Pharmacologic Interaction between Cannabinoid and either Clonidine or Neostigmine in the Rat Formalin Test

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Background: Although spinal cannabinoid receptor agonist (WIN 55,212–2) has been shown to encounter various models of pain, the role of two subtypes of cannabinoid receptor for the antinociceptive effect of cannabinoids has not been investigated at the spinal level. Spinal α2 receptor agonist (clonidine) and cholinesterase inhibitor (neostigmine) are also active in the modulation of nociception. The authors examined the properties of drug interaction after coadministration of WIN 55,212–2-clonidine, and intrathecal WIN 55,212–2-neostigmine, and further clarified the role of cannabinoid 1 and 2 receptors in cannabinoid-induced antinociception at the spinal level.

Methods: Catheters were inserted into the intrathecal space of male Sprague-Dawley rats, and 50 μl of 5% formalin solution was injected into the hind paw to evoke the pain. Isobolographic analysis was used for evaluation of pharmacologic interaction.

Results: Intrathecal 55,212–2, clonidine, and neostigmine dose-dependently suppressed the flinching observed during phase 1 and 2 in the formalin test. Isobolographic analysis revealed a synergistic interaction after intrathecal delivery of WIN 55,212–2-clonidine or WIN 55,212–2-neostigmine mixture in both phases. The antinociceptive effect of WIN 55,212–2 was antagonized by cannabinoid 1 receptor antagonist (AM 251) but not by cannabinoid 2 receptor antagonist (AM 630). No antinociceptive effect was seen after intrathecal administration of cannabinoid 2 receptor agonist (JWH 133).

Conclusions: Intrathecal 55,212–2, clonidine, and neostigmine attenuated the facilitated state and acute pain. WIN 55,212–2 interacts synergistically with either clonidine or neostigmine. The antinociception of WIN 55,212–2 is mediated through the cannabinoid 1 receptor, but not the cannabinoid 2 receptor, at the spinal level.

Cannabinoid receptor agonists produce antinociception in animal models of acute pain and inflammatory pain by a direct spinal action, which is mediated cannabinoid receptors, mainly cannabinoid 1 (CB1) receptor. Clonidine and neostigmine reverse not only acute nociception but also tissue injury hyperalgesia through the action on spinal α2 adrenoceptor and cholinergic receptor, respectively. These findings suggest that cannabinoid receptor agonists, clonidine, and neostigmine may have a comparable profile of antinociceptive actions at the spinal level regardless of their different binding sites. However, there is little information or data about the pattern of their interaction with the other drugs. Although the CB1 receptor, which is located in the central nervous system, has been identified in the spinal cord using autoradiography and immunohistochemistry and the presence of cannabinoid 2 (CB2) receptor in the spinal cord and its role for the nociceptive transmission at the spinal level have not been evaluated.

Therefore, the aim of the present study was to determine the characteristics of the drug interaction between intrathecal cannabinoid receptor agonist (WIN 55,212–2) and clonidine, and between WIN 55,212–2 and neostigmine, using the formalin test, which shows tissue-injury pain leading to the facilitated state and acute pain. In addition, we sought to further clarify the role of cannabinoid receptor subtypes on the antinociceptive effects of intrathecal cannabinoid receptor agonist using the selective cannabinoid receptor antagonists.

Materials and Methods

Animal Preparation

The studies were reviewed and approved by the Institutional Animal Care Committee, Research Institute of Medical Science, Chonnam National University. Male Sprague-Dawley rats (250–300 g) were used for all experiments. The rats were maintained on a 12-h night/day cycle and allowed free access to food and water at all times. For drug administration, an intrathecal catheter was implanted during enflurane anesthesia, as previously described. The catheter was advanced caudally by 8.5 cm through an incision in the atlantooccipital membrane to the lumbar enlargement. The external end of the catheter was tunneled subcutaneously and exited at the top of head and plugged with a piece of steel wire. The skin was closed with 3-0 silk sutures. After surgery, rats were kept in individual cages. Only rats that displayed no post-surgical motor or sensory deficits were used. Animals showing neurologic dysfunction postoperatively were killed immediately. Studies were performed at least 4 or 5 days after intrathecal catheterization.

