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Cannabinoids attenuate capsaicin-evoked hyperalgesia through spinal and peripheral mechanisms

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Abstract

Previous studies in our laboratory have demonstrated that cannabinoids administered intravenously attenuate the duration of nocifensive behavior and block the development of hyperalgesia produced by intraplantar injection of capsaicin. In the present study, we extended these observations and determined whether cannabinoids attenuate capsaicin-evoked pain and hyperalgesia through spinal and peripheral mechanisms, and whether the antihyperalgesia was receptor mediated. Separate groups of rats were pretreated 7 min before capsaicin with an intrathecal injection of vehicle or the cannabinoid receptor agonist WIN 55,212-2 at doses of 0.1, 1.0 or 10 μg in 10 μl. Although the intrathecal application of WIN 55,212-2 did not alter nocifensive behavior following capsaicin, it produced a dose-dependent decrease in hyperalgesia to heat and mechanical stimuli. Intrathecal pretreatment with the CB1 receptor antagonist SR141716A (10 μg) blocked the antihyperalgesia produced by WIN 55,212-2. The ability of intrathecal administration of WIN 55,212-2 to attenuate hyperalgesia was not due to motor deficits since the highest dose of WIN 55,212-2 did not alter performance on the rota-rod test. To investigate whether cannabinoids attenuated capsaicin-evoked hyperalgesia through peripheral mechanisms, separate groups of rats were pretreated with an intraplantar injection of WIN 55,212-2 at doses of 0.1, 1.0, 10 or 30 μg in 100 μl 5 min before capsaicin. Intraplantar pretreatment with WIN 55,212-2 produced a dose-dependent attenuation of hyperalgesia to heat, but did not attenuate mechanical hyperalgesia or the duration of nocifensive behavior. The inactive enantiomer WIN 55,212-3 did not alter the development of hyperalgesia. SR141716A (100 μg) co-injected with WIN 55,212-2 (30 μg) partially attenuated the effects of WIN 55,212-2 on hyperalgesia to heat. Intraplantar injection of the highest dose of WIN 55,212-2 did not interfere with the development of hyperalgesia following capsaicin injection into the contralateral paw. These data show that cannabinoids possess antihyperalgesic properties at doses that alone do not produce antinociception, and are capable of acting at both spinal and peripheral sites.

Keywords: Pain; WIN 55,212-2; Hyperalgesia; Rats

1. Introduction

Cannabinoid receptors located in the central nervous system (CB1) and in peripheral tissues (CB2) have been identified and cloned (Matsuda et al., 1990; Kaminski et al., 1992; Munro et al., 1993; Pertwee, 1993; Howlett, 1995; Abood and Martin, 1996), and endogenous ligands have been discovered (Devane et al., 1992; Facci et al., 1995). Although it is well known that cannabinoids produce antinociception following systemic administration (Kaymakcalan and Deneau, 1971; Buxbaum, 1972; Chesher et al., 1973; Sofia et al., 1973; Bloom et al., 1977; Martin et al., 1991, 1996), various lines of evidence suggest that cannabinoids attenuate nociception through brain-stem and spinal mechanisms. For example, cannabinoid receptors are found in areas known to be involved in pain modulation, such as the periaqueductal gray and the dorsal horn of the spinal cord (Herkenham et al., 1990; Jansen et al., 1992; Tsou et al., 1998). In addition, behavioral studies indicate that injections of cannabinoids into these regions suppress responses to noxious stimuli (Yaksh, 1981; Lichtman and Martin, 1991; Lichtman et al., 1996; Martin et al., 1999a). Moreover, electrophysiological studies have shown that cannabinoids suppress noxious stimulus-evoked activity of nociceptive neurons in the spinal cord (Hohmann et al.,

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1995, 1998) and in the ventroposterolateral nucleus of the thalamus (Martin et al., 1996).

Recent evidence suggests that cannabinoids can modulate nociception peripherally by acting on CB1 cannabinoid receptors located on primary afferent fibers or through CB2 receptors located on immune cells. Studies employing in situ hybridization identified CB1 cannabinoid receptor mRNA in dorsal root ganglion (DRG) cells (Hohmann and Herkenham, 1999), while immunohistochemical studies describe CB1 receptor protein on DRG neurons and peripheral nerves (Sanudo-Pena et al., 1999). In addition, peripheral antinociceptive effects of cannabinoids were shown using a variety of nociceptive measures (Calignano et al., 1998; Richardson et al., 1998b; Ko and Woods, 1999; Hedley et al., 1999).

Hyperalgesia, defined as a decrease in pain threshold and/or increased pain to normally painful stimuli, often occurs following tissue injury and inflammation. Hyperalgesia can be localized to both the site of injury (primary hyperalgesia) and within surrounding, uninjured tissue (secondary hyperalgesia; Lewis, 1936; Hardy et al., 1950). Capsaicin, the pungent ingredient in hot peppers, has been used to produce hyperalgesia. When applied topically or injected into the skin of humans, capsaicin produces burning pain, hyperalgesia to heat within the immediate vicinity of capsaicin application, and mechanical hyperalgesia within a large surrounding area (Szolcsányi, 1977; Carpenter and Lynn, 1981; Simone et al., 1989; Culp et al., 1989; LaMotte et al., 1991). In rats, intraplantar (i.pl.) injection of capsaicin elicits nocifensive behavior characterized by licking, lifting, and shaking the injected paw (Gilchrist et al., 1996). In addition, capsaicin produces a decrease in withdrawal latency to radiant heat (heat hyperalgesia), as well as a decrease in the threshold (mN) for withdrawal from punctate stimuli and an increase in withdrawal frequency to suprathreshold mechanical stimuli applied to the injected paw (mechanical hyperalgesia).

Recently, we investigated whether cannabinoids attenuated nocifensive behavior and hyperalgesia produced by capsaicin. The capsaicin model of hyperalgesia was chosen because of its applicability to humans, the known physiological effects on nociceptors and other afferent fibers, and because it is a model of hyperalgesia associated with neurogenic inflammation. Intravenous (i.v.) administration of the cannabinoid receptor agonist WIN 55,212-2 dose-dependently attenuated capsaicin-evoked nocifensive behavior, mechanical hyperalgesia, and thermal hyperalgesia without producing motor deficits (Li et al., 1999). However, it is uncertain whether the effect of WIN 55,212-2 on hyperalgesia occurred at the level of the central or peripheral nervous system, or both. We hypothesized that separate spinal and peripheral mechanisms would contribute to the antihyperalgesia seen with i.v. WIN 55,212-2 administration. Therefore, in the present study, we investigated the contribution of spinal and peripheral mechanisms to attenuation of capsaicin-evoked nocifensive behavior and hyperalgesia by WIN 55212-2. A preliminary report has appeared (Johanek et al., 2000).

2. Methods

2.1. Subjects

A total of 116 adult, male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN) weighing 250–300 g were used. Animals were paired in plastic cages with soft bedding, maintained on a 12:12 light–dark cycle, and allowed access to food and water ad libitum. Animals that were implanted with an intrathecal (i.t.) catheter were housed individually following surgery. Each animal was used in one experiment only. All protocols were approved by the Animal Care Committee of the University of Minnesota.

2.2. Intrathecal catheterization

Animals were anesthetized with a combination of ketamine hydrochloride (100 mg/kg and acepromazine (1 mg/kg, i.m.)) and placed in a stereotaxic frame. A Polyethelene catheter (PE-10, Clay Adams, Parsippany, NJ), measuring 8.2 cm in length, was inserted through the atlanto-occipital membrane and directed to the lower lumbar spinal cord (L4–L5). A minimum recovery time of 4 days was allowed, and only animals that did not exhibit any motor deficits were used. The catheter was flushed with normal saline every other day to maintain patency, and was plugged with 30 gauge stainless steel wire. Appropriate localization of the i.t. injection was verified at the end of each experiment by injecting 30 μl lidocaine (1%) through the catheter. Only data obtained from animals showing reversible hindlimb motor deficits immediately following the lidocaine injection were used.

