



SPINALLY MEDIATED ANALGESIC INTERACTION BETWEEN GLUTAMATE RECEPTOR ANTAGONISTS AND BUPIVACAINE, MORPHINE, EPIBATIDINE, OR SEROTONIN IN RATS: WHICH IS THE MOST EFFECTIVE COMBINATION

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ABSTRACT

Background: The purpose was to find out the best combination between intrathecal N-methyl-D-aspartate (NMDA) receptor antagonist (AP-5), or α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist (YM-872) and bupivacaine, morphine, serotonin, or nicotinic acetylcholine receptor agonist (epibatidine) in analgesic effects. **Methods:** Male Sprague-Dawley rats implanted with lumbar intrathecal catheters were given intrathecal combination of AP-5 or YM872 and bupivacaine, morphine, serotonin, or epibatidine, then tail flick test or formalin test was performed. Isobolographic analysis was done using 50 % effective doses, and total fractional dose values were calculated. **Results:** In the tail flick test, AP-5 and bupivacaine, and YM 872 and epibatidine showed additive effects, and in the phase 2 of the formalin test, AP-5 and epibatidine had antagonistic effects. Other combinations in both tail flick test and formalin test showed synergistic effects. Morphine showed smallest total fractional dose value in both tail flick test and formalin test. **Conclusions:** When combined with intrathecal AP-5 or YM872, intrathecal morphine showed most potent analgesia in the agents tested for acute thermal and inflammatory acute and facilitated pain. Intrathecal epibatidine with AP-5 enhanced inflammation induced facilitated pain.

KEYWORDS: NMDA receptor, AMPA receptor, Local anesthetic, μ -opioid receptor, Nicotinic acetylcholine receptor, Serotonin.

INTRODUCTION

There are many mechanisms in the spinal cord regulating pain transmission. We have already shown analgesic effects of N-methyl-D-aspartate (NMDA) receptor antagonist,^[1] α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist,^[2] μ opioid receptor agonist,^[3] sodium channel blocker,^[4] nicotinic acetylcholine (nACh) receptor agonist,^[5] serotonin receptor agonist,^[6] α 2 receptor agonist,^[1] γ -aminobutyric acid A receptor agonist,^[5] etc. Then interaction of some of these agents were also investigated in our previous studies.^[1,3-7] However, the best combination is not known. The present study was performed to compare analgesic effects of the combination of μ opioid receptor agonist, sodium channel blocker, nACh receptor agonist, or serotonin receptor agonist, and NMDA receptor antagonist or AMPA receptor antagonist using the same models we have used in our previous studies to save the number of animals.

MATERIALS AND METHODS

After obtaining the approval of the Research Committee of the University of Tokyo, male Sprague-Dawley rats (280-300 g; Nippon Bio-Supply, Tokyo, Japan) were implanted with lumbar intrathecal catheters under halothane (2 %) anesthesia. The experiment procedures are the same as our previous study.^[2] Briefly, an 8.5 cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ) was inserted caudally to the thoracolumbar level in the intrathecal space through atlanto-occipital membrane. The rostral part of the catheter was plugged with a 28-gauge steel wire and put through to the top of the skull. Only rats with normal motor function and behavior and increase in body weight seven days later were used for experiments. After the study, rats were euthanized under halothane 5% and the location of the catheter was confirmed anatomically and the data of the rats with mal location of the catheter was excluded and another rat was added to fill the number of each group.

Drug preparation

Morphine (μ opioid receptor agonist, Merck, Sharpe and Dohme, West Point, PA), serotonin (serotonin receptor agonist, Sigma, St. Louis, MO), bupivacaine (sodium channel blocker, Sigma), epibatidine (nACh receptor agonist, Sigma), and AP-5 (2-amino-5-phosphonovaleic acid, NMDA receptor antagonist, Sigma) were dissolved in normal saline to get the concentrations used in each test. YM872 ([2,3-Dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxaliny] acetic acid, AMPA receptor antagonist, Yamanouchi Pharmaceutical Co. Ltd., Tsukuba, Ibaraki, Japan) 10 mg was dissolved in 0.97 mL distilled water with 30 μ L 1N NaOH added to adjust pH between 7.3 – 7.5, then dissolved in normal saline to get the concentrations used in each test just before administration.

Nociceptive test

According to our previous study,^[2] we used the same two classic methods as nociceptive tests.

