Synergistic Effects Between Intrathecal Clonidine and Neostigmine in the Formalin Test

Spinal alpha-2 adrenoceptors and cholinergic receptors are involved in the regulation of acute nociception and the facilitated processing. The aim of this study was to examine the pharmacological effect of an intrathecal alpha-2 agonist and a cholinesterase inhibitor on the facilitated pain model induced by formalin injection and to determine the nature of drug interaction using an isobolographic analysis. Both intrathecal clonidine and neostigmine dose-dependently suppressed the flinching during phase 1 and phase 2. Intrathecal pretreatment with atropine reversed the antinociceptive effects of clonidine and neostigmine in both phases. Pretreatment with intrathecal yohimbine attenuated the effect of clonidine. The antinociception of clonidine and neostigmine was not reversed by mecamylamine. Isobolographic analysis showed that intrathecal clonidine and neostigmine acted synergistically in both phase 1 and 2. Intrathecal pretreatment with atropine and yohimbine antagonized the effect of the mixture of clonidine and neostigmine in both phases, but no antagonism was observed with mecamylamine pretreatment. These data indicate that spinal clonidine and neostigmine are effective to counteract the facilitated state evoked formalin stimulus, and these two drugs interact in a synergistic fashion. In addition, the analgesic action of intrathecal clonidine is mediated by spinal muscarinic receptors as well as alpha-2 adrenoceptors.

Key Words : *Analgesics; Antinociception; Intrathecal; Clonidine; Neostigmine; Drug Interactions*

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INTRODUCTION

Spinal administration of alpha-2 agonists and cholinesterase inhibitors produces a behavioral analgesia in thermal nociceptive test and formalin test (1-3). This fact implies that these two drugs may modulate both acute noxious state and the facilitated state which occurs secondary to the persistent afferent input generated by a tissue injury. Biochemically, intrathecal alpha-2 agonists stimulate norepinephrine release from the spinal cord dorsal horn (4), and cholinesterase inhibitors inhibit the breakdown of spinally released acetylcholine and increase the aceytylcholine concentration in cerebrospinal fluid (5). Anatomical studies have confirmed the localization of alpha-2 adrenergic and cholinergic ligand binding in the spinal cord dorsal horn (6, 7).

Therefore, it appears that these drugs exert their antinociceptive effect by mimicking the action of spinally released norepinephrine or acetylcholine, which acted on spinal alpha-2 adrenoceptors or cholinergic receptors, respectively.

Several lines of evidence suggest spinal pharmacologic interactions between alpha-2 agonists and cholinesterase inhibitors in acute noxious stimuli. An additive antinoci-ceptive interaction is found between intrathecal clonidine and physostigmine in the tail immersion test and the antinociceptive effect of clonidine is attenuated by muscarinic antagonist (8). On the other hand, spinal coadministration of clonidine and neostigmine reveals a synergistic antinociceptive interaction in the thermal nociceptive test and the antinociception of clonidine is reversed by neither muscarinic nor nicotinic antagonists (9). Meanwhile the characteristics of a spinal interaction between an adrenergic system and a cholinergic system on the facilitated state have not definitely established.

Therefore, we sought to investigate the pharmacologic properties of spinal alpha-2 agonist, clonidine and spinal cholinesterase inhibitor, neostigmine and determine the nature of interaction between these two drugs in rats on a model of facilitated processing, the formalin test.

MATERIALS AND METHODS

All studies were conducted according to a protocol approved by the Institutional Animal Care Committee, Research

Institute of Medical Science, Chonnam National University. Male Sprague-Dawley rats (250-300 g) were kept in group cages with two or three rats and maintained on a 12 hr night/day cycle with access to food and water at all times.

Chronic intrathecal catheters were implanted according to a modification of the method described by Yaksh and Rudy (10). Briefly, the rats were placed in a stereotaxic head holder during enflurane anesthesia. A polyethylene (PE-10) catheter was advanced 8.5 cm caudally through an incision in the atlantooccipital membrane to the level of the lumbar enlargement. The exposed end of the catheter was tunneled subcutaneously and externalized on the top of the skull and plugged with a piece of steel wire. The skin was closed with 3-0 silk sutures. Rats showing motor dysfunction postoperatively were sacrificed. After surgery, rats were housed in the individual cages and allowed to recover for 4-5 days.

