Pain-Modulating Effects of Peripheral (CB_2) Cannabinoid Receptors

By

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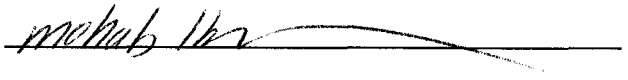
SIGNED: 

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A person's true wealth is not measured in money, but in friends. True friendship is one of two things that can withstand the test of time and distance. If someone asked me about my proudest achievement, I would not hesitate in answering. While I'm proud of the degrees kindly bestowed upon me and publications and awards, the achievement that I'm most proud of is having such wonderful friends. My duties are what bring me to work everyday, but it is my friends that make me stay and enjoy the day. The dissertation in your hands is not just a product of my work; it is the collective work and help from all my friends. I hope that whoever reads this dissertation keeps in mind that I could not have done it without my friends. It is truly my honor and pleasure to have the privilege of acknowledging and thanking my friends. Dr. Phil Malan (to whom I owe everything and more), Carole and Tim Malan, Eddie Mata (for always rescuing me), Aline, Arash, Melissa Brym, Mr. Justin Kowal (for so many things), Dr. Frank Porreca, Dr. Ed French, Dr. Dan Stammer, Dr. Gary Wenk, Dr. John Law, Dr. Rolf Ziegler, Dr. Todd Vanderah (for always making the time to help and advise me), Dr. Mike Ossipov, Dr. Josephine Lai, Dr. Hank Yamamura, Dr. Sloviter, Dr. Alex Makriyannis (for kindly and generously providing the AM drugs), Dr. Anne Cress and Mike Pennington (for their extremely generous help with the HaCat cells), Dr. Thomas Davis, Hamid Badghisi, Qingmin Chen, Peg Davis (for always correcting my English), Milena De Felice, Kerry Gilbraith, Wenhong Guo, Morten Hadsel, David Herman, Yuan Yuan, Rachel Johnson, Tamara King, Miaw-Chyi Luo, Jack Luo, Dr. Ma, Lisa Majuta (for not hurting me), Ohannes Melemedjian, Tammi and Steve Morrissy, Dr. Anders Nylen, Jill Roberts, Anna and Marina Vardanyan, Lou and Shannon Gardell, Kerry Vault, Louis Vera-Portocarrero, Ruizhong Wang (for always being nice), Jennifer Xie, Dongqin Zhang, En-Tan Zhang, Wenjun Zhang, Susan Anderson, Tony Rao, Kirk Salvador, Ken Tolman, Tina and Kevin Douglas, Mark Willer, Will Bevill, Trisha Stanley, Anita Finnell, Patti Pergin and Marilda Swiltick. Of course, both of my wonderful and nice brothers; Wael and Amal (I couldn't have done it without your endless help and support…thank you), and the most patient and wonderful person in the whole world, my mother. Thank you all so much. Not only do you command my respect and appreciation, but also I shall forever be grateful and in your debt.

DEDICATION

I dedicate this study and degree to my father who passed away at a young age before I got to know him well. At a very young age, my father taught me that medicine is not just about adding more years to someone's life, but it is also about adding more life to someone's years. May this work help shed some light on the field of pain and bring relief to those in need of it.

I also dedicate this whole work to the person with great patience who took a chance on me, gave me every reason to excel, and guided me when the wind was blowing me off course. I dedicate this dissertation and degree to Phil Malan, a man that I strive to follow in his foot steps.

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ABSTRACT

Cannabinoid receptor agonists diminish responses to painful stimuli. Extensive evidence implicates the CB_1 receptor in the production of antinociception, inflammatory hyperalgesia, and peripheral nerve injury-induced sensory hypersensitivity. In previous work included in my masters dissertation, our laboratory has demonstrated the capacity of CB_2 receptors located outside the central nervous system (CNS) to inhibit acute nociception and inflammatory hyperalgesia. In this dissertation, I use AM1241, a CB_2 receptor-selective agonist to test the hypothesis that CB_2 receptor activation reverses the tactile and thermal hypersensitivity characteristic of neuropathic pain in L_5/L_6 spinal nerve ligation model. The CB_2 receptor-mediated nature of these effects was demonstrated using receptor-selective antagonists, as well as mice deficient in the genes coding for CB_1 or CB_2 receptors. Experiments using site-specific injections suggest AM1241 acts peripherally at the site of nerve injury and the site of application of the sensory stimulus. The peripheral nature of the effects of AM1241 is consistent with the peripheral distribution of CB_2 receptors. Given the peripheral actions of AM1241, I hypothesized and demonstrated that topical application of

AM1241 modulates pain responses. Additionally, I began to examine the mechanisms by which CB_2 receptor activation modulates pain responses. The effects of AM1241 were reversed by the opioid receptor antagonist, naloxone and by a sequestering antiserum to β-endorphin. In addition, the effects of AM1241 were not observed in μ-opioid receptor knockout mice. These results suggest that the endogenous opioid peptide, β-endorphin plays an essential role in CB_2 receptor mediated pain inhibition. Further, AM1241 stimulated release of β-endorphin from rat skin tissue and cultured human keratinocytes. The stimulation of β-endorphin release by AM1241 was inhibited by the CB_2 receptor-selective antagonist, AM630, and was not observed in skin from CB_2 receptor knockout mice, demonstrating that it is mediated by CB_2 receptor. These results suggest that CB_2 receptor activation produces antinociception by stimulating the release of β-endorphin from local cells and that β-endorphin released acts at μ-opioid receptors to inhibit the responsiveness of primary afferent neurons.

CHAPTER 1

INTRODUCTION

Pain is the gift that no one wants. It can be beneficial, serving to protect an injured body part from further damage. For example, pain causes one to immobilize an injured body part preventing further tissue damage, and enabling it to heal. However, there are times when pain is exaggerated or fails to serve a protective function such as when nervous system responses become exaggerated in patients with neuropathic pain. Current pain treatment is not consistently effective. Currently available pain medication may not fully relieve pain, or may be associated with major side effects. Therefore, we are in need of additional options for pain treatment.

The ancient Egyptians, Indians and Chinese utilized marijuana for its recreational and medicinal properties. Indeed, its medical use predates recorded history. The earliest written reference can be found in the fifteenth-century B.C. Chinese Pharmacopeia, the Ry-Ya (Anonymous, 1975).

The West got its first "puff" of marijuana in the mid-nineteenth century when a British army surgeon who learned of its medicinal application in India introduced its use in

England (Mikuriya, 1973; Mathre, 2001). For over seventy years, the medical community accepted marijuana as a form of medication and prescribed it for the treatment of pain. However, in the 1930s marijuana was identified as an intoxicating drug. In 1937, the Marihuana Tax Act outlawed social use of the drug, and the passage of the Controlled Substances Act of 1970 effected complete prohibition of *Cannabis* by banning its medicinal use. Currently the United States Drug Enforcement Administration considers marijuana a schedule I drug, indicating that it has no medical use (Mathre, 2001).

In 1964 the main psychoactive ingredient in marijuana was identified as Δ^9-tetrahydrocannabinol (Δ^9-THC) (Hively *et al.*, 1966 and Gaoni and Mechoulam, 1964). In 1990, the cannabinoid receptor CB_1 was isolated and cloned (Matsuda *et al.*, 1990). This was followed shortly by the cloning and isolation of the second cannabinoid receptor, CB_2, (Munro *et al.*, 1993).

CB_1 receptors were identified in the central nervous system (CNS) and spleen (Schatz *et al.*, 1997). The greatest concentrations of CB_1 receptors in the CNS are located in the hippocampus, cortex, olfactory areas, basal

ganglia, cerebellum, and spinal cord (Romero *et al.*, 1997). In addition, CB_1 receptors have been identified in nociceptive pathways such as the spinal cord dorsal horn and peripheral neurons (Sanudo-Pena *et al.*, 1999).

Activation of the CB_1 receptor modulates adenylyl cyclase activity (Rhee *et al.*, 1998), inhibits presynaptic calcium channel activation (Wilson *et al.*, 2001), activates potassium A channels, and activates inwardly rectifying potassium channels (Felder et *al.*, 1995).

