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Antinociceptive Effect of the Intrathecal Phosphodiesterase Inhibitor, Zaprinast, in a Rat Formalin Test

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= **Abstract** =

Background: Cyclic guanosine monophosphate (cGMP) and opioid receptors are involved in the modulation of nociception. Although the opioid receptors agonists are active in pain, the effect of an phospodiesterase inhibitor (zaprinast) for increasing the level of cGMP has not been thoroughly investigated at the spinal level. This study examined the effects of intrathecal zaprinast and morphine in a nociceptive test and we also examined the nature of the pharmacological interaction after the coadministration of zaprinast with morphine. The role of the nitric oxide (NO)-cGMP-potassium channel pathway on the effect of zaprinast was further clarified.

Methods: Catheters were inserted into the intrathecal space of male SD rats. For the induction of pain, 50 μl of 5% formalin solution was applied to the hindpaw. Isobolographic analysis was used for the evaluation of the drug interaction between zaprinast and morphine. Furthermore, NO synthase inhibitor (L-NMMA), guanylyl cyclase inhibitor (ODQ) or a potassium channel blocker (glibenclamide) were intrathecally administered to verify the involvement of the NO-cGMP- potassium channel pathway on the antinociception effect of zaprinast.

Results: Both zaprinast and morphine produced an antinociceptive effect during phase 1 and phase 2 in the formalin test. Isobolographic analysis revealed a synergistic interaction after the intrathecal administration of the zaprinast-morphine mixture in both phases. Intrathecal L-NMMA, ODQ and glibenclamide did not reverse the antinociception of zaprinast in either phase.

Conclusions: These results suggest that zaprinast, morphine and the mixture of the two drugs are effective against acute pain and they facilitated pain state at the spinal level. Thus, the spinal combination of zaprinast with morphine may be useful for the management of pain. However, the NO-sensitive cGMP-potassium channel pathway did not contribute to the antinocieptive mechanism of zaprinast in the spinal cord. **(Korean J Pain 2005; 18: 99-106)**

Key Words: antinociception, interaction, morphine, NO-cGMP-potassium channel pathway, spinal cord, zaprinast.

INTRODUCTION

It has been proposed that cyclic guanosine monophosphate (cGMP) is involved in antinociception.[1-6] Guanylyl cyclase catalyzes the formation of cyclic GMP from GTP, leading to the synthesis of cGMP, whereas cyclic GMP-specific phosphodiesterase catalyzes the hydrolysis of cGMP to GMP, thereby ending signal transduction.[7] Therefore, intracellular cGMP concentrations are regulated by the action of guanylyl cyclase and the rate of degradation by cGMP-specific phosphodiesterase.[7,8] Additionally, guanylyl cyclase is activated by nitric oxide (NO) playing an important role in various physiological functions such as neurotransmission.[9] Recent studies indicate that NO modulates synaptic transmission in the nervous system by acting as a retrograde messenger.[10,11] NO is produced within the nervous system from L-arginine by NO synthase and increased NO production has been shown to produce antinocoieption.[12,13] The NO-cGMP signaling pathway participates in the antinociceptive mechanism of several drugs.[2,3,5,6,12-15]

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It has been reported that potassium channels are also involved in the peripheral antinociceptive effect of diclofenac, morphine and ketorolac.[16-19] And the activation of potassium channels might enhance the antinociceptive effects of fentanyl, clonidine and bethanechol at the spinal level.[20] Furthermore, it has been suggested that potassium channels are opened as a result of the activation of the L-arginine-NO-cGMP pathway.[18,19]

Zaprinast is a novel inhibitor of cGMP-specific phosphodiesterase 5, 6 and 9. Local injection of zaprinast does not affect the prostaglandin E_2 or carrageenan-induced hyperalgesia.[21,22] However, there has been little data regarding the effect of zaprinast in a nociceptive state at the spinal level.

The objective of the present study was to investigate the effect of zaprinast on a rat formalin-induced nociception in the spinal cord and also to determine the characteristics of the drug interaction between zaprinast and morphine. Finally, I sought to examine the sequential participation of NO and cGMP synthesis followed by potassium channel opening relative to the effect of intrathecal zaprinast.