The following drugs were used in this study: clonidine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), neostigmine bromide (Research Biochemical Internationals [RBI], Natick, MA, U.S.A.), atropine sulfate (RBI), yohimbine hydrochloride (Sigma), and mecamylamine hydrochloride (RBI). Yohimbine was prepared by dissolving the drug in distilled water and other drugs were dissolved in physiologic saline. Intrathecal delivery of these drugs was performed using a hand-driven, gear-operated syringe pump. All drugs were administered in a volume of 10 μL solution, followed by an additional 10 μL of saline to flush the catheter. Physiologic saline was injected for control experiments.

The formalin test was used as a nociceptive test. 50 μL of 5% formalin solution was injected subcutaneously into the plantar surface of the hindpaw with a 30-gauge needle. Pain behavior was quantified by periodically counting the incidence of flinching/shaking of the injected paw. The flinches were counted for 5 min periods from 0-60 min. Two phases of spontaneous flinching were observed after the formalin injection. The interval from 0-10 min was defined as phase 1 of the formalin test and the interval 10-60 min was defined as phase 2. After experiments, the rats were immediately sacrificed.

The first series of experiments were performed to determine the time course and dose-dependency of antinociceptive effects of intrathecally administered clonidine (0.3, 1, 3, 10 μg) and neostigmine (0.1, 0.3, 1, 3 μg). Two drugs were administered 10 min before formalin injection. To determine the antagonistic properties for each agent, alpha-2 (yohimbine 30 μg), muscarinic (atropine 15 μg), and nicotinic (mecamylamine 15 μg) antagonists were administered 10 min before intrathecal delivery of clonidine and neostigmine. Formalin test was performed 10 min after intrathecal administration of clonidine and neostigmine. To define the nature of interaction between clonidine and neostigmine at the spinal level, an isobolographic analysis was done (11). At first, each ED_{50} value (effective dose produc-

ing a 50% reduction of control formalin response) was determined from the dose-response curves of each of two agents. Next, the respective ED_{50} values of each drug were coadministered based on the fractions of this dose combination. Thus, clonidine ED_{50}+neostigmine ED_{50}, (clonidine ED_{50}+neostigmine ED_{50})/2, (clonidine ED_{50}+neostigmine ED_{50})/4 and (clonidine ED_{50}+neostigmine ED_{50})/8 were injected. 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 study, the ED_{50} values were determined separately in two phases in the formalin test. 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 fractional 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 antagonism of the mixture of intrathecal clonidine and neostigmine was evaluated. Yohimbine (30 μg), atropine (15 μg), and mecamylamine (15 μg) were administered intrathecally 10 min before intrathecal mixture (clonidine ED_{50}+neostigmine ED_{50}) injection, respectively. Formalin was injected 10 min after the administration of intrathecal mixture.

All data are expressed as means±SEM. The time response data are presented as the number of flinches per 5 min. The dose-response data are presented as the sum of flinches in each phase. To obtain ED_{50}, the flinches were converted to percentage maximal possible effect (%MPE).

$$\%MPE = \frac{\text{Sum of phase 1 (2) count with drug}}{\text{Sum of control phase 1 (2) count}} \times 100$$

Dose-response data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni 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 (12).

The difference between theoretical ED_{50} and experimen-

tal ED$_{50}$ was examined by t-test. The effect of antagonist on the agonist mixture was analyzed by ANOVA. $p<0.05$ was considered statistically significant.

RESULTS

No motor impairment was observed in rats after intrathecal administration of clonidine and neostigmine. Some rats receiving intrathecal clonidine and atropine showed urination or vocalization. Neither yohimbine nor mecamylamine

affected general behavior or motor function.