It has been clearly demonstrated that activation of cannabinoid receptors produce antinociception (Monhemius *et al.*, 2001), reversal of inflammatory hyperalgesia (Guhring et al., 2002), and inhibition of neuropathic pain responses (Fox *et al.*, 2001). These effects were prevented by CB_1 receptors-selective antagonists, suggesting that they are mediated by CB_1 receptors. These findings suggested that cannabinoids might be used clinically for pain relief. However, activation of cannabinoid receptors in the CNS produces a wide spectrum of side effects including hypothermia, euphoria, anxiety, sedation, and addiction (Balint, 2001). These effects have limited the medicinal use of cannabinoids to the treatment of wasting in AIDS and cancer patients, and chemotherapy induced emesis.

Therefore, to avoid unwanted side effects of cannabinoids, it is desirable to avoid activation of CNS cannabinoid receptors. One way to achieve that goal is by using an agonist that does not cross the blood brain barrier. Unfortunately, virtually all available cannabinoids are predicted to cross the blood-brain barrier due to their extremely lipophilic nature.

A second approach would be to use drugs that selectively target CB_2 receptors. CB_2 receptors are not present in the central nervous system (Munro *et al.*, 1993; Facci *et al.*, 1995; Galiegue *et al.*, 1995; Schatz *et al.*, 1997). Rather, they are primarily located on immune cells in the periphery. Activation of peripheral CB_2 receptors produces antinociception (Malan et al., 2001) and inhibits inflammatory hyperalgesia (Quartilho et al. 2003).

Since the brain lacks CB_2 receptors, it is hypothesized that CB_2 receptor activation should not produce any central nervous system effects. Consistent with this hypothesis, doses of the CB_2 receptors-selective agonist AM1241 producing maximum possible antinociception did not produce the CNS cannabinoid receptor mediated effects of catalepsy, hypothermia, or decreased spontaneous activity. Therefore,

selectively CB_2 receptors agonists may prove to be a novel way of inhibiting pain responses without CNS side effects.

Neuropathic pain is chronic in nature and may be detrimental to the life quality of the effected individuals and to their support system. It is estimated that 1-2 % of the population is suffering form neuropathic pain. Treatments currently available for the treatment of neuropathic pain do not show complete clinical effectiveness mainly due to their adverse side effects.

The purpose of this dissertation is to test the hypothesis that CB_2 receptors-activation will inhibit neuropathic pain. Additionally, I use mice deficient in the genes coding for CB_1 or CB_2 cannabinoid receptors to further characterize the receptors responsible for the actions of the CB_2 receptors-selective agonist AM1241. Finally, I test the hypothesis that CB_2 receptors activation inhibits pain responses by stimulating the release from neighboring cells of endogenous opioid receptor agonists.

CHAPTER 2

PRESENT STUDY

The studies in this dissertation employed the following methods to address the questions of interest. Spinal nerve ligation L5/L6 spinal nerve ligation was performed as described by Kim and Chung (1992). Animals were anesthetized with halothane. An incision was made lateral to the lumbar spine. The right L5 and L6 spinal nerves were isolated and tightly ligated distal to the dorsal root ganglion. The incision was closed and animals were allowed to recover for 10 days. Sham-operated animals were prepared in an identical fashion except that the spinal nerves were not ligated.

Measurement of tactile withdrawal threshold was determined as described by Chaplan *et al.* (1994). Animals were acclimated for 30 min in suspended cages with wire mesh bottoms. The hindpaw was probed with calibrated von Frey filaments (Stoelting, Wood Dale, IL) applied perpendicularly to the plantar surface. A positive response was indicated by a sharp withdrawal of the paw. The 50% paw withdrawal threshold was determined by the non-parametric method of Dixon (1980), in which the stimulus is incrementally increased until a positive response is

obtained, then decreased until a negative result is observed. The protocol was repeated until three changes in behavior were determined. A maximal cut-off value of 15 g was used for rats and 3.5 g for mice. The 50% paw withdrawal threshold was determined as $(10^{[Xf+k\delta]})/10,000$, where Xf = the value of the last von Frey filament employed, k = Dixon value for the positive/negative pattern, and δ = the logarithmic difference between stimuli.

Measurement of thermal withdrawal latency was done by using the method of Hargreaves *et al.* (1988). Animals were acclimated within plexiglass enclosures on a clear glass plate maintained at 30°C. A radiant heat source (high-intensity projector lamp) was focused onto the plantar surface of the paw. When the paw was withdrawn, a motion detector halted the stimulus and a timer. A maximal cut-off of 40 sec for rats and 30 sec for mice was used to prevent tissue damage.

Hank's balanced salt solution, HBSS, <$CaCl_2$ 1.26mM, KCl 5.33mM, KH_2PO_4 0.44mM, $MgCl_2$-$6H_2O$ 0.5mM, $MgSO_4$ 0.41mM, NaCl 138mM, $NaHCO_3$ 4mM, Na_2HPO_4 0.3mM, Glucose 5.6mM; PH 7.4> was used for tissue release assays. AM1241 was dissolved dimethyl sulfoxide at a concentration of

2.5µg/ul. One hundred µl of AM1241 solution was then dissolved into 1ml of HBSS containing 1%BSA. Subsequent dilutions were made in HBSS to achieve the desired final concentrations of AM1241. DMSO was added so that each diluted sample contained an equivalent concentration of DMSO. The same method was employed to prepare AM630.

Animals were euthanized using 4% halothane. Skin from the dorsal surface of the hind paw was quickly collected and placed in HBSS at 37°C. A punch of 8 mm in diameter was used to prepare skin samples of equivalent surface area. Each skin sample was cut in half and equilibrated in HBSS 30 minutes at 37°C.

Each skin sample was placed in a 1.5 ml plastic tube containing 120µl HBSS + 30µl of HBSS containing DMSO or AM1241. AM1241 was present at a final concentration of 10, 3.3, or 1 µM. DMSO was present at a final concentration of 0.2%. Tubes containing both 10µM AM1241 + 10µM AM630 were prepared in an analogous manner. Tissue was placed in 90 µl of HBSS + 30µl 50µM AM630. Five min later, 30µl of 50 µM AM1241 was added to the tubes, to reach final concentration of 10 µM AM630 and 10 µM AM1241. The tubes containing the tissue were incubated at 37°C for 30 minutes with periodic gentle agitation to improve oxygenation. The

supernatant was collected and placed on ice. Beta-endorphin content was measured immediately by enzyme immunoassay (Peninsula Laboratories, San Carlos, CA.)

HaCat cells were grown in 12-well plates in a Iscove's Modified Dulbecco's Medium supplemented with 10% Fetal Bovine Serum and Penicillin-Streptomycin (Invitrogen, Carlsbad, CA.) at 37°C. Each well contained a final volume of 350 µl for release assay. AM1241 and AM630 were dissolved in DMSO, and subsequently diluted in culture medium. Following addition of AM1241, AM630, or vehicle, plates were incubated for 30 min. The media was then collected. Beta-endorphin was measured by enzyme immunoassay.

HaCat cells were grown in 12-well plate in medium at 37°C. For testing of agonist induced desensitization they were treated in final volume of 350 µl at 37°C with vehicle or 1µM AM1241. In the vehicle group, cells were incubated with vehicle for 30 min. In the control group, HaCat cells were incubated for 30 minutes in 1 µM AM1241 before the media was collected and β-endorphin was measured. In the desensitized group, cells were incubated with 1 µM AM1241 for 2 hours. Cells were then washed and the wells were replaced with fresh media containing 1 µM AM1241. After 30

minutes, the samples of medium were collected and β-endorphin was measured by enzyme immunoassay.

Total RNA was isolated from homogenized tissue and from cells using RNAqueuos ™ (Ambion, Austin, TX.) following the manufacturer's instructions.

