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A cannabinoid agonist differentially attenuates deep tissue hyperalgesia in animal models of cancer and inflammatory muscle pain

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Abstract

Pain associated with cancer and chronic musculoskeletal disorders can be difficult to control. We used murine models of cancer and inflammatory muscle pain to examine whether the cannabinoid receptor agonist WIN55,212-2 reduces hyperalgesia originating in deep tissues. C3H/He mice were anesthetized and implanted with osteolytic NCTC clone 2472 cells into the humeri or injected with 4% carrageenan into the triceps muscles of both forelimbs. At the time of peak hyperalgesia, WIN55,212-2 (1–30 mg/kg) or vehicle was administered intraperitoneally and forelimb grip force was measured 0.5–24 h later. WIN55,212-2 produced time- and dose-related antihyperalgesia in both models. A 10 mg/kg dose of WIN55,212-2 fully reversed carrageenan-evoked muscle hyperalgesia. However, 30 mg/kg of WIN55,212-2 attenuated tumor-evoked hyperalgesia only ~50%. After controlling for the difference in magnitude of hyperalgesia between the two models, WIN55,212-2 was still more potent at reducing hyperalgesia in the inflammatory model. In the cancer pain model, the antihyperalgesic effect of WIN55,212-2 was partially blocked by pretreatment with the selective CB1 (SR141716A) but not the CB2 (SR144528) receptor antagonist. In contrast, both antagonists blocked antihyperalgesic effects of WIN55,212-2 on carrageenan-evoked muscle hyperalgesia. Catalepsy and loss of motor coordination, known side effects of cannabinoids, did not account for the antihyperalgesia produced by WIN55,212-2. These data show that cannabinoids attenuate deep tissue hyperalgesia produced by both cancer and inflammatory conditions. Interestingly, cannabinoids differentially modulated carrageenan- and tumor-evoked hyperalgesia in terms of potency and receptor subtypes involved suggesting that differences in underlying mechanisms may exist between these two models of deep tissue pain.

Keywords: Hyperalgesia; Carrageenan inflammation; Bone cancer; Cannabinoid receptors; WIN 55,212-2; Grip force

1. Introduction

Chronic, unremitting pain in deep tissues that results from cancer or musculoskeletal disorders adversely affects a disproportionately large portion of the population. Approximately 14 million people have cancer worldwide (Carver and Foley, 2000). More than 70% of patients in advanced stages of this disease report pain (Brescia et al., 1992; World Health Organization, 1996), and more than 36% of patients with cancer experience pain sufficiently severe to interfere with normal function (Cleeland et al., 1994). In particular, pain is most severe for patients with bone metastases, the most common cause of pain in advanced stage cancer patients (Mercadante et al., 1992), and is often difficult to manage adequately (Janjan et al., 1998). Non-malignant musculoskeletal pain affects an even larger share of the population and is one of the most frequent symptoms for which medical assistance is sought (Kantor, 1990). However, chronic pain of non-malignant origin is more likely to be inadequately managed than malignant pain (Cleeland et al., 1994), and like malignant pain can significantly impact the lives of affected individuals (Helig, 1988; Hitchcock et al., 1994; Holzberg et al., 1996).

Unfortunately, current therapeutic approaches for treating chronic pain of deep tissue origin are somewhat limited due

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to a lack of understanding of their underlying biological mechanisms (Strang, 1998; Mense and Simons, 2001). To address this problem, animal models of cancer (Schwei et al., 1999; Wacnik et al., 2000, 2001; Medhurst et al., 2002; Shimoyama et al., 2002; Wacnik et al., 2002) and inflammatory muscle (Kehl et al., 2000) pain were recently developed. These models mimic the movement-associated hyperalgesia characteristic of many types of cancer and muscle pain (McCain, 1994; Portenoy et al., 1999; Kehl et al., 2000; Wacnik et al., 2000). This type of breakthrough pain is considered to be a predictor of a poor response to routine pharmacotherapy in cancer patients (Mercadante et al., 1992; Bruera et al., 1995; Portenoy et al., 1999) and is particularly difficult to relieve in patients with bone metastases (Banning et al., 1991).

Currently, opioids are the principal agents employed for management of cancer pain but the therapeutic benefit of their prolonged use is frequently offset by the development of undesirable side effects such as constipation, sedation, respiratory depression and tolerance (Cherny, 2000). These side effects and concerns regarding the development of tolerance and/or dependence have hindered the widespread acceptance of long-term opioid use for management of chronic non-malignant pain (Dunajcik, 1999). A potential alternative to the use of opioids that has not yet been fully investigated is the use of cannabinoids. These compounds are derivatives of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive component of marijuana. Scattered reports suggest that cannabinoids exhibit analgesic effects in patients with cancer (Noyes et al., 1975; Staquet et al., 1978) and chronic muscle pain (Petro, 1980), but these evaluations have been of a limited nature. Studies to determine the mechanisms mediating their analgesic effects on deep tissue pain and direct comparisons of possible differences in the effectiveness of cannabinoids for reversing malignant and non-malignant pain have not been reported previously.

Cannabinoids exert their effects via activation of CB1 receptors, located in the central nervous system (CNS) and on primary afferent fibers, and CB2 receptors located primarily on immune cells (Matsuda et al., 1990; Gerard et al., 1991; Munro et al., 1993). These two receptor subtypes have been cloned (Matsuda et al., 1990; Gerard et al., 1991; Munro et al., 1993) and endogenous ligands have been identified for both (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995; Hanus et al., 2001; Porter et al., 2002). The non-selective endogenous agonist anandamide blocks carrageenan-evoked thermal hyperalgesia (Richardson et al., 1998a,b), nocifensive behavior following subcutaneous formalin injection (Calignano et al., 1998; Jagger et al., 1998) and mechanical hyperalgesia associated with bladder inflammation (Jagger et al., 1998). Similarly, the non-selective synthetic cannabinoid WIN55,212-2 attenuated nocifensive behaviors and both thermal and mechanical hyperalgesia produced by nerve injury (Herzberg et al., 1997). The recently identified endocannabinoid noladin ether binds to CB1 but not CB2 receptors and produces analgesia to heat stimuli (Hanus et al., 2001). Similarly, the selective CB2 agonists HU-308 and AM1241 have also been shown to produce antinociception to thermal stimuli (Hanus et al., 1999; Malan et al., 2001). Thus, the antinociceptive effects of cannabinoids occur through binding at both CB1 and CB2 receptors.

