

# Morphine Can Enhance the Antiallodynic Effect of Intrathecal R-PIA in Rats with Nerve Ligation Injury

Jai-Hyun Hwang, MD, Gyu-Sam Hwang, MD, Sung-Kang Cho, MD, and Sung-Min Han, MD

Department of Anesthesia and Pain Medicine, Asan Medical Center, Seoul, Korea

Nerve ligation injury may produce a tactile allodynia. Intrathecal adenosine receptor agonists or morphine have an antiallodynic effect. In this study, we examined the effect of intrathecal morphine on the antiallodynic state induced by the adenosine A1 receptor agonist, *N*<sup>6</sup>-(2-phenylisopropyl)-adenosine *R*-(-) isomer (R-PIA), in a rat model of nerve ligation injury. Rats were prepared with ligation of left L5–6 spinal nerves and intrathecal catheter implantation. Tactile allodynia was measured by applying von Frey filaments to the lesioned hindpaw. Thresholds for withdrawal response were assessed. Morphine and R-PIA were administered to obtain the dose-response curve and the 50% effective dose (ED<sub>50</sub>). Fractions of ED<sub>50</sub>s were administered concurrently to establish the ED<sub>50</sub> of the drug combination. The drug interaction

was analyzed using the isobolographic method. Intrathecal 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an A1 receptor antagonist, and naloxone were administered to examine the reversal of the antiallodynic effect. Side effects were also observed. Intrathecal morphine and R-PIA and their combination produced a dose-dependent antagonism without severe side effects. Intrathecal morphine synergistically enhanced the antiallodynic effect of R-PIA when coadministered. Intrathecal naloxone and DPCPX reversed the maximal antiallodynic effect in the combination group. These results suggest that activation of  $\mu$ -opioid and A1 receptors at the spinal level is required for the synergistic interaction on tactile allodynia.

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**P**eripheral nerve injury may result in a condition of extreme cutaneous sensitivity to normally innocuous mechanical stimuli, termed “tactile allodynia.” Unilateral ligation of L5 and L6 spinal nerves produces some signs that seem representative of neuropathic pain (1,2). Signs of tactile allodynia were most evident in the nerve ligation model among several experimental animal models (3). The spinal pharmacology at spinal nerve ligation-induced allodynia has been shown to be distinct from that associated with acute nociceptive input. In general, the intrathecal administration of adenosine receptor agonists has an antiallodynic effect, which is mediated by the spinal adenosine A1 receptor system in a dose-dependent manner, in rats with nerve ligation injury (4–6). Many experimental studies suggest that morphine induces the release of adenosine (7–10). However,

a previous study reported that reduced morphine-induced spinal release of adenosine may be attributed to a dipyrindamole-sensitive disruption in the opioid-adenosine link in the spinal cord of neuropathic rats (11). The adenosine A1 receptor is mainly involved in antinociception and antiallodynia without severe motor weakness (4,12).

*N*<sup>6</sup>-(2-phenylisopropyl)-adenosine *R*-(-) isomer (R-PIA), a selective adenosine A1 agonist, has an antiallodynic effect (4,13–16), which is antagonized by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an A1 receptor antagonist (6). In experimental animal models, R-PIA has shown a dose-dependent antiallodynic effect to light touch (14,16). Although the synergistic analgesic interaction of spinal adenosine with opioids has been demonstrated in a mouse model of nociceptive pain (17), there is no study on the antiallodynic interaction of morphine and the adenosine A1 receptor agonist, R-PIA, in the spinal nerve ligation rat model. In addition, a previous study suggested that minimal reduction in allodynia by spinal morphine is enhanced in an additive manner by spinal adenosine in rats with spinal nerve ligation (5). However, adenosine simultaneously acts on three adenosine receptor subtypes. As the administered dose increases, each drug alone may have side effects other than its own

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Address correspondence and reprint requests to Jai-Hyun Hwang, MD, Department of Anesthesia and Pain Medicine, University of Ulsan, College of Medicine, Asan Medical Center, 388-1 Pungnap-Dong, Songpa-Gu, Seoul 138-736, Korea. Address e-mail to jhhwang@amc.seoul.kr.

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antiallodynic effect in large dose. Instead, combination treatment may have the advantage of a synergistic effect and reduced side effects.