Total RNA was reverse-transcribed using oligo dT and random decamers methods of priming. These reactions were carried out using the following conditions: initial incubation of the reaction cocktail at 44°C for 1 hour followed by an incubation at 92°C for 10 min.

DNA amplification was carried using commercially available kit (RETROscript ™, Ambion, Austin, TX.). The conditions for DNA amplification were as follows: Initial incubation at 92°C for 2 minutes followed by 40 cycles of 94°C incubation for 30 sec, 60°C incubation for 30 sec, and 72°C incubation for 1 min. After the completion of the 40 cycles, the reaction cocktail was incubated at 72°C for 7 min and held at a 4°C until samples are frozen in the -20°C.

For detection of CB_2 receptor mRNA in rats tissue, we used the primers designed by Porcella et al., 1998. The sense primer was 5'-TTTCCCACTGATCCCTAACG-3'. The antisense primer was 5'-AGTTAACAAGGCACAGCATG-3'. The expected

amplicon size is 328 bp. For detection of CB_2 receptor mRNA from human cells, we used those primers designed for human tissue designed by Emmanuel et al., 1999. The sense primer was 5'-GCATCATGTGGGTCCTCTC-3'. The antisense primer was 5'-TCTCCACTCCGTAGAGCATAG-3'. The expected amplicon size is 451 bp. A negative control consisted of the RT-PCR reaction in the absence of homogenized tissue

Genetically engineered mice were used to address some of the questions. CB_1, CB_2, and μ-opioid receptor deficient mice were used.

Differences between groups were tested using ANOVA followed by post-hoc testing with Students't-test with Bonferroni's correction. Significance was defined as $P < 0.05$.

The results collected support the hypothesis that the activation of the CB_2 cannabinoid receptors modulates pain responses in rats. Using site-specific injection of AM1241, we investigated the drug's site of action. AM1241 was effective when injected locally in the paw on the side of nerve injury and sensory testing. Additionally, the lipophilic nature of AM1241 made it a candidate for external topical administration. Additionally, β-endorphin seems to play a central role in mediating the actions of CB_2

cannabinoid receptors activation by AM1241. The effects of β-endorphin did not produce maximal possible effects in models of neuropathic pain, suggesting that other mediators may participate in the activation of CB_2 receptor by using AM1241.

The final conclusion of this dissertation is that CB_2 receptor agonists such as AM1241 offer a choice for treatment of acute, inflammatory, and neuropathic pain. AM1241 produces pain relief without CNS side effects, consistent with the fact that the effects of the CB_2 receptor agonists are local rather than systemic

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CHAPTER 3

ACTIVATION OF CB_2 CANNABINOID RECEPTORS BY AM1241 INHIBITS EXPERIMENTAL NEUROPATHIC PAIN: PAIN INHIBITION BY RECEPTORS NOT PRESENT IN THE CENTRAL NERVOUS SYSTEM

Introduction

Neuropathic pain is defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system (Merskey & Bogduk, 1994). It affects approximately 1% of the population and results from a variety of etiologies including trauma, infection, diabetes, immune deficiencies, ischemic disorders, and toxic neuropathies (Merskey & Bogduk, 1994 and Bowsher, 1991). It can be excruciating and some patients are unable to work or to perform normal daily activities. Neuropathic pain often responds poorly to medical therapy (Kingery, 1997, and Jensen et al, 2001). This may be due, in part, to adverse side effects of available medications that limit drug dosage (Sindrup and Jensen, 1999). Medications currently used for the treatment of neuropathic pain act on neurotransmitter systems or ion channels and typically produce significant central nervous system (CNS) side-effects. For example, gabapentin, a drug commonly employed to treat neuropathic

pain due to its modest side effect profile compared to other therapeutic options, produces somnolence in 19% of patients and dizziness in 17% (Neurontin prescribing information, Parke Davis, Morris Plains, New Jersey). A therapy directed at targets not found in the CNS would avoid these problems. CB_2 cannabinoid receptors are one such potential target.

CB_2 receptor mRNA is not detected in brain (Munro et al, 1993 and , Griffin et al, 1999). In addition, the CB_2 receptor-selective antagonist SR144528 did not displace the non-selective cannabinoid ligand [^{3}H]-CP55,940 from binding to rat brain (Griffin et al, 1999). Finally, binding of [^{3}H]-CP55,940 to mouse brain was eliminated by disruption of the CB_1 receptor gene (Zimmer et al, 1999), but was not affected by disruption of the CB_2 receptor gene (Buckley, et al, 2000). These studies suggest that CB_2 receptors are not found in the normal CNS, although they do not fully exclude the possibility that CB_2 receptors are expressed in the CNS in small, but functionally significant, amounts. CB_2 receptor-selective agonists do not produce CNS effects typically caused by non-selective cannabinoid agonists (Malan et al, 2001 and Hanus et al, 1999). CB_2 receptors are found primarily in peripheral tissues with immune

functions (Munro et al, 1993, Galiegue et al, 1995, Facci et al, 1995, and Schatz et al, 1997).

We designed AM1241, a selective CB_2 receptor agonist, and used it to test the hypothesis that CB_2 receptor activation will reverse the sensory hypersensitivity observed in neuropathic pain states without producing CNS side-effects. These experiments were performed in light of the previous demonstration that AM1241 inhibits acute thermal nociception (Malan et al, 2001).

AM1241 belongs to a class of cannabergic ligands known as aminoalkylindoles. Early on, a number of these compounds were shown to bind to the CB_1 receptor and elicit the characteristic effects produced by Δ^9-tetrahydrocannabinol, the key psychoactive ingredient in *Cannabis sativa*. Following the discovery of the CB_2 receptor (Munro et al, 1993), the second cannabinoid receptor subtype, our group has sought to develop novel aminoalkylindole analogs possessing high potency and selectivity for this receptor. Our efforts were rewarded with the design and synthesis of AM1241, a compound possessing high affinity and selectivity for the CB_2 receptor.

METHODS

Binding assays: Binding to cannabinoid receptors was tested using competition-equilibrium binding *vs.* [^{3}H]-CP55,940 as described in Lan *et al.* (1999). AM1241 was diluted into 25 mM Tris-base, pH 7.4; 5 mM $MgCl_2$; 1 mM EDTA; 0.1% essentially fatty acid-free bovine serum albumin (BSA) and transferred to Regisil-treated 96-well plates. [^{3}H]-CP55,940 (Dupont NEN, Boston, MA; specific activity 100-180 Ci/mmol) was added to make a concentration of 0.8 nM. Membranes prepared from rat brain (containing CB_1 receptors) or mouse spleen (containing CB_2 receptors) were added (~50 µg membrane protein/well), plates were incubated at 30 °C for 1 hr and the contents were filtered over Packard Unifilter GF/B 96-well filters (Perkin-Elmer, Boston, MA) using a Packard Filtermate 196 Cell Harvester. Filters were washed with ice-cold 50 mM Tris-base, 5 mM $MgCl_2$, 0.5% BSA and dried. Bound radioactivity was quantitated and corrected for non-specific binding, and results were normalized between 0 and 100% [^{3}H]-CP-55,940 specifically bound. IC_{50} was determined by non-linear regression analysis using GraphPad Prism and transformed to a K_i value (Cheng and Prusoff, 1973). All data were collected in

duplicate. IC_{50} and K_i values were determined from three independent experiments.

Animals: All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the guidelines of the International Association for the Study of Pain and the National Institutes of Health. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) were 250-350 g at the time of testing. Mice were 20-25 g at the time of testing. Animals were maintained in a climate-controlled room on a 12-h light/dark cycle and allowed food and water *ad libitum*.