Evidence exists that cannabinoids produce antinociceptive effects via supraspinal, spinal and peripheral mechanisms. For example, intracerebroventricular (i.c.v.) administration of cannabinoid agonists produces antinociception in the tailflick and hotplate tests (Welch et al., 1995b; Raffa et al., 1999). Cannabinoids also exert antinociceptive actions at the spinal level in response to noxious thermal stimuli (Hohmann et al., 1998), formalin (Hohmann et al., 1999), capsaicin (Johanek et al., 2001), carrageenan (Richardson et al., 1998a), complete Freund's Adjuvant (Martin et al., 1999b), and intraabdominal phenylbenzoquinone (Welch et al., 1995a). Cannabinoids have been shown to decrease evoked activity of nociceptive dorsal horn neurons and to block 'wind-up' (Hohmann et al., 1998; Strangman and Walker, 1999). Cannabinoids also exert antinociceptive effects through peripheral mechanisms as evidenced by their capacity to attenuate capsaicin- and carrageenan-evoked mechanical and thermal hyperalgesia following intraplantar administration (Richardson et al., 1998b; Ko and Woods, 1999; Johanek et al., 2001). Furthermore, intraplantar administration of cannabinoids also reverses mechanical hyperalgesia in a model of neuropathic pain (Fox et al., 2001).

Although the reports summarized above indicate that cannabinoids are antinociceptive and antihyperalgesic in a variety of animal models, no prior studies have evaluated the antinociceptive properties of cannabinoids in animal models of cancer or muscle pain. We performed the studies described here to evaluate the effectiveness of the non-selective cannabinoid agonist WIN55,212-2 in blocking movement-associated hyperalgesia in murine models of these prevalent clinical pain states. We also investigated which cannabinoid receptor subtypes mediate effects of cannabinoids in these models. In addition, since cannabinoids are known to produce catalepsy and decrease motor coordination (Little et al., 1988), which may confound behavioral measures of nociception, we determined whether impaired motor function contributed to antinociception in these models. Preliminary reports have appeared in abstract form (Croft et al., 2001; Norsted et al., 2001).

2. Methods

All studies conformed to guidelines put forth by the International Association for the Study of Pain for the use of experimental animals (Zimmermann, 1983) and were approved by the University of Minnesota's Institutional Animal Care and Use Committee. For all experiments, data were collected by observers blinded to treatment allo-

cation including administration of carrageenan or tumor cells as well as drug administration.

2.1. Subjects

Male C3H/He mice (National Cancer Institute), 8–10 weeks old and weighing 20–28 g, were used in all experiments. The mice were housed in boxes of ten with free access to mouse chow and water in a temperature- and humidity-controlled environment maintained on a 12 h light/dark cycle.

2.2. Preparation of tumor cells and carrageenan

NCTC clone 2472 osteolytic fibrosarcoma cells (tumor cells), obtained from the American Type Culture Collection (Rockville, MD, USA), were grown in 75 cm^2 flasks containing NCTC 135 media (pH 7.35, Sigma Chemical, St. Louis, MO, USA) and supplemented with 10% horse serum (HyClone Laboratories, Logan, UT, USA) as previously described (Clohisy et al., 1996). For implantation, tumor cells were trypsinized, pelleted, washed once with phosphate-buffered saline (PBS) and counted. Cells were pelleted and resuspended in PBS at a concentration of (20×10^7/ml) and held on ice until implantation. Carrageenan (4%; Sigma Chemical, St. Louis, MO, USA) was suspended in PBS, briefly sonicated (<2 min), centrifuged at 3000 rpm (30 min at 4°C) and stored at 4°C for up to 24 h prior to injection.

2.3. Tumor cell implantation and injection of carrageenan

Mice were briefly anesthetized using 1–2% halothane in air until deep anesthesia was demonstrated by the absence of a withdrawal response to paw pinch. Tumor cells (2×10^5 in 10 μl PBS/humerus) were implanted into the medullary canals of both humeri as follows: the proximal end of each humerus was identified by flexing the limb, which caused the joint to protrude. The joint was probed with a 29 gauge needle attached to a 0.3 ml insulin syringe containing the cells until the proximal end of the humerus was identified. Once identified, the needle was positioned parallel to the long axis of the humerus and the needle was used to bore a hole into the medullary space. After the needle passed through the bone and into the medullary canal, the cells were injected. Carrageenan (4% in 40 μl PBS/triceps) was injected into both triceps muscles in mice under halothane anesthesia.

2.4. Measurement of grip force

Measurement of forelimb grip force was made using a grip force analyzer as previously described (Kehl et al., 2000). This apparatus measured the amount of tensile force each mouse exerted against a wire mesh grid (12×7 cm^2) attached to a force transducer (DFIS series; Chatillon, Greensboro, NC). During testing, each mouse was held by its tail, allowed to grasp the wire mesh grid with its forepaws, and gently pulled (~10 cm/s) in a caudal direction. The peak force exerted by the mouse before it released its grasp of the wire mesh grid was registered by a force transducer and recorded in grams. Importantly, the force each mouse applied to the mesh grid was determined by the mouse itself. Therefore, the amplitude of force exerted was subject to factors, such as hyperalgesia, which influenced the behavioral performance of each mouse.

Three consecutive forelimb grip force measurements (~10 s apart) were obtained for each mouse at each time point; the average of these three measurements was used to represent each mouse's grip force for each time point. Reduction in grip force following intramuscular (i.m.) carrageenan or intraosseous tumor cell implantation relative to baseline grip force levels provides an index of the reduction in nociceptive threshold to mechanical stimulation of deep tissues in the forelimb. Mice that failed to demonstrate at least a 33 g reduction in grip force following tumor cell implantation or at least a 19 g reduction in grip force after i.m. carrageenan were eliminated from the study because they had not developed a sufficient level of hyperalgesia to assess the ability of the cannabinoid to produce antihyperalgesia. We selected these cutoff levels of reduction in grip force because they were two standard deviations below the average reduction in grip for each model in our preliminary studies.