MATERIALS AND METHODS

1. Animal Preparation

The Institutional Animal Care Committee, Research Institute of Medical Science, Chonnam National University approved all of the experimental procedures.

Adult male Sprague-Dawley rats weighing 250−300 g were used in all experiments. The animals were housed in groups of four, with free access to standard rat diet and tap water in a room under 12 : 12 h light/dark cycle. For the purpose of drug administration, an intrathecal catheter was implanted under enflurane anesthesia and aseptic surgical conditions as described previously.[23] A polyethylene-10 tube was inserted into the subarachnoid space through a slit made in the atlantooccipital membrane. The catheter was advanced caudally 8.5 cm to reach the level of the lumbar enlargement. The external end of the catheter was tunneled subcutaneously, exiting at the top of the head and plugged with a piece of steel wire. The skin was closed using 3-0 silk sutures. After catheter implantation, rats were housed in individual cages. All animals with neurological deficit postoperatively were rejected from further study and killed immediately with an overdose of volatile anesthetics. At least 5 days of postsurgical recovery were allowed before the behavioral study.

2. Drugs

The following drugs were used in this study: zaprinast (Tocris Cookson Ltd., Bristol, UK), morphine sulfate (Sigma, St Louis, MO, USA), N^G-monomethyl-$_L$-arginine acetate ($_L$-NMMA, Sigma), 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ, Sigma) and glibenclamide (Sigma). Zaprinst, ODQ and glibenclamide were dissolved in dimethylsulfoxide (DMSO). The remaining drugs were dissolved in 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

Antinociception was assessed with the formalin test. The rats were subcutaneously injected into the plantar surface of the hind paw with 50 μl of 5% formalin solution using a 30 gauge needle. The formalin injection produces specific pain behavior readily discriminated and characterized as rapid and brief withdrawal or flexing of the injected paw. This behavior was called a flinching response. Such pain behavior was therefore quantified by periodically counting the number of flinches of the injected paw after injection. The number of flinches was counted for 1 min periods at 1 and 5 min and at 5 min intervals from 10 to 60 min. Formalin-induced flinching behavior is biphasic. The initial acute phase (0−9 min) is followed by a relatively short quiescent period, which is then followed by a prolonged tonic response (10−60 min). At the end of the experiment, the rats were killed with volatile anesthetics.

4. Experimental Paradigm

On the day of experiments, the rats were placed in a restraint cylinder and allowed to adapt for 20 min. Rats were then allocated to receive one of the experimental drugs. The control study was done using intrathecal DMSO or saline, depending on the solvent for the agents. Animals were tested only once. The total number of rats used was 135 with 4−7 rats per group. The investigator responsible for assessing behavioral testing was blind to the drugs given to each animal.

5. Effects of Zaprinast and Morphine

The rats received intrathecal saline and increasing doses of either zaprinst (10, 30 and 100 μg) or morphine (1, 3, 10 and 30 μg) 10 min before formalin injection, and the effects of zaprinast and morphine were examined. Each ED_{50} value (effective dose producing a 50% reduction in control formalin response) for the agents was calculated separately in two phases. Moreover, zaprinast was intrathecally delivered 9 min after formalin injection to assess the effect of posttreatment

with zaprinast (100 μg).

6. Drug Interaction

Isobolographic analysis[24] was used to determine the nature of pharmacologic interaction between zaprinast and morphine in the formalin test. This method is based on the comparison of doses determined to be equieffective. At first, each ED_{50} value was determined from the dose-response curves of agents alone. Next, zaprinast and morphine were intrathecally coadministered at a dose calculated using the ED_{50} values and fractions (1/2, 1/4 and 1/8) of ED_{50} for each drug. The ED_{50} values of the mixture were calculated from the dose-response curves of the combined drugs, and the combinations were used to plot the isobologram. In this experiment, the isobolograms were used to express the effect of the zaprinast-morphine combinations. An 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 then 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 an 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. Mechanisms of Zaprinast