Subcutaneous formalin injection resulted in a biphasic flinching response of the injected paw (Fig. 1). Intrathecal administration of clonidine and neostigmine produced a dose-dependent suppression of the flinching during phase 1 and phase 2 in the formalin test (Fig. 2). The ED$_{50}$ of clonidine and neostigmine alone were 1.8 and 0.7 μg in phase 1 and 1.6 and 0.43 μg in phase 2 (Table 1). Thus, the calculated dose ratio for clonidine and neostigmine were 2.5:1 in phase 1 and 3.7:1 in phase 2.

The antinociceptive effect of intrathecal clonidine was antag-

Table 1. Effect of intrathecal clonidine and neostigmine alone or combination in the formalin test

	Phase 1		Phase 2	
	ED$_{50}$ (95% CI) μg	Slope (95% CI) μg	ED$_{50}$ (95% CI) μg	Slope (95% CI) μg
Clonidine	1.8 (1.2~2.7)	-50.9 (-66.4~-35.5)	1.6 (1.1~2.1)	-51.7 (-64.5~-38.9)
Neostigmine	0.72 (0.53~0.98)	-51.8 (-64.0~-39.5)	0.43 (0.31~0.58)	-52.5 (-64.7~-40.3)
Clonidine#	0.38 (0.32~0.45)	-66.0 (-79.2~-52.8)	0.35 (0.29~0.41)	-67.6 (-79.7~-55.4)
Dose-Ratio	2.5:1		3.7:1	
Total Fraction Value	0.43		0.35	

ED$_{50}$, effective dose resulting in a 50% reduction of control response; CI, confidence intervals; #, the value in the mixture of clonidine and neostigmine; Dose-Ratio, a ratio for clonidine and neostigmine
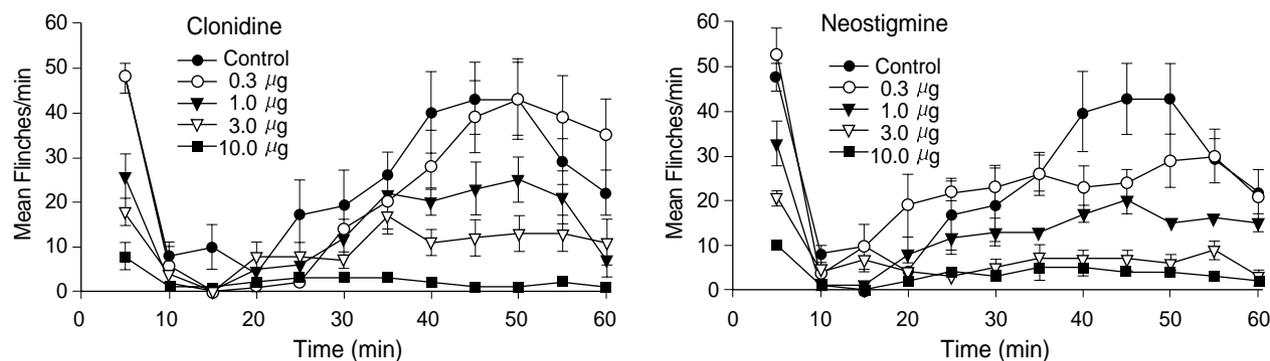

Fig. 1. Time course effect of intrathecal clonidine and neostigmine on the flinching in the formalin test. The number of flinches per 5 min is plotted versus time. Each point on the graph represents the mean ±SEM of 5-6 rats.
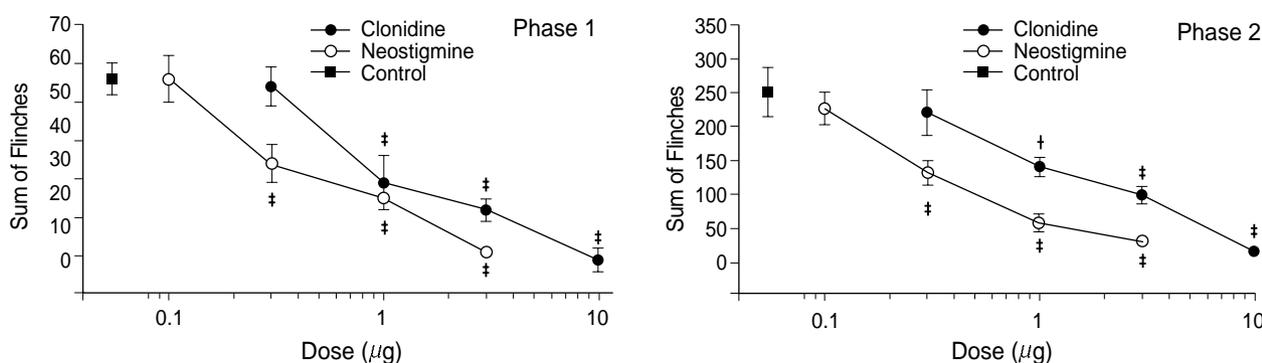

