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The Antiallodynic Effects of Intrathecal Cholinesterase Inhibitors in a Rat Model of Neuropathic Pain

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Background: **This study determined the effect of intrathecally administered cholinesterase inhibitors, edrophonium and neostigmine, on nerve injury–induced, touch-evoked allodynia and identified the pharmacologic characteristics of this action.**

Methods: **Rats were prepared with tight ligation of the left L5 and L6 spinal nerves and with lumbar intrathecal catheters fitted for long-term monitoring. Edrophonium (3, 10, 30, or 100 μg) or neostigmine (0.3, 1, 3, or 10 μg) was administered intrathecally. Tactile allodynia and motor weakness were assessed. To evaluate the pharmacologic characteristics of the activity, a muscarinic receptor antagonist or a nicotinic receptor antagonist was administered intrathecally before edrophonium or neostigmine was injected. To compare the action of subtype antagonists, the M_1 muscarinic receptor antagonist pirenzepine, the M_2 antagonist methoctramine, the M_3 antagonist 4-DAMP (diphenylacetoxy-N-methypiperidine), and the M_4 antagonist tropicamide were administered intrathecally before cholinesterase inhibitors were injected.**

Results: **Intrathecal edrophonium or neostigmine produced a dose-dependent antagonism of the touch-evoked allodynia. Neostigmine resulted in a moderate effect on motor weakness at doses of 3 and 10 μg. Pretreatment with intrathecal atropine but not mecamylamine yielded a complete antagonism of the effects of the cholinesterase inhibitors. In addition, antiallodynia produced by edrophonium (100 μg) was reversed by pretreatment with methoctramine, 4-DAMP, tropicamide, and pirenzepine. In the neostigmine (10 μg) group, only the M_1 antagonist pirenzepine had a moderate effect on reversal of increased allodynic threshold.**

Conclusions: **These experiments suggest that intrathecal edrophonium or neostigmine produces an antagonism on touch-evoked allodynia at the spinal level in a rat model of neuropathic pain and that the antiallodynic action of cholinesterase inhibitors is probably mediated by a spinal muscarinic system, especially at the M_1 receptor subtype. (Key words: Allodynia; antagonists; receptor; subtype.)**

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 11A.

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INTRATHECAL administration of cholinergic receptor agonists or cholinesterase inhibitors have an antinociceptive effect in a dose-dependent manner, which is mediated by spinal muscarinic receptors in animals, and this analgesic effect has been confirmed in human studies.[1–7] The mechanisms of this spinal action are not clear, but autoradiographic studies have shown the existence of muscarinic receptors, both M_1 and M_2, in laminae II and III of the spinal cord. Because dorsal rhizotomy lead to their rapid disappearance, they are believed to be primary afferent terminals.[8]

Peripheral nerve injury may produce a syndrome consisting of spontaneous pain and hyperalgesia and tactile and thermal allodynia. Kim and Chung[9] and Sheen and Chung[10] described a rat model of neuropathy in which tight ligatures are placed around the L5 and L6 spinal nerves. Within a day or two, these animals display profound and long-lasting tactile allodynia.

The spinal pharmacologic nature of this nerve injury–induced allodynia has been shown to be distinct from that associated with acute nociceptive input. Thus, α_2-adrenergic receptor agonists, N-methyl D-aspartate receptor antagonists, γ-aminobutyric acid receptor agonists, and adenosine receptor agonists have been shown to have an antiallodynic effect at the spinal cord level.[11–15]

Therefore, the current study was designed (1) to characterize the effects of spinal cholinesterase inhibitors on

nerve injury-induced tactile allodynia and (2) to define the pharmacologic nature of the cholinergic receptors that mediate the effects of this family of agents.

Materials and Methods

Animal Preparation

The following investigations were performed under a protocol approved by the Animal Care Committee, Asan Life Science Institute, University of Ulsan. Experiments were conducted in male Sprague Dawley rats (weight, 140–160 g; Asan LSI, Seoul, Korea), which were housed individually in a temperature-controlled (21 ± 1°C) vivarium and allowed to acclimate for 5 days in a 12-h light–dark cycle.