Therefore, to identify the role of the A1 receptor more relevant to the sensory component, other than the effect of nonspecific adenosine in the interaction with morphine, a series of investigations using a behavioral method were designed to examine the potential enhancement of morphine in antiallodynia induced by the A1 selective agonist R-PIA in a rat model of spinal nerve ligation. Whether either DPCPX or naloxone alone reverses the antiallodynic effect by combination of each agonist was also examined.

## Methods

This study was performed under a protocol approved by the Animal Use and Care Committee at Asan Institute for Life Science. The experiments were conducted in male Sprague-Dawley rats (weight 160–180 g), which were housed individually in a temperature-controlled vivarium and allowed to acclimate for 3 days in a 12/12-h light/dark cycle. For creating the neuropathic rat model, a surgical procedure was performed (1). Under halothane anesthesia, the left L5 and L6 spinal nerves were gently isolated and ligated tightly with 6-0 black silk distal to the dorsal root ganglion and proximal to the formation of the sciatic nerve. After a 7-day postoperative period, implantation of the intrathecal catheter was performed if the rat showed a withdrawal threshold of  $\leq 4.0$  g by postoperative day 7. These rats were defined as demonstrating tactile allodynia. For spinal drug administration, the rats were chronically implanted with catheters as previously described (18). Intrathecal PE-10 tubing was passed caudally from the cistern magna to the spinal cord level of lumbar enlargement. The catheter was externalized through the skin. Proper location was confirmed by a temporary motor block of both hindlimbs after injection of 2% lidocaine 7  $\mu$ L, followed by saline. Only animals with no evidence of neurologic deficit after the operation were studied. Tactile allodynia develops within 1 wk after nerve ligation surgery and it lasts for 6–8 wk. All experiments were conducted 2 wk after spinal nerve ligation. At least a 5-day recovery period was allowed before the animals were used in experiments. The animals were 10–12 wk of age at the time of drug testing.

For intrathecal administration, the drugs were given by using a microinjection syringe over a 60-s interval in a volume of 10  $\mu$ L, followed by a 10- $\mu$ L flush. The drugs given were blind to the experimenter. For the determination of the time to peak effect and the dose ( $ED_{50}$ ) estimated to produce 50% maximal possible effect (%MPE) for each drug, morphine sulfate (Sigma,

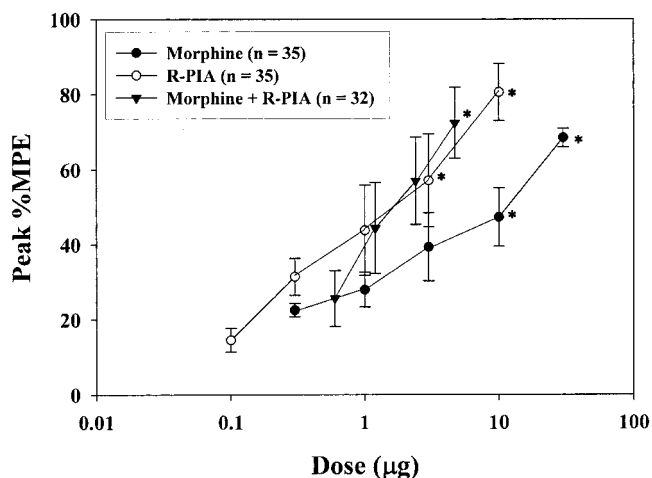
St. Louis, MO) and R-PIA (Sigma) were administered intrathecally. The doses of 0.3, 1, 3, 10, and 30  $\mu$ g ( $n = 7$  per subgroup) were injected for morphine, 0.1, 0.3, 1, 3, and 10  $\mu$ g ( $n = 7$  per subgroup) for R-PIA, respectively. Although R-PIA acts on the A1 receptor more selectively and loses selectivity in large doses, antinociceptive doses of 3–30  $\mu$ g usually maintain its selectivity on the A1 receptor (12). Thus, we used the doses of 0.1–10  $\mu$ g in this study. Because the A1 receptor is involved in antiallodynia and the A2 receptor is more involved in motor weakness (4), we used only the A1 receptor agonist R-PIA. Fractions of  $ED_{50}$ s (1/2, 1/4, 1/8, and 1/16;  $n = 8$  per subgroup) were administered intrathecally in an equal dose ratio to establish the  $ED_{50}$  of the drug combination. When the drug combinations were given, the intrathecal injections were concurrent because the times of the peak effect of intrathecal morphine and R-PIA coincided. For the evaluation of an antagonistic effect in each pretreatment group, the A1 antagonist DPCPX (Sigma) 10  $\mu$ g ( $n = 6$ ) or naloxone (Sigma) 10  $\mu$ g ( $n = 6$ ) was administered intrathecally 5 min before injections of the combination of the two. To identify whether the vehicles and DPCPX have an effect on antiallodynia, normal saline ( $n = 5$ ), dimethyl sulfoxide (DMSO) ( $n = 6$ ), and DPCPX ( $n = 6$ ) were administered. DPCPX was dissolved in DMSO (minimum 99.5%; Sigma) and diluted with 0.9% sodium chloride solution. All other drugs were dissolved in 0.9% sodium chloride solution. There was at least a 5-day interval between drug injections of successive experiments to minimize any possibility of tolerance development and to eliminate the residual effects of a drug. Each animal received a maximum of three injections.