Tail-flick test

The tail-flick test was performed with the Tail-Flick Analgesia Meter (MK-330A; Muromachi Kikai Co. Ltd., Tokyo, Japan). Rats were placed in a clear plastic cage with their tails extending through a slot located of the rear of the cage. Thermal stimulation was given by a beam of high intensity light focused on the tail 2 to 3 cm proximal to the end. The time between the start of the stimulation and tail withdrawal response was measured as a tail-flick latency. The cut-off time in the absence of a response was set to 14 seconds to prevent tissue injury of the tail. The test was done at 5, 10, 15, 30, 60, 90, 120, 180, and 240 minutes after drug injection. The data were shown as the % of maximum possible effect (% MPE): $\% \text{ MPE} = (\text{post-drug latency} - \text{pre-drug latency at time 0}) \times 100 / (\text{cut-off time (14 sec)} - \text{pre-drug latency at time 0})$.

Formalin test

The formalin test was performed 10 minutes after intrathecal drug injection. Fifty μ L of 5 % formalin was injected subcutaneously into the dorsal surface of the right hind paw with a 30 G needle. Immediately after injection, the rat was placed in an open clear plastic chamber and their flinching or shaking paw response was observed for 60 minutes. The number of flinches was counted for 1 minute. Usually two phases were observed: phase 1, during 0 to 6 minutes after formalin injection; and phase 2, beginning about 10 minutes after injection with the interval of no flinches between both phases.

Protocol

Each drug and combination intrathecally administered were adjusted in 10 μ L and after injection of the drug, the catheter was flushed with normal saline 10 μ L to clear the dead space of the catheter. In each test, 8 rats were used.

At first, tail flick test was performed with intrathecal morphine 1, 3, 10, and 30 μ g and ED50 was calculated. The ED50s of the tail flick test and the formalin test were already obtained with intrathecal AP-5,^[1] YM 872,^[2] serotonin,^[6] epibatidine,^[5] bupivacaine,^[4] and the ED50 of the formalin test with intrathecal morphine^[3] was also obtained in our previous studies, therefore, we used those data.

The combination of 1/2, 1/4, 1/8, and 1/16 ED50s of AP-5 or YM872 and serotonin, bupivacaine, or epibatidine in 10 μ L were studied in the tail flick test and the formalin test. For morphine, tail flick test with AP-5 adding 1/32 ED50 and YM872, and formalin test with YM872 were done. The formalin test with the combination of morphine and AP-5 was already done,^[3] therefore, we used those data.

The ED50 was obtained using the maximum effects in the tail flick test and the area under the curve of the number of flinches in the formalin test. To compare the theoretical additive point with experimentally derived ED50, isobolographic analysis was used. Total fractional dose values were calculated to describe the magnitude of the interaction as follows: $(\text{ED50 dose of drug 1 in combination}) / (\text{ED50 dose of drug 1 alone}) + (\text{ED50 dose of drug 2 in combination}) / (\text{ED50 dose of drug 2 alone})$. The value was normalized by assigning the ED50 value of each drug given alone as 1. Values near 1 suggest an additive interaction, values > 1 implies an antagonistic interaction, and values < 1 indicate a synergistic interaction.

Data analysis

The data are shown as mean $\pm$ standard deviation or 95% confidential interval (CI). Statistical analysis was performed with the analysis of variance (ANOVA) followed by Neuman-Keuls test to compare the calculated ED50 values with the theoretical additive values. A p value less than 0.05 was considered to be statistically significant.

RESULTS

The ED50s were shown in the Table 1, while isobolographs were not shown. Total dose fractional values were indicated in the Table 2. In the tail flick test, AP-5 and bupivacaine, and YM 872 and epibatidine showed additive effects, and in the phase 2 of the formalin test, AP-5 and epibatidine had antagonistic effects. Other combinations in both tail flick test and formalin test showed synergistic effects. Morphine showed smallest total dose fractional values in both tail flick test and formalin test.