Surgical Preparation

Neuropathic Model. For creating the neuropathic rat model, the surgical procedure was performed according to the previous description by Kim and Chung.[9] Briefly, during halothane anesthesia (2% in 100% oxygen), a partial excision of the left L6 transverse process was made, and the left L5 and L6 spinal nerves were isolated and ligated tightly with 6–0 black silk distal to the dorsal root ganglion and proximal to the formation of the sciatic nerve. Animals that could not flex the left hind limb after operation, indicating damage to the L4 nerve, were killed. After a 7-day postoperative period, the intrathecal catheter was implanted if the rat shows a withdrawal threshold of 4 g or less by postoperative 7 day. Such rats were defined as showing the appropriate tactile allodynia.

Implantation of Intrathecal Catheters. For spinal drug administration, rats were implanted long-term with catheters, as previously described.[16] Briefly, during halothane anesthesia the rats were placed in a stereotaxic head holder. Polyethylene tubing (PE-10; Becton Dickinson, Sparks, MD) was passed caudally from the cisterna magna to the T12-L1 spinal cord level (8.5 cm) and externalized through the skin. Animals with evidence of neuromuscular dysfunction were killed promptly. At least 5 days of postsurgical recovery were allowed before animals were used in experiments. There was at least a 3-day interval between successive experiments with any rat after intrathecal administration of drug and each animal received, in total, three or four injections.

Behavioral Measures

Behavioral testing was performed during the day portion of the circadian cycle (6:00 AM to 6:00 PM). To measure the tactile threshold, rats were placed in individual plastic cages with wire mesh bottoms. After 20 min, the mechanical threshold was measured by applying a von Frey hair (Stoelting, Wood Dale, IL) to the midplantar surface of the hind paw with the lesion until a positive sign for pain behavior was elicited. The up-down paradigm for stimulus delivery was used to assess the threshold. This model has been described in detail elsewhere.[12,17] Briefly, von Frey hairs with logarithmically incremental stiffness (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g) were applied serially to the paw in ascending order of strength, perpendicular to the plantar surface, with sufficient force to cause slight bending against the paw and held for 5 s. A positive response was recorded if the paw was sharply withdrawn. Flinching on removal of the hair was also considered a positive response. Based on observations on normal, unoperated rats, the cutoff of a 15.1-g hair (approximately 10% of the body weight of our smaller rats) was selected as the upper limit for testing, because stiffer hairs tended to raise the entire limb rather than to buckle, substantially changing the nature of the stimulus. The resulting pattern of positive and negative responses was tabulated, and the 50% response threshold was interpolated using the following formula: 50% g threshold = $(10^{[Xf+k\delta]})/ 10{,}000$, where Xf is the value (in log units) of the final von Frey hair used; k is a tabular value for the pattern of positive–negative responses from the calibration table; and δ is the mean difference (in log units) between stimuli (here, 0.224)[17] (see also experimental paradigm).

Motor weakness was also assessed by observing ambulation, posture, abnormal weight bearing, and righting-stepping reflexes and was graded as follows: score 0, no motor change (normal symmetrical ambulation); score 1, mild motor weakness (normal posture and ambulation, normal weight bearing, and reduced righting and stepping reflex responses); and score 2, moderate to severe motor weakness (abnormal weight bearing, paw withdrawal to pinch but attenuated).

Drug Administration

Intrathecal drug was delivered manually using a microinjection syringe (Hamilton Company, Reno, NV) during a 60-s interval in a volume of 10 μl of solution. Drug solution was immediately followed by 10 μl of physiologic saline to flush the catheter.