Behavioral testing was performed during the day portion of the circadian rhythm. To undertake these measurements of a tactile threshold, the rats were placed in an individual plastic cage with a wire mesh bottom. After 20 min, tactile threshold was measured by applying a series of 8 calibrated von Frey filaments (0.40, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.1 g; Stoelting Co., Wood Dale, IL) to the midplantar surface of the hindpaw ipsilateral to the nerve injury until a positive sign for pain behavior was elicited. It was held for 6 s. A brisk withdrawal or paw flinching was considered as positive responses, in which case the next filament tested was the next lower force. In the absence of such response, the next filament tested was the next greater force. In the absence of a response at 15 g of pressure, the animals were assigned to this cutoff value. The tactile stimulus producing a 50% likelihood of withdrawal was determined by using the up-down method (19). Measurements were taken before and 15, 30, 45, 60, 90, 120, and 180 min after an intrathecal dose of the drug(s). Baseline threshold value for each animal at each drug trial was determined by checking responses to von Frey filaments on

the same day just before drug injection. Side effects were simply assessed by observing the presence of sedation and motor weakness. Severe sedation was defined as a significant decrease in spontaneous activity and a loss of the orienting response to the light-touch stimulation. Motor weakness was evaluated by observing the righting and placing/stepping reflexes, abnormal weight bearing, and normal ambulation.

The first series of experiments defined the dose-response curves of intrathecal morphine, R-PIA, and their combinations from the mean %MPE. The second series of experiments, fractions of  $ED_{50}$ s (1/2, 1/4, 1/8, and 1/16) were administered concurrently to establish the  $ED_{50}$  for the combination group. Thereafter, the interaction between these two drugs was assessed isoblographically. In the third series of the experiments, to investigate a possible mechanism of the spinal interaction between morphine and R-PIA, the relatively selective A1 antagonist DPCPX 10  $\mu$ g or  $\mu$ -opioid receptor antagonist naloxone 10  $\mu$ g was delivered intrathecally 5 min before injection of a combination dose (1/2  $ED_{50}$ s). The maximal reversal from the peak effect for the combination group for each antagonist group was assessed and compared with peak %MPE. In the last series of experiments, in which we sought to identify whether the vehicles and DPCPX had an effect on antiallodynia, normal saline, DMSO, and DPCPX were administered.