Drugs

The following drugs were used in this study: WIN 55,212–2 mesylate (Tocris Cookson Ltd., Bristol, UK), clonidine hydrochloride (Sigma Chemical Co., St., Louis, MO), neostigmine bromide (Research Biochemical International, Natick, MA), AM 251 (Tocris), AM 630 (Tocris), and JWH 133 (Tocris). WIN 55,212–2, AM 251, AM 630 and JWH 133 were dissolved in 100% dimethylsulfoxide, and clonidine and neostigmine were dissolved in...
normal saline. Intrathecal administration of these agents was performed using a hand-driven, gear-operated syringe pump. All drugs were delivered in a volume of 10 μl solution.

**Nociceptive Test**

For the formalin test, 50 μl of 5% formalin solution was injected subcutaneously into the plantar surface of the hind paw using a 30-gauge needle. The formalin-injection produces characteristic pain behavior: biphasic flinching or shaking of the injected paw. Such pain behavior was therefore quantified by periodically counting the incident of spontaneous flinching or shaking of the injected paw. The number of flinches was counted for 1-min periods at 1 and 5 min and at 5-min intervals from 10 to 60 min. Two phases of spontaneous flinching were observed after the formalin injection. Phase 1 and 2 were defined as 0–9 and 10–60 min after formalin injection, respectively. After the observation period of 1 h, the animals were immediately killed.

**Experimental Paradigm**

Four to 5 days after surgery, rats were placed in a restraint cylinder for the experiment. After a 15- to 20-min adaptation, rats were then assigned to one of the drug treatment groups. The control study was done using intrathecal saline or dimethylsulfoxide depending on the solvent for experimental drug. Each animal was used in one experiment only. The total number of rats used was 298, and each group comprised 6–9 rats. The investigator was unaware of which drug was administered to each animal.

**Effects of Intrathecal WIN 55,212-2, Clonidine, and Neostigmine**

For evaluation of the dose-response of the antinociceptive action of cannabinoid receptor agonist (WIN 55,212-2 0.3, 1, 3, 10 μg), α2 receptor agonist (clonidine 1, 3, 10, 30 μg), and cholinesterase inhibitor (neostigmine 0.1, 0.3, 1, 3 μg), each of three agents was intrathecally administered. Intrathecal drugs were injected 10 min before formalin injection. Each ED50 value (effective dose producing a 50% reduction of control formalin response) of three agents was separately determined in two phases.

**Drug Interaction**

An isobolographic analysis was used to determine the nature of pharmacologic interaction between spinal WIN 55,212-2 and clonidine, and spinal WIN 55,212-2 and neostigmine. This method is based on comparisons of doses that are determined to be equieffective. First, each ED50 value was determined from the dose-response curves of three agents alone. Next, WIN 55,212-2 and either clonidine or neostigmine were intrathecally coadministered at a dose of the ED50 values and fractions (1/2, 1/4, 1/8) of ED50 of each drug. From the dose-response curves of the combined drugs, the ED50 values of the mixture were calculated and these dose combinations were used for plotting the isobologram. In this experiment, the isobolograms were undertaken to characterize the effect of WIN 55,212-2-clonidine and WIN 55,212-2-neostigmine combinations. The isobologram was constructed by plotting the ED50 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:

\[
\text{Total fraction value} = \frac{\text{ED50 of drug 1 combined with drug 2}}{\text{ED50 for drug 1 given alone}} + \frac{\text{ED50 of drug 2 combined with drug 1}}{\text{ED50 for drug 2 given alone}}
\]

The fractional values indicate what portion of the single ED50 value was accounted for by the corresponding ED50 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 mixture was delivered intrathecally 10 min before the formalin test.