2.5. Drug preparation and administration

WIN55,212-2 (0.1–3 mg/ml; Sigma Chemical Co., St. Louis, MO), a non-selective cannabinoid agonist, and its inactive enantiomer WIN55,212-3 (1 and 3 mg/ml; Sigma Chemical Co., St. Louis, MO), were prepared in 10% emulphor in 0.9% saline. The selective CB1 receptor antagonist SR141716A (1 mg/ml) and the selective CB2 receptor antagonist SR144528 (0.3 mg/ml) (National Institute of Drug Abuse) were each prepared in a 1:1:18 mixture of ethanol, emulphor, and 0.9% saline. Drug solutions were vortexed and then sonicated until completely dissolved. All drugs were administered by intraperitoneal (i.p.) injection.

2.6. Experimental design

Forelimb grip force measurements for all mice were obtained on 3 consecutive days. Following acquisition of the third set of grip force measurements (baseline), tumor cells were implanted into half of the mice. Then grip force measurements were obtained for all mice (implanted and not implanted) on the third and sixth days after tumor implantation. On the seventh day after implantation, all mice were again tested for grip force. Mice that had not received tumor cells were given i.m. carrageenan. Forty-eight hours later, pre-drug grip force measurements were obtained for both groups. Previous studies (Wacnik et al., 2002) demonstrated that this time course for tumor cell implantation and carrageenan injection allowed peak hyper-

algesia for each model to occur on the same day and to occur without observable motor deficit.

Immediately following acquisition of pre-drug grip force measurements, WIN55,212-2 (1–30 mg/kg), WIN55,212-3 (10 or 30 mg/kg), or vehicle was administered by i.p. injection to test their capacity to attenuate the reduction in grip force. Forelimb grip force was measured at 0.5, 1.5, 3 and 24 h following administration of WIN55,212-2 and compared to pre-drug values. In additional experiments, we evaluated whether the effects of WIN55,212-2 were mediated through CB1 or CB2 receptor subtypes. After measurement of pre-drug grip force and 15 min prior to administration of WIN55,212-2, the selective cannabinoid receptor antagonists SR141716A (10 mg/kg) and SR144528 (3 mg/kg) were administered by i.p. injection. Again, forelimb grip force was measured 30 min following administration of WIN55,212-2 and compared to pre-drug values.

When we performed experiments using the time course outlined above, the reduction in grip force following tumor cell implantation was approximately two times greater than that after carrageenan injection. Therefore, to control the effect that this difference in amplitude might have on the apparent capacity of WIN55,212-2 to attenuate tumor-evoked hyperalgesia, we tested the effect of WIN55,212-2 (3, 10, or 30 mg/kg) on tumor-evoked hyperalgesia at an earlier time point, 7 days following implantation, when the amplitude of grip force reduction was the same as that following the injection of carrageenan.

2.7. The bar test

Cannabinoids are well known to produce catalepsy, a state of decreased responsiveness to external stimuli associated with a waxy rigidity of the extremities. Therefore, the possibility existed that increased forelimb muscle rigidity would result in increased grip force (i.e. reversing the reduction in grip force) that could mistakenly be interpreted as reversal of hyperalgesia. To distinguish between the antihyperalgesic and cataleptic effects of WIN55,212-2 on grip force, we evaluated the effect of catalepsy on grip force in mice that had not received hyperalgesic stimuli (i.e. tumor cell implantation or i.m. carrageenan).

We used the bar test, a standard test for catalepsy (Sanudo-Pena et al., 2000), to measure the level of catalepsy produced by WIN55,212-2. During the bar test, each mouse was placed with its forelimbs on a plastic bar (diameter = 1 cm) positioned 5 cm above and parallel to a countertop with its hind paws resting on the countertop. The total amount of time each mouse spent with its forelimbs on the bar in 3 consecutive 60 s trials was summed. To evaluate the effect of catalepsy on grip force, bar test measurements were collected just prior to grip force measurements at each time point. Mice were tested before and at 30 min after injection of WIN55,212-2 (30 mg/kg i.p.), haloperidol (1 mg/kg i.p., a positive control), or vehicle.

2.8. Rota-rod test

In a separate experiment conducted to test for the effect of WIN55,212-2 on motor coordination, mice tested for catalepsy with the bar test were also evaluated for performance on the Rota-rod. Mice were trained by undergoing two, 5 min trials on an accelerating rotating rod (0–30 rpm), earlier, on the day of the experiment. Then, each mouse was placed on the rotating rod for a 5 min period before and 30 min after administration of WIN55,212-2 (30 mg/kg, i.p.), haloperidol (1 mg/kg, i.p.), or vehicle. The time to first fall was recorded at each time point.

2.9. Data analysis

Grip force (g), reduction in grip force (g), time on the bar (s) and time to first fall (s) were analyzed by two-way analysis of variance (ANOVA) with repeated measures followed by Student–Newman–Keuls post-hoc tests to determine if differences occurred between groups at each time point or between time points for each group. To examine the dose–response relationship of WIN55,212-2's reversal of carrageenan- and tumor-evoked hyperalgesia, data were converted to % maximum possible effect (%MPE):

$$\%\text{MPE} = \frac{\text{Pre-drug grip force} - \text{Post-drug grip force}}{\text{Pre-drug grip force} - \text{Baseline grip force}} \times 100$$

A one-way ANOVA was performed to determine if the reduction in grip force differed among the doses of WIN55,212-2 administered. Then, the ED_{50} (50% effective dose) and 95% confidence intervals were calculated from the %MPE using a custom designed Microsoft Excel macro program based on the method of Tallarida and Murray (1987).

The abilities of the two cannabinoid receptor antagonists to block the antihyperalgesic effects of WIN55,212-2 were assessed using a two-way ANOVA with repeated measures followed by Student–Newman–Keuls post-hoc tests to determine if differences in the reduction in grip force existed between mice that received cannabinoid antagonists or vehicle followed by administration of WIN55,212-2. All data are expressed as mean ± SEM. A probability value <0.05 was considered significant.