Generation of $CB_1^{-/-}$ mice: CB_1-deficient mice were generated in concert with Deltagen, Inc. (Redwood City, CA), using the 1.4 kb mouse CB_1 cDNA sequence as a starting point for the generation of a CB_1 targeting vector. The CB_1 targeting vector includes 5.8 kb of genomic sequence and a 7 kb IRES-LacZ-Neo-pA cassette. Homologous recombination between the targeting vector and the wild type CB_1 allele results in deletion of 1223 bp of the mouse CB_1 coding sequence, encompassing bp 26-1248, and replacement of this sequence with the IRES-LacZ-Neo-pA cassette. Linear CB_1 targeting vector was electroporated into 129/SvJ-derived R1 embryonic stem cells. Homologous recombinant clones were identified

by PCR and confirmed by Southern blot analysis using 5´ and 3´ probes outside of the targeting vector homology arms. Targeted clones were injected into C57BL/6 blastocysts, and chimeras were bred to 129/SvJ (Jackson Laboratory, Bar Harbor, ME) mice. Germline transmission was confirmed by Southern blot analysis using a 3´ outside-region probe to confirm the presence of the predicted 5 kb and 11 kb HindIII fragments diagnostic of the wild type and mutant CB_1 alleles (Figure 3*A*). All mice analyzed in this study have the genetic background 129/SvJ, and were derived from heterozygous breeding. Genotyping of CB_1 wild type ($CB_1^{+/+}$), heterozygous ($CB_1^{+/-}$) and knockout ($CB_1^{-/-}$) mice was carried out using a 3-primer PCR assay (Fig. 3*A*), where primer pair 9739 and 9740 generates a 199 bp amplicon indicative of the CB_1 wild-type allele, primer pair 3195 and 9740 generates a 314 bp amplicon indicative of the mutant allele, and the presence of both of amplicons is indicative of a heterozygous compliment of both alleles. Primer sequences are as follows: 9740 - 5´-TATCTAGAGGCTGCGCAGTGCCTTC-3´; 9739 - 5´-CCCTCTGCTTGCGATCATGGTGTATG-3´; 3195 - 5´-GGG CCAGCTCATTCCTCCCACTCAT-3´. PCR genotyping of tail DNA was carried out in a total reaction volume of 50 µl using ExTaq polymerase (PanVera, Madison, WI) with 10 µl of 9740 (10

µM), 5 µl of 9739 (10 µM) and 5 µl of 3195 (10 µM). PCR conditions were 94 °C for 5 min; 14 cycles at 98°C for 20 sec, 68°C for 45sec; 16 cycles at 98°C for 20 sec, 68°C for 45 sec plus 15 sec/cycle; 72°C for 10 minutes.

Receptor autoradiography: Autoradiography was conducted as described by Herkenham *et. al.* (1991) with minor modifications. Mouse brain or spinal cord cryosections (15 µm) were thaw-mounted on Superfrost Plus slides and stored at -80 °C until the day of the experiment. Slides were pre-incubated in 50 mM Tris-HCl, pH 7.4 at 25°C for 30 minutes. 50 mM Tris-HCl, pH 7.4; 5% BSA containing 10 nM [^{3}H]-CP55,940 (specific activity, 165 Ci/mmol; Dupont NEN, Boston, MA) was applied at 25°C to the tissue sections and placed horizontally in a humid chamber for 2 hours at room temperature. 10 µM CP55,940 was used to define non-specific binding. Slides were washed, dried and exposed as described previously (Herkenham et al, 1991).

Drug administration: Cannabinoid drugs were dissolved in dimethyl sulfoxide. AM630 is a CB_2 receptor-selective antagonist with 70- to 165-fold selectivity for binding to the CB_2 receptor *in vitro* (Ross et al, 1999 and Hosohata et al, 1997). AM251 is a 300-fold selective CB_1 receptor

antagonist (Gatley et al, 1996 and Gatley et al, 1997). Drugs were administered intraperitoneally 15 min before behavioral testing.

Spinal nerve ligation: L5/L6 spinal nerve ligation was performed as described by Kim and Chung (1992). Animals were anesthetized with halothane. An incision was made lateral to the lumbar spine. The right L5 and L6 spinal nerves were isolated and tightly ligated distal to the dorsal root ganglion. The incision was closed and animals were allowed to recover for 10 days. Sham-operated animals were prepared in an identical fashion except that the spinal nerves were not ligated.

Measurement of tactile withdrawal threshold: Tactile withdrawal threshold was determined as described by Chaplan *et al.* (1994). Animals were acclimated for 30 min in suspended cages with wire mesh bottoms. The hindpaw was probed with calibrated von Frey filaments (Stoelting, Wood Dale, IL) applied perpendicularly to the plantar surface. A positive response was indicated by a sharp withdrawal of the paw. The 50% paw withdrawal threshold was determined by the non-parametric method of Dixon (1980), in which the stimulus is incrementally increased until a positive response is obtained, then decreased until a negative

result is observed. The protocol was repeated until three changes in behavior were determined. A maximal cut-off value of 15 g was used for rats and 3.5 g for mice. The 50% paw withdrawal threshold was determined as $(10^{[Xf+k\delta]})/10,000$, where Xf = the value of the last von Frey filament employed, k = Dixon value for the positive/negative pattern, and δ = the logarithmic difference between stimuli.

Measurement of thermal withdrawal latency: The method of Hargreaves *et al.* (Hargreaves et al, 1988) was used. Animals were acclimated within plexiglass enclosures on a clear glass plate maintained at 30°C. A radiant heat source (high-intensity projector lamp) was focused onto the plantar surface of the paw. When the paw was withdrawn, a motion detector halted the stimulus and a timer. A maximal cut-off of 40 sec for rats and 30 sec for mice was used to prevent tissue damage.

Data analysis: Differences in responses between groups was tested using ANOVA followed by post-hoc testing with Students' t-test with Bonferroni's correction. Significance was defined as $P < 0.05$.

RESULTS

AM1241 is an aminoalkylindole analog substituted at the 1-position with a methylene group linked to an N-methylpiperidine ring at the 2-position (Fig. 1*A*), while the 3-indole substitutent is a 2-iodo-5-nitrobenzoyl group. The compound has favorable physicochemical properties and can be crystallized both as the free base and its water-soluble hydrochloride salt.

The affinity of AM1241 (K_i) for the CB_2 receptor in a mouse spleen preparation was 3.4 ± 0.5 nM (mean ± SEM) and its affinity (K_i) for the CB_1 receptor in a rat brain preparation was 280 ± 41 nM (mean ± SEM) (Fig. 1*B*), indicating an 82-fold selectivity for the CB_2 receptor in rodent tissue.

AM1241 produced a dose-dependent inhibition of spinal nerve ligation (SNL)-induced tactile and thermal hypersensitivity in rats (Fig. 2). The effect of AM1241 was completely blocked by the CB_2 receptor-selective antagonist, AM630, but was not affected by the CB_1 receptor-selective antagonist, AM251.

To further test whether activation of the CB_2 receptor is sufficient to inhibit nerve injury-induced sensory hypersensitivity, we constructed mice deficient in the CB_1

receptor (Fig. 3*A*) and studied the effects of AM1241 on SNL-induced tactile and thermal hypersensitivity in $CB_1^{-/-}$ mice. Disruption of the CB_1 receptor gene eliminated binding of the non-selective cannabinoid ligand [^{3}H]-CP55940 in brain and spinal cord (Fig. 3*B*), indicating a total absence of cannabinoid receptors (CB_1 and CB_2) in the central nervous system. WIN55,212-2, a mixed CB_1/CB_2 receptor agonist, produced significant catalepsy in $CB_1^{+/+}$ mice, but did not produce catalepsy in $CB_1^{-/-}$ mice, demonstrating a functional lack of CB_2 receptor activity *in vivo* (data not shown).

Pre-surgical tactile withdrawal thresholds were lower for $CB_1^{-/-}$ than for $CB_1^{+/+}$ (wild-type) mice (Fig. 4). In contrast, pre-surgical thermal withdrawal latencies did not differ between $CB_1^{-/-}$ and $CB_1^{+/+}$ animals (Fig. 5).