**Role of Cannabinoid Receptor Subtypes**

To determine whether the effect of intrathecal WIN 55,212-2 was mediated through certain subtypes of cannabinoid receptors, CB1 receptors antagonist (AM 251, 30 μg) and CB2 receptors antagonist (AM 630, 100 μg) were intrathecally administered 10 min before the delivery of WIN 55,212-2 (ED50). The maximal doses of cannabinoid receptor antagonists without affecting the control formalin response were determined from the pilot experiments. The formalin test was done 10 min after administration of WIN 55,212-2. These experiments were conducted in phase 1 and 2, respectively. Furthermore, we examined the effect of the CB2 receptor agonist (JWH 133). Because rats showed motor dysfunction at more than 100 μg of JWH 133, we evaluated its effect at 100 μg.

**General Behavior**

For evaluation of behavioral change of WIN 55,212-2, clonidine, and neostigmine, additional rats were received the highest doses of three agents used in this study, and examined at 5, 10, 20, 30, 40, 50, and 60 min after intrathecal administration. Motor function was assessed by the righting reflex and placing-stepping reflex. The former was evaluated by placing the rat horizontally with its back on the table, which normally gives rise to
an immediate coordinated twisting of the body to an upright position. Drawing the dorsum of either hind paw across the edge of the table evoked the latter. Normally, rats try to put the paw ahead into a position to walk. Changes in motor function were scored as follows: 0, normal; 1, slight deficit; 2, moderate deficit; 3, severe deficit. Pinea reflex and corneal reflex were also evaluated and judged as present or absent.

**Statistical Analysis**

Data are expressed as means ± SD. The time–response data are presented as the number of flinches, whereas the dose–response data are presented as percentage of control in each phase. To calculate the ED$_{50}$ values of each drug, the number of flinches was converted to percentage of control as follows:

\[
\frac{\text{Sum of phase 1 (2) flinching count with drug}}{\text{Sum of control phase 1 (2) flinching count}} \times 100
\]

Dose–response data were analyzed by one-way ANOVA with Scheffé for post hoc. The dose–response lines were fitted using least-squares linear regression and the ED$_{50}$ and its 95% confidence intervals were calculated according to the method described by Tallarida and Murray. The difference between theoretical and experimental ED$_{50}$, the antagonism for the effect of WIN 55,212–2, and the effect of CB2 receptor agonist were analyzed by t test. $P < 0.05$ was considered statistically significant.

**Results**

No change in pinna reflex, corneal reflex, and motor function was seen after intrathecal administration of WIN 55,212–2, clonidine, and neostigmine. The sum of the number of flinches in the saline or dimethylsulfoxide control group was not statistically different from each other in phase 1 (21 ± 4 vs. 19 ± 3) or phase 2 (166 ± 44 vs. 158 ± 38). Figure 1 displays the time course of intrathecal WIN 55,212–2, clonidine, and neostigmine, administered 10 min before formalin injection.

Intrathecal WIN 55,212–2, clonidine, and neostigmine produced a dose-dependent suppression of the flinching response during phase 1 and 2 in the formalin test (fig. 2). The ED$_{50}$ values (95% confidence intervals) of WIN 55,212–2, clonidine, and neostigmine in phase 1 were 1.4 (0.7–2.8), 10.3 (4.4–23.8), and 0.6 μg (0.4–0.8 μg), respectively. The phase 2 ED$_{50}$ values (95% confidence intervals) of WIN 55,212–2, clonidine, and neostigmine were 2.8 (1.3–6.1), 4.7 (3.1–7.2), and 0.3 μg (0.2–0.4 μg), respectively.

Isobolographic analysis revealed a synergistic interaction between intrathecal WIN 55,212–2 and clonidine, as well as intrathecal WIN 55,212–2 and neostigmine during phase 1 and 2 in the formalin test (figs. 3 and 4).