Table 1: ED50

	Tail flick	Formalin phase 1	Formalin phase 2
AP-5 (μg) ^[2]	5.5 (2.2-13.8)	7.6 (4.3-13.6)	1.4 (0.05-35.9)
YM 872 (μg) ^[1]	1.0 (0.4-2.8)	0.24 (0.08-0.75)	0.21 (0.06-0.73)
Bupivacaine (μg) ^[5]	6.9 (3.8-10.0)	5.4 (1.9-9.6)	3.6 (1.4-6.1)
Bupivacaine + AP-5			
Bupivacaine (μg)	3.0 (1.5-5.9)	0.9 (0.4-2.1)*	0.9 (0.5-1.6)*
AP-5 (μg)	2.4 (1.3-4.7)	0.37 (0.16-0.85)*	0.37 (0.2-0.67)*
Bupivacaine + YM 872			
Bupivacaine (μg)	0.9 (0.62-1.31)*	0.14 (0.02-1.04)*	0.37 (0.03-5.04)*
YM 872 (μg)	0.13 (0.09-0.19)*	0.01 (0.001-0.06)*	0.02 (0.002-0.3)*
Morphine (μg)	1.4 (1.1-1.9)	7.1 (4.6-13.5)[6]	3.6 (1.3-9.5)[6]
Morphine + AP-5			
Morphine (μg)	0.002 (0.001-0.007)*	0.082 (0.036-0.135)*[6]	0.022 (0.007-0.067)*[6]
AP-5 (μg)	0.008 (0.002-0.01)*	0.032 (0.014-0.072)*[6]	0.009 (0.002-0.026)*[6]
Morphine + YM872			
Morphine (μg)	0.13 (0.08-0.22)*	0.005 (0.002-0.008)*	0.002 (0.0001-0.008)*
YM872 (μg)	0.096 (0.07-0.12)*	0.0025 (0.0001-0.0009)*	0.00011 (0.00002-0.0009)*
Serotonin (μg) ^[3]	34.4 (21.3-55.6)	12.6 (5.5-31.0)	1.3 (0.04-8.2)
Serotonin + AP-5			
Serotonin (μg)	3.7 (3.56-3.83)*	0.3 (0.02-4.85)*	0.22 (0.15-0.46)*
AP-5 (μg)	0.59 (0.57-0.68)*	0.3 (0.01-5.7)*	0.25 (0.125-0.33)*
Serotonin + YM872			
Serotonin (μg)	6.02 (5.1-8.3)*	0.2 (0.01-0.41)*	0.12 (0.08-0.19)*
YM872 (μg)	0.17 (0.14-0.21)*	0.002 (0.001-0.004)*	0.002 (0.0013-0.0035)*
Epibatidine (ng) ^[4]	32.0 (22.2-46.5)	38.0 (21.5-65.1)	27.1 (10.4-43.5)
Epibatidine + AP-5			
Epibatidine (ng)	4.6 (3.3-6.4)	4.6 (1.5-15.6)	52.0 (3.9-691)
AP-5 (μg)	0.8 (0.6-1.1)	0.25 (0.08-0.82)	2.7 (0.2-36.2)
Epibatidine + YM872			
Epibatidine (ng)	14.9 (5.9-37.7)	0.3 (0.03-3.8)	0.04 (0.002-0.85)
YM872 (μg)	0.46 (0.18-1.2)	0.002 (0.0002-2.8)	0.0003 (1.5×10^{-5} -0.006)

Mean (95% confidence interval) *: P < 0.05 vs. theoretical additive value

Table 2: Total dose fractional values.

	Tail flick	Formalin phase 1	Formalin phase 2
Bupivacaine + AP-5	0.94 (0.24-3.69)*	0.22 (0.05-1.3)	0.51 (0.088-1.45)
Bupivacaine + YM 872	0.26 (0.16-0.82)	0.07 (0.003-1.3)	0.10 (0.008-3.6)
Morphine + AP-5	0.003 (0.0007-0.01)	0.02 (0.0014-0.041)	0.013 (0.0016-0.33)
Morphine + YM872	0.19 (0.07-0.5)	0.002 (0.0003-0.013)	0.001 (0.00004-0.006)
Serotonin + AP-5	0.22 (0.11-0.47)	0.063 (0.00001-0.22)	0.33 (0.023-1.75)
Serotonin + YM872	0.35 (0.27-0.91)	0.024 (0.001-0.12)	0.1 (0.011-1.33)
Epibatidine + AP-5	0.29 (0.11-0.79)	0.15 (0.05-0.92)	3.85 (0.095-7.3) ⁺
Epibatidine + YM872	0.93 (0.21-1.7)*	0.065 (0.0009-1.68)	0.003 (0.00007-0.32)

Mean (95% confidence interval) *: additive effect, ⁺: antagonistic effect, Others show synergistic effect.

DISCUSSION

The present study showed morphine had the strongest synergistic analgesia with AP-5 and YM 872 in the tail flick test and both phases of the formalin test. Epibatidine had the antagonistic effect with AP-5 in the phase 2 of the formalin test. We used tail flick test and formalin test in this study because we have used these two methods in the previous studies, therefore, we could use some data from these previous studies^[1-6] to save animals. The tail flick test is a model of acute pain and the formalin test is

a model of prolonged pain with the phase 2 response associated with a peripheral inflammation and central hypersensitivity.