Spinal nerve ligation decreased tactile withdrawal threshold in both $CB_1^{-/-}$ and $CB_1^{+/+}$ mice (Fig. 4 *B, D*), while sham operation had no effect (Fig. 4 *A, C*). Intraperitoneal injection of AM1241 reversed SNL-induced tactile hypersensitivity. The effects of AM1241 were completely blocked by AM630, but not by AM251. In spinal nerve-ligated animals AM1241 returned tactile withdrawal thresholds in $CB_1^{-/-}$ mice to preligation values of $CB_1^{+/+}$

animals, while in sham-operated $CB_1^{-/-}$ mice the compound increased tactile withdrawal thresholds until they equaled those observed in $CB_1^{+/+}$ animals.

Similarly, spinal nerve ligation decreased thermal withdrawal latency in both $CB_1^{-/-}$ and $CB_1^{+/+}$ mice (Fig. 5 *B*, *D*). Conversely, sham operation had no effect (Fig. 5 *A*, *C*). Intraperitoneal injection of AM1241 reversed SNL-induced thermal hypersensitivity. AM1241 also prolonged thermal withdrawal latency beyond pre-surgical baseline values in both nerve-ligated and sham-operated animals. Again, the effects of AM1241 were completely inhibited by AM630, but not by AM251.

DISCUSSION

Ligation of the L5 and L6 spinal nerves in experimental animals is used to model human neuropathic pain resulting from injury or disease of primary afferent neurons. Spinal nerve ligation (SNL) increases sensitivity to tactile and thermal stimuli (Kim and Chung, 1992), two features commonly observed in human neuropathic pain (Rowbotham, 1995).

AM1241 reversed SNL-induced tactile and thermal hypersensitivity in rats and in mice. The effects of AM1241 were inhibited by the CB_2 receptor-selective antagonist AM630, but not by the CB_1 receptor-selective antagonist AM251, indicating that they are mediated by the CB_2 receptor. Further, the reversal by AM1241 of SNL-induced tactile and thermal hypersensitivity in $CB_1^{-/-}$ mice confirms that AM1241 acts through a mechanism independent of the CB_1 receptor. AM1241 inhibited both thermal hypersensitivity, which is dependent upon intact capsaicin-sensitive, small, unmyelinated C-fiber afferents as well as tactile hypersensitivity, a condition which does not appear to be mediated by C-fiber afferents, but may be mediated by large, myelinated Aβ fibers (Ossipov et al., 1999).

In addition to reversing nerve injury-induced thermal hypersensitivity, AM1241 prolonged thermal withdrawal latencies beyond pre-ligation baseline values. This increase is consistent with the increased thermal withdrawal latencies observed in sham-operated animals and with the previously demonstrated thermal antinociceptive effects of CB_2 receptor activation (Malan et al., 2001).

The greater tactile sensitivity in $CB_1^{-/-}$ mice compared to $CB_1^{+/+}$ animals suggests that CB_1 receptors modulate basal tactile sensitivity through the action of endogenous cannabinoid agonists and/or by intrinsic activity of the receptor. In contrast, the equivalent thermal sensitivity observed in $CB_1^{-/-}$ and $CB_1^{+/+}$ mice suggests either that CB_1 receptors do not participate in the modulation of basal thermal sensitivity or that compensatory changes in other regulatory systems return thermal sensitivity to normal levels in $CB_1^{-/-}$ animals. Our finding of equivalent thermal sensitivity in $CB_1^{-/-}$ and $CB_1^{+/+}$ in mice is consistent with the observation by Zimmer *et al.* (Zimmer et al., 1999) that withdrawal latencies in the tail flick assay do not differ between wild-type and CB_1 knockout mice. AM1241 returned thermal withdrawal latencies in $CB_1^{-/-}$ mice to preligation values of $CB_1^{+/+}$ animals, demonstrating that it is capable of

reversing the tactile hypersensitivity produced by disruption of the CB_1 receptor gene, as well as that induced by spinal nerve ligation. This is consistent with the finding that in sham-operated $CB_1^{-/-}$ mice, AM1241 increased tactile withdrawal thresholds until they equaled those observed in $CB_1^{+/+}$ animals.

The mechanism by which AM1241 acts to reverse SNL-induced somatosensory hypersensitivity is not known. The effects of this compound are unlikely to be the result of actions in the CNS because considerable evidence suggests that CB_2 receptors are not present in the CNS (Munro et al., 1993, Griffin et al., 1999, Zimmer et al., 1999, and Buckley et al., 2000). A site of action outside the CNS is consistent with our previous findings where, using site-specific injections of agonist and antagonists, we showed that the antinociceptive actions of AM1241 appear to be mediated at peripheral sites (Malan et al., 2001).

One possibility for a peripheral mechanism of action is direct inhibition of primary afferent neurons. However, CB_2 receptor mRNA was not found in dorsal root ganglia, while CB_1 receptor mRNA was readily detected (Hohmann and Herkenham, 1999), suggesting that CB_2 receptors are not expressed in primary afferent neurons. This does not,

however, exclude the possibility that CB_2 receptors are expressed in small, but functionally significant, amounts.

A second possibility for a peripheral mechanism is an indirect action of AM1241 to decrease the sensitivity of primary afferent neurons. Indeed, CB_2 receptors are expressed primarily on mast and immune cells (Munro et al., 1993, Galiegue et al., 1995, Facci et al., 1995, and Schatz et al., 1997) and CB_2 receptor agonists, including AM1241, have been demonstrated to have anti-inflammatory effects (Hanus et al., 1999 and Quartilho et al., 2003). It is also known that mast and immune cells release mediators that are capable of sensitizing primary afferent neurons, including histamine, serotonin, prostaglandins, interleukin 1β, tumor necrosis factor-α, and nerve growth factor (Dray, 1995). Therefore, activation of peripheral CB_2 receptors might decrease the sensitivity of primary afferent neurons by inhibiting the release of sensitizing substances from neighboring mast and immune cells. Congruent with the above is the observation that neurogenic inflammation appears to take place after spinal nerve ligation at the site of spinal nerve ligation and in peripheral tissues surrounding the distal terminals of primary afferent neurons. Cyclooxygenase 2-immunoreactive cells, including

macrophages, are increased at the injury site in spinal nerve-ligated rats (Ma and Eisenach, 2002), and peripheral nerve injury results in polymorphonuclear leukocyte accumulation in the periphery (Daemen et al., 1998 and Daemen et al., 1998). Alternatively, CB_2 receptor activation may reverse sensory hypersensitivity by inhibiting input into a nervous system sensitized at more central locations. Sensory hypersensitivity resulting from enhanced responses to normal levels of input from peripheral terminals of sensory neurons might be blocked by inhibition of this peripheral input. CB_2 receptor activation appears to be capable of inhibiting the sensory sensitivity of peripheral terminals of primary afferent neurons in the absence of peripheral inflammation or nerve injury, as demonstrated by the ability of AM1241 to inhibit acute thermal nociception by acting at peripheral CB_2 receptors (Malan et al., 2001).

Our data demonstrate that activity of CB_1 cannabinoid receptor is not required for the inhibition of neuropathic pain by AM1241. They do not, however, fully exclude the involvement of other receptors. For example, a putative receptor has recently been described in brain that is modulated by the cannabinoid receptor agonist WIN55,212-2,

but has different pharmacological properties from the CB_1 receptor and is not inhibited by the CB_1 receptor-selective antagonist AM251 (Hajos and Freund, 2002). To date this receptor has not been cloned and its interactions with cannabinoid ligands have not been characterized.

In previous work, we have shown that AM1241 does not produce catalepsy, hypothermia, inhibition of spontaneous locomotor activity, or inhibition of performance on the rotarod apparatus (Malan et al, 2001). Earlier data with the CB_2 receptor-selective but less potent agonist HU-308 also showed that this compound does not produce catalepsy, hypothermia, or inhibition of spontaneous locomotor activity (Hanus et al, 1999).