Neostigmine bromide (molecular weight [MW] = 303.21; Research Biochemicals International [RBI], Natick, MA) and edrophonium chloride (MW = 201.69;

RBI) were administered in doses of 0.3, 1, 3, or 10 μg and 3, 10, 30, or 100 μg, respectively. Either 10 μg of the muscarinic antagonist atropine sulfate (MW = 676.81; RBI) or 10 μg of the nicotinic antagonist mecamylamine hydrochloride (MW = 203.76; RBI) were injected intrathecally with 3 μg of the muscarinic subtype M_1 antagonist pirenzepine dihydrochloride (MW = 424.31; RBI), 3 μg of the M_2 antagonist methoctramine tetrahydrochloride (MW = 728.77; RBI), 3 μg of the M_3 antagonist 4-DAMP methiodide (MW = 451.45; RBI), and 3 μg of the M_4 antagonist tropicamide (MW = 284.35; RBI). To evaluate receptor antagonism, cholinergic antagonists were delivered and then cholinesterase inhibitors were injected intrathecally 5 min later. All agents were dissolved in 0.9% sodium chloride solution.

Experimental Paradigm

For time-*versus*-effect curves, threshold testing and motor function were assessed at 15, 30, 45, 60, and 90 min for edrophonium and, in addition, at 120 and 180 min for neostigmine. Additional testing was conducted if the effects of the drugs were sustained. In the antagonist studies, we administered either a muscarinic receptor antagonist or a nicotinic receptor antagonist in the first series and then selective muscarinic receptor antagonists in the second series.

For pretreatment, we measured threshold levels 5 min before and immediately after injection of the antagonist for baseline values and then at 15, 30, 45, 60, 90, 120, and 180 min after injection of edrophonium or neostigmine.

Data Analysis and Statistics

Threshold data from von Frey hair testing were presented as the actual threshold in grams or were converted to percentages of the maximum possible effect (%MPE), where %MPE for antiallodynia =

$$\frac{(\text{post drug threshold} - \text{baseline threshold})}{(15\text{ g} - \text{baseline threshold})} \times 100$$

The cutoff value was defined as a stimulus intensity of 15 g for the tactile threshold (*i.e.*, %MPE = 100).

In the analysis of motor dysfunction, %MPE was calculated using the following formula: %MPE for motor weakness =

$$\frac{(\text{sum of individual scores over observation interval})}{\text{maximal possible score}} \times 100$$

(*e.g.*, here the maximal possible score is 10 for edrophonium and 14 for neostigmine).

The peak effect observed in each rat after drug delivery was recorded. This peak drug effect was used to calculate a %MPE and these data were used to plot a %MPE-*versus*-log dose curve. Data from these dose–response curves were analyzed using the dose–response analysis of Tallarida and Murray.[18] Median effective dose (ED_{50}) values, slope, and 95% confidence intervals (CIs) were determined. Data were expressed as the mean ± SD. The results were analyzed using a one-way analysis of variance (Scheffé test for *post hoc* analysis of means) to compare the data obtained after antagonist administration at each time interval or by a paired Student's *t* test to compare the data with the baseline threshold for each dose group. $P < 0.05$ was considered significant.

Results

After the spinal nerve ligation, all rats entered into this study displayed a significant decrease in the magnitude of mechanical stimulus (from 15 g cutoff to the range of 1 to 4 g) necessary to evoke a brisk withdrawal response in the injured hind paw in response to von Frey hair stimulation.

Antiallodynic Effect of Intrathecal Cholinesterase Inhibitors

Figure 1 shows the time course of the increase in mechanical threshold produced by intrathecal injection of edrophonium. Threshold was increased maximally by 15 min of the injection of 30 and 100 μg edrophonium ($P < 0.05$) and then gradually decreased to baseline level during a 90-min period, depending on the dose.

A longer antiallodynic time course was observed after intrathecal injection of neostigmine (0.3 to 10 μg), as shown in figure 2. The onset of action was slower than that of edrophonium. The threshold was maximally increased within 15 to 30 min after injection of 3 and 10 μg neostigmine ($P < 0.05$) and then gradually decreased to near baseline threshold levels after 5 h (data not shown).

The antiallodynic effects of the intrathecal drugs were dose dependent, as illustrated in figure 3. The ED_{50} (95% CI) and slope (95% CI) for edrophonium were 12 μg (range, 5.5 to 26 μg) and 45.5 (range, 20.3 to 70.7). For neostigmine, the ED_{50} (95% CI) and slope (95% CI) were 0.3 μg (0.1 to 0.8 μg) and 36.4 (21.1 to 51.7).