2.3. Cannabinoid preparation and administration

For i.t injection, the cannabinoid receptor agonist WIN 55,212-2 (Research Biochemicals, Inc.) was dissolved in a vehicle containing 50% dimethyl sulfoxide (DMSO) in normal saline, and the CB1 receptor antagonist SR141716A (provided by Sanofi Recherche, Montpellier, France) was dissolved in a vehicle containing 5% polyxyethylene(20)sorbitan monooleate (Tween 80) and 20% DMSO in normal saline. WIN 55,212-2 (0.1, 1 and 10 μg), SR141716A (10 μg), and the vehicle were administered intrathecally using a 50 μl microsyringe (Hamilton Company, Reno, NV). All injections were administered in a volume of 10 μl, followed by 10 μl of normal saline.

For i.pl. injection, WIN 55,212-2, its enantiomer WIN 55,212-3, and SR141716A were dissolved in a vehicle containing 5% Tween 80 and 5% DMSO in normal saline. Higher concentrations of DMSO were found to produce a transient hyperalgesia when injected into the skin. WIN 55,212-2 (0.1, 1, 10 and 30 μg), WIN 55,212-3 (100 μg),

and the vehicle were each administered in a volume of 100 μl. In order to determine whether any peripheral effects of WIN 55,212-2 were mediated via the CB1 receptor, WIN 55,212-2 (30 μg) and SR141716A (100 μg) were co-injected in a volume of 100 μl. Intraplantar injections were given into a randomly selected hindpaw using a 0.5 ml insulin syringe with a 28-gauge needle. Before any injection, a 5-mm diameter circle was marked on the center of the plantar surface of the paw to denote the drug or vehicle injection site, and subsequently, the capsaicin injection site.

2.4. Intraplantar injection of capsaicin

Capsaicin (8-methyl-*N*-vanillyl 6-nonamide; Sigma) at a concentration of 1 mg/ml (0.1%) was prepared as described previously (Gilchrist et al., 1996). Capsaicin was dissolved in a vehicle containing 7% Tween 80 in normal saline. The capsaicin solution was passed through a Millipore filter (0.22 μm) and stored in a sterile glass injection vial. Intraplantar injections of 10 μg were given in a volume of 10 μl using a 0.5 ml insulin syringe with a 28-gauge needle.

During the capsaicin injection, the needle penetrated the skin just distal to the marked area on the hindpaw, and the injection was made into its center. Care was taken to try to enter the same puncture hole made by the previous i.pl. drug or vehicle injection, and to deliver each injection superficially in the skin. The appearance of a bleb within the marked injection site on the hindpaw indicated a successful injection.

2.5. Withdrawal responses to mechanical stimuli

Rats were placed on an elevated plastic mesh (1 cm^2 perforations) under a clear plastic cage (23 × 13 × 13 cm) and were allowed to acclimate to the testing environment for at least 15 min. A calibrated von Frey monofilament with a bending force of 65 mN was used to deliver punctate stimuli to the plantar surface of the hindpaw and was applied from below the mesh floor. The stimulus was applied for a duration of approximately 1 s, with an interstimulus interval of approximately 5 s. The frequency of withdrawal responses evoked by the monofilament was obtained from ten consecutive trials. Random locations proximal and distal to the marked injection site on the plantar surface were stimulated, and the toes, heel, and injection site were avoided. Only robust and immediate withdrawal responses from the stimulus were counted as withdrawal responses. Mechanical hyperalgesia was defined as an increase in the frequency of withdrawal evoked by mechanical stimuli.

2.6. Withdrawal responses to heat stimuli

Rats were placed under a clear plastic cage (23 × 13 × 13 cm) on a 3-mm thick glass plate that was elevated to allow maneuvering of a radiant heat source from beneath. Rats were acclimated to the testing environment for at least 15 min prior to any stimulation. Radiant heat was applied to the plantar surface of each hindpaw, and the latency to withdrawal was determined according to a method similar to that described previously (Hargreaves et al., 1988). Heat stimuli were produced by a 50 W light bulb placed in a custom-built case which allowed focusing of the light source. The heat source was positioned such that the focused beam of radiant heat (8 mm diameter) was applied to the middle of the plantar surface at and surrounding the capsaicin injection site. Withdrawal latencies to the nearest 0.1 s were measured automatically by the use of a photocell that terminated each trial and stopped the timer upon withdrawal of the hindpaw. A 19 s cut-off was imposed on the stimulus duration to prevent tissue damage. Four stimuli were applied to each hindpaw, alternating between paws, with an interstimulus interval for each paw of at least 1 min. Withdrawal latency for each paw was defined as the mean of the last three trials. Withdrawal latencies were measured daily for each hindpaw during a training period of at least 4 consecutive days or until latencies were stable. The heat source was maintained at a constant intensity that produced stable withdrawal latencies of approximately 12 s during the training period. Hyperalgesia to heat was defined as a decrease in withdrawal latency.

2.7. Experimental design

Following baseline measures of withdrawal responses evoked by mechanical and heat stimuli, animals were divided into groups that were matched according to their withdrawal latencies to heat. For studies of antinociception produced by spinal administration of WIN 55,212-2, animals received vehicle (50% DMSO in saline) or WIN 55,212-2 at doses of 0.1, 1 or 10 μg ($n = 8$/group). In our earlier study (Li et al., 1999), it was found that i.v. administration of WIN 55,212-2 attenuated capsaicin-evoked hyperalgesia when given 5 min before capsaicin. Therefore, a similar injection protocol was used in this study, whereby WIN 55,212-2 was administered 7 min prior to the capsaicin injection to allow for the slow rate of i.t. injection (1–2 min). Additional groups were given the CB1 antagonist SR14716A (10 μg; $n = 6$) or vehicle (5% Tween 80 and 20% DMSO in saline; $n = 6$), followed by WIN 55,212-2 (10 μg) 5 min later, with a subsequent capsaicin injection given 7 min after the WIN 55,212-2 injection.

For studies of i.pl. cannabinoid administration, separate groups of animals were pretreated 5 min before capsaicin with a subcutaneous i.pl. injection of either vehicle (5% Tween 80 and 5% DMSO in saline; $n = 7$), WIN 55,212-2 at does of 0.1, 1, 10 or 30 μg ($n = 6$–8/group), 100 μg of WIN 55,212-3 ($n = 6$), or 30 μg WIN55,212-2 co-injected with 100 μg of SR141716A ($n = 6$). During the first 5 min after capsaicin, the duration of nocifensive behavior, defined as lifting and guarding the capsaicin-injected hindpaw, was determined. Withdrawal responses to mechanical and heat stimuli were obtained 30 min before (baseline)

capsaicin and at 5 (the time of peak hyperalgesia; Gilchrist et al., 1996) and 30 min after capsaicin.

To ensure that any antinociceptive effects of i.pl. administration of WIN 55,212-2 were mediated peripherally rather than centrally, WIN 55,212-2 (or vehicle) was injected into the hindpaw contralateral to the capsaicin injection. A randomly selected hindpaw was pretreated with either vehicle ($n = 6$) or 30 μg of WIN 55,212-2 ($n = 9$) in a volume of 100 μl. The experimental design presented above was followed with the exception that capsaicin was injected in the contralateral paw. To maximize our ability to detect a contralateral effect, mechanical hyperalgesia was evaluated using a von Frey with a greater bending force (116 mN) since the magnitude of mechanical hyperalgesia is a function of stimulus intensity (Gilchrist et al., 1996).