3. Results

3.1. Attenuation of carrageenan- and tumor-evoked hyperalgesia by WIN55,212-2

Systemic administration of WIN55,212-2 attenuated carrageenan-evoked hyperalgesia in a time-dependent manner ($F_{(5,90)} = 3.19$, $P < 0.05$). Prior to the i.m. injection of carrageenan, mice exhibited a baseline grip force of ~160 g (Fig. 1). Forty-eight hours after injection of carrageenan into both triceps muscles, grip force decreased to ~110 g. WIN55,212-2 (10 mg/kg) completely reversed

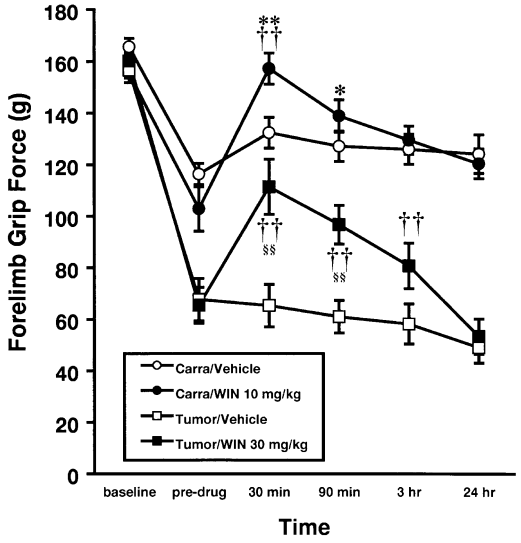

Fig. 1. Effect of WIN55,212-2 on carrageenan-evoked (circles) and tumor-evoked (squares) hyperalgesia. Forelimb grip force was obtained at baseline (prior to tumor cell implantation and carrageenan administration), 9 days later (pre-drug), and then at subsequent intervals after i.p. administration of WIN55,212-2 (filled symbols) or vehicle (open symbols). The * (for carrageenan-evoked hyperalgesia) and the § (for tumor-evoked hyperalgesia) indicate significant differences from pre-drug levels ($^{*}P < 0.05$; $^{**}P < 0.01$; §§$P < 0.01$). Crosses indicate significant differences from vehicle ($^{\dagger\dagger}P < 0.01$). Data shown represent mean ± SEM. ($n = 10$–11 mice/group).

carrageenan-evoked hyperalgesia 30 min following its administration (157.2 ± 6.1 g; $P < 0.01$). This antihyperalgesic effect persisted for 90 min ($P < 0.05$) after which time hyperalgesia returned and there was no significant difference in grip force relative to the vehicle-treated group. In contrast, vehicle did not significantly attenuate carrageenan-evoked hyperalgesia.

Systemic administration of WIN55,212-2 also attenuated tumor-evoked hyperalgesia in a time-dependent manner ($F_{(5,95)} = 6.73$, $P < 0.01$). Prior to injection of tumor cells into the humeri, the mice exhibited a baseline grip force that was not significantly different from that of mice prior to carrageenan (Fig. 1). Nine days after injection of tumor cells into the humeri, grip force decreased from ~160 g to ~65 g. Administration of vehicle did not significantly alter grip force. In contrast, WIN55,212-2 (30 mg/kg) attenuated tumor-evoked hyperalgesia by ~50% (111.4 ± 10.7 g) 30 min after administration ($P < 0.01$). The antihyperalgesic effect of WIN55,212-2 persisted for up to 3 h ($P < 0.01$), after which time hyperalgesia returned and there was no significant difference in grip force relative to the vehicle-treated group.

These results show that both i.m. injection of carrageenan and intraosseous implantation of tumor cells reduced forelimb grip force. However, at the time of peak hyperalgesia the reduction in grip force was 1.8 times greater in the tumor model as compared to the carrageenan model. I.p. administration of the cannabinoid WIN55,212-2 attenuated both carrageenan- and tumor-evoked hyperalgesia in a time-dependent manner. The antihyperalgesic effect of WIN55,212-2 peaked at 30 min after administration in both models.

3.2. Dose-dependent attenuation of deep tissue hyperalgesia by WIN55,212-2

To determine the relationship between the dose of WIN55,212-2 and its antihyperalgesic effects, the data were converted to reduction in grip force (g) with zero reduction signifying the return to baseline grip force. Fig. 2 shows that 30 min after administration, WIN55,212-2 dose-dependently attenuated both carrageenan- ($F_{(5,50)} = 3.09$, $P < 0.05$) and tumor- ($F_{(5,55)} = 2.71$, $P < 0.05$) evoked hyperalgesia. WIN55,212-2 at a dose of 10 mg/kg completely reversed the reduction in grip force produced by carrageenan, whereas after administration of vehicle, grip force remained reduced by 33.3 ± 7.7 g. In contrast, attenuation of tumor-evoked hyperalgesia required higher doses of WIN55,212-2 (3–30 mg/kg). The highest dose of WIN55,212-2 resulted in ~50% reversal of the reduction in grip force compared to vehicle ($P < 0.01$). Hence, WIN55,212-2 appeared to be more efficacious in carrageenan- than tumor-evoked hyperalgesia.

Fig. 2 also shows that administration of the inactive enan-

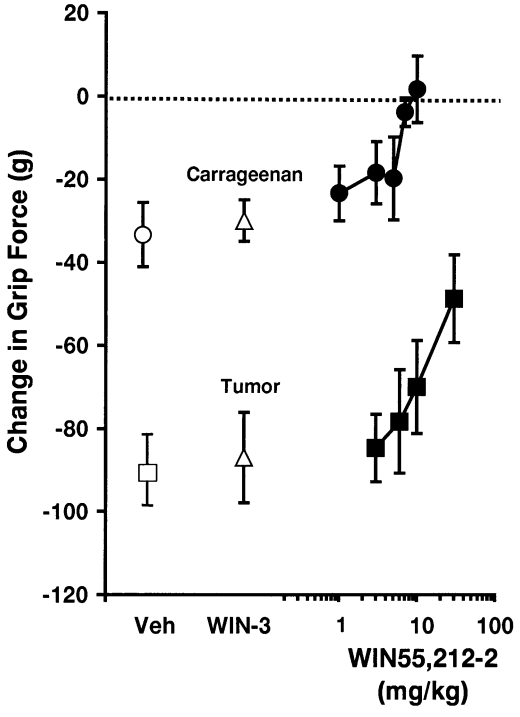

Fig. 2. Relationship between the dose of WIN55,212-2 and its reversal of carrageenan- (filled circles) and tumor-evoked (filled squares) hyperalgesia. Reduction in grip force 30 min after administration of WIN55,212-2 (filled symbols), its inactive enantiomer WIN55,212-3 (WIN-3, open triangles), or vehicle (open circle or square). Zero reduction in grip force (dotted line) indicates complete attenuation of hyperalgesia. Data shown represent mean ± SEM ($n = 9$–11 mice/group).

tiomer, WIN 55,212-3, did not alter the reduction in grip force produced by either carrageenan or tumor. In the carrageenan model, the most effective dose of WIN55,212-2 was 10 mg/kg. However, 30 min after administration of WIN55,212-3, at the same dose, mice exhibited a grip force that was not different from that exhibited by mice treated with vehicle. In the tumor model, the most effective dose of WIN55,212-2 was 30 mg/kg. Similarly, 30 min after administration of WIN55,212-3 (30 mg/kg), mice exhibited a reduction in grip force that was not different from the reduction in grip force of mice that received vehicle. Thus, stereospecificity of the reversal of the reduction in grip force produced by WIN55,212-2 suggests that this effect is receptor-mediated.