In order to determine the nature of the L-arginine NO-cGMP-potassium channel pathway's participation in the antinociceptive effect of zaprinast, rats were pretreated with L-NMMA (NO synthase inhibitor), ODQ (guanyly cyclase inhibitor) or glibenclamide (potassium channel blocker). These three drugs were intrathecally administered 10 min before intrathecal zaprinast (100 μg) or a combination of zaprinast with morphine (each ED_{50} value in both phases), respectively, and formalin was injected 10 min later. The maximal doses of L-NMMA (10 μg), ODQ (4 μg) and glibenclamide (3 μg) were selected based on their lack of significant effect on the control formalin response from the pilot experiments.

8. General Behavior

In order to evaluate the behavioral changes of zaprinast and morphine, additional rats (n = 10) received the highest doses of agents used, and were examined 5, 10, 20, 30, 40, 50 and 60 min after intrathecal administration. Motor functions were assessed by examining the righting and placing-stepping reflexes. 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 their paws forward into a position for walking. Changes in motor functions were scored as: 0, normal; 1, slight deficient; 2, moderate deficient; and 3, severely deficient. Pinna and corneal reflexes were also evaluated and judged as present or absent.

9. 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 flinches or as percentage of control in each phase. To calculate the ED_{50} values for each drug, the number of flinches were converted to percentage of control: % of control = ([sum of phase 1 or 2 flinching count with drug) / (sum of control phase 1or 2 flinching count]) × 100.

Dose-response data were analyzed by one-way analysis of variance 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.[25] The difference between theoretical ED_{50} and experimental ED50 and the antagonism with regard to the effect of the mixture of zaprinast and morphine were analyzed by *t*-test, with $P < 0.05$ being considered statistically significant.

RESULTS

Pinna, corneal reflexes and motor functions were normal after intrathecal delivery of zaprinst, morphine and the mixture of both drugs with the doses used in this study. No other side effects were observed in either the control or the treated group.

In control groups, the sum of the number of flinches did not differ from each other in both phases (saline: DMSO; 18

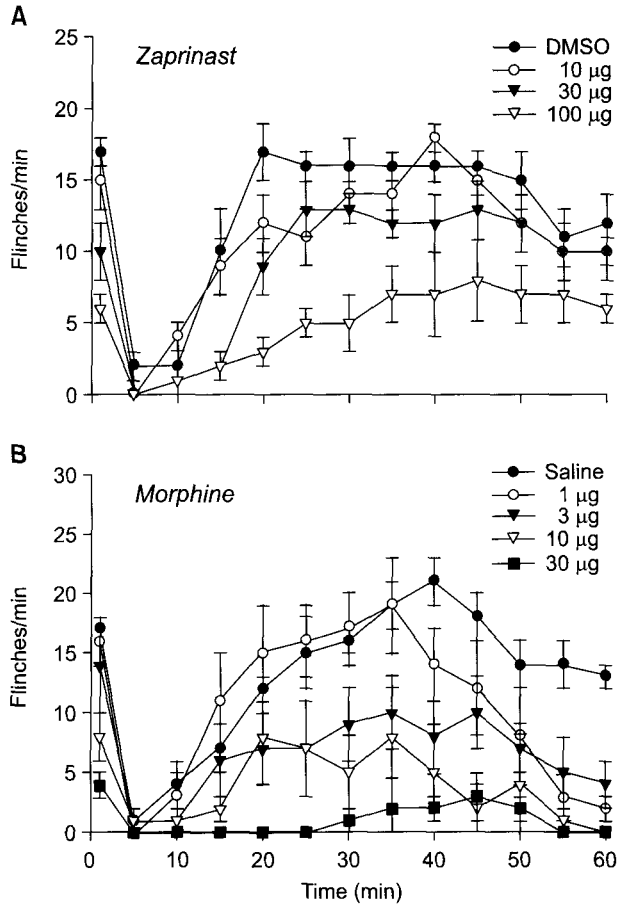

Fig. 1. Time effect curve of intrathecal zaprinast (A) and morphine (B) for flinching in the formalin test. Each drug was administered 10 min before the formalin injection. Formalin was injected at time 0. Data are presented as the number of flinches. Each line represents the mean ± SEM of 5−7 rats.