2.8. Effect of intrathecal WIN 55,212-2 on motor function

The effect of i.t. administration of WIN 55,212-2 (10 μg) on motor function was determined using the rota-rod test (Dunham and Miya, 1957; Kinnard and Carr, 1957). Animals were trained to run continuously on a rota-rod treadmill (Stoelting, Chicago, IL) for a maximum period of 5 min with a treadmill speed of 16 revs./min. The retention time on the treadmill was recorded 15 min before and 7 min after treatment with vehicle or WIN 55,212-2.

2.9. Statistical analysis

The effect of cannabinoids on nocifensive behavior was determined using one-way ANOVAs. To determine the effects of WIN 55,212-2 on heat and mechanical hyperalgesia following capsaicin injection, between and within subject analysis of variance (also termed split-plot ANOVA) was used. The subjects were rats, the between-subjects factor was drug treatment, and within-subject factors were paw (ipsilateral or contralateral) and time (baseline, 5 min after capsaicin, and 30 min after capsaicin). All comparisons were pre-specified and were treated as contrasts in the three-way interaction, drug treatment by paw by time, using the appropriate error terms from the ANOVA. For a given comparison, e.g. among treatment groups, the 0.05 significance level was spread among the different pairwise comparisons using the Bonferroni inequality. The amount of time animals treated with vehicle or WIN 55,212-2 were able to remain on a treadmill was compared using the Student's *t*-test. A probability value of 0.05 was considered significant. All data are expressed as the mean (±SEM).

To further determine the ability of WIN 55,212-2 to attenuate hyperalgesia following capsaicin, we determined the percentage of the maximal possible effect (%MPE) using the following formula in Eq. (1):

$$\%\text{MPE} = \frac{\text{test response} - \text{control response}}{\text{maximal response} - \text{control response}} \quad (1)$$

The test response represents the mean withdrawal frequency or withdrawal latency at 5 min after capsaicin injection in cannabinoid-treated animals; the control response is the mean response at 5 min after capsaicin in vehicle-treated animals, and the maximal response is the mean baseline response of all animals (i.e. 8.8% withdrawal frequency and 11.7 s withdrawal latency for the groups that received i.t. injections). Data are expressed as mean %MPE (±SEM) values. Dose response curves were generated where possible and the ED_{50} (with 95% confidence levels), defined as the dose producing 50% MPE, was determined.

3. Results

3.1. Studies of intrathecal administration of WIN 55,212-2

3.1.1. Nocifensive behavior following capsaicin

The duration of nocifensive behavior, defined as the amount of time spent guarding or avoiding pressure by raising the paw, was recorded within the first 5 min following capsaicin injection. Injection of capsaicin produced characteristic nocifensive behaviors persisting for an average of 209.4 ± 18.7 s in animals pretreated intrathecally with vehicle (50% DMSO in saline). Pretreatment with WIN 55,212-2 produced mean durations of nocifensive behavior ranging from 246.9 ± 13.4 (0.1 μg) to 168.4 ± 18.6 s (10 μg). A one-way ANOVA revealed no statistical difference in the duration of nocifensive behavior between vehicle and cannabinoid-treated groups. Thus, i.t. administration of WIN 55,212-2 did not attenuate the duration of nocifensive behavior relative to the vehicle-treated group.

3.1.2. Withdrawal responses to mechanical stimuli following capsaicin

Mechanical hyperalgesia, defined as an increase in withdrawal response frequency evoked by a von Frey monofilament with a bending force of 65 mN, developed in the injected paw following capsaicin. Before capsaicin, no differences in withdrawal response frequency were observed between the groups. As shown in Fig. 1A, the mean frequency of withdrawal increased from 10.0 ± 5.3% before injection to 56.3 ± 7.5% ($P < 0.001$) at 5 min and 45.0 ± 5.7% ($P < 0.001$) at 30 min after injection in animals pretreated with vehicle. Intrathecal administration of WIN 55,212-2 produced a dose-dependent decrease in withdrawal frequency as compared with the vehicle-treated group with an ED_{50} of 0.8 μg (0.4–1.6 μg). At 5 min following capsaicin, the mean frequency of withdrawal for animals pretreated with 0.1 and 1.0 μg increased significantly ($P < 0.001$) relative to baseline values, however, the increase to 33.8 ± 5.3% observed by the group pretreated with 1.0 μg was less than that observed for the vehicle-treated group ($P < 0.001$). At 30 min following capsaicin, withdrawal frequencies of animals pretreated with vehicle or 0.1 μg, but not with 1.0 μg WIN 55,212-2, remained elevated relative to baseline values ($P < 0.001$). Animals pretreated with the 10 μg

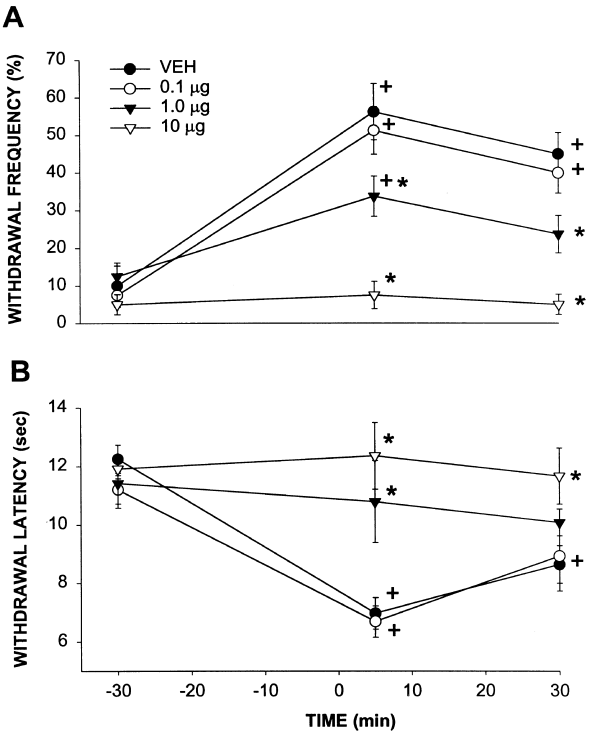

Fig. 1. Effect of i.t. application of vehicle or WIN 55,212-2 (0.1–10 μg) on capsaicin-evoked mechanical hyperalgesia (A) and thermal hyperalgesia (B). (A) Mean (±SEM) frequency of withdrawal evoked by mechanical stimuli before and at 5 and 30 min after i.pl. injection of capsaicin. (B) Mean (±SEM) withdrawal latency evoked by radiant heat before and at 5 and 30 min after. Data are shown for the injected paw only. In this and in subsequent figures, '−30' represents baseline values, and capsaicin was given at time '0'. A plus sign s(+) indicates a significant difference from baseline (−30 min); and an asterisk (*) indicates a significant difference from the vehicle-treated group.

dose of WIN 55,212-2 did not exhibit any increase in withdrawal response frequency after capsaicin. These data indicate that the 1.0 μg dose of WIN 55,212-2 attenuated the development of mechanical hyperalgesia (mean %MPE = 47.4 ± 11.2%), while the 10 μg dose completely blocked hyperalgesia (mean %MPE = 102.6 ± 7.7%).

