-opioid receptors modulate nociceptive transmission. It has been shown that intrathecal morphine suppressed the formalin- and thermal-induced nociception.[15,16] The above findings suggest that mGluRs compounds and morphine may have a different profile for the regulation of the nociception. Understanding the functional role of these receptors in altered spinal nociception may help provide novel targets for the therapy of pain. Moreover, there is little information or data about the pattern of their interaction.

Therefore, the aim of the present study was to delineate the involvement of each of the three groups of mGluRs for the nocieptive stmuli. In addition, the author sought to determine the characteristics of the drug interaction between intrathecal mGluRs compounds and morphine.

MATERIALS AND METHODS

1. Animal Preparation

The studies were reviewed and approved by the Institutional Animal Care Committee, Research Institute of Medical Science, Chonnam National University.

Male Sprague-Dawley rats (250−300 g) were used. The rats were maintained on a 12 h night/day cycle and allowed free access to food and water at all times. For drug administration, an intrathecal catheter was implanted during enflurane anesthesia, as previously described.[17] The catheter was advanced caudally by 8.5 cm through an incision in the atlantooccipital membrane to the lumbar enlargement. The external end of the catheter was tunneled subcutaneously and exited at the top of head and plugged with a piece of steel wire. The skin was closed with 3−0 silk sutures. After surgery, rats were kept in individual cages. Only rats that displayed no postsurgical motor or sensory deficits were used. Animals showing neurologic dysfunction postoperatively were killed immediately. Studies were performed at least 4−5 days following intrathecal catheterization.

2. Drugs

The following drugs were used in this study: t-ADA (Tocris Cookson Ltd., Bristol, UK), LY 367385 (Tocris), 2-methyl-6-(phenylethynyl)-pyridine (MPEP, Tocris), APDC (Tocris), LY 341495 (Tocris), ACPT-III (Tocris), UBP1112 (Tocris), morphine sulfate (Research Biochemical Internationals [RBI], Natick, USA). t-ADA, LY 367385, LY 341495 and UBP1112 were dissolved in NaOH. MPEP was dissolved in 20% dimethylsulfoxide (DMSO). APDC, ACPT-III and morphine were dissolved with normal saline. Intrathecal administration of these agents was performed using a hand-driven, gear-operated syringe pump. All drugs were delivered in a volume of 10 μl solution.

3. Nociceptive Test

For the formalin test, 50 μl of 5% formalin solution was injected subcutaneously into the plantar surface of the hind paw using a 30 gauge needle. The formalin injection produces characteristic pain behavior; biphasic flinching/shaking of the injected paw. Such pain behavior was therefore quantified by periodically counting the incident of spontaneous flinching/shaking of the injected paw. The number of flinching was counted for 1 min periods at 1 and 5 min and at 5 min intervals from 10 to 60 min. Two phases of spontaneous flinching were observed after the formalin injection. Phase 1 and phase 2 were defined as 0-9 and 10−60 min after formalin injection, respectively. After the observation period of 1 hr, the animals were immediately killed.

A modified Hargreaves-type thermal testing device was used to evaluate the effect of drugs on acute nociception.[18,19] In brief, the animal was placed in a clear plastic cage on an elevated glass surface with a radiant heat source located beneath the surface. The temperature of the surface was maintained at 30°C throughout the experiment. Activation of the stimulus simultaneously activated a timer. Both bulb and timer were turned off by paw withdrawal or after 20 sec (cutoff time). After habituation for 15−20 min, a measurement was taken for each hindpaw to determine an average baseline latency. The response latency was determined by exposing the plantar surface of the hindpaw to radiant heat. The mean of the response latencies from each paw

was taken as the latency. The withdrawal response latency was measured 15, 30, 60, 90, and 120 min after injection

4. Experimental Paradigm

Four to five days after surgery, rats were placed in a restraint cylinder for the experiment. After a 15−20 min adaptation, rats were then assigned to one of the drug treatment groups. The control study was done using intrathecal saline, DMSO or NaOH depending on the solvent for experimental drug. Each animal was used in one experiment only. The total number of rats used was 194 and the number of rat per group was 6−9. The investigator was unaware of which drug was administered into each animal.