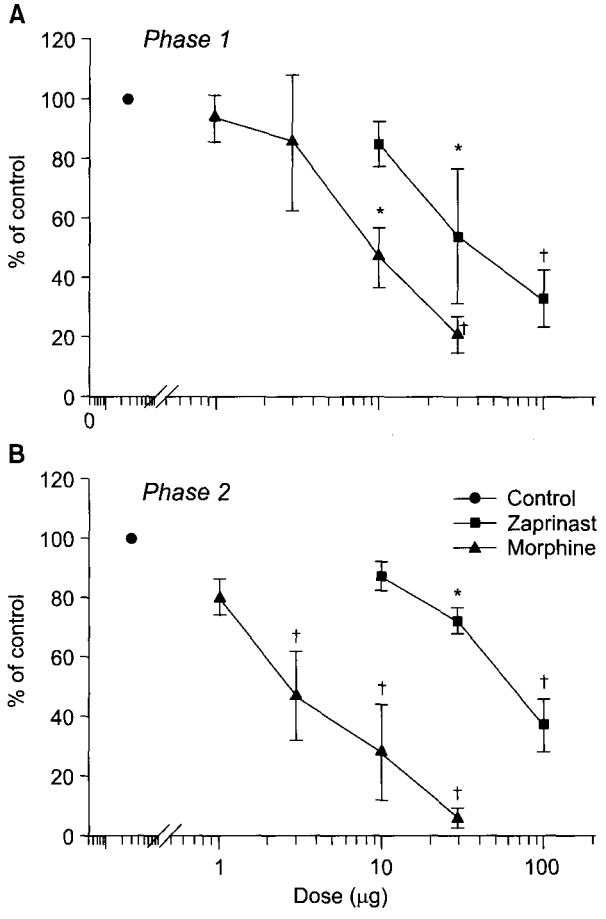

Fig. 2. Dose response curve of intrathecal zaprinast and morphine for flinching in the formalin test. Data are presented as the percentage of control. Zaprinast and morphine produced a dose-dependent inhibition of flinches in phase 1 (A) and phase 2 (B). Each line represents the mean ± SEM of 5−7 rats. Compared with control, *P < 0.01, †P < 0.001.

± 2: 18 ± 1 in phase 1, 152 ± 7: 147 ± 7 in phase 2).

Fig. 1 displays the time course of intrathecal zaprinast and morphine, administered 10 min before formalin injection. Intrathecal zaprinast and morphine resulted in the dose-dependent inhibition of the flinching response during phase 1 and phase 2 in the formalin test (Fig. 2). The phase 1 ED_{50} values (95% confidence intervals) of zaprinast and morphine were 43.8 (23.8−80.8) and 9.5 μg (4.3−21.1 μg), respectively. The ED_{50} values (95% confidence intervals) of zaprinast and morphine for phase 2 were 62.2 (38.6−62.2) and 3.4 μg (1.8−6.5 μg), respectively. Posttreatment with intrathecal zaprinast reduced the flinching behavior during phase 2 (Fig. 3).

Isobolographic analysis revealed a synergistic interaction between intrathecal zaprinast and morphine during phase 1 and 2 in the formalin test (Fig. 4). The experimental ED_{50} value was significantly lower than the theoretical ED_{50} value. Accordingly, the ED_{50} values (95% confidence intervals) of zaprinast in the mixture of zaprinast and morphine for phase 1 and phase 2 were 3.8 (1.3−10.9) and 2.8 μg (0.8−9.7 μg), respectively. Each total fraction value for the mixture of zaprinast and morphine in phase 1 and phase 2 were 0.14 and 0.08, indicating a synergistic interaction.

The antinociceptive effect of zaprinast was not inhibited by intrathecal L-NMMA, ODQ or glibenclamide (Fig. 5).