3.1.3. Withdrawal responses to heat stimuli following capsaicin

Hyperalgesia to heat, defined as a decrease in withdrawal latency from a radiant heat source, developed in the injected paw following capsaicin. Prior to capsaicin, withdrawal latencies did not differ between the groups. As illustrated in Fig. 1B, animals pretreated with vehicle exhibited a decrease in withdrawal latency to heat from 12.3 ± 0.5 before capsaicin to 7.0 ± 0.5 s at 5 min after capsaicin, and remained decreased relative to baseline values at 30 min after capsaicin injection (8.6 ± 0.6 s). Pretreatment with WIN 55,212-2 attenuated hyperalgesia to heat in a dose-dependent manner as compared with animals pretreated with the vehicle. The ED_{50} for WIN 55,212-2 was 0.6 μg (0.2–1.8 μg). At 5 min following capsaicin, the mean withdrawal latency for animals pretreated with the 0.1 μg dose of WIN 55,212-2 did not differ from that obtained for the vehicle-treated group. In contrast, animals pretreated with either the 1.0 or 10 μg dose did not exhibit a decrease in withdrawal latency to heat after capsaicin, as evidenced by %MPE values of 80.6 ± 29.8 and 113.8 ± 24.2%, respectively. The mean latencies of 10.8 ± 1.4 and 12.4 ± 1.1 s, respectively, were greater than that exhibited by the vehicle group (7.0 ± 0.5 s; $P < 0.001$). At 30 min following capsaicin, only the vehicle-treated group exhibited a mean withdrawal latency (8.6 ± 0.6 s) that was decreased relative to baseline values ($P < 0.001$). Thus, doses of 1.0 and 10 μg WIN 55,212-2 given intrathecally blocked the development of hyperalgesia to heat produced by capsaicin.

3.1.4. Withdrawal responses to mechanical and heat stimuli in the contralateral paw

Intrathecal administration of WIN 55,212-2 did not alter withdrawal responses in the paw contralateral to the capsaicin injection. The frequency of withdrawal for the contralateral paw was not altered after capsaicin and did not differ among the groups (Fig. 2A). Mean withdrawal frequencies ranged from 6.3 ± 2.6 to 15.0 ± 3.8% across all groups at all times. Similarly, withdrawal latencies for the contralateral paw were not altered by capsaicin and did not differ between the groups (Fig. 2B). Withdrawal latencies for the contralateral paw ranged from 10.8 ± 0.7 to 13.8 ± 1.2 s across all groups and all times. The lack of effect of i.t. WIN 55,212-2 on the non-injected paw demonstrates that the doses of WIN 55,212-2 used in the present study did not produce antinociception in the absence of capsaicin.

3.1.5. Intrathecal administration of the CB1 antagonist SR141716A

SR141716A attenuated the antihyperalgesic effects of WIN 55,212-2 on mechanical hyperalgesia (Fig. 3A). Prior to capsaicin, the mean withdrawal response frequencies did not differ between groups that received vehicle (5% Tween and 20% DMSO in saline) or SR141716A (6.7 ± 3.3 and 6.6 ± 3.3%, respectively), each followed by WIN 55,212-2. At 5 min after capsaicin, the withdrawal frequency for animals pretreated with vehicle and WIN 55,212-2 increased from baseline values to 26.7 ± 6.1% ($P < 0.001$). Animals pretreated with SR141716A and WIN 55,212-2 exhibited a greater increase in withdrawal response frequency (41.7 ± 7.9%; $P < 0.01$). At 30 min after capsaicin, no differences in response frequency were observed between the groups or relative to baseline values.

SR141716A also attenuated the antihyperalgesic effect of WIN 55,212-2 on hyperalgesia to heat. Prior to treatment, there was no difference in the heat withdrawal latencies between the group that received SR141716A and WIN 55,212-2 and the group that received vehicle and WIN 55,212-2 (12.0 ± 0.5 and 12.7 ± 0.6 s, respectively; Fig. 3B). The mean withdrawal latency of animals pretreated

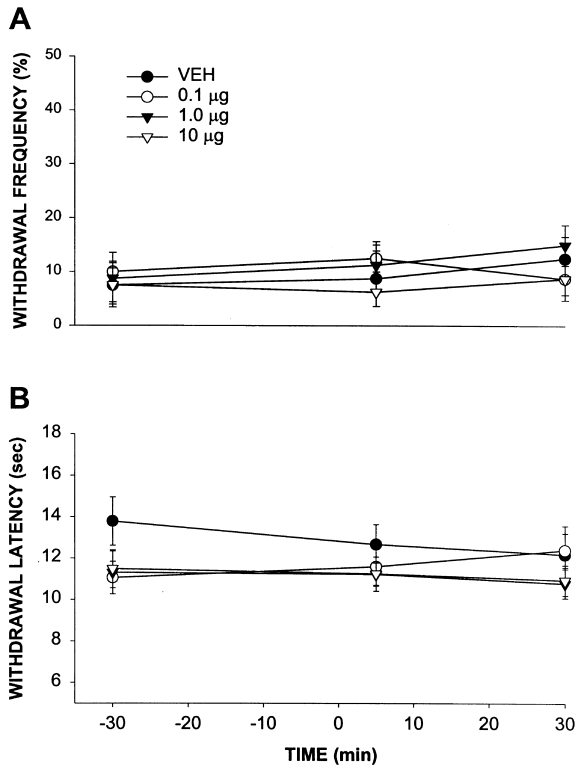

Fig. 2. Effect of i.t. application of vehicle or WIN 55,212-2 (0.1–10 μg) on withdrawal responses to mechanical (A) and heat (B) stimuli applied to the paw contralateral to the capsaicin injection. (A) Mean (±SEM) frequency of withdrawal evoked by mechanical stimuli before and at 5 and 30 min after i.pl. injection of capsaicin in the opposite paw. (B) Mean (±SEM) withdrawal latency evoked by radiant heat before and at 5 and 30 min after capsaicin injection in the contralateral paw. Data are shown for the paw contralateral to the capsaicin injection. No significant differences were found among the groups or across time.

with vehicle and 10 μg WIN 55,212-2 did not change at 5 min following capsaicin (12.3 ± 0.4 s), again demonstrating that this dose blocked the hyperalgesia to heat that occurs following capsaicin. However, a marked decrease in the withdrawal latency to heat was observed in animals pretreated with 10 μg SR141716A followed by administration of 10 μg WIN 55,212-2. At 5 min after capsaicin, a withdrawal latency of 6.1 ± 0.5 s was observed, which was shorter than that obtained for animals pretreated with vehicle and WIN 55,212-2 ($P < 0.002$), and which is consistent with the decrease in latency typically produced by capsaicin. Thus, SR141716A completely blocked the antihyperalgesia produced by WIN 55, 212-2. Collectively, these data suggest that a CB1 receptor mechanism is involved in the attenuation of capsaicin-evoked hyperalgesia by WIN 55,212-2.

3.1.6. Effect of intrathecal WIN 55,212-2 on motor function

Intrathecal administration of 10 μg of WIN 55,212-2 had no effect on the amount of time animals were able to run on a treadmill. Before administration of vehicle (50% DMSO) or WIN 55,212-2, all animals remained on the treadmill for the entire test period of 300 s (Fig. 4). After vehicle or WIN 55,212-2, all but one animal in each group remained on the treadmill for the entire 300 s period. It was therefore concluded that the effects of i.t. WIN 55,212-2 on capsaicin-evoked hyperalgesia were not attributed to motor deficits.