Withdrawal threshold data from von Frey hair testing were obtained as the actual threshold in grams and were converted to %MPE using the formula: %MPE for antiallodynia =  $([\text{postdrug threshold} - \text{baseline threshold}] / [15 \text{ g} - \text{baseline threshold}]) \times 100$ , where postdrug threshold = the largest threshold observed after intrathecal injection. The cutoff value was defined as a stimulus intensity of 15 g for the tactile threshold (i.e., %MPE = 100). The peak drug effect was used to calculate a %MPE, and these data were used to plot a %MPE versus log dose curve. The  $ED_{50}$  values, slopes, and 95% confidence intervals are calculated using dose-response data. Variances and its 95% confidence intervals for the theoretical  $ED_{50}$  may also be calculated from the variances of each component administered alone (20). To determine whether the drug interaction is additive or synergistic, isoblographic analysis was performed. An isoblogram was constructed by plotting the  $ED_{50}$  value for morphine on the x axis and the  $ED_{50}$  values for R-PIA on the y axis. Individual  $ED_{50}$  values for each agonist were resolved from the combination dose required to cause 50% MPE and were plotted on the isoblogram as the experimental combination dose.  $ED_{50}$  was defined separately for each drug. Fractions (1/2, 1/4, 1/8, and 1/16) of the  $ED_{50}$  of each drug were then administered concurrently and the  $ED_{50}$  of the morphine-R-PIA combination was determined. The theoretical additive dose combination was calculated.



**Figure 1.** Dose-response curves from the peak effects of percent maximal possible effect (%MPE) for antiallodynia in the morphine,  $N^6$ -(2-phenylisopropyl)-adenosine R(-)-isomer (R-PIA), and morphine + R-PIA groups. These curves show a dose-dependent antiallodynic effect. Data are expressed as mean  $\pm$  SEM. Doses ( $\mu$ g) are represented logarithmically on the x axis and peak %MPE is represented on the y axis. Asterisks indicate that the mean %MPE of each group is significant compared with the smallest dose. \* $P < 0.05$ ; unpaired  $t$ -test.

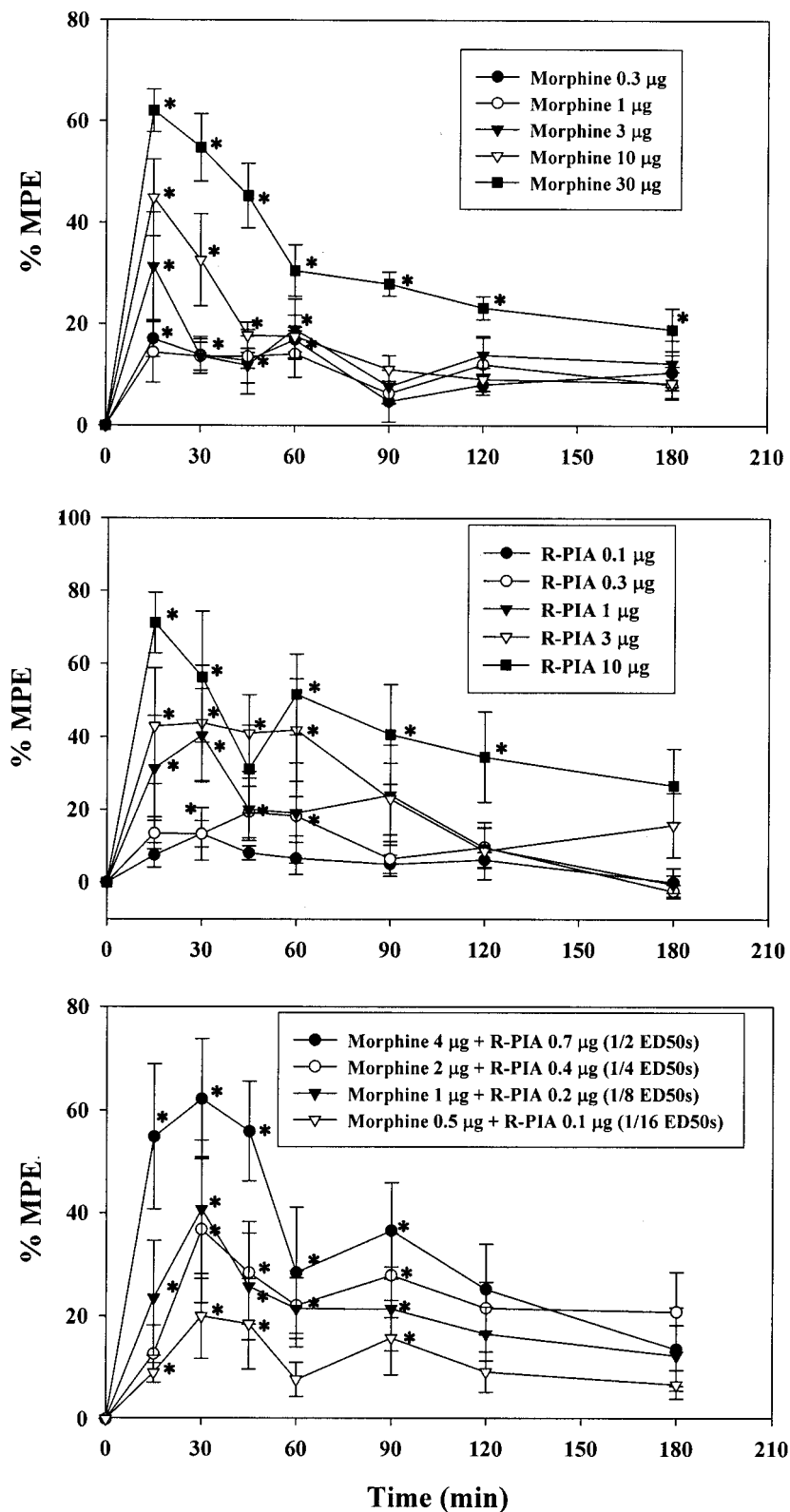
Experimental values were compared with theoretical additive values as defined by the theoretical additive line. The theoretical additive point lies on a line connecting the  $ED_{50}$  values of the individual drugs, and experimental values that lie below and to the left of this additive line are considered to be synergistic.