Although WIN55,212-2 appeared to differentially attenuate tumor- and carrageenan-evoked hyperalgesia, the difference in magnitude of hyperalgesia between the two models made comparison of WIN55,212-2's potency difficult. Analysis of normalized dose–response curves (not shown) indicated that WIN55,212-2 was approximately four times more potent in reversing carrageenan-evoked hyperalgesia (5 doses from 1 to 10 mg/kg; ED_{50}, 5.6 mg/kg; 95% C.L., 3.4–9.6 mg/kg) than 9-day tumor-evoked hyperalgesia (4 doses from 3 to 30 mg/kg; ED_{50}, 23.3 mg/kg; 95% C.L., 13.6–40.0 mg/kg). This increased susceptibility of carrageenan-evoked hyperalgesia was also evident in separate studies when we tested WIN55,212-2 (3, 10, and 30 mg/kg) against hyperalgesia of a comparable intensity at an earlier time point in the tumor model. Seven days after implantation of tumor cells, mice ($n = 34$) exhibited a reduction in grip force (45.7 ± 2.1 g) that was similar to the reduction exhibited by mice that received i.m. carrageenan in the previous experiment (48.5 ± 2.1 g). Thirty minutes after administration, WIN55,212-2 (30 mg/kg) returned grip force (140.8 ± 3.2 g) to baseline levels (150.6 ± 2.7 g, $P = 0.05$). Thus, the difference in magnitude of hyperalgesia appeared to account for the difference in efficacy of WIN55,212-2 between the two models of hyperalgesia. However, the dose of WIN55,212-2 that fully reversed carrageenan-evoked hyperalgesia (10 mg/kg) was not able to fully reverse tumor-evoked hyperalgesia 7 days after implantation of tumor cells (148.6 ± 1.9 g before and 131.9 ± 4.1 g after; $P < 0.05$). Thus, the difference in magnitude of hyperalgesia between the carrageenan and tumor models contributed to the difference in efficacy but could not fully account for the difference in potency of WIN55,212-2 between the two models.

3.3. The role of CB1 and CB2 receptors in attenuation of inflammatory and tumor-evoked deep tissue hyperalgesia

Pretreatment with either the selective CB1 receptor antagonist SR141716A (10 mg/kg) or the selective CB2 receptor antagonist SR144528 (3 mg/kg) significantly blocked the reversal of carrageenan-evoked muscle hyperalgesia produced by 10 mg/kg of WIN55-212 (Fig. 3A; $F_{(2,39)} = 14.0$, $P < 0.01$). Prior to administration of WIN55,212-2, mice in the control group exhibited a reduction in grip force to 53.4 ± 5.6 g below baseline values. Pretreatment with vehicle failed to block the reversal of the reduction in grip force produced by administration of 10 mg/kg of WIN55,212-2 ($P < 0.01$). In contrast, pretreatment with SR141716A attenuated the reversal in the reduction in grip force produced by WIN55,212-2 by approximately 70% compared to vehicle ($P < 0.01$). Pretreatment with the selective CB2 receptor antagonist, SR144528, decreased the antihyperalgesic effect of WIN55,212-2 by approximately 25% compared to vehicle ($P < 0.01$). Therefore, the antihyperalgesic effect of WIN55,212-2 in the model of deep tissue inflammation appeared to be mediated by both the CB1 and CB2 receptors, with a greater effect being mediated through the CB1 receptor.

Fig. 3B shows that pretreatment with SR141716A

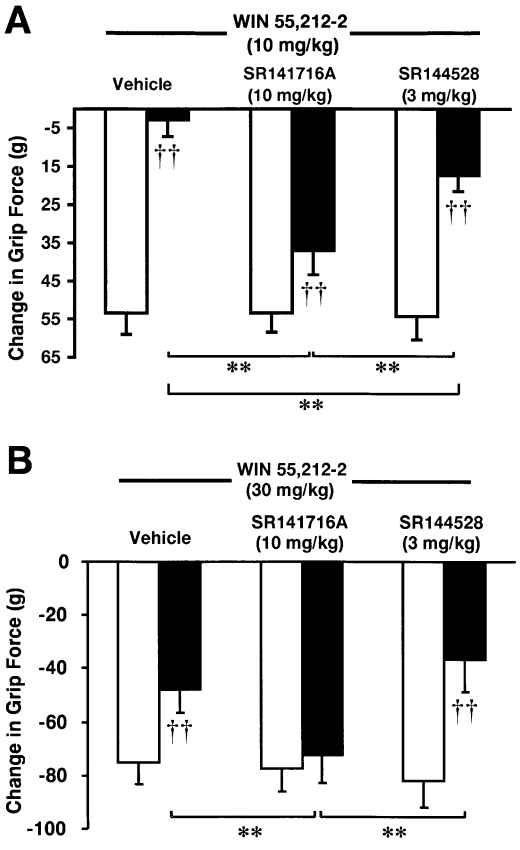

Fig. 3. Contributions of CB1 and CB2 receptor subtypes to the antihyperalgesic effects of WIN55,212-2 in the carrageenan (A) and tumor (B) models of hyperalgesia. Reduction in grip force (open columns) was determined for each group. Then mice were pretreated with the CB1 antagonist (SR141716A), the CB2 antagonist (SR144528) or vehicle. Thirty minutes after administration of WIN55,212-2 (filled columns), the reduction in grip force was again determined. Asterisks indicate a significant difference between levels of reduction in grip force after drug administration (**$P < 0.01$). Crosses indicate a significant difference in the level of reduction in grip force before versus after drug administration ($^{\dagger\dagger}P < 0.01$). Data shown represent mean ± SEM (n = 13–15 mice/group).