3.2. Studies of intraplantar administration of WIN 55,212-2

3.2.1. Nocifensive behavior following capsaicin

The duration of nocifensive behavior was recorded for 5 min following capsaicin injection. Injection of capsaicin produced characteristic nocifensive behavior that persisted for an average duration of 175.0 ± 20.6 s in animals pretreated with vehicle (5% Tween 80 and 5% DMSO in saline). Pretreatment with WIN 55,212-2 produced mean durations of nocifensive behavior ranging from 214.2 ± 33.4 (1 μg) to 135.5 ± 27.7 s (30 μg), while the enantiomer WIN 55,212-3 produced nocifensive behavior lasting for an average of 211.0 ± 19.5 s. A one-way

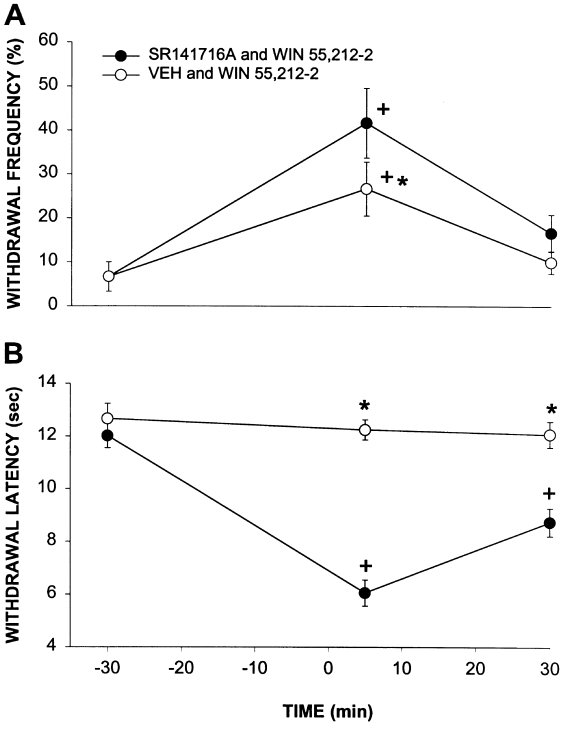

Fig. 3. Effect of the CB1 antagonist SR141716A on the ability of WIN 55,212-2 to attenuate hyperalgesia produced by capsaicin. Animals were pretreated intrathecally with vehicle or SR141716A, followed by WIN 55,212-2. (A) Mean (±SEM) withdrawal response frequency evoked by mechanical stimuli before and at 5 and 30 min after capsaicin. The withdrawal frequency increased for both groups; however, the increase was greater following SR141716A. (B) Mean (±SEM) withdrawal latency from radiant heat before and at 5 and 30 min after i.pl. injection of capsaicin. Animals pretreated with vehicle and WIN 55,212-2 did not become hyperalgesic to heat, while latencies for animals pretreated with SR141716A and WIN 55,212-2 decreased at 5 and 30 min after capsaicin. A plus sign (+) indicates a significant difference from baseline; and an asterisk (*) indicates a significant difference between the groups at 5 min after capsaicin.

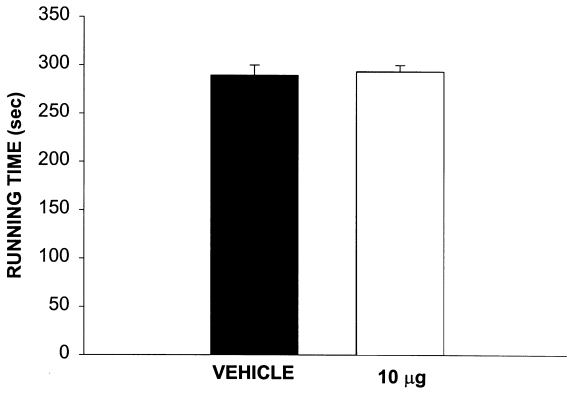

Fig. 4. Mean (±SEM) duration of running time on a treadmill at 7 min after i.t. application of vehicle or WIN 55,212-2 (10 μg). Prior to treatment, all animals were trained to run on the treadmill continuously for 5 min.

ANOVA did not reveal significant differences between groups in the duration of nocifensive behavior. Thus, i.pl. injection of WIN 55,212-2 was not effective in attenuating nocifensive behavior produced by capsaicin.

3.2.2. Withdrawal responses to mechanical stimuli following capsaicin

Before capsaicin, no differences between the groups were observed in the frequency of withdrawal responses. Animals that received i.pl. pretreatment with vehicle displayed an increase in the mean frequency of withdrawal from 5.7 ± 2.0% before capsaicin injection to 37.1 ± 6.1% ($P < 0.001$) at 5 min and to 15.7 ± 5.3% at 30 min after injection. Intraplantar pretreatment with WIN 55,212-2 did not significantly alter the increase in withdrawal frequency following capsaicin (Fig. 5A) and none of the %MPE values were significantly elevated above vehicle. Although groups pretreated with WIN 55,212-2 doses of 1.0 and 30 μg had mean withdrawal frequencies of 21.7 ± 4.0 and 23.3 ± 7.6%, respectively, at 5 min after capsaicin injection, these did not differ from that of vehicle-treated group (37.1 ± 6.1%). Pretreatment with the enantiomer WIN 55, 212-3 did not alter the development of mechanical hyperalgesia (Fig. 6A). No differences in mean withdrawal frequency were observed between the groups at 30 min following capsaicin. The frequency of withdrawal for the contralateral paw was not altered after capsaicin and did not differ among the groups treated with WIN 55,212-2.

3.2.3. Withdrawal responses to heat stimuli following capsaicin

Before capsaicin, no differences in the baseline withdrawal latencies were observed among the groups. As illustrated in Fig. 5B, animals pretreated with vehicle exhibited a decrease in withdrawal latency to heat from 12.7 ± 0.3 to 5.7 ± 0.5 s at 5 min after capsaicin ($P < 0.001$). At 30 min after capsaicin, the withdrawal latency increased to 9.9 ± 0.7 s, but was still less than baseline values ($P < 0.002$). Intraplantar injection of WIN 55,212-2 dose-dependently attenuated capsaicin-evoked hyperalgesia to heat with an ED_{50} of 6.9 μg (2.1–22.6 μg). Withdrawal latencies of animals pretreated with 0.1, 1.0 or 10 μg decreased at 5 min following capsaicin relative to baseline measures ($P < 0.001$). However, the decrease in latency to 8.8 ± 1.1s exhibited by animals pretreated with the 10 μg dose was less than that observed for the vehicle-treated group ($P < 0.001$; %MPE = 47.2 ± 15.3%). Withdrawal latencies for animals that received the 30 μg dose did not decrease after capsaicin relative to baseline values (%MPE = 71.5 ± 13.6%). At 30 min following capsaicin injection, animals pretreated with 0.1, 1.0 or 30 μg WIN 55,212-2 had increased withdrawal latencies as compared with the vehicle-pretreated group (9.9 ± 0.7 s), with mean latencies of 13.3 ± 1.3 ($P < 0.001$), 13.4 ± 1.4 ($P < 0.001$) and 12.9 ± 1.5 s ($P < 0.003$), respectively. Pretreatment with the enantiomer WIN 55,212-3 did not attenuate capsaicin-induced thermal hyperalgesia (Fig. 6B), as latencies decreased from 12.7 ± 0.3 s before capsaicin to 5.6 ± 0.6 and 10.3 ± 1.1 s at 5 and 30 min after capsaicin, respectively. Withdrawal latencies for the contralateral paw did not differ among groups pretreated with vehicle or WIN 55,212-2. These data demonstrate that i.pl. pretreatment with WIN 55,212-2 attenuated or blocked the development of hyperalgesia to heat produced by capsaicin.