These results provide evidence that CB_2 receptor-selective agonists may be effective in treating neuropathic pain without CNS side effects. Such a therapeutic profile offers significant advantages over current therapies. In addition, the value of CB_2 receptor-selective agonists, such as AM1241, in pain therapy is enhanced by their observed effectiveness against nociceptive (Malan et al., 2001), inflammatory (Quartilho et al., 2003), and neuropathic pain. Although multiple pain mechanisms may be active in the same patient (as, for example, in cancer pain), there

are presently no single therapies that are consistently effective against these diverse types of pain. Our data suggest the importance of the development of CB_2 receptor-selective agonists for human therapeutics.

List of Illustrations

Figure 1

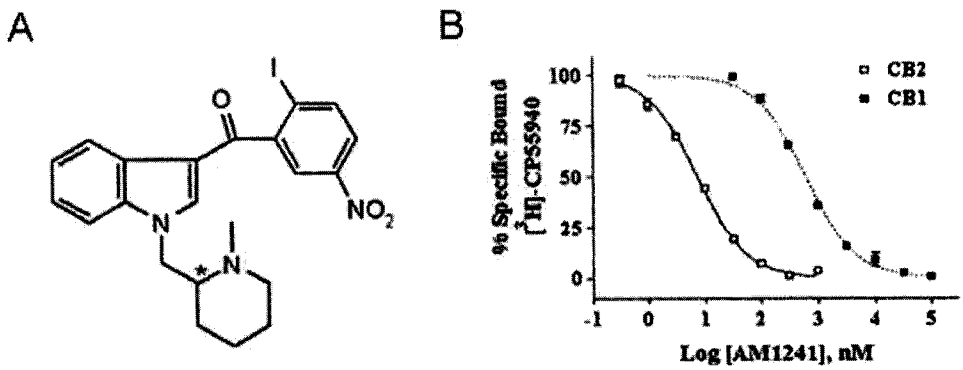

Fig. 1. AM1241 is a CB_2 cannabinoid receptor-selective ligand. A. Structure of AM1241. B. Equilibrium-competition binding of AM1241 *vs.* [^{3}H]-CP55940 using either rat brain synaptosomal membranes (CB_1) or mouse spleen homogenates (CB_2) illustrates the selectivity of this ligand for CB_2. The curves shown are representative of single experiments that were independently replicated three times.

Figure 2

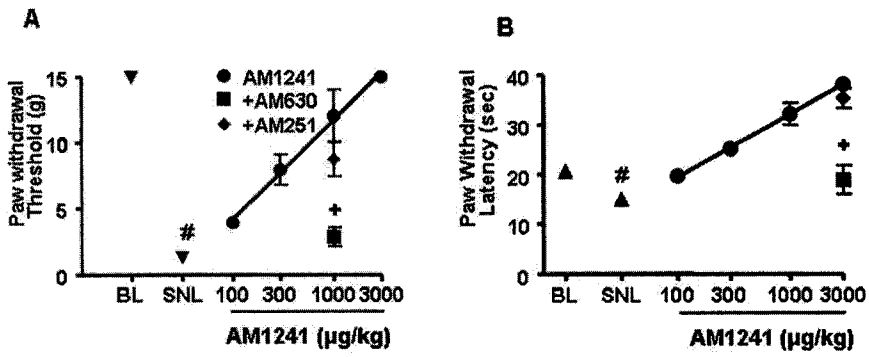

Fig. 2. AM1241 dose-dependently inhibits sensory hypersensitivity in rats. A, inhibition of tactile hypersensitivity. B, inhibition of thermal hypersensitivity. BL = pre-surgical baseline. SNL = after spinal nerve ligation. All drugs were administered i.p. AM630 and AM251 were administered at a dose of 300 µg/kg. Data expressed as mean ± S.E.M. n = 6 per group. #, $P < 0.05$ compared to pre-surgical baseline; +, $P < 0.05$ compared to AM1241 alone.

Figure 3

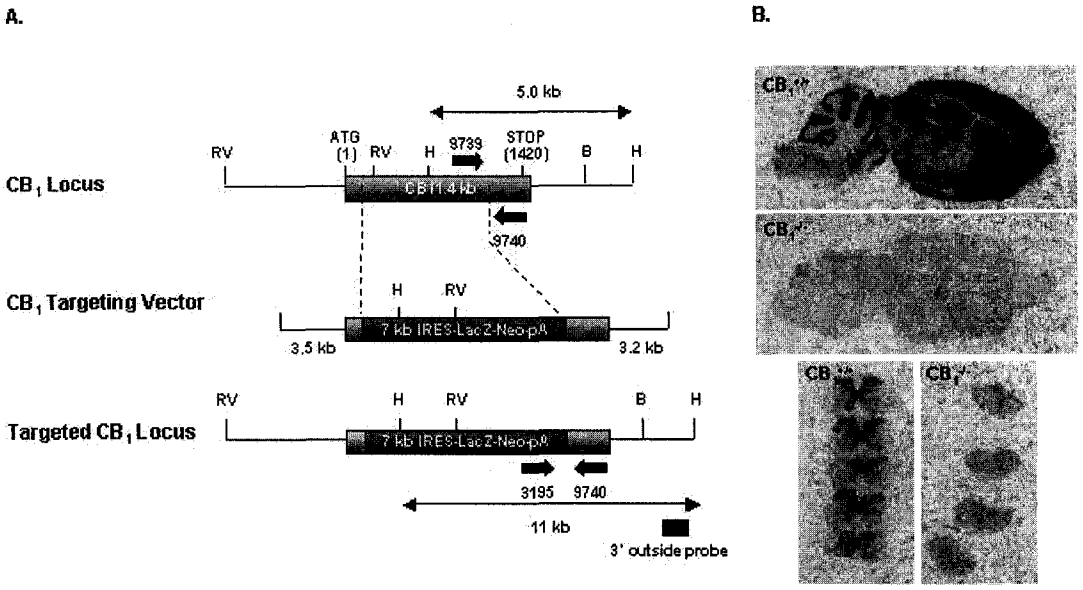

Fig 3. A. Generation of CB_1 receptor-deficient mice. B. Disruption of the CB_1 receptor gene eliminated binding of the non-selective cannabinoid ligand [^{3}H]CP55,940. Autoradiography of 10 nM [^{3}H]-CP55,940 binding in $CB_1^{+/+}$ and $CB_1^{-/-}$ sagital sections of mouse brain (upper panels) and transverse sections of lumbar spinal cord (lower panels).

Figure 4

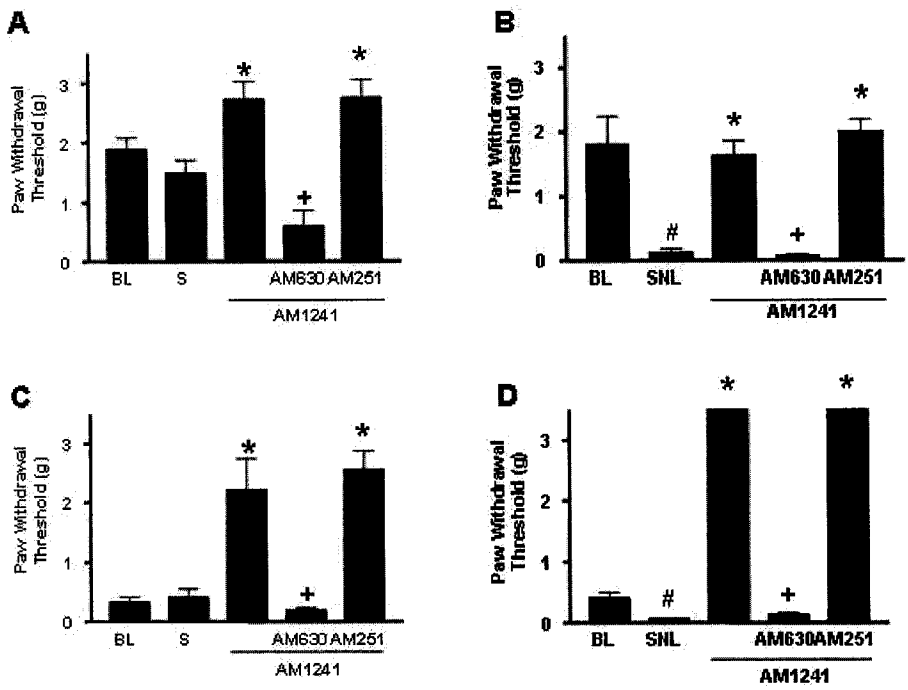

Fig. 4. AM1241 inhibits tactile hypersensitivity in mice lacking the CB_1 receptor. A, sham-operated wild-type mice. B, spinal nerve-ligated wild-type mice. C, sham-operated $CB_1^{-/-}$ mice. D, spinal nerve-ligated $CB_1^{-/-}$ mice. BL = pre-surgical baseline. S = after sham operation. SNL = after spinal nerve ligation. Doses used were: AM1241, 1 mg/kg; AM 630, 1 mg/kg; AM251, 300 µg/kg. Data expressed as mean ± S.E.M. n = 6 per group. #, $P < 0.05$ compared to pre-surgical baseline; *, $P < 0.05$ compared to post-surgical values; +, $P < 0.05$ compared to AM1241 alone.