5. Effects of Intrathecal t-ADA, LY 367385, MPEP, APDC, LY 341495, ACPT-III, UBP1112 and Morphine

The effects of group I mGluRs agonist (t-ADA, 100 μg), group I mGluRs antagonists (mGlu1a: LY 367385, 200 μg; mGlu5: MPEP, 300 μg), group II mGluRs agonist (APDC, 100 μg), group II mGluRs antagonist (LY 341495), group III mGluRs agonist (ACPT-III, 10 μg), group III mGluRs antagonist (UBP 1112, 200 μg) and morphine were investigated in the formalin test. Intrathecal drugs were injected 10 min before formalin injection. Each ED_{50} value (effective dose producing a 50% reduction of control formalin response) of agents was separatedly determined in two phases.

6. Drug Interaction

An isobolographic analysis[20] was used to determine of the nature of pharmacologic interaction between mGluRs compounds and morphine in the formalin test. Because mGluRs compounds did not produce an antinociceptive effect during phase 1, an isobolographic analysis was performed during phase 2.

This method is based on comparisons of doses that are determined to be equieffective. At first, each ED_{50} value was determined from the dose-response curves of agents alone. Next, mGluRs compounds and morphine were intrathecally coadministered at a dose of the ED_{50} values and fractions (1/2, 1/4, 1/8) of ED_{50} of each drug. From the dose-response curves of the combined drugs, the ED_{50} values of the mixture were calculated and these dose combinations were used for plotting the isobologram. In this experiment, the isobolograms were undertaken to characterize the effect of LY 341495- morphine combination. The isobologram was constructed by plotting the ED_{50} values of the single agents on the X and Y axes, respectively. The theoretical additive dose combination was calculated. From the variance of the total dose, individual variances for the agents in the combination were obtained. Furthermore, to describe the magnitude of the interaction, a total fraction value was calculated.

$$\text{Total fraction value} = \frac{ED_{50} \text{ of drug 1 combined with drug 2}}{ED_{50} \text{ for drug 1 given alone}} + \frac{ED_{50} \text{ of drug 2 combined with drug 1}}{ED_{50} \text{ for drug 2 given alone}}$$

The fraction values indicate what portion of the single ED_{50} value was accounted for by the corresponding ED_{50} value for the combination. Values near 1 indicate additive interaction, values greater than 1 imply an antagonistic interaction and values less than 1 indicate a synergistic interaction. The mixture was delivered intrathecally 10 min before the formalin test.

7. General Behavior

For evaluation of behavioral change of mGluRs compounds and morphine, additional rats received the highest doses of agents used here, and examined at 5, 10, 20, 30, 40, 50 and 60 min after intrathecal administration. Motor function was assessed by the righting reflex and placing-stepping reflex. The former was evaluated by placing the rat horizontally with its back on the table, which normally gives rise to an immediate coordinated twisting of the body to an upright position. The latter was evoked by drawing the dorsum of either hind paw across the edge of the table. Normally rats try to put the paw ahead into a position to walk. Changes in motor function were scored as follows: 0, normal; 1, slight deficit; 2, moderate deficit; 3, severe deficit. Pinna reflex and corneal reflex were also evaluated and judged as present or absent.

8. Statistical Analysis

Data are expressed as means ± SEM. In the formalin test, the time response data or the dose-response data are presented as the number of flinching or as the sum of flinches in each phase. To calculate the ED_{50} values of each drug, the number of flinching was converted to percentage of control as follows: % of control = [(sum of phase 1 or 2 flinching count with drug)/(sum of control phase 1 or 2 flinching count)] × 100. In the thermal test, the time response data or the dose-response data are presented as the withdrawal latency or as %MPE (percent maximum possible effect). The withdrawal response latency was converted to %MPE as follows: %MPE = [(postdrug latency-baseline latency)/(cutoff time-baseline latency)] × 100 and the ED_{50} value was calculated by a computer program.

Dose-response data were analyzed by one-way analysis of variance ANOVA with Scheffe for post hoc. The dose-response lines were fitted using least-squares linear regression and ED_{50} and its 95% confidence intervals were calculated according to the method described by Tallarida and Murray.[21]

The difference between theoretical ED_{50} and experimental ED_{50} was analyzed by *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

No change of pinna reflex, corneal reflex, and motor function was seen after intrathecal administration of mGluRs compounds and morphine.

The sum of the number of flinching in saline, DMSO or NaOH control group was not statistically different from each other in both phases (saline: DMSO: NaOH; 17 ± 1: 17 ± 1: 14 ± 1 in phase 1, 141 ± 7: 145 ± 8: 134 ± 18 in phase 2). The baseline withdrawal latency was 6.2 ± 0.1 sec and did not differ in experimental groups.