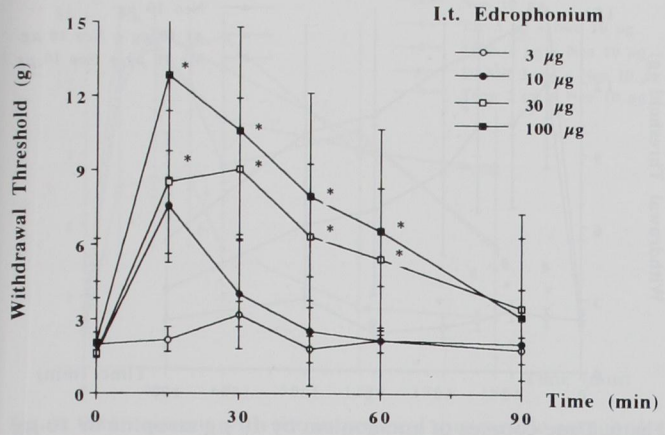

Fig. 1. Time courses of the antiallodynic effects of edrophonium administered intrathecally in rats made allodynic by L5 and L6 spinal nerve ligation. The results are expressed as the mean ± SD of five rats in each group. The asterisk shows the value of $P < 0.05$ compared with baseline. I.t. = intrathecal.

Motor Effect of Intrathecal Cholinesterase Inhibitors

Both intrathecal cholinesterase inhibitors resulted in a dose-dependent hind limb motor weakness, which, although detectable at the higher doses examined, were not sufficient to produce a significant effect on ambulation (fig. 3). At antiallodynic doses, the drugs did not obtund the brisk hind paw withdrawal induced by the greater von Frey hair stimuli. These general observations are consistent with the numeric rating of motor weakness provided by the drugs (see behavioral measures).

After intrathecal injection of 3 and 10 μg neostigmine, a detectable motor weakness occurred within 5–30 min, and the residual effect persisted for several hours. In contrast, intrathecal injection of edrophonium did not show marked motor weakness even at the highest dose (fig. 3). Intrathecal injection of edrophonium had a minimal effect on motor function, as shown in the motor weakness scores. Although only 20% of rats showed motor dysfunction even at 100 μg of edrophonium, more than 80% of rats given 3 μg neostigmine showed motor weakness with score 2, and this motor effect lasted for as long as 90 min (fig. 4).

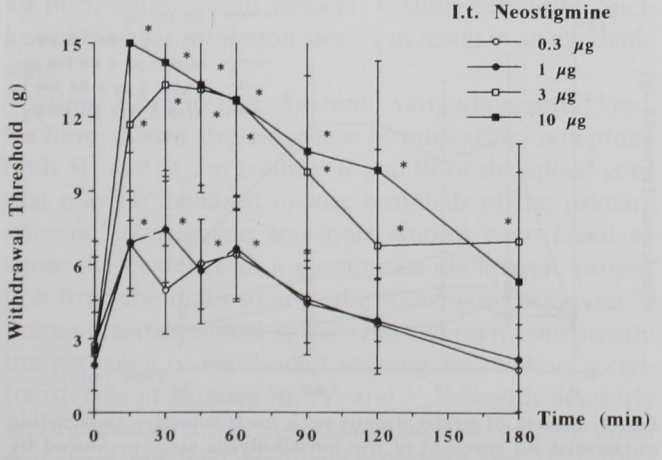

Fig. 2. Time courses of the antiallodynic effects of neostigmine administered intrathecally in rats made allodynic by L5 and L6 spinal nerve ligation. The results are expressed as the mean ±SD of four to six rats in each group. The asterisk shows the value of $P < 0.05$ compared with baseline. I.T. = intrathecal.