Fig. 2. Dose-response curves of intrathecal clonidine and neostigmine on the flinching during phase 1 and phase 2 in the formalin test. Data are expressed as the sum of flinches versus log dose in micrograms. Each point on the graph represents the mean±SEM of 5-6 rats. Intrathecal clonidine and neostigmine resulted in a dose-dependent suppression of the flinching in both phases. †$p<0.01$, ‡$p<0.001$ compared with control.
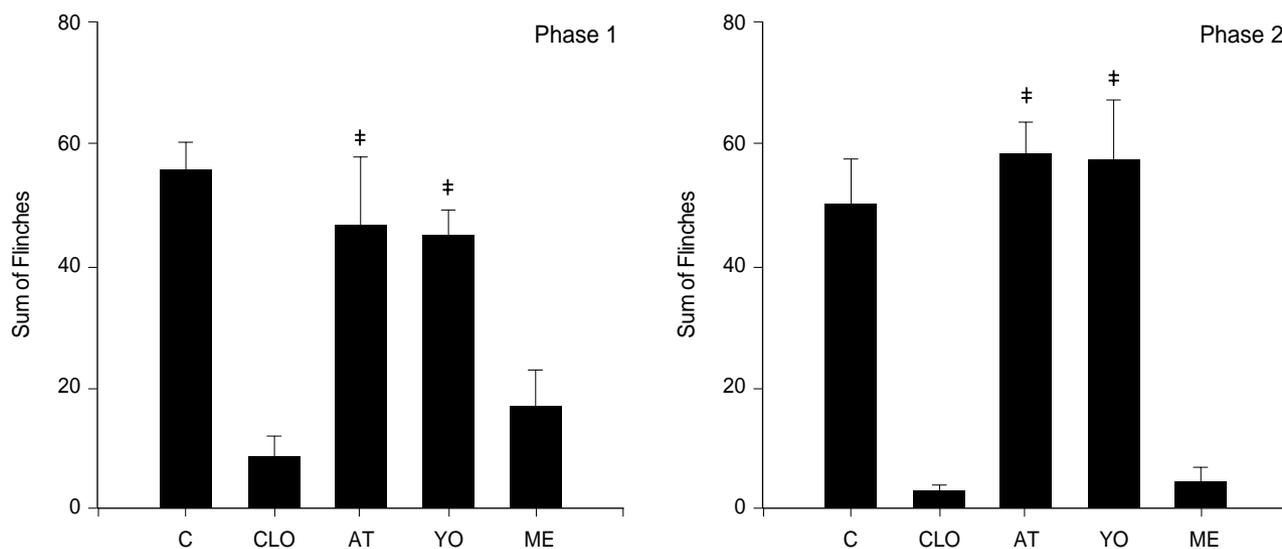
Fig. 3. Antagonistic effect of atropine (AT, 15 μg), yohimbine (YO, 30 μg), and mecamylamine (ME, 15 μg) on the antinociception of clonidine (CLO, 10 μg) in the formalin test. AT, YO and ME were delivered intrathecally either 10 min before CLO administration, and formalin was injected 10 min later. Each bar expressed as the sum of flinches on the graph represents the mean ±SEM of 5-6 rats. Intrathecal atropine and yohimbine reverse the effect of clonidine in both phases. ‡$p < 0.001$ compared with control (C).
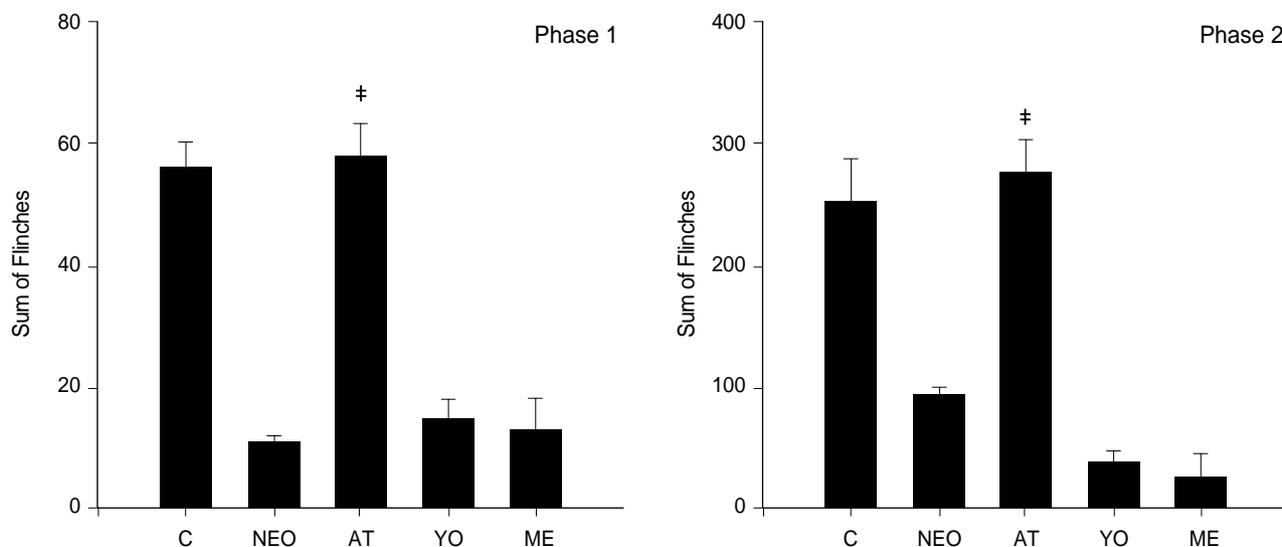