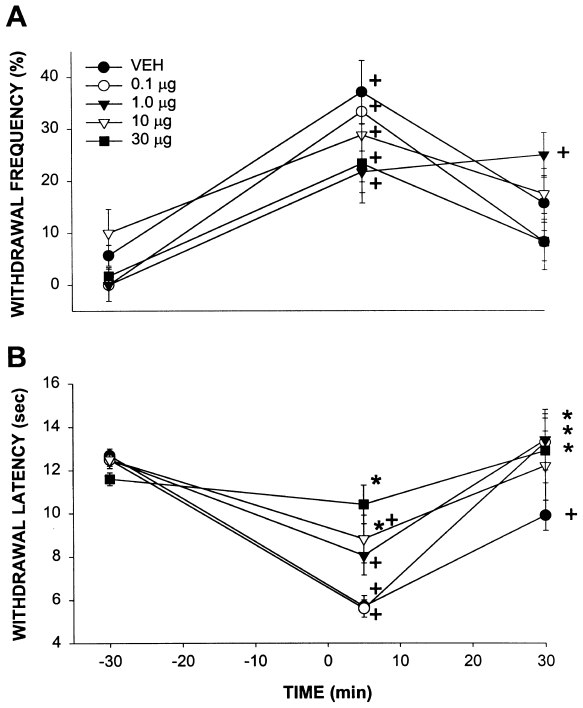

Fig. 5. Effect of i.pl. pretreatment with vehicle, WIN 55,212-2 (0.1–30 μg) on capsaicin-evoked mechanical hyperalgesia (A) and thermal hyperalgesia (B). (A) Mean (±SEM) frequency of withdrawal evoked by mechanical stimuli; and (B), mean (±SEM) withdrawal latency evoked by radiant heat, before and at 5 and 30 min after capsaicin. A plus sign (+) indicates a significant difference from baseline; and an asterisk (*) indicates a significant difference from the vehicle-treated group.

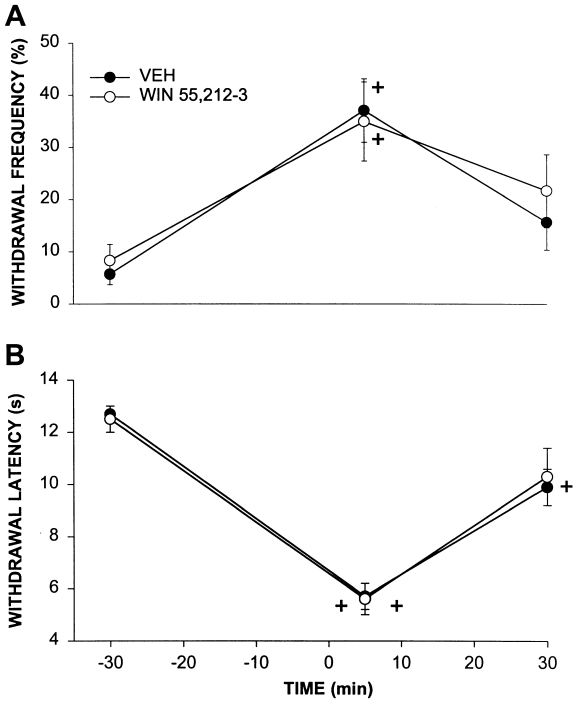

Fig. 6. Effect of i.pl. pretreatment with vehicle or the inactive enantiomer WIN 55,212-3 (100 μg) on mechanical and thermal hyperalgesia produced by capsaicin. (A) Mean (±SEM) frequency of withdrawal evoked by mechanical stimuli; and (B), mean (±SEM) withdrawal latency evoked by radiant heat stimuli, before and at 5 and 30 min capsaicin. A plus sign (+) indicates a significant difference from baseline.

3.2.4. Effect of intraplantar administration of SR141716A

At 5 min following capsaicin, animals pretreated with SR141716A (100 μg) co-administered with WIN 55,212-2 (30 μg) or with WIN 55,212-2, alone exhibited an increase in withdrawal frequency from baseline values ($P < 0.001$; Fig. 7A). No significant differences were observed between the groups in withdrawal responses at 5 min following capsaicin. However, animals pretreated with the antagonist co-administered with WIN 55,212-2 exhibited increased withdrawal frequency at 30 min following capsaicin ($P < 0.002$), whereas the frequency of withdrawal returned to baseline measures in animals pretreated with WIN 55,212-2 only. Differences in the mean frequency of withdrawal between the groups, however, were not observed at the 30 min time-point.

Intraplantar co-administration of SR141716A with WIN 55,212-2 partially attenuated the effect of WIN 55,212-2 on hyperalgesia to heat (Fig. 7B). While mean withdrawal latencies for animals that received the 30 μg dose of WIN 55,212-2 did not change from baseline after capsaicin, co-administration with SR141716A resulted in a reduction in withdrawal latency at 5 min following capsaicin ($P < 0.002$). Animals that received WIN 55,212-2 alone exhibited a 9.9 ± 8.2% decrease in withdrawal latency after capsaicin, and just one animal displayed a decrease in withdrawal of more than 30%. In contrast, animals that received co-administration of SR141716A and WIN 55,212-2 displayed a 29.5 ± 4.8% mean decrease in latency and four of six animals exhibited latencies that were reduced by more than 30%. However, the mean withdrawal latencies at 5 min after capsaicin did not differ between the groups. In addition, the mean withdrawal latency for animals that received SR141716A with WIN 55,212-2 (8.4 ± 0.9 s) was greater than the latency obtained following i.pl. administration of vehicle alone (5.7 ± 0.5; $P < 0.05$; see Fig. 5B). WIN 55,212-2 exhibited a %MPE of 71.5 ± 13.6%, while WIN 55,212-2 with SR141716A produced a %MPE of 41.4 ± 14.0%. Thus, SR141716A attenuated, but did not completely block, the antihyperalgesia produced by WIN 55,212-2.

3.2.5. Effect of WIN 55,212-2 injected into the paw contralateral to the capsaicin injection

To ensure that the effect of i.pl. administration of WIN 55,212-2 was local and not due to systemic diffusion of the drug, the highest dose of WIN 55,212-2 (30 μg) or the vehicle (5% Tween 80 and 5% DMSO) was injected into the paw contralateral to the capsaicin injection. It was found that injection of either vehicle or WIN 55,212-2 did not alter withdrawal response frequency for the capsaicin-injected

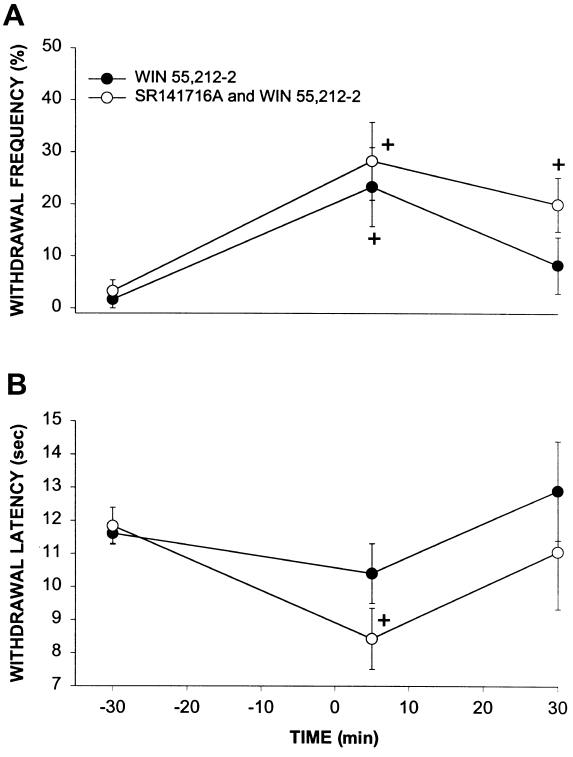

Fig. 7. Effect of i.pl. injection of the CB1 antagonist SR141716A on the ability of i.pl. injection of WIN 55,212-2 to attenuate hyperalgesia produced by capsaicin. Animals were pretreated with an i.pl. injection of either WIN 55,212-2 (30 μg) or a co-injection of WIN 55,212-2 (30 μg) and SR141716A (100 μg), followed by capsaicin injection. (A) Mean (±SEM) withdrawal response frequency evoked by mechanical stimuli; (B), mean (±SEM) withdrawal latency from radiant heat, before and at 5 and 30 min after capsaicin. A plus sign (+) indicates a significant difference from baseline.