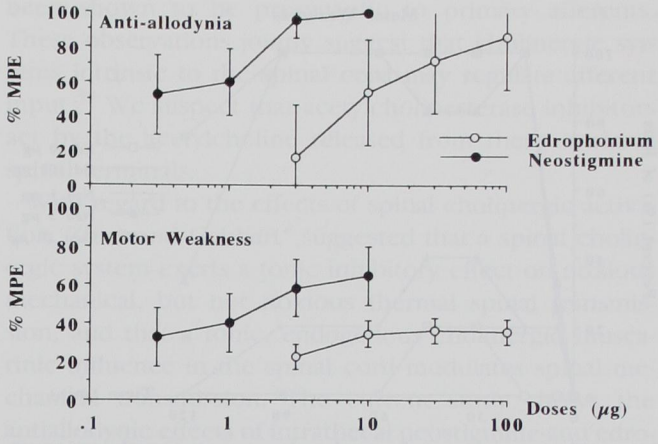

Fig. 3. Dose–response curves showing the peak effect observed after drug injection were expressed as percentages of maximal possible effect (%MPE) for antiallodynia (*upper*) and for motor weakness (*lower*) of intrathecally administered edrophonium or neostigmine. The results are expressed as the mean ± SD. Doses (μg) are represented logarithmically on the X axis, and %MPE is represented on the Y axis.

Effects of Antagonists

Muscarinic *versus* Nicotinic. To identify the characteristics of the receptor effect of edrophonium and neostigmine, we administered intrathecally a nonselective muscarinic receptor antagonist (10 μg atropine) or a nicotinic receptor antagonist (10 μg mecamylamine) 5 min before either edrophonium or neostigmine were given. As illustrated in figures 5 and 6, the effects of the maximally effective dose of either edrophonium or neostigmine were significantly antagonized by intrathecal atropine ($P < 0.05$) but not by mecamylamine. The baseline threshold was not changed or even mildly decreased after intrathecal injection of atropine. Although there was a modest reduction in the response with mecamylamine, this difference was not statistically significant. Threshold values at 45 and 60 min in the edrophonium group and 45 min in the neostigmine group were significant but beyond the peak of spinal action duration.

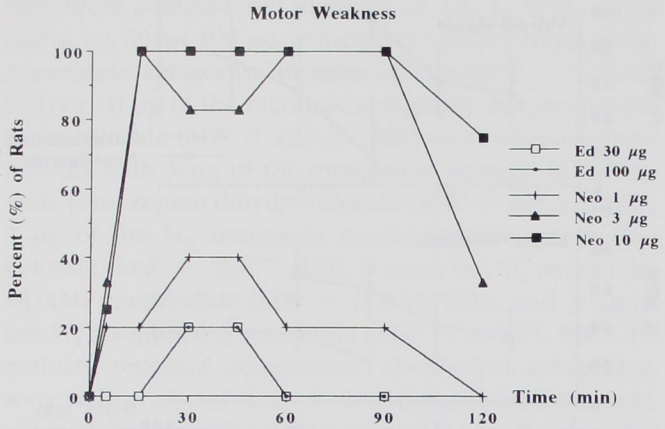

Fig. 4. The percentage of rats showing a motor weakness score of 2 after intrathecal injection of edrophonium or neostigmine. Ed = edrophonium; Neo = neostigmine.

Muscarinic Subtype. In figure 7, antiallodynia produced by intrathecal edrophonium (100 μg) was reversed by pretreatment with intrathecal methoctramine, 4-DAMP, tropicamide, and pirenzepine ($P < 0.05$). In contrast, the antiallodynic state produced by intrathecal neostigmine (10 μg) was not antagonized by methoctramine, 4-DAMP, or tropicamide. Only the M_1 antagonist pirenzepine had a moderate effect on reversal of the increased allodynic threshold ($P < 0.05$; fig. 8), but atropine showed complete antagonism (fig. 6). The magnitude of difference for the reversal of the antiallodynic effect between atropine and selective muscarinic antagonists was significant ($P < 0.05$).