The experimental ED$_{50}$ values were significantly lower than the calculated ED$_{50}$ values. Accordingly, the phase 1 ED$_{50}$ values (95% confidence intervals) of the WIN 55,212–2 in the mixture of WIN 55,212–2-clonidine and WIN 55,212–2-neostigmine were 0.02 (0.01–0.02), 0.08 μg (0.01–1.0 μg), respectively. The phase 2 ED$_{50}$ values (95% confidence intervals) of the WIN 55,212–2 in the mixture of WIN 55,212–2-clonidine and WIN 55,212–2-neostigmine were 0.3 (0.03–1.7), 0.6 μg (0.2–1.8 μg), respectively. The total fraction values of the mixture of WIN 55,212–2-clonidine were 0.03 in phase 1 and 0.19 in phase 2, and those of WIN 55,212–2-neostigmine were 0.12 in phase 1 and 0.41 in phase 2, indicating synergistic interactions in both phases.

Intrathecal AM 251 and AM 630 alone did not affect the control flinching response evoked by formalin injection (fig. 5, A). Intrathecal AM 251 reversed the antinociceptive effect of intrathecal WIN 55,212–2 during phase 1 and 2 in the formalin test (fig. 5, B and C). However, the antinociceptive effect of intrathecal WIN 55,212–2 was not antagonized by intrathecal AM 630 in both phases.
Intrathecal JWH 133 did not alter the formalin-evoked flinching response in both phases (fig. 6).

Discussion

In the current study, intrathecal WIN 55,212–2 attenuated the flinching response during phase 1 and 2 in the formalin test. In the formalin test, phase 1 response seems to result from the immediate and intense increase of primary afferent activity. However, phase 2 response mirrors the activation of wide dynamic range of dorsal horn neurons with very low level of ongoing activity of primary afferent. Therefore, phase 2 reflects a facilitated state that seems to be prominent, considering the decreased level of afferent input. These observations suggest that WIN 55,212–2 may alter the facilitated state and the acute nociception at the spinal level. An interesting aspect of the results was the relative effectiveness of WIN 55,212–2 on phase 1 and 2 of the formalin response. A twofold-higher ED_{50} for phase 2 was observed compared to that for phase 1 with WIN 55,212–2. This effect was further evident in the combination studies when WIN 55,212–2 was combined with clonidine or neostigmine. The phase 2 to phase 1 ratio was 8–15 under the combined administrations. However, for both clonidine and neostigmine, the phase 2 to phase 1 ratio was 0.5. These data suggest that WIN 55,212–2 seems to be much more effective on the acute pain than on the facilitated state. However, previous studies showed different effects on formalin-induced response, in which injection of a cannabinoid receptor agonist attenuated phase 2 response, but not phase 1 or vice versa. Such discrepancy may be caused by the use of a different drug, the route of drugs given, the kind of animal, the concentration of formalin solution, and the type of measures made.

Cannabinoids are involved in the control of nociception at almost all relays of pain transmission, especially at the spinal level where they act through cannabinoid receptors. Cannabinoids activate two receptor subtypes, the CB1 and CB2 receptors. Expression of the CB1 receptor is essentially restricted to neurons and is coupled with G-proteins, whereas CB2 receptor expression is essentially restricted to immune cell lines. Moreover, CB1 receptor has been identified in the dorsal horn of the spinal cord intimately concerned with the processing of nociceptive information and the modula-
tion.\textsuperscript{1,12-15} However, no reports have been made to date regarding the expression of the CB2 receptor at the spinal cord. The antinociceptive actions of peripheral CB1 and CB2 receptor agonists have been reported,\textsuperscript{19,24} which implicates that peripheral CB1 and CB2 receptors participate in the control of nociception. However, the role of each subtype of CB receptors was not examined at the spinal level. In the current experiments, the CB1 antagonist, not the CB2 antagonist, antagonized the antinociceptive effect of WIN 55,212-2, and further administration of intrathecal CB2 agonist did not produce antinociception. These findings suggest that the antinociceptive action of cannabinoids is mediated through just the CB1 receptor at the spinal level.