Intrathecal group I mGluRs agonist (t-ADA), group I mGluRs antagonists (mGlu1a: LY 367385, mGlu5: MPEP), group II mGluRs agonist (APDC), group III mGluRs agonist (ACPT-III) and group III mGluRs antagonist (UBP1112) did not suppress flinching response in both phases in the formalin test (Fig. 1−3).

Group II mGluRs antagonist (LY 341495) resulted in dose-dependent inhibition of the phase 2 response without affecting phase 1 response or withdrawal latency of thermal stimulus (Fig. 4, 5). Intrathecal morphine dose-dependently increased the withdrawal latency (Fig. 5) and attenuated flinching response in both phases (Fig. 6). The phase 2 ED_{50} values (95% confidence intervals) of LY 341495 and morphine were 46.2 (22.3−95.5) and 3.9 μg (2.2−7.1 μg), respectively. The ED_{50} values (95% confidence intervals) of morphine for phase 1 and the thermal stimulus were 8.7 (5.1−14.8) and 8.4 μg (6.5−11 μg).

Isobolographic analysis revealed a synergistic interaction between intrathecal LY 341495 and morphine during phase 2 in

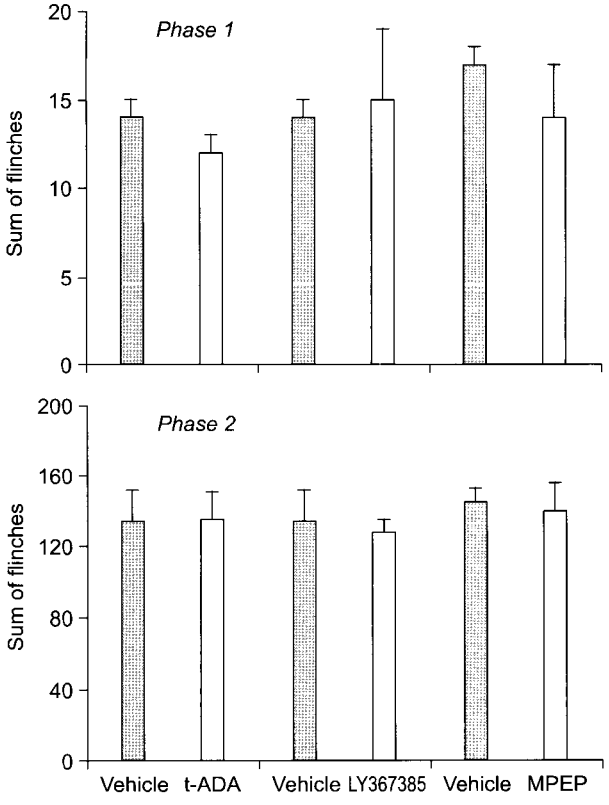

Fig. 1. Effect of intrathecal t-ADA (100 μg), LY 367385 (200 μg) and MPEP (300 μg) for flinching during phase 1 and phase 2 in the formalin test. The drug was administered 10 min before formalin injection. Data are presented as the sum of the number of flinches. Each bar represents the mean ± SEM of 5−8 rats.