Data were expressed as mean  $\pm$  SE because of the small number of rats in each group. The difference between the theoretical additive  $ED_{50}$  value and the experimental  $ED_{50}$  value was compared using a Student's  $t$ -test. The least antagonistic effect for each pretreatment group was compared with the peak agonistic effect of the combination group using the unpaired  $t$ -test. A  $P$  value  $< 0.05$  was considered to be statistically significant.

## Results

After spinal nerve ligation, most rats displayed normal general behavior and weight gain. After catheter implantation in the animals with nerve ligation, the thresholds for evoking hindpaw withdrawal were in the range of 1–4 g for all rats.

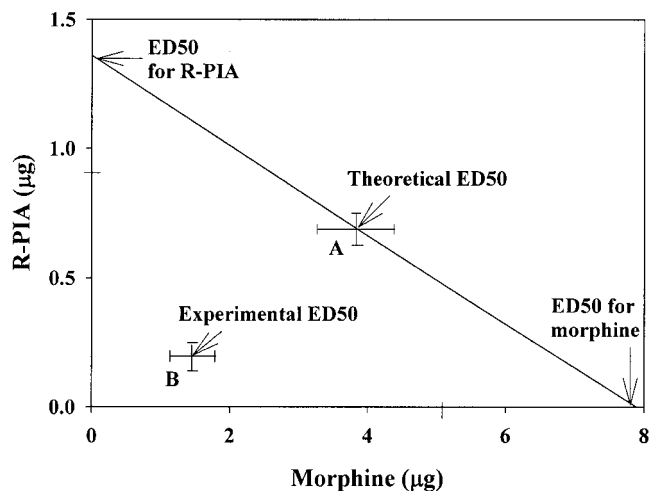
Intrathecal morphine, R-PIA, and their combination resulted in a dose-dependent antiallodynic effect (Fig. 1). Although not being closely paralleled, the slope of the combination group was shifted to the left side in larger doses compared with morphine (1–30  $\mu$ g) and R-PIA (3 and 10  $\mu$ g), respectively (Fig. 1). The  $ED_{50}$  values and slopes (95% confidence intervals) are as follows: 7.9 (3.9–15.9) and 19.3 (13.9–24.6)  $\mu$ g for morphine, 1.4 (0.8–2.4) and 31.6 (20.6–42.5)  $\mu$ g for R-PIA,



**Figure 2.** Time course of antiallodynic effects by intrathecal injection of morphine (7 rats per each dose), *N*<sup>c</sup>-(2-phenylisopropyl)-adenosine R(-)-isomer (R-PIA) (7 rats per each dose), and R-PIA + morphine (8 rats per each dose). These curves show a dose-dependent antiallodynic effect in each group. Data are expressed as mean ± SEM. Asterisks indicate that mean percent maximal possible effect (%MPE) of each group for antiallodynia at that time point is significant compared with baseline value. \**P* < 0.05; one-way repeated-measures analysis of variance followed by multiple comparisons (Dunnett's method).

and 1.7 (1.1–2.7) and 51.2 (20.6–81.9) µg for their combination, respectively. The high slope value of the combination group could reflect an increased efficacy.