Fig. 4. Antagonistic effect of atropine (AT, 15 μg), yohimbine (YO, 30 μg), and mecamylamine (ME, 15 μg) on the antinociception of clonidine (CLO, 10 μg) in the formalin test. AT, YO and ME were delivered intrathecally either 10 min before NEO administration, and formalin was injected 10 min later. Each bar expressed as the sum of flinches on the graph represents the mean ±SEM of 5-6 rats. Intrathecal atropine reverses the effect of neostigmine in both phases. ‡$p < 0.001$ compared with control (C).

onized by pretreatment with intrathecal atropine and yohimbine during phase 1 and phase 2 in the formalin test (Fig. 3).

Pretreatment with intrathecal atropine reversed the antinociception of intrathecal neostigmine in both phases, but intrathecal yohimbine was ineffective in antagonizing the effect of neostigmine (Fig. 4).

As shown in Fig. 3 and 4, intrathecal mecamylamine did not affect the antinociceptive effect of intrathecal clonidine and neostigmine. Isobolographic analysis revealed a synergistic interaction between intrathecal clonidine and neostig-

mine in two phases of the formalin test (Fig. 5).

The experimental ED_{50} was significantly less than the calculated ED_{50}. Thus, the ED_{50} of the clonidine-neostigmine mixture was 0.38 μg in phase 1 and 0.35 μg in phase 2 (Table 1). Each total fraction value of phase 1 and phase 2 was 0.43 and 0.35, respectively, which corresponded to the synergistic interaction. Intrathecal atropine and yohimbine reversed the effect of the mixture (clonidine ED_{50}+neostigmine ED_{50}), while mecamylamine did not show such antagonism (Fig. 6).
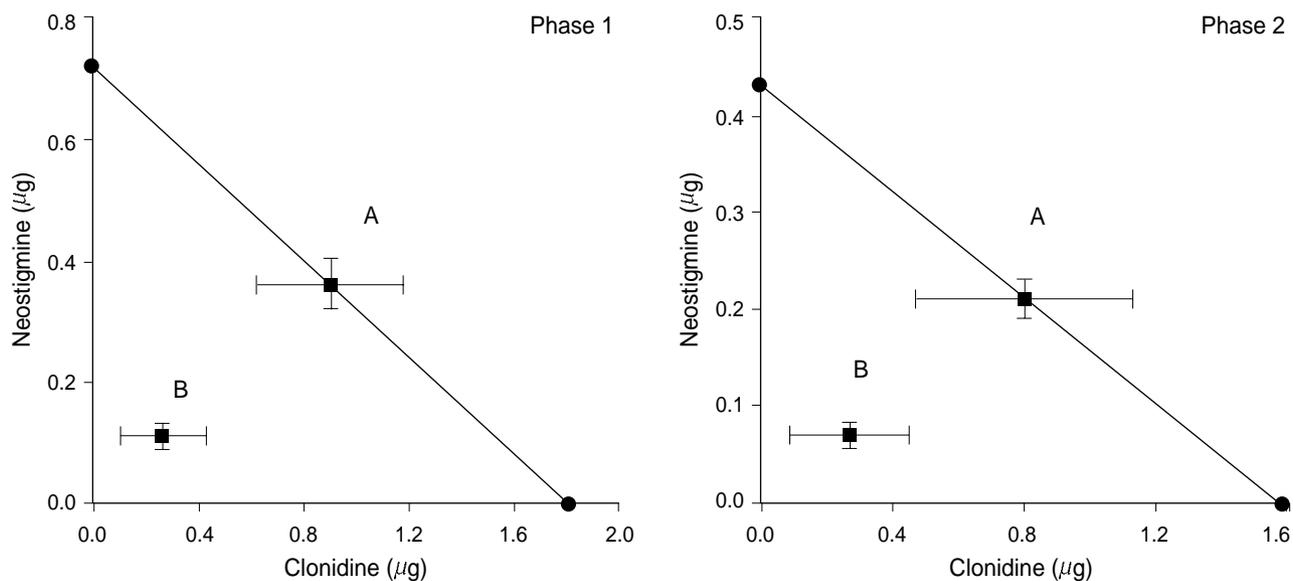
Fig. 5. Isobologram for the interaction between intrathecal clonidine and neostigmine in the formalin test. The straight line connecting the single drug ED50 points is the theoretical additive line, and the point on this line is the theoretical ED50 (A). The heavy lines on the axes represent the SEM of ED50. The experimental ED50 point (B) for the mixture lies below the theoretical additive point in both phases, indicating a synergistic interaction. Each point on the graph represents the mean ±SEM of 5-6 rats.
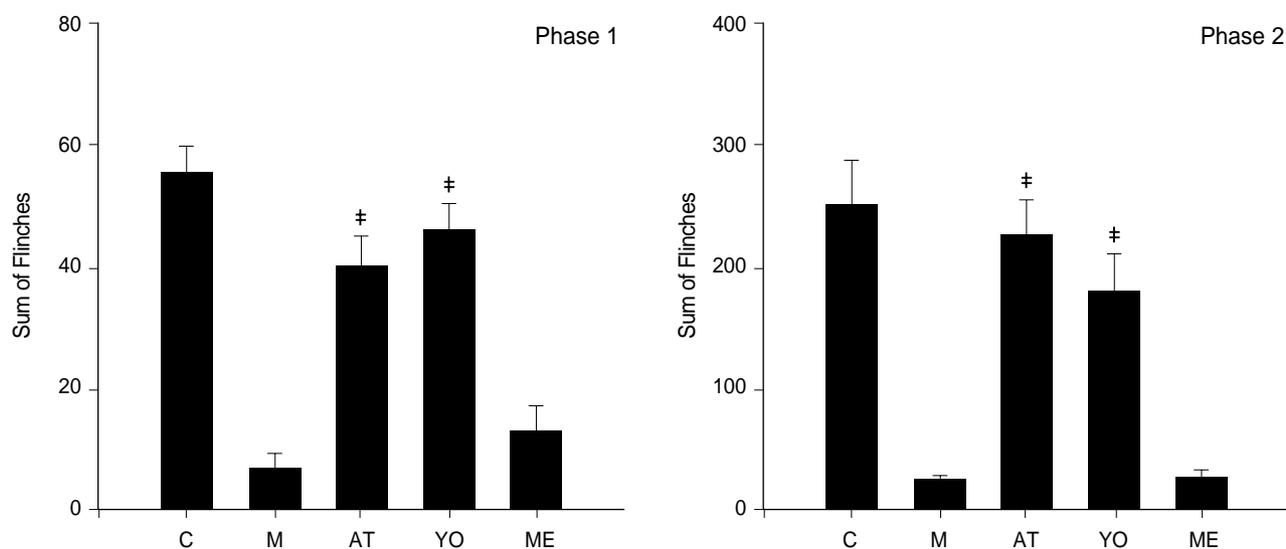