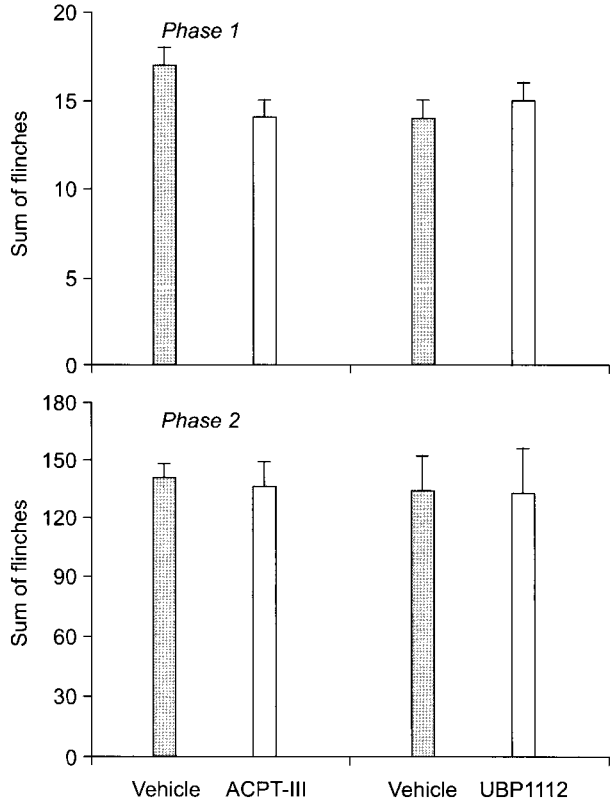

Fig. 2. Effect of intrathecal ACPT-III (10 μg) and UBP1112 (200 μg) for flinching during phase 1 and phase 2 in the formalin test. The drug was administered 10 min before formalin injection. Data are presented as the sum of the number of flinches. Each bar represents the mean ± SEM of 5−7 rats.

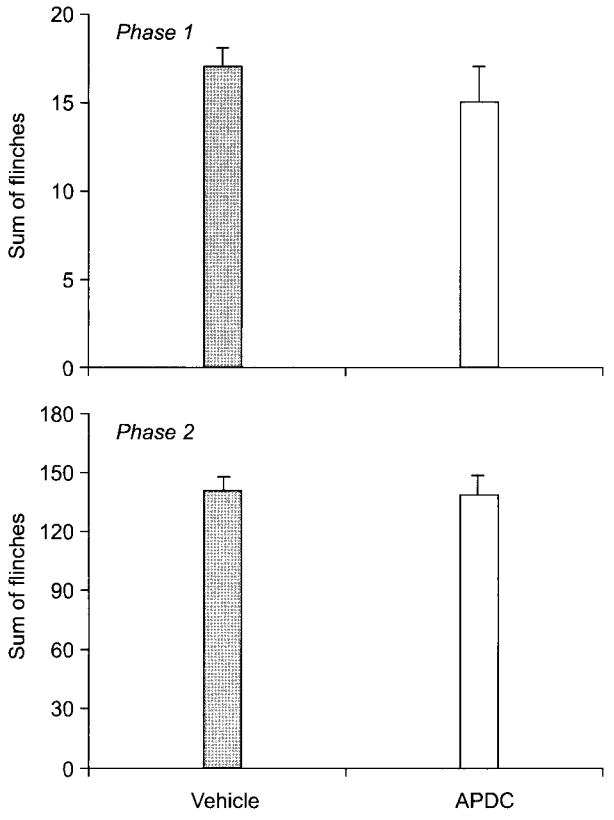

Fig. 3. Effect of intrathecal APDC (100 μg) for flinching during phase 1 and phase 2 in the formalin test. The drug was administered 10 min before formalin injection. Data are presented as the sum of the number of flinches. Each bar represents the mean ± SEM of 5 −7 rats.

the formalin test. The experimental ED_{50} value was significantly lower than the calculated ED_{50} value. Accordingly, the phase 2 ED_{50} value (95% confidence intervals) of LY 341495 in the mixture of LY 341495 and morphine was 6.3 μg (2.8−13.9 μg). The total fraction value of the mixture of LY 341495 and morphine was 0.25 in phase 2, indicating a synergistic interaction.

DISCUSSION

In the current study, intrathecal LY 341495 decreased the flinching response during phase 2, but not during phase 1 in the formalin test. No antinociceptive effect of LY 341495 was observed in the thermal stimulus. The other mGluRs compounds did not affect formalin-induced nociceptive behavior. These findings suggest that just group II mGluRs (mGluR2 and mGluR3) are involved in the processing of formalin-induced nociception at the level of the spinal cord. Of particular interest was that blockade of group II mGluRs was effective only for