DISCUSSION

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

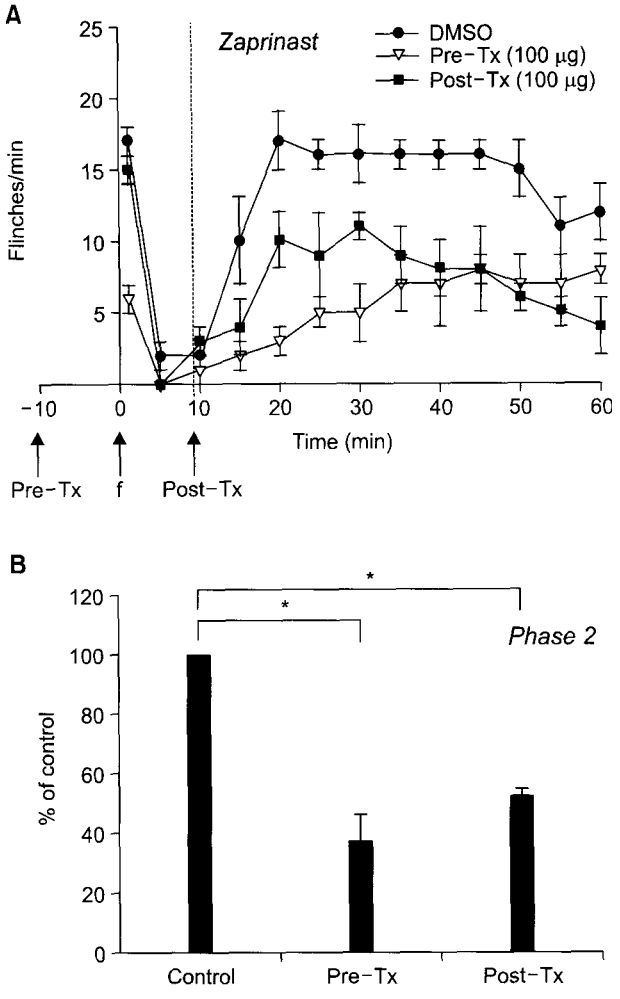

Fig. 3. Comparison of pretreatment (Pre-Tx) and posttreatment (Post-Tx) of zaprinast in the formalin test. Drugs were administered intrathecally 10 min before or 9 min after the f (formalin) injection. Data are presented as the number of flinches (A) or as the percentage of control during phase 2 (B). Each bar represents mean ± SEM of 5–6 rats. Compared with control, *P < 0.001.