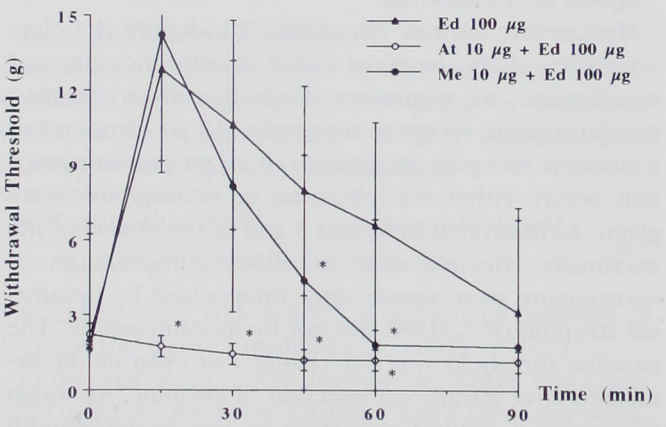

Fig. 5. Time courses of antagonism by 10 μg atropine or 10 μg mecamylamine administered intrathecally duirng the course of antiallodynia produced by 100 μg intrathecally administered edrophonium. The results are expressed as the mean ± SD of four or five rats in each group. The asterisk shows the value of $P < 0.05$ compared with edrophonium group. Ed = edrophonium; At = atropine; Me = mecamylamine.

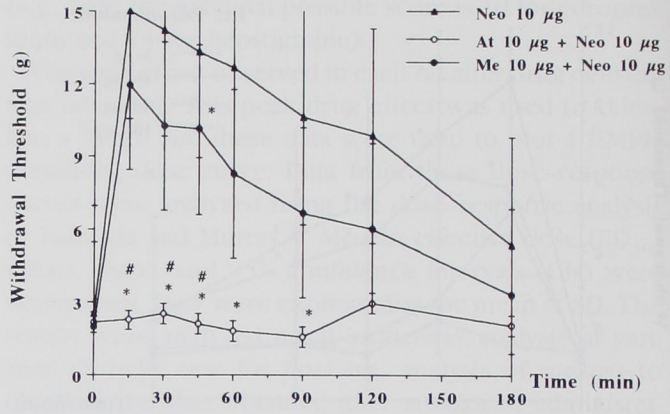

Fig. 6. Time courses of antagonism by 10 μg atropine or 10 μg mecamylamine administered intrathecally during the course of antiallodynia produced by 10 μg intrathecally administered neostigmine. The results are expressed as the mean ± SD of four or five rats in each group. The asterisk shows the value of $P < 0.05$ compared with the neostigmine group. The number sign shows the value of $P < 0.05$ compared with mecamylamine group. Neo = neostigmine; At = atropine; Me = mecamylamine.

Discussion

Post-Nerve Injury Allodynia

Peripheral nerve lesions may generate a syndrome in which innocuous low-intensity mechanical stimuli can evoke a powerful escape behavior (*i.e.*, tactile allodynia).[19,20] This abnormal pain state may be diminished by sympathectomy[21,22] and is considered to be a component of sympathetically dependent pain. Thus, it is relatively less sensitive to opiates[12,23] but may be diminished by α_2 agonists.[12,24,25]

The mechanisms underlying the nerve injury-induced

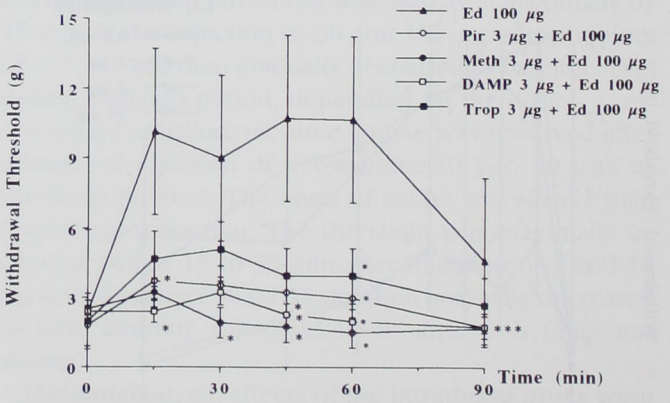

Fig. 7. Effects of pretreatment with each selective muscarinic antagonist on reversal of the antiallodynic state produced by 100 μg intrathecally administered edrophonium. The results are expressed as the mean ± SD of six to eight rats in each group. The asterisk shows the value of $P < 0.05$ compared with edrophonium group. Ed = edrophonium; Pir = pirenzepine; Meth = methoctramine; Trop = tropicamide.