Figure 5

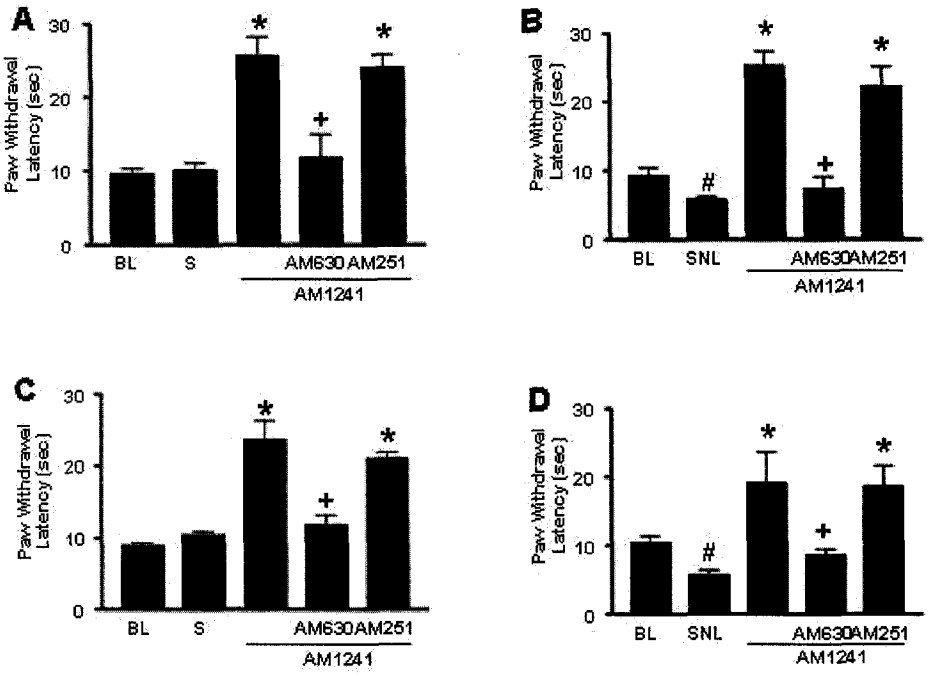

Fig. 5. AM1241 inhibits thermal hypersensitivity in mice lacking the CB_1 receptor. A, sham-operated wild-type mice. B, spinal nerve-ligated wild-type mice. C, sham-operated $CB_1^{-/-}$ mice. D, spinal nerve-ligated $CB_1^{-/-}$ mice. BL = pre-surgical baseline. S = after sham operation. SNL = after spinal nerve ligation. Doses used were: AM1241, 3 mg/kg; AM 630, 1 mg/kg; AM251, 300 µg/kg. Data expressed as mean ± S.E.M. n = 6 per group. #, $P < 0.05$ compared to pre-

surgical baseline; *, $P < 0.05$ compared to post-surgical values; +, $P < 0.05$ compared to AM1241 alone.

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CHAPTER 4

CB_2 CANNABINOID RECEPTORS ARE NECESSARY FOR THE PAIN-MODULATING EFFECTS OF THE PUTATIVE CB_2 RECEPTOR-SELECTIVE AGONIST AM1241

Introduction

Activation of the cannabinoid system produces antinociception (Monhemius *et al.*, 2001), blocks tactile and thermal hypersensitivity produced by peripheral inflammation (Quartilho et al., 2003) and blocks sensory hypersensitivity produced by nerve injury (Fox *et al.*, 2001). Early data suggested that cannabinoid-induced pain modulation is the result of CB_1 cannabinoid receptor activation, because cannabinoid effects were blocked by the CB_1 receptor-selective antagonist SR141716A (Monhemius et al., 2001). The CB_1 receptor was first isolated and cloned in 1990 (Matsuda *et al.*, 1990). It is located in the central nervous system (CNS) and in peripheral tissues (Schatz *et al.*, 1997). In addition to producing antinociception, agonists with activity at the CB_1 receptors produce a constellation of CNS effects in animal models, including hypothermia, catalepsy, decreased spontaneous

activity, impaired motor coordination, and increased appetite (Balint, 2001).

In contrast, the CB_2 cannabinoid receptor is not located in the CNS. Rather, it is located peripherally on immune and skin cells (Munro *et al.*, 1993; Facci *et al.*, 1995; Galiegue *et al.*, 1995; Schatz *et al.*, 1997, Casanova et al., 2003). Using the CB_2 receptor-selective agonist AM1241 in naïve, carrageenan-inflamed and L_5/L_6 SNL rats previously showed that activation of CB_2 receptors produces antinociception (Malan et al., 2001), inhibits thermal hypersensitivity produced by peripheral inflammation (Quartilho et al. 2003), and reverses sensory hypersensitivity in a model of neuropathic pain (Ibrahim et al., 2003), respectively. The effects of AM1241 were prevented by the CB_2 receptor-selective antagonist AM630. AM251, a CB_1 receptor-selective antagonist, had no effect on AM1241 (Malan et al., 2001, Ibrahim et al., 2003, and Quartilho et al. 2003). In addition, AM1241 inhibited peripheral nerve injury-induced tactile and thermal hypersensitivity in mice lacking the CB_1 cannabinoid receptors (Ibrahim et al., 2003), demonstrating that CB_1 receptors are not required for the actions of AM1241.

These experiments strongly suggested that the actions of AM1241 were mediated by CB_2 receptors. However, since CB_2 receptors are not found on CNS or peripheral neurons, the mechanism by which they inhibit pain responses has not been evident. This has led to speculation that AM1241 inhibits nociception by acting at an additional class of non CB_1, non CB_2 cannabinoid receptors located on neurons that were also inhibited by AM630. Recently, evidence supporting the existence of additional (non- CB_1, non- CB_2) cannabinoid receptors has been reported, providing an additional potential target for AM1241. Therefore, to definitively test the hypothesis that CB_2 receptors mediate the actions of AM1241, we tested the prediction that AM1241 would not inhibit antinociception and reverse sensory sensitivity in mice lacking the CB_2 receptor. The strain of CB_2 receptor knockout mice used has been developed and previously reported (Buckley et al, 2000).

Methods:

Animals: All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the guidelines of the International Association for the Study of Pain and the National Institutes of Health. Mice were 20-30 g at the time of testing. Breeding pairs of mice heterozygous for the deleted CB_2 cannabinoid receptor gene ($CB_2^{+/-}$ mice) were kindly provided by Nancy Buckley (California State Polytechnic University, Pomona, CA and Andreas Zimmer (Laboratory of Genetics, NIMH, Bethesda, MD). The method for producing the CB_2 knockout mice is previously described (Buckley et al. 2000). Briefly, the targeting vector was derived from the plasmid pPNT by introducing upstream of the neomycin gene, 731 base pair of the CB_2 cannabinoid receptor genomic fragment containing the 5′ region of the axon which has the entire coding sequence. Another non-coding cannabinoid CB_2 receptor gene sequence was introduced downstream of the neomycin gene. Chimeric mice were generated by morula aggregation or blastocyst injection with the targeted embryonic stem cell line 129. Chimeric mice were backcrossed with C57BL/6 mice. Southern blot hybridization followed by polymerase chain reaction were used to asses the presence of the CB_2 cannabinoid

receptor gene. Animals were maintained in a climate-controlled room on a 12-h light/dark cycle and allowed food and water *ad libitum*.