(10 mg/kg) blocked the reversal of tumor-evoked reduction in grip force produced by administration of 30 mg/kg of WIN55,212-2 ($F_{(2,38)} = 8.74$, $P < 0.01$). Prior to administration of WIN55,212-2, mice in the control group exhibited a reduction in grip force to 74.9 ± 8.1 g below baseline values. Pretreatment with vehicle failed to block the partial reversal of the reduction in grip force produced by administration of 30 mg/kg of WIN55,212-2 ($P < 0.01$). In contrast, after pretreatment with SR141716A, WIN55,212-2 did not reverse the reduction in grip force, which was still 72.4 ± 10.4 g below baseline values. Pretreatment with SR144528 (3 mg/kg) did not block the reversal in the reduction in grip force produced by administration of WIN55,212-2. Therefore, the antihyperalgesic effects of WIN55,212-2 in the tumor model appeared to be mediated exclusively through the CB1 receptor whereas both CB1 and CB2 receptors appear to contribute to its antihyperalgesic effects in the carrageenan model.

3.4. Effect of catalepsy on grip force

One of the adverse effects of cannabinoids is catalepsy, a state of non-responsiveness associated with waxy rigidity of the extremities. Because rigidity of the forelimbs may increase grip force and be misinterpreted an antihyperalgesia, we examined the effect of catalepsy on grip force in naive mice. We administered haloperidol (1 mg/kg) as a positive control because it has been shown to produce catalepsy in rats (Costall et al., 1972) and mice (Ushijima et al., 1997; Costa-Campos et al., 1998). Fig. 4A shows that both haloperidol and WIN55,212-2 produced catalepsy ($F_{(2,17)} = 17.5$, $P < 0.01$). Prior to administration of WIN55,212-2, haloperidol, or vehicle, mice spent a total of 8.2–8.6 s in contact with the bar in 3 consecutive 1 min bar test trials. Thirty minutes following administration of the vehicle, there was no significant change in time spent on the bar. In contrast, time spent on the bar increased from 8.2 ± 1.3 s to 129.7 ± 17.4 s, 30 min after administration of 1 mg/kg of haloperidol ($F_{(2,17)} = 17.5$, $P < 0.01$). Similarly, time spent on the bar increased from 8.6 ± 1.7 s to 145.6 ± 6.9 s, 30 min after administration of 30 mg/kg of WIN55,212-2 ($P < 0.01$). However, even though these drugs produced catalepsy, they failed to alter forelimb grip force (Fig. 4B). Therefore, catalepsy was not associated with increased grip force in mice that had not received carrageenan or tumor cells.

3.5. Effect of WIN55,212-2 on motor coordination

To further evaluate the effect of WIN55,212-2 on motor function, we conducted the same experimental paradigm with the exception that motor function was evaluated by measuring performance on a Rota-rod apparatus. Neither WIN55,212-2 (30 mg/kg), haloperidol (1 mg/kg), nor vehicle significantly changed the time to the first fall as compared to pre-drug levels (data not shown; $F_{(2,19)} = 0.62$, $P > 0.05$). However, although mice that received WIN 55,212-2 or haloperidol did not fall sooner, we noted that they were more likely to 'wedge' themselves between the partitions of the apparatus and rotate passively. Thus, WIN55,212-2 decreased the ability of mice to walk on the apparatus but these mice still had sufficient strength and coordination to 'wedge' themselves and avoid falling.

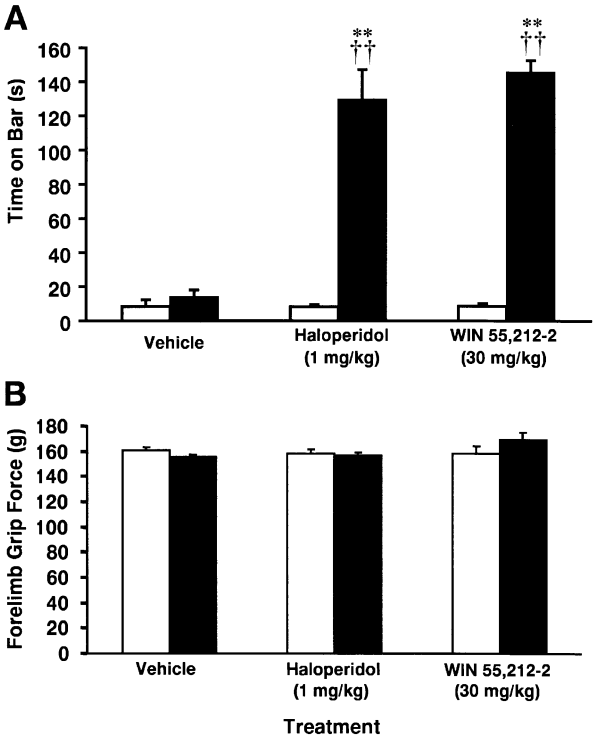

Fig. 4. Effect of WIN55,212-2-evoked catalepsy (A) on grip force (B). Baseline bar test and grip force measurements were obtained in mice that had not received carrageenan or tumors (open columns). Thirty minutes after administration of WIN55,212-2, haloperidol, or vehicle, bar test and grip force measurements were again obtained (filled columns). Asterisks indicate a significant difference in time on the bar after drug administration from the vehicle-treated group (**$P < 0.01$). Crosses indicate a significant difference in time on the bar before versus after drug administration ($^{\dagger\dagger}P < 0.01$). Data shown represent mean ± SEM ($n = 5$–10 mice/group).