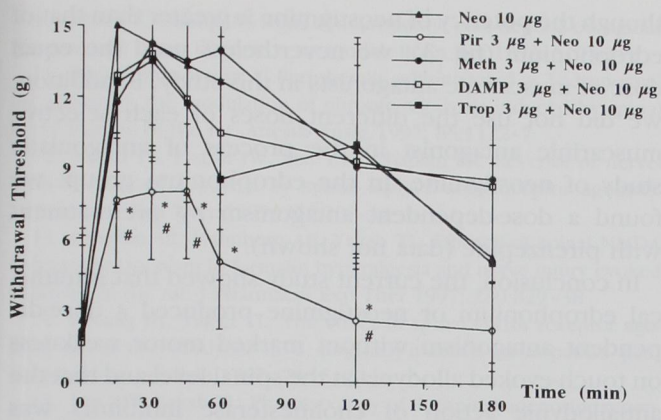

Fig. 8. Pretreatment with the intrathecal muscarinic antagonist pirenzepine only reversed the antiallodynic state produced by intrathecal neostigmine. The results are expressed as the mean ± SD of four to six rats in each group. The asterisk shows the value of $P < 0.05$ compared with the neostigmine group. The pirenzepine group also shows the value of $P < 0.05$ compared with the other three antagonist groups (#). Neo = neostigmine; Pir = pirenzepine; Meth = methoctramine; Trop = tropicamide.

miscoding of low-threshold afferent information are not fully understood, but several events are relevant. First, after local axon injury, spontaneous activity in mid-axon sprouts and dorsal root ganglion,[26,27] trans-synaptic changes in the appearance of dorsal horn neurons,[28–30] sprouting of large afferents,[31] and *de novo* sympathetic innervation of dorsal root ganglion neurons[32] have been reported. Second, peripheral axotomy has been shown to cause long-lasting sprouting of A fibers into lamina II, an area where they do not normally terminate.[31,33,34] Finally, peripheral nerve injury can result in loss of dorsal horn neurons and reduced GABAnergic tone. Such loss of intrinsic inhibition alone can result in an allodynic state.[35]

Spinal Cholinergic System. Autoradiographic studies have shown the existence of muscarinic receptors, both M_1 and M_2, in laminae II and III of the spinal cord that may be localized on the terminals of the primary afferent.[8] Retrograde transport studies have failed to show the evidence of a continuous cholinergic projection from the brain stem to the spinal cord in the rat.[36] Immunohistochemical studies have shown consistently the presence of cell bodies staining for choline acetyltransferase in laminae III, IV, and V,[37,38] with dendritic arborizations that spread to laminae I, II, and III, areas that were involved predominantly with the processing of afferent pain impulses. These results indicate that the muscarinic cholinergic system of the lumbar spinal cord is intrinsic. Intrinsic spinal cholinergic terminals have been shown to be presynaptic to primary afferents. These observations jointly suggest that cholinergic systems intrinsic to the spinal cord may regulate afferent input.[39] We suspect that acetylcholinesterase inhibitors act by the acetylcholine released from these intrinsic spinal terminals.

With regard to the effects of spinal cholinergic activation, Zhuo and Gebhart[2] suggested that a spinal cholinergic system exerts a tonic inhibitory effect on noxious mechanical, but not noxious thermal spinal transmission, and that a tonic, endogenous cholinergic muscarinic influence in the spinal cord modulates spinal mechanical transmission. The current study shows the antiallodynic effects of intrathecal neostigmine and edrophonium in a dose-dependent manner, and the results of this study may imply that muscarinic cholinergic stimulation of critical spinal sites results in the activation of local lumbar cholinergic circuits that may modulate the transmission of afferent allodynic information.