Fig. 6. Antagonistic effect of atropine (AT, 15 μg), yohimbine (YO, 30 μg), and mecamylamine (ME, 15 μg) on the antinociception of mixture of clonidine and neostigmine (M, ED50) in the formalin test. AT, YO and ME were delivered intrathecally either 10 min before the mixture administration, and formalin was injected 10 min later. Each bar expressed as the sum of flinches on the graph represents the mean ±SEM of 5-6 rats. Intrathecal atropine and yohimbine reverse the effect of mixture in both phases. [‡]$p < 0.001$ compared with control (C).

DISCUSSION

Rats injected with formalin into the paw showed the characteristic biphasic pain behavior, a flinching response. Mechanistically, phase 1 flinching seems to result from the immediate and intense increase of primary afferent activity. On the other hand, phase 2 response mirrors the activation of wide dynamic range dorsal horn neurons with a very low level of ongoing activity of primary afferents (13). Thus, the neural process of each phase is fundamentally different. Particularly, phase 2 reflects a facilitated state that occurs secondary to the persistent afferent input generated by a local tissue injury.

Formalin stimulus evokes a spinal release of glutamate and substance-P, through their respective N-methyl D-aspartate and neurokinin-1 receptors, which initiates a cascade, including an increase of intracellular calcium and the activation of kinases. Subsequent complex cascade leads to a

state of facilitation (14).

In the current study, intrathecal clonidine and neostigmine resulted in the antinociception during phase 1 and phase 2 in the formalin test. This observation implicates that clonidine and neostigmine are effective for the facilitated state as well as acute nociception at the spinal level. Autoradiographic studies have demonstrated a high density of alpha-2 adrenoceptors, muscarinic and nicotinic receptors in lamina I and II of the dorsal horn, areas important in nociceptive processing (6, 7). Rhizotomies have been shown to reduce alpha-2 and muscarinic binding in the spinal dorsal horn (15, 16). Previous studies showed that intrathecal administration of clonidine and neostigmine increases the concentration of cerebrospinal norepinephrine or acetylcholine (4, 5).

These findings jointly suggest that intrathecal clonidine and neostigmine increase the level of spinal norepinephrine or acetylcholine, thereby producing an antinociceptive effect, which is mediated by the spinal alpha-2 adrenoceptor or cholinergic receptor, in acute noxious stimuli and the facilitated state.

Another possibility that explains this antinociception of intrathecal clonidine and neostigmine would be the role of the descending inhibitory pathway. Noxious stimulation is able to activate the intrinsic pain inhibitory system, thereby increasing the release of inhibitory neurotransmitters such as norepinephrine and acetylcholine (17, 18). In a microdialysis study, Eisenach et al. (19) confirmed that cerebrospinal concentration of norepinephrine and acetylcholine increased during acute pain. Accordingly, the activation of the descending inhibitory pathway by a formalin stimulus may increase the level of spinal norepinephrine and acetylcholine, thus enhancing the antinociceptive effect of intrathecal clonidine and neostigmine.