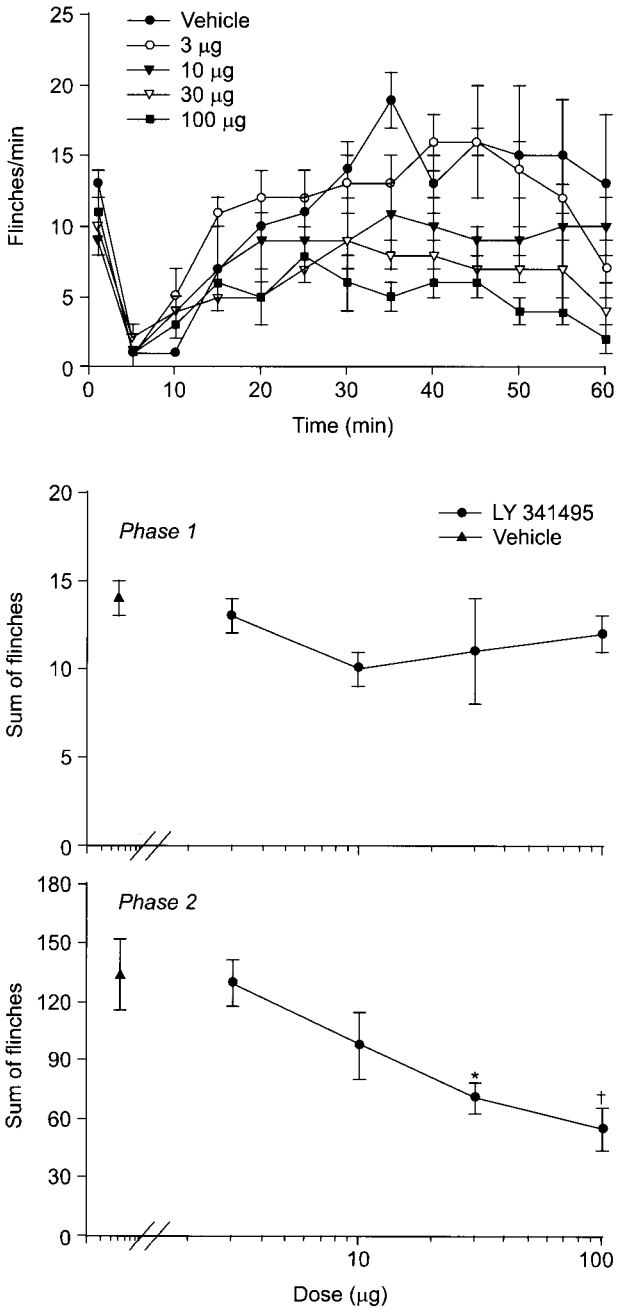

Fig. 4. Time effect curve (top) and dose response curve (middle and bottom) of intrathecal LY 341495 for flinching in the formalin test. Drug was administered 10 min before formalin injection. Data are presented as the number of flinches or the sum of flinches. LY 341495 dose-dependently decreased flinches during phase 2, but not phase 1. Each line represents the mean ± SEM of 5−7 rats. Compared with vehicle, *$P < 0.05$. †$P < 0.01$.

phase 2 response of the formalin stimulus, which suggests that group II mGluRs are active in the facilitated processing without affecting acute nociception. No effect of LY 341495 in the thermal test also indicates that group II mGluRs do not play a