The time-effect courses as a function of the intrathecal doses of these two agonist groups and their combination groups were similar in general (Fig. 2). The max-



**Figure 3.** Isobologram for the intrathecal interaction of morphine and *N*<sup>6</sup>-(2-phenylisopropyl)-adenosine *R*(-)-isomer (R-PIA). Horizontal and vertical bars indicate SEM. The diagonal line connecting 2 50% effective dose (ED<sub>50</sub>) points is the theoretical additive line. The ED<sub>50</sub> point A is calculated from the ED<sub>50</sub> values and 95% confidence intervals of each drug. The experimental ED<sub>50</sub> point B lies far below the line of additivity, indicating significant synergism.

imal effects occurred within 15–30 min and then gradually decreased up to the previous baseline level over time for all doses of each group. There was a dose-dependent increase in magnitude and duration of the effect. A somewhat longer antiallodynic time course was observed in some rats after the injections of R-PIA 3 or 10 µg and morphine 10 or 30 µg. Intrathecal normal saline and DMSO (vehicle groups) produced only a slight increase in withdrawal response, which means that vehicles do not have an effect on the action of each drug and their combination.

A synergistic effect was found in the morphine-R-PIA combination group (Fig. 3). The experimentally determined morphine-R-PIA combination ED<sub>50</sub> (±SEM) was 1.45 (±0.34) µg for morphine and 0.25 (±0.06) µg for R-PIA. The theoretical additive ED<sub>50</sub> was calculated to be 3.95 (±0.95) µg for morphine and 0.68 (±0.16) µg for R-PIA. The experimental value of the morphine-R-PIA combination group was significantly smaller than the calculated theoretical additive value ( $P < 0.05$ ). The standard errors of these two points on the isobologram show that they do not overlap, which supports a significant synergistic interaction.

Pretreatment with either naloxone or DPCPX remarkably attenuated the maximal antiallodynic effect produced by the intrathecal morphine-R-PIA combination after 30 and 45 min ( $P < 0.05$ ) (Fig. 4). In the DPCPX and naloxone pretreatment groups, any significant increase was not shown during the entire experiment. DPCPX alone produced only a slight increase in %MPE and this suggests that DPCPX does not have an effect on antiallodynia.

Some rats showed mild-to-moderate motor weakness or sedation with a large dose of each drug, but no severe motor weakness or sedation was observed in any rats. The incidence and magnitude of side effects were considerably reduced in the combination group (Table 1). Moderate motor weakness was observed in 2 rats (1 in the morphine 30-µg group and 1 in the R-PIA 10-µg group). No other adverse effects were noted. The occurrence of mild-to-moderate motor weakness returned to the baseline level within 3 h, whereas the sedative effect, although the magnitude was reduced, did not return to the previous level up to 3 h in most sedated rats.