In the present study, the antinociceptive effect of the intrathecal clonidine was antagonized by not only intrathecal alpha-2 antagonist but also intrathecal muscarinic antagonist during phase 1 and phase 2 in the formalin test. These results are in accord with the previous findings of Gordh et al. (8) who have shown an attenuation of clonidine-induced antinociception by intrathecal atropine. These findings imply that the antinociceptive action of intrathecal clonidine is mediated by spinal muscarinic receptor as well as alpha-2 adrenoceptor. However, contrary to our findings, others have not observed such antagonism (9, 20). Although the reasons for these differences among experiments are unclear, the facts obtained from our data suggest a possibility of neuronal connection in the spinal cord. Detweiler et al. (20) suggested that adrenergic neurons may cause presynaptic excitation of muscarinic neuron, not vice versa. This suggestion is supported by our data because the antinociception of clonidine was reversed by atropine, but that of neostigmine was not reversed by yohimbine. Additionally, in this study, the antinociception of intrathecal clonidine

and neostigmine was not reversed by intrathecal nicotinic antagonist, which suggests that spinal nicotinic receptor may not be involved in the analgesic action of intrathecal clonidine and neostigmine in acute nociception and the facilitated state. Furthermore, intrathecal nicotinic agonist, epibatidine, does not alter pain behaviors of the formalin test (3). However, the antiallodynic effect of intrathecal clonidine on neuropathic pain is attenuated by spinal muscaric and nicotinic antagonists (21). Therefore, further evaluation of spinal interaction among adrenergic, muscarinic, and nicotinic systems will be necessary.

Isobolographic analysis used here demonstrated the synergistic interaction between intrathecal clonidine and neostigmine in both phases. Previous studies showed that the antinociceptive effect of intrathecal clonidine was additively enhanced by intrathecal physostigmine and neostigmine in acute nociceptive situations (8, 20). On the other hand, the coadministration of intrathecal clonidine and neostigmine or edrophonium exhibited a synergistic effect in acute thermal stimulus (9). Our results suggest that the synergism between clonidine and neostigmine at the spinal level occurs in the facilitated state as well as in acute nociception. Several explanations would be possible for this synergistic interaction. First, agents may interact by changing the kinetic variables of each other at the target site. Second, 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 (22). It has been known that an alpha-2 agonist has a presynaptic inhibitory effect on the terminals of the primary afferent in the spinal cord and decreases the release of excitatory neurotransmitters such as substance-P and calcitonin gene-related peptide (CGRP) (23). Postsynaptically, it inhibits the activity of wide dynamic range dorsal horn neurons, which transmit nociceptive information (24). Further, histochemical studies have shown that the choline acetyltransferase positive cells are distributed in dendrites and axons within the substantia gelatinosa of the dorsal horn (25). Immunopositive varicosities are found pre- and post-synaptic to the central varicosities associated with large and small axons. These observations suggest that spinal cholinesterase inhibitor may regulate the nociceptive transmission and processing by pre- and post-synaptic mechanisms. Therefore, simultaneous engagement of pre- and post-synaptic mechanisms may augment the antinociceptive action produced by either drug acting at one site independently (26). Third, spinal clonidine is a cholinesterase inhibitor (27) and increases acetylcholine concentration in cerebrospinal fluid (28). Hence, the coadministration of clonidine and neostigmine increases acetylcholine more than either drug alone and may lead to a synergism. Finally, norepinephrine has been reported to inhibit cholinesterase activity (29). This action would further increase the acetylcholine level and promote antinociception after concurrent delivery of clonidine and neostigmine.

In conclusion, the present study demonstrated that intrathecal clonidine and neostigmine attenuate both acute noxious stimulus and the facilitated state generated by formalin injection, and these two drugs act synergistically. The spinal alapha-2 and partly muscarinic receptor system is associated with the intrathecal clonidine-induced antinociception. However that of intrathecal neostigmine matched to only spinal muscarinic receptor system.

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