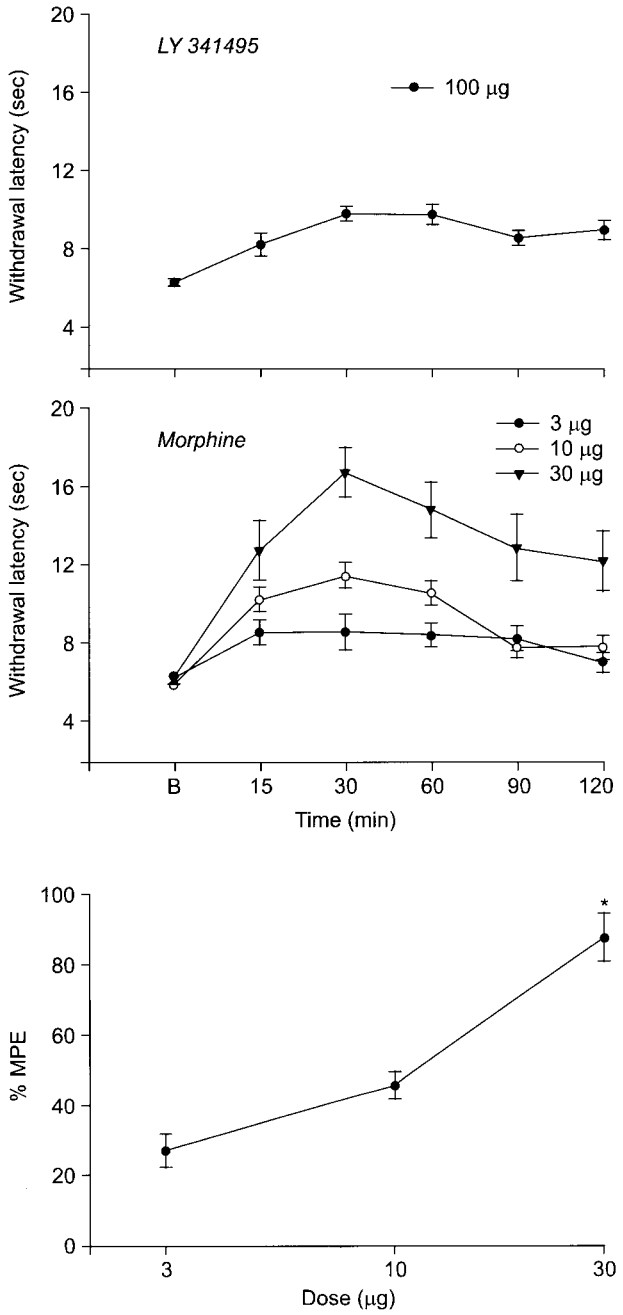

Fig. 5. Time effect curve (top and middle) and dose response curve (bottom) of intrathecal LY 341495 and morphine on thermal stimulation in rats. Each line represents the mean ± SEM of 5−7 rats. % MPE: percent maximum possible effect. Compared with 3 μg. *P < 0.001.

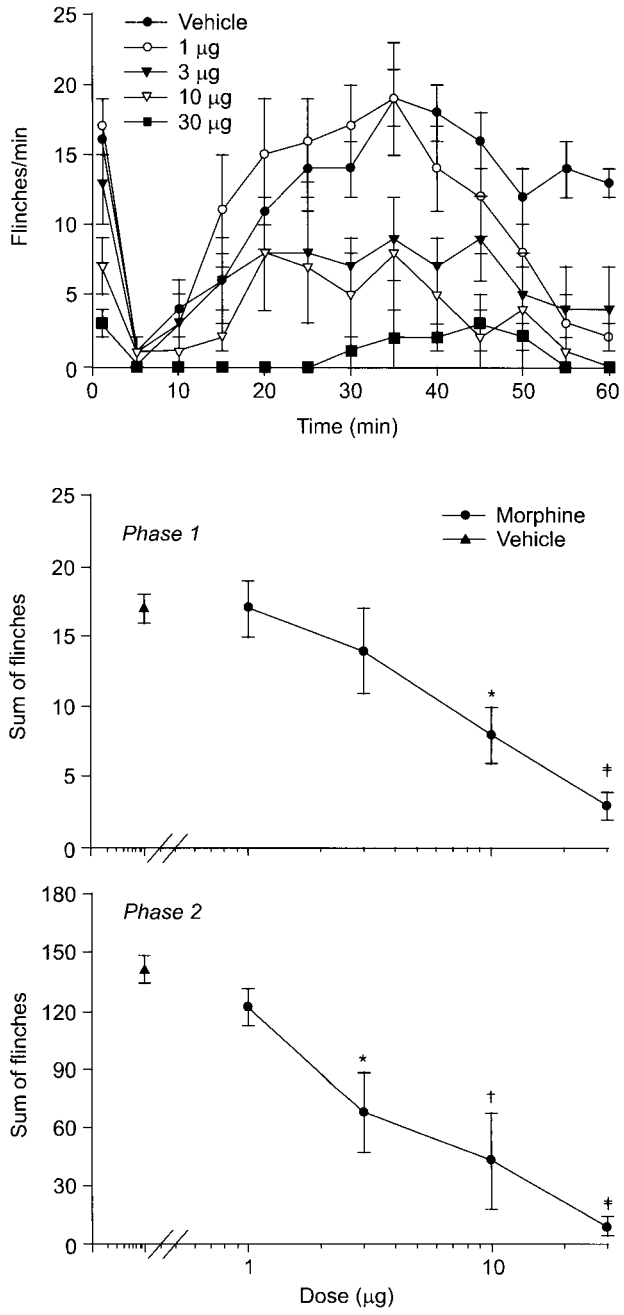

Fig. 6. Time effect curve (top) and dose response curve (middle and bottom) of intrathecal morphine for flinching in the formalin test. Drug was administered 10 min before formalin injection. Data are presented as the number of flinches or the sum of flinches. Morphine produced a dose-dependent suppression of flinches in both phases. Each line represents the mean ± SEM of 5−7 rats. Compared with vehicle, *P < 0.05. †P < 0.01, ‡P < 0.001.

crucial role in modulating acute pain.