4. Discussion

Results of the present study demonstrate that cannabinoids have the capacity to attenuate hyperalgesia in animal models of deep tissue pain produced by cancer and inflammation. Interestingly, there appears to be a difference in the antihyperalgesic effects of the non-selective cannabinoid agonist, WIN55212-2, in the two models of deep tissue hyperalgesia. WIN55,212-2 appeared to produce greater antihyperalgesia in the inflammatory model than the tumor model. This was in part due to the greater magnitude of hyperalgesia produced by the tumor model because when we examined the effect of WIN55,212-2 on tumor-evoked hyperalgesia at an earlier time point (i.e. 7 days after implantation), when the magnitude of hyperalgesia was

similar to that produced by carrageenan, WIN55,212-2 attenuated tumor-evoked hyperalgesia to the same level that it did for carrageenan-evoked hyperalgesia. However, the potency of WIN55,212-2 was still modestly (i.e. ~4 times) greater in the carrageenan than the tumor model. Furthermore, the dose of WIN55,212-2 that completely reversed carrageenan-evoked hyperalgesia only partially attenuated tumor-evoked hyperalgesia 7 days after implantation. Finally, the antihyperalgesic effects of WIN55,212-2 appeared to be mediated by both CB1 and CB2 receptors in the carrageenan model compared to only the CB1 receptor in the tumor model. Thus, the cannabinoid, WIN55,212-2, was antihyperalgesic in both inflammatory and tumor models of deep tissue hyperalgesia. Moreover, our results suggest that cannabinoids may differentially modulate carrageenan- and tumor-evoked hyperalgesia and that differences in mechanisms underlying hyperalgesia may exist between these two models of deep tissue pain.

Although numerous studies have shown that cannabinoids are antinociceptive in models of acute cutaneous pain (Welch et al., 1995b, 1998; Edsall et al., 1996; Lichtman et al., 1996; Hohmann et al., 1998; Hohmann et al., 1999; Martin et al., 1999a; Raffa et al., 1999) and attenuate cutaneous hyperalgesia (Herzberg et al., 1997; Richardson et al., 1998a,b; Li et al., 1999; Martin et al., 1999b; Mao et al., 2000; Fox et al., 2001; Johanek et al., 2001), less is known regarding the ability of cannabinoids to attenuate hyperalgesia associated with deep tissues. For example, intraabdominal administration of *p*-phenylquinone produces a writhing response that can be blocked by intrathecal administration of the endogenous cannabinoid agonist anandamide (Welch et al., 1995a). Similarly, anandamide blocked mechanical hyperalgesia associated with bladder inflammation (Jagger et al., 1998) and both anandamide and Δ^9-THC reversed mechanical hyperalgesia evoked by paw pressure in polyarthritic rats (Smith et al., 1998). The results of the present study provide further evidence that cannabinoids may be useful for attenuating both inflammation and cancer-associated deep tissue hyperalgesia.

In the present study, the highest dose of WIN55,212-2 (30 mg/kg) failed to fully reverse tumor-evoked hyperalgesia, but we were unable to get higher doses of WIN55,212-2 into solution in a vehicle that did not itself produce adverse effects. At least two potential explanations may account for this observation. The first is that tumor cells may have damaged the muscle fibers or motor innervation required to produce forelimb grip force. This possibility is unlikely, however, because of the following two observations. First, histologic studies demonstrated that on the seventh day following implantation in the humerus, tumor cells had eroded bone but typically had not broken out of bone to invade the surrounding muscle and connective tissue. By the tenth day following implantation, bone remodeling was extensive and osteolysis had occurred to the point that in some cases tumor cells were found outside the bone. However, when the latter occurred, it was not associated with destruction of muscle cells, but rather these tumor cells appeared to form small pockets along connective tissue planes (Wacnik et al., 2003). Second, co-administration of WIN55,212-2 with morphine (i.p.) fully reversed tumor-evoked hyperalgesia 9 days following tumor cell implantation (Nguyen et al., 2002). This finding suggests that at 9 days after implantation of tumor cells into the humeri, muscle fibers and motor innervation were not damaged to the extent that mice could not produce grip forces similar to that prior to implantation, because mice that received an adequate level of analgesia were able to generate grip forces similar to baseline levels. Taken together, these findings suggest that the reason we did not see full reversal of tumor-evoked hyperalgesia was not because mice could not produce grip forces similar to baseline levels, but rather because the highest dose of WIN55,212-2 we tested (30 mg/kg) was not able to produce complete antihyperalgesia.

A second possible explanation for the inability of WIN55,212-2 to completely reverse tumor-evoked hyperalgesia may be due to the magnitude of hyperalgesia at the time point studied (9 days post-implantation). Compared to the carrageenan model, in which WIN55,212 completely reversed the hyperalgesia, the magnitude of tumor-evoked hyperalgesia was almost two times greater. This explanation appears plausible because when we tested the effect of WIN55-212,2 7 days post-implantation, at a time when the magnitude of hyperalgesia was similar to the magnitude evoked by carrageenan, WIN55,212-2 (30 mg/kg) was able to fully attenuate tumor-evoked hyperalgesia. Thus, the greater magnitude of hyperalgesia 9 days after implantation contributed to the inability of WIN55,212-2 to fully attenuate tumor-evoked hyperalgesia. However, the dose of WIN55,212-2 (10 mg/kg) that completely reversed carrageenan-evoked hyperalgesia did not completely reverse tumor-evoked hyperalgesia 7 days following tumor cell implantation suggesting that WIN55,212-2 was more potent in producing antihyperalgesia in the carrageenan than in the tumor model. Unfortunately, we were not able to directly answer the question of whether doses greater than 30 mg/kg could fully reverse tumor-evoked hyperalgesia 9 days post-implantation because these higher doses were insoluble in vehicles that did not impair motor function.

WIN55,212-2 was approximately four times more potent in reversing carrageenan- than tumor-evoked hyperalgesia. One potential mechanism underlying this difference may be a difference in the activity of cannabinoid receptor subtypes mediating the antihyperalgesic effects of WIN55,212-2. The data shown in Fig. 3A provide evidence that reversal of carrageenan-evoked hyperalgesia by WIN55,212-2 was mediated by CB1 and, to a lesser extent, CB2 receptors. The dose of the CB1 selective antagonist, SR141716A, (10 mg/kg i.p.) that blocked the antihyperalgesic effects of WIN55,212-2 in both models of hyperalgesia was the same as that used to block the antihyperalgesic effects of cannabinoids in arthritic rats (Smith et al., 1998). In contrast, we

found that CB1 but not CB2 receptors mediated reversal of tumor-evoked hyperalgesia by WIN55,212-2. This result suggests that the absence of CB2 receptor-mediated activity may, at least partially, account for the inability of WIN55,212-2 to completely reverse hyperalgesia 9 days after tumor cell implantation. If such a difference in CB2 receptor activity exists between inflammatory hyperalgesia, which was modeled in this study by carrageenan administration, and cancer pain, which was modeled by tumor cell implantation, it would suggest that different mechanisms might be involved in the modulation of these two pain states. This interpretation is supported by our similar findings when comparing morphine's antihyperalgesic potency and efficacy between tumor and inflammation models (Wacnik et al., 2003).