In our study, rats given neostigmine showed some side effects, such as salivation, tremor, and mild to moderate rigidity to movement of the hind limb. Lauretti *et al.*[3,4] reported that intrathecal neostigmine produced a dose-dependent antinociceptive effect with troublesome side effects in human studies, so they recommended the combined administration with other drugs to reduce the side effects. A finding of particular interest in this study was that more than half of the rats pretreated with intrathecal atropine showed, spontaneously or by evoked stimuli, intermittent pain behaviors, such as vocalization, tremor, agitation, and a moderate writhing action, for as long as 30 to 45 min. Several reports indicate that the spinal delivery of atropine appears to result in only modest hyperalgesia.[1,2,5] Intrathecal pretreatment with atropine, which attenuates the antinociceptive effect of intrathecal administration of carbachol, physostigmine, and neostigmine, also attenuates the antiallodynic effects of intrathecal administration of neostigmine or edrophonium.[1,2,40] Atropine has the highest potency or affinity with regard to all subtypes of receptors and, for example, is 10 times more potent than pirenzepine for the M_1-receptor subtype.[41] Thus, complete reversal of antiallodynia has been shown in pretreatment with atropine but not with subtype antagonists (figs. 5 and 6). Although there was a modest reduction in response with mecamylamine, this difference appeared to be caused by the reduction of moderate motor dysfunction produced by intrathecal administration of maximally effective doses of cholinesterase inhibitors.

Muscarinic Receptor Subtype. Muscarinic receptors are examples of G-protein-coupled receptors. The second messenger is subtype selective with M_1, M_3, and M_5 coupling to phospholipase C to generate inositol triphosphate, with M_2 and M_4 subtypes negatively coupled to adenylate cyclase to reduce the formation of cyclic adenosine monophosphate.[42] Höglund and Baghdoyan[43] reported that M_2-, M_3-, and M_4-, but not M_1-, muscarinic receptor subtypes are present in the rat spinal cord, showing that M_2 binding sites were distributed throughout the dorsal and ventral horns, whereas M_3 binding sites were localized to laminae I to III of the dorsal horn.

Several studies have tried to investigate the muscarinic receptor subtypes involved in the antinociception evoked by muscarinic agonists. Iwamoto and Marion[44] suggested that M_1-, M_2-, or both receptor subtypes were involved, whereas Sheardown *et al.*[45] suggested that M_1-receptor agonist activity was not a requirement for muscarinic antinociception. Using selective receptor antagonists, Naguib and Yaksh[46] showed that spinal antinociceptive effects were produced by intrathecal muscarinic cholinergic agonists in a dose-dependent manner and were likely mediated by spinal M_1-, M_3-, or both receptor subtypes.

We used only single doses of subtype antagonists, because, first, we thought our results could suggest a trend about the involvement of muscarinic receptor subtype, and second, these antagonists given are not more selective. In other words, it is important to note that muscarinic antagonists are only relatively selective, not exclusively specific, for individual subtypes.[47] In the current study in which we used selective muscarinic antagonists, we found that the antiallodynic effect of neostigmine was likely mediated by the spinal M_1-muscarinic subtype and that of edrophonium was reversed by four muscarinic subtype antagonists.

The M_1-receptor subtype is thought to be involved in antiallodynia at the spinal level. It is unclear why the results of the antagonistic study in the neostigmine group were different from those of edrophonium. Our study shows differences in the pattern of M_1-M_4 antagonist effects for edrophonium *versus* neostigmine. Such differences may be caused by different mechanisms of action of two cholinesterase inhibitors,[7] different affinities of each antagonist for receptor subtype, the doses of antagonists given, and different tissue localization or distribution patterns of receptor subtypes in the spinal cord. Therefore, these factors may affect the local concentration of acetylcholine at the action sites. Even though the potency of neostigmine is greater than that of edrophonium (fig. 3), we nevertheless used the equal dose of muscarinic antagonists in this study. In addition, we did not use the different doses of each selective muscarinic antagonist in the process of antagonistic study of neostigmine. In the edrophonium group, we found a dose-dependent antagonism by pretreatment with pirenzepine (data not shown).

In conclusion, the current study showed that intrathecal edrophonium or neostigmine produced a dose-dependent antagonism without marked motor weakness on touch-evoked allodynia at the spinal level and that the antiallodynic action of cholinesterase inhibitors was probably caused by modulation of the spinal muscarinic system, especially at the M_1 subtype. The magnitude of difference for the reversal of antiallodynic effects between atropine and selective muscarinic antagonists was significant. This finding may indicate that another muscarinic effect by the action of receptor sites other than spinal neuron may be involved, in part, in the antiallodynia.

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