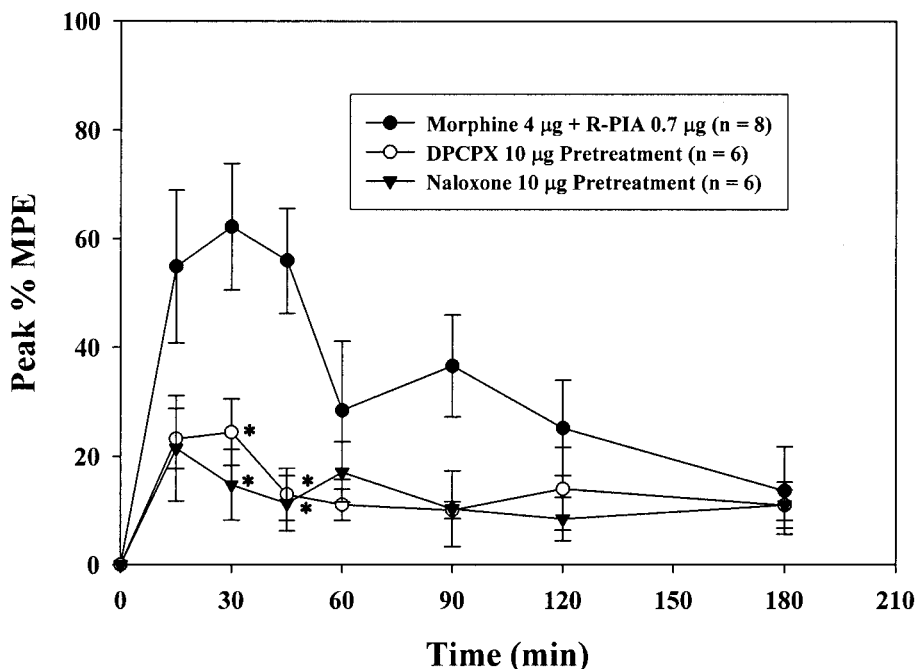
## Discussion

We found that intrathecal morphine, R-PIA, and their combination produced a dose-dependent increase of withdrawal threshold for a spinally-mediated tactile allodynia and that morphine enhanced the effect of R-PIA synergistically when coadministered intrathecally.

Previous observations indicate that intrathecally administered R-PIA antagonizes the allodynic response to tactile stimuli in a dose-dependent manner without marked motor weakness (14,16). The selective antagonism confirms that such an antiallodynic effect is mediated by the spinal adenosine A1 receptor subtype (6,14). Several animal neuropathic pain models induced by sciatic chronic constriction injury and intrathecal strychnine suggest an antiallodynic effect mediated by the spinal adenosine A1 receptor (14,16,21). Likewise, we observed a similar result in this spinal nerve ligation injury model using intrathecal R-PIA.

Previous studies demonstrated that both A1 and A2 receptor subtypes are concentrated in a very small area of the dorsal horn and are only localized diffusely throughout the ventral horn (22,23). Bantel et al. (24) reported that there was no evidence for up-regulation in spinal A1 receptors after spinal nerve ligation and that there was a depletion of spinal cord adenosine after spinal nerve ligation.

Morphine produces a dose-dependent release of adenosine from the spinal cord (8). However, morphine-induced spinal release of adenosine is reduced after spinal nerve ligation (11). Additionally, intrathecal adenosine does not relieve allodynia-like behavior in spinally injured rats. In contrast, one study suggests that exogenous and endogenous adenosine enhances the spinal antiallodynic effects of morphine in a rat model of neuropathic pain (5). Therefore, we do not think that spinal adenosine release caused by intrathecal morphine had an effect on the antiallodynic effect of intrathecal R-PIA because antagonism by naloxone pretreatment was not as significant as that of DPCPX and each agonist had a similar peak time and was administered concurrently.



**Figure 4.** Antagonism by either naloxone or 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) pretreatment. Pretreatment with naloxone 10 µg or DPCPX 10 µg was performed 5 min before injection of a large dose in the morphine + N<sup>6</sup>-(2-phenylisopropyl)-adenosine R(-)isomer (R-PIA) group. Graphs show the time course of the antiallodynic effect in the combination group and pretreatment groups. Data are expressed as mean ± SEM. Maximal antagonism is seen after 30 and 45 min. Asterisks indicate that mean percent maximal possible effect (%MPE) of pretreatment groups for antiallodynia at that time point is significantly less compared with the morphine + R-PIA group. \*P < 0.05; unpaired t-test.

**Table 1.** Incidence of Side Effects

Drug	Dose (µg)	No. of rats	Side effects (%)	
			Motor weakness	Sedation
Morphine	0.3	7	—	—
	1	7	—	1 (14)
	3	7	—	1 (14)
	10	7	—	2 (29)
	30	7	1 (14)	4 (57)
R-PIA	0.1	7	—	—
	0.3	7	—	—
	1	7	—	1 (14)
	3	7	—	1 (14)
	10	7	1 (14)	2 (29)
Morphine + R-PIA	0.5 + 0.1	8	—	—
	1 + 0.2	8	—	1 (13)
	2 + 0.4	8	—	1 (13)
	4 + 0.7	8	—	2 (25)

R-PIA = N<sup>6</sup>-(2-phenylisopropyl)-adenosine R(-)isomer.