CB2 receptors are present on mast cells in the periphery where their activation can inhibit degranulation (Facci et al., 1995). The cannabinoid Δ^9-THC has been reported to suppress T lymphocyte and macrophage function (Hollister, 1988; Klein et al., 1991; McCoy et al., 1995, 1999). Since injection of carrageenan elicits a characteristic inflammatory response associated with hyperalgesia, our observation that SR144528 partially blocked attenuation of carrageenan-evoked muscle hyperalgesia by WIN55,212-2 is consistent with these anti-inflammatory effects of CB2 receptor activation.

In contrast, the CB2 antagonist did not block the reversal of tumor-evoked hyperalgesia by WIN55,212-2, 9 days following implantation. This is somewhat surprising because bone tumors are known to release inflammatory substances such as interleukin-1 (Fonsatti et al., 1997), interleukin-6 (Nasu et al., 1998), tumor necrosis factor-α (Glezerman et al., 1998), prostaglandins (Hida et al., 1998) and endothelin-1 (Wacnik et al., 2001). In fact, increased release of endothelin-1 after implantation of osteolytic fibrosarcoma cells (the same cell line used in the present study) into the calcaneus bones of mice (Wacnik et al., 2001), coincided with electrophysiological evidence of sensitization of nociceptors (Cain et al., 2001). It is possible that the inability of SR144528 to block WIN55,212-2's antihyperalgesic effect in the tumor model may have been due, in part, to the limited solubility of SR144528 in a vehicle that did not produce motor deficits. Perhaps if we had been able to test higher doses of the antagonist we would have seen some effect in the tumor model. Yet the dose tested (3 mg/kg) was sufficient to partially block the antihyperalgesic effect of WIN55,212-2 in the carrageenan model suggesting that differences in mechanisms mediating inflammatory compared to tumor-evoked deep hyperalgesia may exist.

An alternative explanation for the lack of effect of the CB2 antagonist in the tumor model is that, although inflammatory mediators are released from tumor cells (Chirgwin and Guise, 2000), inflammation may be a relatively small component of tumor-evoked hyperalgesia. It is also possible that different mediators with different actions may play primary roles in malignant versus non-malignant inflammation, or that factors released by tumor cells may alter the density or binding properties of CB2 receptors on immune cells. However, in addition to producing inflammation, bone tumors also directly stimulate periosteal nociceptors, increase intraosseous pressure (Chang, 1999) and produce morphological evidence of neuropathy (marked reduction in epidermal nerve fibers; Cain et al., 2001). These reports and others provide evidence that multiple mechanisms contribute to cancer pain and that differences may exist in the processing of cancer pain when compared to other pain models (e.g. inflammatory, neuropathic) (Honore et al., 2000).

Collectively, the results in Figs. 1–3 provide converging lines of evidence that cannabinoids have the capacity to attenuate both carrageenan- and tumor-evoked hyperalgesia. Yet cannabinoids are well known to produce catalepsy, evidenced by waxy rigidity of the extremities and decreased spontaneous locomotion (Little et al., 1988). Consequently, the possibility existed that the increased grip force that was interpreted as antihyperalgesia may have been due to forelimb rigidity secondary to catalepsy. However, our results in the bar test experiment demonstrated that the catalepsy did not alter forelimb grip force in mice without hyperalgesia. We did not use mice with carrageenan- or tumor-evoked hyperalgesic because then we would have been unable to separate the antihyperalgesic effects from the cataleptic effects of WIN55,212-2 on grip force. Furthermore, reports exist to show that cannabinoid associated motor dysfunction and antinociception can be dissociated, providing evidence that motor impairment is not responsible for the apparent antinociceptive effects. For example, injection of cannabinoid agonists into the periaqueductal gray or rostal ventromedial medulla, brain regions known to modulate nociception, blocks the tail-flick reflex while administration into CNS areas not involved in pain modulation has no effect (Martin et al., 1995; Lichtman et al., 1996; Martin et al., 1998). Likewise, spinal administration of the α2-adrenergic antagonist yohimbine or the κ opioid antagonist norbinaltorphimine blocked cannabinoid-mediated antinociception in the tail-flick test; yet these antagonists had no effect on the associated motor dysfunction (Lichtman and Martin, 1991; Smith et al., 1993). Conversely, cannabinoid administration into the striatum produces catalepsy but does not increase tail-flick latency (Gough and Olley, 1978; Lichtman et al., 1996). These reports, in conjunction with results of the present study, provide evidence that cannabinoid associated antinociception can be dissociated from motor dysfunction providing evidence that motor impairment is not responsible for their apparent antinociceptive effects.

If cannabinoids are to be considered as potential therapeutic agents for management of chronic malignant or non-malignant pain, certain issues will need to be addressed. First, although tumor-suppressing activities have been attributed to this class of compounds (DePetrocellis et al.,

1998), cannabinoids have also been reported to promote tumor growth (Zhu et al., 2000). Secondly, cannabinoids exert undesirable side effects such as sedation and catalepsy at higher doses, which currently limits their utility as therapeutic agents. However, evidence exists that cannabinoids potentiate the antinociceptive effects of opioids (Welch and Stevens, 1992; Welch et al., 1995b; Cichewicz et al., 1999). Therefore, co-administration of low doses of a cannabinoid drug with an opioid would theoretically allow the achievement of analgesia equivalent to that of relatively higher doses of either agent. Such an administration strategy may produce fewer undesirable side effects. Another therapeutic strategy may include development of new pharmacologic agents, such as the compounds AM381 or AM404, that inhibit tissue uptake or metabolism of endogenously produced cannabinoids (Pertwee, 1999). Finally, peripheral administration of low doses of these compounds, or of selective CB2 agonists that are thought to be devoid of the CNS side effects, may also prove to be a useful therapeutic approach (Ward, 2000; Pertwee, 2001). Future studies, using animal models of malignant and non-malignant pain are needed to investigate these therapeutic strategies further if cannabinoids are to be seriously considered as therapeutic agents for management of clinical pain.

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