paw. Before capsaicin, no differences in the withdrawal response frequency were observed between the groups. An increase in the frequency of withdrawal 5 min after capsaicin was apparent in animals that received either a contralateral i.pl. injection of vehicle or WIN 55,212-2 (Fig. 8A). The mean withdrawal frequency increased from 6.7 ± 3.3% before capsaicin to 35.0 ± 4.3% ($P < 0.001$) at 5 min after capsaicin for vehicle-pretreated animals, while frequencies increased from 5.6 ± 2.4% before to 40.0 ± 6.2% ($P < 0.001$) in animals pretreated with WIN 55,212-2. Similarly, both groups exhibited a comparable increase in withdrawal frequency at 30 min following capsaicin. Thus, capsaicin produced similar mechanical hyperalgesia in animals given a contralateral injection of either vehicle or 30 μg of WIN 55,212-2.

Withdrawal latencies from radiant heat for the capsaicin-injected paw were also not altered by a contralateral i.pl. injection of WIN 55,212-2. Capsaicin produced a significant decrease in latency at 5 min after capsaicin in animals with a contralateral injection of either vehicle ($P < 0.001$) or 30 μg of WIN 55,212-2 ($P < 0.001$; Fig. 8B). Withdrawal latencies decreased from 12.4 ± 0.4 s before capsaicin to 7.8 ± 0.9 s at 5 min after capsaicin in vehicle-pretreated animals, and decreased from 11.9 ± 0.2 to 7.7 ± 0.6 s in WIN 55,212-2-pretreated animals. There was no difference at 5 or 30 min between vehicle and WIN 55,212-2-pretreated animals in their mean withdrawal latencies. Collectively, these data suggest that the antihyperalgesic effects observed following i.pl. administration of WIN 55,212-2 are not due to central nervous system mechanisms resulting from systemic uptake of WIN 55,212-2, but rather result from local peripheral mechanisms. Intraplantar injection of WIN 55,212-2 at doses higher than 30 μg attenuated hyperalgesia following capsaicin injection in the contralateral paw (data not shown).

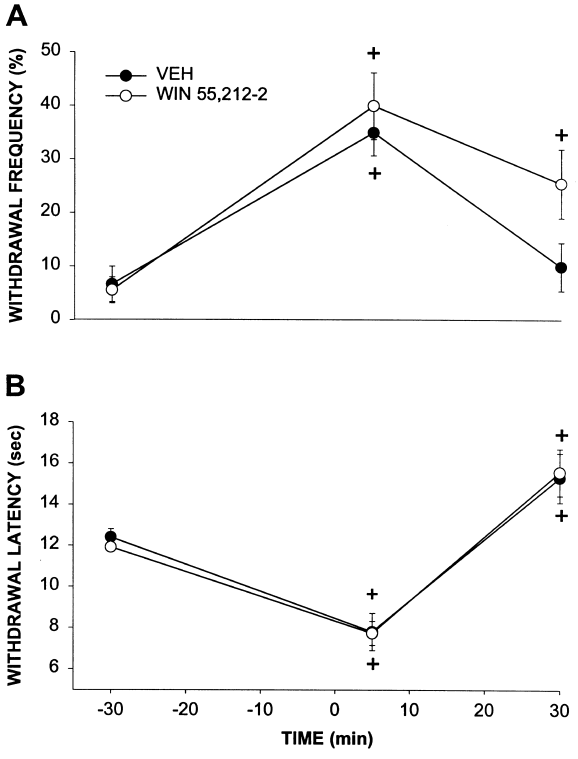

Fig. 8. Effect of i.pl. injection of WIN 55,212-2 on hyperalgesia produced by contralateral i.pl. injection of capsaicin. Animals were pretreated with either vehicle or WIN 55,212-2 (30 μg), and subsequently received capsaicin in the contralateral paw. (A) Mean (±SEM) withdrawal response frequency evoked by mechanical stimuli; and (B), mean (±SEM) withdrawal latency from radiant heat, before and at 5 and 30 min after capsaicin. Withdrawal frequencies increase for both the vehicle group and the WIN 55,212-2 group. Data are shown for the capsaicin-injected paw only. A plus sign (+) indicates a significant difference from baseline.

4. Discussion

In a previous study, we reported that i.v. administration of the cannabinoid agonist WIN 55,212-2 attenuated capsaicin-evoked nocifensive behavior and hyperalgesia (Li et al., 1999). Results of the present study show that WIN 55,212-2 attenuated capsaicin-evoked hyperalgesia through both spinal and peripheral mechanisms. Heat hyperalgesia was attenuated following both spinal and peripheral administration. In contrast, mechanical hyperalgesia was attenuated only by spinal administration of WIN 55,212-2. Interestingly, neither spinal nor peripheral cannabinoid administration notably attenuated nocifensive behavior evoked by capsaicin. Rather, the doses of WIN 55,212-2 used in this study produced 'antihyperalgesia' only, which is consistent with a previous report showing that systemic administration of cannabinoids attenuated hyperalgesia following peripheral nerve injury (Herzberg et al., 1997) or inflammation (Richardson et al., 1998a; Martin et al., 1999b). The antihyperalgesic effect of i.t. administered WIN 55,212-2 was supported in the present study by its lack of effect on withdrawal responses of the hindpaw contralateral to the capsaicin injection. Although cannabinoids have the ability to depress motor function (Compton et al., 1993), i.t. administration of WIN 55,212-2 had no effect on the ability of animals to perform on the rota-rod test. This is consistent with the earlier study in our laboratory showing that i.v. administration of WIN 55,212-2 (100 μg/kg) did not impair motor coordination (Li et al., 1999). It is therefore unlikely that the antihyperalgesic effects observed in the present study were attributed to depressed motor function.

The endogenous cannabinoid anandamide has been shown to decrease the second phase of formalin-evoked nocifensive behavior when administered intraperitoneally (Jaggar et al., 1998), and i.v. administration of WIN 55,212-2 decreased the duration of capsaicin-evoked nocifensive behavior (Li et al., 1999). Therefore, it was surprising that neither i.pl. nor i.t. WIN 55,212-2 attenuated the duration of nocifensive behavior in the present study. Attenuation of nocifensive behavior following i.v. administration of WIN 55,212-2 might be due to supraspinal site(s) of action. Microinjection of WIN 55,212-2 into supraspinal

areas involved in descending modulation, such as the dorsal lateral periaqueductal gray, dorsal raphe, or rostral ventral medulla, has been shown to elevate tail flick latencies (Martin et al., 1995, 1998). Cannabinoid actions occurring concurrently at a number of sites may be more effective in attenuating capsaicin-evoked nocifensive behavior than when administered only spinally or peripherally. However, anandamine has been shown to decrease both phases of formalin-evoked nocifensive behavior when injected into the paw (Calignano et al., 1998). The lack of effect of peripherally-applied WIN 55,212-2 on nocifensive behavior produced by capsaicin may be due to different populations of nociceptors that are activated by the two algesic chemicals. The distribution of cannabinoid receptors on peripheral terminals of various functional subtypes of primary afferent fibers remains unknown.