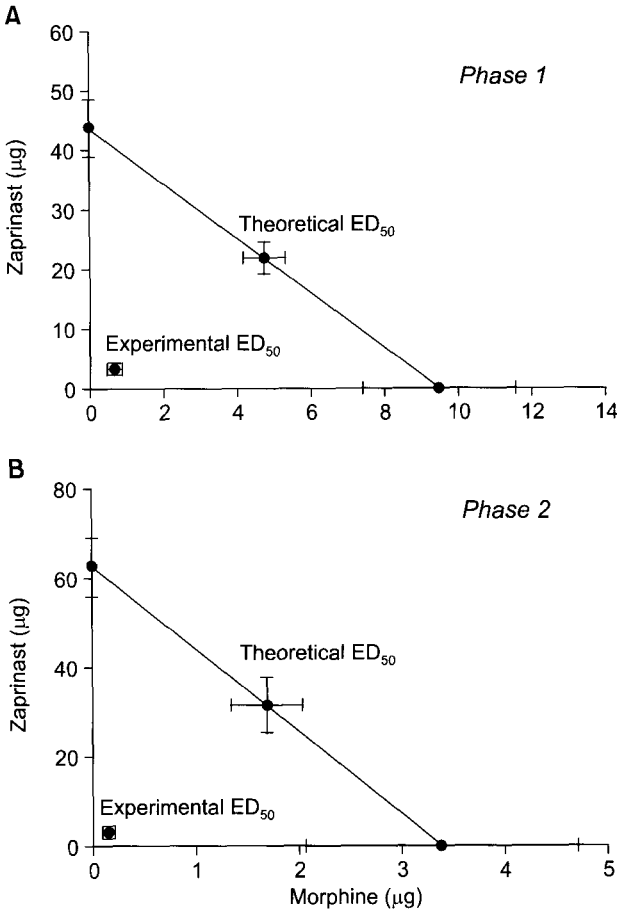

Fig. 4. Isobologram for the interaction between intrathecal zaprinast and morphine during phase 1 (A) and phase 2 (B) in the formalin test. The ED_{50} values for each agent are plotted on the x- and y-axes, respectively. Horizontal and vertical bars indicate confidence intervals. The straight line connecting each ED_{50} value is the theoretical additive line and the point on this line is the theoretical additive ED_{50}. The experimental ED_{50} point was significantly different from the theoretical ED_{50} points, indicating a synergistic interaction.

the decreased level of afferent input.

Results of the current study showed that intrathecal zaprinast, administered before the formalin injection, decreased the flinching response during phase 1 and phase 2 in the formalin test. These findings suggest a significant participation of spinal phosphodiesterase 5, 6 and 9 in the formalin-induced nociception, and the inhibition of these enzymes being effective in attenuating acute pain and the facilitated state in the spinal cord. Interestingly, posttreatment with intrathecal zaprinast also suppressed the flinching response during phase 2, suggesting that zaprinast can reduce the nociceptive response even after the initiation of nociceptive processing in the spinal cord. Intrathecal morphine, given after formalin stimulation, also decreased the second flinching response.[26] As such, there may be therapeutic uses in cases of chronic pain since we can modulate the nociception established already.

Phosphodiesterase enzymes occur widely in biological systems and are present in mammalian tissues.[27] The cyclic nucleotide phosphodiesterase is responsible for degrading the second messenger nucleotides cAMP and cGMP. To date, at least nine distinct nucleotide phospodiesterase isoenzymes (1 to 9) have been identified on the basis of their functional characteristics, such as substrate specificity, cellular distribution and susceptibility to selective inhibitors.28) It has been reported that phosphodiesterases 5, 6 and 9 are specific for cGMP.[7]

cGMP-specific phosphodiesterase catalyzes the hydrolysis of cGMP to GMP. In particular, cGMP may play a critical role in the modulation of nociception. This proposal was based on the observation that local injection of dibutyryl-cGMP produ-