There are at least three possible mechanisms by which cannabinoid may act spinally to reduce the nociceptive transmission.\textsuperscript{1} First, it may prevent the afferent barrage by acting presynaptically to inhibit neurotransmitter release. Activation of CB1 receptor leads to reduction of calcium channel currents through N-type\textsuperscript{25,26} and P/Q-type\textsuperscript{27} channels. Activation of the cannabinoid receptor has been reported to inhibit adenyl cyclase activity.\textsuperscript{28}

In addition, CB1 receptor activation has been shown to enhance potassium channel “A” currents\textsuperscript{29} and activate potassium currents\textsuperscript{30} through G-protein–coupled inwardly rectifying K⁺ channels. Initiating these currents or second messenger systems aids in decreasing the cellular excitability and reducing subsequent neurosecretion. Indeed, activation of the CB1 receptor inhibits the release of transmitter from primary afferent fibers in the dorsal horn of the spinal cord.\textsuperscript{1,3} Electrophysiologic studies have shown that cannabinoids suppress noxious stimulus-evoked activity of nociceptive neurons in the spinal cord,\textsuperscript{31} and that activity-dependent facilitation of nociceptive dorsal horn neurons is decreased after the application of cannabinoids to the spinal cord.\textsuperscript{32} Second, cannabinoids may act postsynaptically to stabilize membrane potentials at subthreshold levels via the enhancement of K⁺ currents and thus prevent the transduction of the nociceptive message. Third, cannabinoids may lead to disinhibition of an inhibitory circuit. Inhibition of terminal A removes the inhibition of terminal B resulting in the release of an antihyperalgesic substance.
Isobolographic analysis of this study revealed the synergistic interaction between intrathecal WIN 55,212-2 and clonidine, and between intrathecal WIN 55,212-2 and neostigmine during phase 1 and 2 in the formalin test. These results indicate that spinal combination of WIN 55,212-2 with clonidine or neostigmine can augment the antinociceptive effect of each drug alone, in both an acute nociceptive state and a tissue-injury state evoked by formalin stimulus. Although a pharmacologic interaction between two kinds of drugs is most likely complicated to characterize, several explanations could be possible for this synergy. First, drugs may interact by altering the kinetics of each other. One agent may alter the actions of the other agent at the receptor or channel. Second, such interaction may occur when both drugs affect different critical points along a common pathway.53 Cannabinoids, clonidine, and neostigmine act on receptors that are G-protein coupled. Hence, the action of these three agents may independently alter intracellular second messenger systems coupled with G-protein activation and mediate a synergistic interaction.54 Third, functional interaction may result from distinct drug effects at separate anatomic sites that may act independently and together to inhibit spinal nociceptive processing.55 As mentioned previously, cannabinoids, clonidine, and neostigmine possess both presynaptic and postsynaptic actions. Therefore, simultaneous engagement of presynaptic and postsynaptic mechanisms may augment the antinociceptive action produced by either drug acting at one site independently.56

Clinically, spinal cannabinoids are not yet available. However, in the future they can be used with clonidine or neostigmine to manage pain because their combination with clonidine or neostigmine may provide a decreased dose of either drug or an increased maximum achievable. Taken together, intrathecal WIN 55,212-2, clonidine, and neostigmine reduce the pain behavior evoked by formalin stimulus, and WIN 55,212-2 interacts with clonidine or neostigmine in a synergetic fashion. Spinal CB1 receptor, but not CB2 receptor, is involved in the antinociception of intrathecal WIN 55,212-2.

References


Fig. 6. Effect of intrathecal JWH 133 for flinching during phase 1 (A) and phase 2 (B) in the formalin test. The drug was administered 10 min before formalin injection. Data are presented as the sum of the number of flinches. Each bar represents the mean ± SD of six or nine rats.


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