A previous study proposed that there was a possible central interaction between low-threshold mechanoreceptors and nociceptors (25). With this change, tactile stimuli may be transmitted to the superficial area via activated spinal interneurons that might modulate the local transmission of the afferent allodynic information. Therefore, intrathecal morphine might have an effect on antiallodynic action after spinal nerve injury.

Lavand'homme and Eisenach (5) reported that spinal morphine itself produced a minimal reduction in allodynia in rats after spinal nerve injury and that this

was enhanced in an additive manner by spinal adenosine. The maximal effect of the morphine-adenosine combination resulted in <60% reversal of allodynia. In our study, however, intrathecal morphine and the combination with R-PIA produced a more prominent reduction in allodynia without severe side effects. We think that this may be attributable to the difference between adenosine acting simultaneously on all adenosine receptor subtypes and R-PIA predominantly acting on the A1 receptor subtype.

In our experiments, the interaction between morphine and R-PIA was synergistic. We hypothesized that the antiallodynic effect found in the combination group at the spinal level was mediated by the independent receptor systems and there was a reduction in dose for each drug, suggesting a synergistic interaction. The decreased clearance, change in agonist affinity, and functional receptor interaction are possible explanations for this enhanced effect. We do not believe that these results were caused by the altered clearance of either drug because there was no apparent increase in the duration of action in the combination group. With regard to change in agonist affinity, an increase in slope might reflect increased efficacy. In our experiments, the slope was increased in the combination group and was shifted to the left in large doses, which may explain a synergistic interaction. If a functional receptor interaction exists, we would anticipate that the appearance of motor weakness and sedation would have been similarly enhanced. Although there is a synergism in the allodynic component, failure to observe such enhancement likely excludes a facilitation of the receptor interaction. However, the

fact that the receptors for the sensory component are mainly located in the dorsal horn of spinal cord and that there may be an interaction of the A1 receptor with the opioid receptor increases the possibility of a functional receptor interaction. Although two receptors share common second-messenger systems and pain-signaling mechanism at the cellular level (26), the difference in action site and receptor number and function changes after nerve injury may affect the results. Despite all these possible explanations, the exact mechanisms are not yet known.

To investigate the reversal effect, we performed an antagonistic study with pretreatment of either naloxone or DPCPX in the combination subgroup presenting maximal effect. In our experiment, we chose only the A1 antagonist DPCPX because the A1 receptor subtype was most effective in the reversal of tactile allodynia in the nerve ligation injury model (6). Pretreatment with either naloxone or DPCPX remarkably attenuated the maximal antiallodynic effect in the combination group. These findings may suggest that spinal morphine is independently necessary for the optimal function of R-PIA in producing a synergistic effect.

Although not systematically quantified, intrathecal morphine or R-PIA resulted in a dose-dependent reduction in spontaneous activity. The effect on motor performance is particularly crucial in studies of spinal A1 receptor agonists because of the possible action of a large dose of R-PIA in the motor neuron area of the spinal cord (23). Although the A1 receptor is present even in the ventral horn, there was only mild motor weakness in this experiment. Several studies reported that motor weakness is mediated through the adenosine A2 receptor and no significant motor weakness could be seen in R-PIA doses of  $\leq 10 \mu\text{g}$  (4,12). We considered that each test used here was a relatively gross test without any quantification and that side effects in animal studies are difficult to evaluate without blinded objective scoring and quantification. Therefore, we simply assessed the motor weakness by only checking the presence of each component. Although the incidence of rats showing sedation in the combination group was less than that of the morphine group, there may be a synergistic interaction in producing sedation. More worrisome is that the major side effects of morphine in humans are nausea and vomiting, which cannot be evaluated in the rat. Briefly, with respect to reduced side effect and synergistic effect, a combination therapy administering a smaller dose of each drug and a target-specific treatment using the A1 receptor agonist R-PIA may be beneficial to the management of allodynia.

In conclusion, morphine and R-PIA produced a dose-dependent antiallodynia without severe side effects and intrathecal morphine produced a synergistic interaction with R-PIA in a rat model of nerve ligation

injury. Thus, these results suggest that activation of both  $\mu$ -opioid and adenosine A1 receptors is required for the synergistic interaction between morphine and R-PIA in reducing tactile allodynia.

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