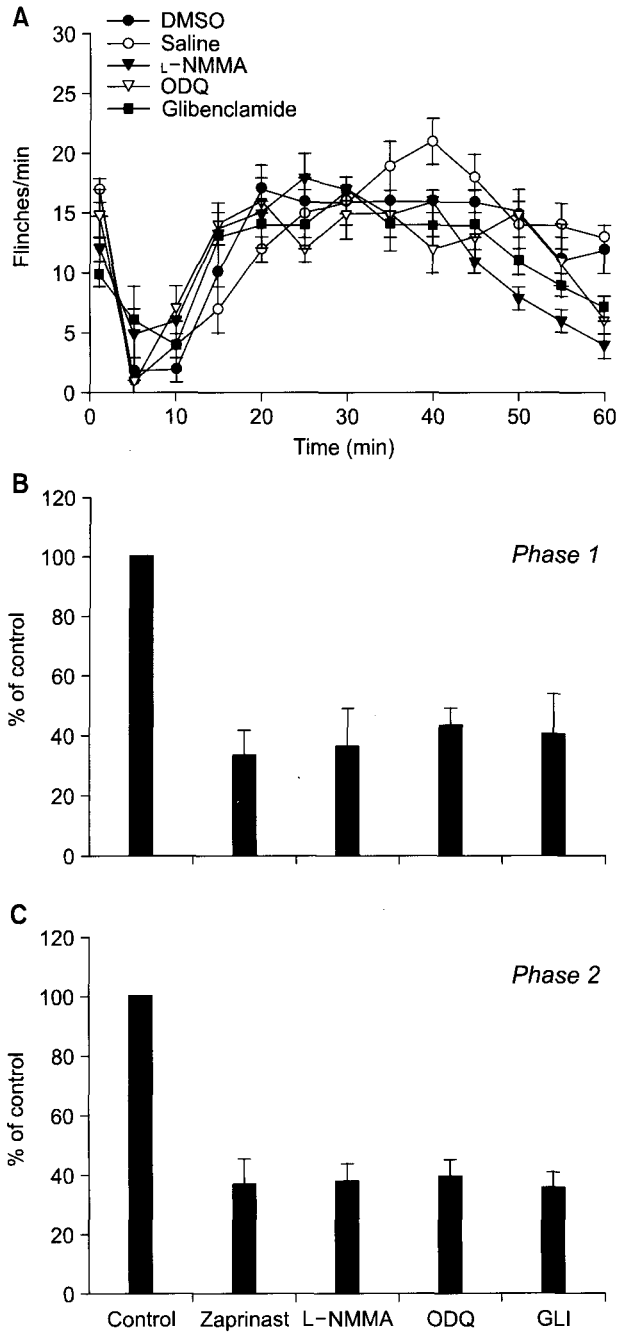

Fig. 5. The antagonistic effects of intrathecal L-NMMA (10 μg), ODQ (4 μg) and glibenclamide (GLI 3 μg) for the antinociceptive action of intrathecal zaprinast (100 μg) in the formalin test. L-NMMA, ODQ and GLI were given 10 min before zaprinast administration, and the formalin test was done 10 min after zaprinast delivery. Data are presented as the number of flinches or as the percentage of control. L-NMMA, ODQ and GLI alone did not affect the control response (A), but none of them reversed the effect of zaprinast (B, C). Each bar represents mean ± SEM of 4−6 rats.

ced antinociception in a modification of the Randall-Selitto hyperalgesia.[1] Furthermore, local sildenafil, a phosphodiesterases 5 inhibitor, caused antinociception in carrageenan-induced hyperalgesia, the writhing test and the second phase of the formalin test.[2-6] Additionally, intrathecal 8-bromo-cGMP reduced mechanical allodynia in neuropathic rats.[29] These findings suggest that inhibition of this enzyme, in turn, may increase the level of cGMP, thereby producing antinociception. However, intraplantar injection of sildenafil did not affect the phase 1 response in the formalin test.[2,4] Additionally, local zaprinast had no effect on prostaglandin E_2 or carrageenan-induced hyperalgesia.[21,22] Moreover, sildenafil did not alter the nociceptive threshold in the tail-flick and hot-plate assays.[3] Although the difference was not assessed in this experiment, this discrepancy could be due to the use of different kinds of animals, difference in drug and dosages, injection sites, the concentration of formalin solution and the nociceptive tests. As described earlier, zaprinast is an inhibitor of cGMP-specific phosphodiesterase 5, 6 and 9, but phosphodiesterase 5 seems to be the most relevant enzyme in cGMP in cells.[7,8] Therefore, it could be assumed that the effect of zaprinast in this research may have been obtained through the blockade of phosphodiesterase 5 isoenzyme. However, it is necessary to further investigate the role of phosphodiesterase 6 and 9 isoenzymes in nociceptive regulation using the selective inhibitor.

On the other hand, intrathecal morphine reduced the flinching response in both phases of the formalin test in the present study, corroborating with previous results.[30] Therefore, opioid receptors are involved in the modulation of acute pain as well as the facilitated state.

Isobolographic analysis conducted in this study revealed the synergistic interaction between intrathecal zaprinast and morphine during phase 1 and phase 2 in the formalin test. These results indicate that the spinal combination of zaprinast with morphine is able to augment the antinociceptive effect for each drug alone, in acute pain and the facilitated state evoked by formalin injection. Although a pharmacological interaction between two kinds of drugs is most likely too complicated to characterize, several explanations may be possible for this synergy. Firstly, drugs may interact by altering each others kinetics. One agent may alter the actions of the other agents at a receptor or channel. Secondly, such interaction may occur when both drugs affect different critical points along a common pathway.[31] Previous reports have shown that sildenafil-induced peripheral analgesia was mediated through the activation of the NO-cGMP pathway.[3] And morphine stimulated the synthesis of cyclic guanylyl cyclase in neuronal tissue and increased peripheral analgesia through the stimulation of the cGMP system via NO release.[14,15,32] Hence, the action of

zaprinast and morphine may independently alter the NO-cGMP pathway and mediate a synergistic interaction.[33] But such mechanism may not be likely in this case, because the effect of zaprinast was not reversed by NO synthase and guanylyl cyclase inhibitors in the spinal cord. Rather, increased cGMP, by zaprinast in a NO-insensitive manner, intensified the effect of morphine, leading to synergism.