In the formalin test, phase 1 response seems to result from the immediate and intense increase of primary afferent activity. On the other hand, phase 2 response reflects the activation of wide dynamic range of dorsal horn neurons with very low level of ongoing activity of primary afferent. Therefore, phase 2 reflects a facilitated state which appears to be prominent, considering the decreased level of afferent input.

Glutamate has long been recognized to play a significant role in the processing of nociceptive information in the spinal cord.[2] The actions of glutamate are mediated either through interaction

with iGluRs or by G protein-coupled mGluRs.[3] To date, eight mGluRs subtypes have been identified, which can be classified into three subgroups based on their sequence similarities and transduction mechanisms.[4] Expression of group I and II mGluRs mRNA has been identified in spinal cord,[22,23] and immunoreactivity detected pre- and postsynaptically in superficial dorsal horn, an area intimately associated with nociceptive processing.[24-27] The group III mGluRs are expressed in the dorsal horn of the spinal cord.[23,28-31] Group I mGluRs are coupled to phospholipase C, which stimulates the production of inositol trisphosphate (IP_3) and diacylglycerol (DAG).[32] DAG, in turn, activates protein kinase C, which has been shown to contribute significantly to the development of pain.[33,34] Hence, blocking agent for group I mGluRs may suppress the nociceptive state. On the other hand, group II and group III mGluRs couple to inhibition of adenylate cyclase, thereby producing the antinociceptive effect.[32] Therefore, it could be supposed that antagonist for group I mGluRs and agonist for group II and III mGluRs attenuate the nociceptive state. However, the results of current study were unanticipated. Interestingly, only group II mGluRs antagonist displayed the antinociceptive effect for the facilitated state, but other mGluRs compounds failed to affect nociceptive behavior. Although some data of this study were consistent with previous findings, others were not. We did not assess the basis for the difference in this experiment, such discrepancy may be caused by the use of kinds of animal, difference of drug and dose, injection site, the concentration of formalin solution and the nociceptive test. mGluRs compounds (t-ADA, LY 367385, MPEP, APDC, LY 341495 and UBP1112) were not soluble at higher doses than those used in this study. ACPT-III caused a motor dysfunction above 30 μg. Hence, the highest doses of mGluRs compounds administered in the present study were regarded as the maximal doses. Furthermore, considering that group II mGluRs agonist enhanced formalin-induced nociception in second phase, it is possible that spinal activation of group II mGluRs produces nociceptive effect.[7] Nociceptive effect of group II mGluRs agonist may be due to a presynaptically-mediated reduction in inhibition due to modulation of the GABAergic system or increase of neuronal excitability of the spinal cord.[35,36] Therefore, antagonist for group II mGluRs could block the nociceptive state, which was supported by our results. On the other hand, intrathecal morphine reduced the flinching response in both phases and increased the withdrawl latency in the present study, which is in agreement with previous results.[12,13] Therefore, opioid receptors are involved in the modulation of acute pain as well as the facilitated state.

Isobolographic analysis of this study revealed the synergistic interaction between intrathecal LY 341495 and morphine during phase 2 in the formalin test. These results indicate that spinal combination of LY 341495 with morphine is able to augment the antinociceptive effect of each drug alone, in the facilitated state evoked by formalin injection. Although a pharmacological interaction between two kinds of drugs is most likely complicated to characterize, several explanations could be possible for this synergy. First, drugs may interact by altering the kinetics of each other. One agent may alter the actions of the other agents at the receptor or channel. Second, such interaction may occur when both drugs affect different critical points along a common pathway.[37] Group II mGluRs and opioid receptors act on receptors which are G-proteins coupled. Hence, the action of LY 341495 and morphine may independently alter intracellular second messenger systems coupled with G-proteins activation and mediate a synergistic interaction.[38] Finally, functional interaction may result from distinct drug effects at separate anatomic sites that may act independently as well as together to inhibit spinal nociceptive processing.[39] As above mentioned, group II mGluRs and morphine located in both pre- and postsynaptic action. Therefore, simultaneous engagement of pre- and postsynaptic mechanisms may augment the antinociceptive action produced by either drug acting at one site independently.[40]

Clinically, spinal group II mGluRs antagonists have not been available, yet. However, in future they can be used with morphine in treatment of pain, because the combination with morphine may provide a decreased dose of either drug or an increased maximum achievable.

Taken together, intrathecal LY 341495 and morphine reduce the facilitated state evoked by formalin stimulus and LY 341495 interacts with morphine in a synergistic fashion.

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