NMDA receptors in the spinal cord have a role in the development of central sensitization and neuropathic pain after formalin injection,^[8] play a role in the induction of inflammatory pain, but have no roles in the maintenance of chronic inflammatory pain.^[9] Increased presynaptic NMDA receptor activity can potentiate glutamate release

from primary afferent terminals to spinal dorsal horn neurons.^[10] Our previous study^[2] showed that intrathecal AP-5, a NMDA receptor antagonist, had analgesic effects on acute thermal and formalin induced acute and facilitated pain. AMPA receptor mediates fast excitatory transmission of acute nociceptive input.^[11] Spinal AMPA receptors have a role in the mechanism of both acute and persistent inflammatory pain.^[12, 13] Our previous study showed that spinally administered YM872, an AMPA receptor antagonist, had analgesic effects on acute thermal and formalin induced acute and facilitated pain in the rat models.^[2] AMPA complexes are physically coupled with NMDA receptors in the dorsal horn.^[14] During inflammation, AMPA receptor/NMDA receptor ratio increased, but AMPA mediated currents did not increase and NMDA mediated currents decreased.^[15] Therefore, for inflammatory pain, AMPA receptor antagonists might be more useful than NMDA receptor antagonists. In our previous study,^[2] the ratio of ED₅₀ of YM872 / AP-5 was smaller in both phases of the formalin test than in the tail flick test, which was consistent with the results of the study by Vikman et al.^[15] The total fractional dose value was significantly lower in YM872 than AP-5 in phase 2 of the formalin test. The AMPA receptors mainly contribute to synaptic transmission and plasticity triggered by different mechanisms.^[16]

Morphine has an action both pre- and postsynaptic to the primary afferent inducing analgesia on acute and tonic pain.^[17] Electrophysiological evidence was observed for interaction between NMDA and μ opioid receptors.^[18] Intrathecal opioid decreases the release of glutamate from small primary afferent fibers.^[19] Block of the NMDA receptor in the spinal dorsal horn potentiates analgesic effects of morphine.^[20] Morphine and AP-5 and an AMPA receptor antagonist, ACEA 2752 showed analgesic synergism in both phases of the formalin test in our previous study.^[3] The present results showed that intrathecal morphine had the strongest analgesic synergism with AP-5 and YM 872 in all the agents studied. For the acute thermal pain, AP-5 might be better, but for the inflammatory pain, YM 872 might be better combination with morphine from our results.

Local anesthetics are neuronal voltage-gated sodium channel blockers and have action on NMDA and AMPA receptors differently according to the type of anesthetics.^[21] Bupivacaine inhibits NMDA or AMPA pathway in postsynaptic neurons.^[22] Bupivacaine decreased peak amplitudes of NMDA-induced currents dose dependently,^[23] and also decreased NMDA-induced phosphorylated extracellular signal regulated kinase activation.^[24]

The antinociceptive effect of bupivacaine maybe due to direct modulation of NMDA receptors in the superficial dorsal horn^[23] as well as through voltage-gated sodium channels. Our present results showed that bupivacaine

had only an additive analgesic effect with AP-5 for acute thermal pain, but synergistic effects for inflammatory pain, and synergistic effects with YM 872 for both acute thermal and inflammatory pain.

Serotonin acts on different subtypes of 5-hydroxytryptamine (HT) receptors in the spinal cord. The activation of 5-HT₃ receptors decreases NMDA-induced motoneuron depolarizations,^[25] and induces analgesic effect.^[26] Serotonin selectively and potently decreased NMDA-induced current in spinal dorsal horn neurons.^[27] In contrast, 5-HT₂ receptor potentiates NMDA receptor mediated ion currents.^[28] Serotonin enhances L-glutamate-induced excitation of motoneurons in the spinal cord.^[29] Therefore, serotonin might have either analgesic or algesic effect. Our previous study^[6] showed analgesic effect of intrathecal serotonin in acute thermal and inflammatory pain. The present results had synergistic analgesia between intrathecal serotonin and AP-5 or YM 872 in both acute thermal and inflammatory pain, especially for inflammatory acute pain.

Epibatidine stimulates nACh receptors.^[30] Intrathecal nACh receptor agonists produce both analgesic and algesic behaviors via stimulation of separable populations of nACh receptors.^[31] The algesic effects of intrathecal epibatidine are mediated by $\alpha 7$ nACh receptors,^[31] but analgesic effects are mediated by different subtypes of nACh receptors.^[31] However, Rowley et al.^[32] reported that $\alpha 7$ nACh receptor activation might provide analgesia after injury. Therefore, different kinds of nACh receptors showed different effects according to the kinds of pain stimuli. Intrathecal nACh receptor agonists induced excitatory amino acid release and produced pain behaviors blocked by NMDA receptor antagonists.^[33] Our results showed intrathecal epibatidine alone showed analgesia in both acute thermal and inflammatory acute and facilitated pain. However, intrathecal epibatidine with AP-5 enhanced inflammation induced facilitated pain, while which nACh receptor had a role in this phenomenon was not clear. This should be further clarified.

In conclusion, when combined with intrathecal MNDA or AMPA receptor antagonist, intrathecal morphine showed more potent analgesia than bupivacaine, serotonin, and epibatidine in acute thermal and inflammatory acute and facilitated pain. Intrathecal epibatidine with NMDA receptor antagonist enhanced inflammation induced facilitated pain.

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