NO is an endogenous activator of guanylyl cyclase, which results in the accumulation of cGMP.[9] cGMP constitutes a common pathway in many processes including vascular smooth muscle cell relaxation, inhibition of platelet activity, inhibition of neutrophil chemotaxis and signal transduction in the central and peripheral nervous system.[34,35] Several lines of evidence have shown that the NO-cGMP signaling pathway have played an antinociceptive role. L-Arginine and NO donors have been demonstrated to produce antinociception.[36] The antinociceptive effects of local L-arginine and NO donor were blocked by NO inhibitors and methylene blue (guanylyl cyclase inhibitor).[12,13] Pretreatment with NO synthase and guanylyl cyclase inhibitors inhibited the antinociception of local phospodiesterase 5 inhibitor.[2,3,5,6] Furthermore, the antinociceptive effects of local cyclooxygenase-2 preferential inhibitor and morphine were reduced by NO synthase and guanylyl cyclase inhibitors.[14,15] Therefore, the NO-cGMP pathway could be proposed as an antinociceptive mechanism of some drugs at the local level.

There is evidence that the opening of potassium channels is involved in antinociceptive effects produced by certain nonsteroidal anti-inflammatory drugs (NSAID), such as ketorolac and diclofenac, and by morphine.[16-19] Moreover, the effects of ketorolac and diclofenac were reverted by NO synthesis inhibitor, guanylyl cyclase inhibitor and potassium channel blockers.[18,19] Additionally, potassium channels have been involved in spinal antinociception by fentanyl, clonidine and bethanechol.[20] Also, it has been reported that glibenclamide, a potassium channel blocker, reduces the antinociceptive effect of a NO donor.[37] The above findings suggest the participation of potassium channels in antinociception, and a link between the activation of the L-arginine-NO-cGMP pathway and potassium channel opening. However, the results of the current study were different from the anticipitation. The antinociceotive effect of intrathecal zaprinast was not reversed by intrathecal L-NMMA (NO synthase inhibitor), ODQ (guanylyl cyclase inhibitor) and glibenclamide (potassium channel blocker). These observations suggest that the antinociceptive action of zaprinast may be not related to the NO-cGMP-potassium channel pathway in the spinal cord. Furthermore, the L-arginine-NO-cGMP-potassium channel pathway does not appear to be a mechanism involved in the antinociceptive effects of all drugs because the blockade of this pathway did not affect the antinociception of local indomethacin.[19] Additionally, the antinociceptive effect of intrathecal ketamine was not modified by the intrathecal NO synthase inhibitor,[38] which suggests that the activation of the NO-cGMP pathway does not contribute to the antinociception of ketamine at the spinal level.

On the other hand, in the present study, it is difficult to explain the failure of NO synthase inhibitor, guanylyl cyclase inhibitor and potassium channel blocker in inhibiting the effect of zaprinast. One possible explanation would be that an increase in intracellular cGMP content by zaprinast does not imply the participation of NO-sensitive guanylyl cyclase and subsequent potassium channel opening.

Spinal phosphodiesterase inhibitors have not yet been available in clinics. However, in the future they may be used in combination with morphine in the treatment of pain, because the combination may result in a decreased dose of either drug or an increased maximum achievable effect.

Taken together, inhibition of phospodiesterase by zaprinast, in turn, increases the level of cGMP in a NO-independent way in the spinal cord, thereby alleviating acute pain and the facilitated state evoked by injection of formalin. And zaprinast interacts with morphine in a synergistic manner. However, the activation of NO-sensitive cGMP-potassium channel pathway may not play a role in the antinociception of zaprinast at the spinal level.

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