The CB1 receptor appears to contribute to the antihyperalgesia produced by i.t. administration of WIN 55,212-2 because antihyperalgesic effects were blocked by the highly selective CB1 receptor antagonist SR141716A (Rinaldi-Carmona et al., 1994). The present data are in agreement with earlier studies that reported attenuation of hyperalgesia through spinal CB1-mediated mechanisms. For example, behavioral evidence demonstrated that spinal administration of cannabinoids attenuates mechanical (Martin et al., 1999b) and thermal (Richardson et al., 1998a) hyperalgesia produced by inflammation of the rodent hindpaw. Studies of Fos expression have shown that formalin-induced Fos labeling in the rat spinal cord is suppressed by systemic WIN 55,212-2 (Tsou et al., 1996) and by i.t. CP55,940 (Hohmann et al., 1999a). Finally, electrophysiological recordings established that activity-dependent facilitation (wind-up) of nociceptive dorsal horn neurons is decreased following the application of cannabinoids to the spinal cord (Strangman and Walker, 1999).

In contrast, SR141716A was unable to attenuate the antihyperalgesic effects of i.pl. administration WIN 55,212-2 when co-administered with WIN 55,212-2. Co-administration of the antagonist may be less effective than pretreating with the antagonist alone, although other studies have found this method effective. Administration of anandamide into the hindpaw attenuated formalin-evoked pain behaviors (Calignano et al., 1998) and reduced thermal hyperalgesia following inflammation (Richardson et al., 1998b). The antihyperalgesic effects in the above studies were presumably mediated via the CB1 receptor because they were blocked by SR141716A. Using a capsaicin model of hyperalgesia in monkeys, Ko and Woods (1999) demonstrated that a 100 μg injection of capsaicin into the tail produced transient hyperalgesia to heat which was eliminated with co-administration of 10–320 μg of Δ^9-tetrahydrocannabinol (Δ^9-THC). This effect was also antagonized by SR141716A, further suggesting a role for CB1 receptors. However, other mechanisms, including the activation of a CB2-like cannabinoid receptor, may be involved in peripheral cannabinoid-mediated antihyperalgesia. CB2 receptors are located on mast and other immune cells (Facci et al., 1995; Galiegue et al., 1995) and may interact with cannabinoid agonists to prevent the release of injury-induced inflammatory mediators that sensitize primary afferent fibers (Mazzari et al., 1996). Interestingly, WIN 55,212-2 has been shown to have a higher affinity for the CB2 receptor over the CB1 receptor, exhibiting IC_{50} values for the functional inhibition of forskolin-stimulated cAMP accumulation in CHO-CB2 and CHO-CB1 cells of 0.407 ± 0.31 and 24.0 ± 3.7 nM, respectively (Felder et al., 1995). In behavioral studies, palmitylethanolamide (PEA), an endogenous agonist that interacts with CB2-like receptors, was effective in reducing formalin-evoked pain behaviors (Calignano et al., 1998) and in attenuating visceral hyper-reflexia following inflammation of the urinary bladder (Jaggar et al., 1998). However, unpublished studies from our laboratory suggest that i.pl. injection of PEA does not attenuate capsaicin-evoked hyperalgesia. It is also possible that cannabinoids can produce their effects through non-specific membrane mechanisms due to their lipophilic nature (Hillard et al., 1985). Although non-receptor mediated mechanisms could be involved in the antihyperalgesia produced by i.pl. administration of WIN 55,212-2, the finding that the enantiomer WIN 55,212-3 did not attenuate capsaicin-induced hyperalgesia argues against a non-receptor mechanism. Thus, although the present studies suggest that cannabinoids attenuate capsaicin-evoked hyperalgesia through peripheral mechanisms, the role of CB1 receptors is less clear.

4.1. Potential mechanisms by which cannabinoids produce antinociception

The spinal and peripheral mechanisms by which cannabinoids produce antihyperalgesia in the present findings are unclear. However, cannabinoids are thought to decrease cellular excitability and reduce neurotransmitter release. CB1 receptor activation has been reported to enhance potassium channel 'A' currents (Deadwyler et al., 1993) and activate potassium currents through G-protein-coupled inwardly rectifying K^+ channels (Henry and Chavkin, 1995). Activation of the CB1 receptor has also been shown to reduce calcium channel currents through N-type and P/Q-type channels (Caulfield and Brown, 1992; Mackie and Hille, 1992; Pan et al., 1996; Twitchell et al., 1997) and inhibit adenylyl cyclase activity (Howlett, 1984). Initiating these currents or second messenger systems aids in decreasing the excitability of the cell and reducing consequent neurotransmitter release. Indeed, cannabinoids have been found to inhibit glutamergic synaptic transmission in hippocampal cultures (Shen et al., 1996; Kim and Thayer, 2000). Moreover, inhibition of neuropeptide release from primary afferents has been documented. Anandamide was found to attenuate capsaicin-evoked CGRP release from isolated rat hindpaw skin (Richardson et al., 1998b) and from primary afferent fibers in the dorsal horn (Richardson et al., 1998a). Recent support for cannabinoid effects on primary afferent

fibers was provided by a study demonstrating that WIN 55,212-2 inhibits voltage-activated calcium current in cultured neonatal DRG neurons and in DRG X neuroblastoma hybridoma cells (Ross et al., 2000).

Since cannabinoids decrease neuronal excitability and neurotransmitter release, there are several potential spinal and peripheral neural mechanisms by which cannabinoids attenuate capsaicin-induced hyperalgesia. Spinal application of cannabinoids may decrease the excitability of second order neurons directly, or by attenuating neurotransmitter release from certain primary afferent fibers. It has been found that cannabinoid receptors are located on the central terminals of primary afferent fibers, as well as postsynaptically in the dorsal horn (Hohmann et al., 1999b). In the periphery, cannabinoids may decrease the responses of nociceptors evoked by capsaicin, or interfere with mechanisms that lead to nociceptor sensitization. Burning pain and hyperalgesia to heat following capsaicin is believed to occur, at least in part, by excitation and sensitization of C-fiber nociceptors (Kenis, 1982; Konietzny and Hensel, 1983; Szolcsányi et al., 1988; Baumann et al., 1991; LaMotte et al., 1992). Capsaicin activates vanilloid (VR1) receptors found on small diameter neurons that are thought to be C-fibers (Caterina et al., 1997; Tominaga et al., 1998). Recent findings have demonstrated co-localization of the VR1 and the CB1 receptors in the trigeminal ganglion (Price et al., 2000) and in the DRG (Ahluwalia et al., 2000). Thus, cannabinoids may diminish the excitability of certain C-fiber nociceptors. Since pretreatment with WIN 55,212-2 did not diminish nocifensive behavior, but attenuated mechanical and heat hyperalgesia, it is possible that WIN 55,212-2 did not attenuate responses of nociceptors excited by the capsaicin injection, but attenuated the sensitization of nociceptors.

4.2. Conclusions

This study provides evidence that cannabinoids act at peripheral and spinal sites to attenuate capsaicin-evoked hyperalgesia. Antinociceptive effects of cannabinoids resulting from interactions with primary afferent neurons are particularly important since unwanted side-effects associated with cannabinergic mechanisms in the CNS would be avoided. However, the types of afferent fibers affected by cannabinoids, and the physiological changes produced by cannabinoids, are still unknown. Electrophysiological studies are needed to determine cannabinoid effects on the excitation and sensitization of primary afferent nociceptors, and the conditions under which modulation of nociceptor excitability occurs.

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