Drug administration

AM1241 is a potent cannabinoid receptor agonist with 70-fold selectivity for the CB_2 receptor *in vitro* (Ibrahim et al. 2003). AM630 is a CB_2 receptor-selective antagonist with 70 to 165-fold selectivity for binding to the CB_2 receptor *in vitro* (Ross *et al.*, 1999; Hosohata *et al.*, 1997). All drugs were dissolved in dimethyl sulfoxide (DMSO). Drugs were injected intraperitoneally (i.p., 50-70 µl). Measurements were taken 20 min after i.p. injection. In preliminary experiments, these were determined to be the times of maximal drug effect.

Measurement of thermal withdrawal latency: The method of Hargreaves et al. (1988) was used. Animals were acclimated within plexiglass enclosures on a clear glass plate maintained at 30°C. A radiant heat source (high-intensity projector lamp) was focused onto the plantar surface of the paw. When the paw was withdrawn, a motion detector halted the stimulus and a timer. A maximal cut-off of 30 sec was used to prevent tissue damage.

Spinal Nerve Ligation (SNL) Injury:

The L5 and L6 spinal nerve were ligated as described by Kim and Chung (Kim and Chung, 1992), except mice were used. Under halothane anesthesia, the dorsal vertebral column was surgically exposed from L4 to S2. The L5 and L6 spinal nerves were exposed and tightly ligated distal to the dorsal root ganglion using 4-O silk suture. Incisions were closed and the animals allowed to recover. Animals that exhibited motor dysfunction or failed to develop tactile hypersensitivity (less than 2% of animals) were excluded from further testing.

Testing of Tactile withdrawal threshold:

The paw ipsilateral to the side of nerve injury was probed with a series of calibrated von Frey filaments. Each filament was applied perpendicularly to the plantar surface of the paws of mice held in suspended wire-mesh cages. The withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength (the "up and down" method) and the data were analyzed using the non-parametric method of Dixon, as described by Chaplan *et al.* (Chaplan et al, 1994).

Data analysis: Differences in responses between groups was tested using ANOVA followed by post-hoc testing with

Students' t-test with Bonferroni's correction. Significance was defined as $P < 0.05$.

Results

In wild-type mice ($CB_2^{+/+}$), AM1241 produced antinociception. Intraperitoneal doses of 0.3, 1, and 3 mg/kg resulted in an increase in paw withdrawal latency 10 ± 1 to 14 ± 1, 19 ± 1, 27 ± 1 seconds, respectively. In CB_2 receptor knockout ($CB_2^{-/-}$) mice, the same doses of AM1241 produced no changes in paw withdrawal latency compared to baseline (Figure 1). In the absence of drug administration, withdrawal latencies were shorter in $CB_2^{-/-}$ mice than in $CB_2^{+/+}$ mice. To test whether the lack of effect of AM1241 in $CB_2^{-/-}$ was simply due to enhanced nociceptive responses in $CB_2^{-/-}$ mice, the intensity of the thermal stimulus was decreased to a point where baseline withdrawal latency in $CB_2^{-/-}$ mice equaled the baseline withdrawal latency at the high intensity in $CB_2^{+/+}$ mice (Figure 2). AM1241 (3mg/kg i.p.) had no effect in $CB_2^{-/-}$ mice at the lower stimulus intensity. Ligation of the L5/L6 spinal nerves decreased the thermal withdrawal latency of $CB_2^{+/+}$ from 10.3 ± 0.9 sec and of $CB_2^{-/-}$ mice from 5.5 ± 0.5 sec to 4.1 ± 0.3 sec, respectively (Figure 3). AM1241 (i.p.) dose-dependently reversed thermal hypersensitivity and prolonged thermal withdrawal latencies beyond pre-nerve injury values in $CB_2^{+/+}$ mice. AM1241 had no

effect in $CB_2^{-/-}$ mice. Similarly, spinal nerve ligation decreased tactile withdrawal threshold from 1.7 ± 0.4 g to 0.08 ± 0.03 g in $CB_2^{+/+}$ mice and from 0.6 ± 0.2 g to 0.08 ± 0.04 g in $CB_2^{-/-}$ mice (Figure 4). AM1241 (i.p.) dose-dependently reversed thermal sensitivity in $CB_2^{+/+}$ mice, but had no effect in $CB_2^{-/-}$ mice.

Discussion

The putative CB_2 cannabinoid receptor-selective agonist AM1241 inhibits pain responses in a variety of pain models. AM1241 produced full effects in CB_1 cannabinoid receptor-knockout mice, demonstrating that AM1241 is capable of producing pain inhibition independently of CB_1 receptors. This is consistent with the findings of Zimmer et al. (1999) who showed that CB_1 receptor-deficient mice showed signs of pain relief. Δ^9-tetrahydrocannabinol increased the tail flick latency as well as decreased abdomen licking in CB_1 cannabinoid receptor deficient mice. The pain-inhibiting effects of AM1241 are blocked by AM630, an antagonist selective for CB_2 over CB_1 receptors. However, the possibility that AM630 also antagonizes additional uncharacterized non-CB_1, non-CB_2 cannabinoid receptors cannot be excluded. While no additional cannabinoid receptors have been cloned, there is pharmacological evidence for their existence in brain (Monory et al., 2001) and peripheral tissues (Mang et al., 2001). WIN55,212-2, a non-selective CB_1/CB_2 cannabinoid receptor agonist, was found to stimulate [^{35}S] GTPγS in cerebellum tissue of $CB_1^{-/-}$ mice which was not blocked by either CB_1 or CB_2 receptor antagonists. In the peripheral tissue, anandamide

increased the basal [^{3}H]-acetylcholine in guinea-pig ileum. The CB_1 and CB_2 receptor antagonists, SR141716A and SR144528, did not modify the effects of anandamide.

We provide evidence that CB_2 receptors are required for the effects of AM1241. $CB_2^{-/-}$ mice did not develop antinociception in response to AM1241, while $CB_2^{+/+}$ mice exhibited dose dependent antinociception. Similarly, AM1241 inhibited sensory hypersensitivity produced by spinal nerve ligation in $CB_2^{+/+}$, but not in $CB_2^{-/-}$ mice.

Interestingly, $CB_2^{-/-}$ mice had lower thermal paw withdrawal latencies and tactile paw withdrawal threshold when compared to $CB_2^{+/+}$ mice suggesting that tonic activity of CB_2 receptor may play a role in the regulation of the basal sensory thresholds. When the intensity of the thermal stimulus was decreased, so that the $CB_2^{-/-}$ mice responded with similar latencies to $CB_2^{+/+}$ mice at the original intensity, AM1241 still failed to produce antinociception. This suggests that the lack of effects of AM1241 in $CB_2^{-/-}$ mice is not due to enhanced nociceptive responsiveness, but is rather due to the absence of CB_2 receptors. Spinal nerve ligation is an accepted model of neuropathic pain and results in thermal and tactile

hypersensitivity, two common features of human neuropathic pain (Rowbotham, 1995).

In summery, these data support the hypothesis that the pain-inhibiting actions of AM1241 are mediated by CB_2 cannabinoid receptors. By doing so, they provide support for the hypothesis that CB_2 receptor activation inhibits acute, inflammatory and neuropathic pain. CB_2 receptor activation may be an effective method for the treatment of pain. Given the location of CB_2 receptors exclusively outside the normal CNS, CB_2 receptor agonists are predicted to be without CNS side effects. This hypothesis is supported by data showing that AM1241 does not produce CNS effects produced by cannabinoid receptor agonists retaining activity at CB_1 receptors (Malan et al., 1999).

List of Illustrations

Figure 1

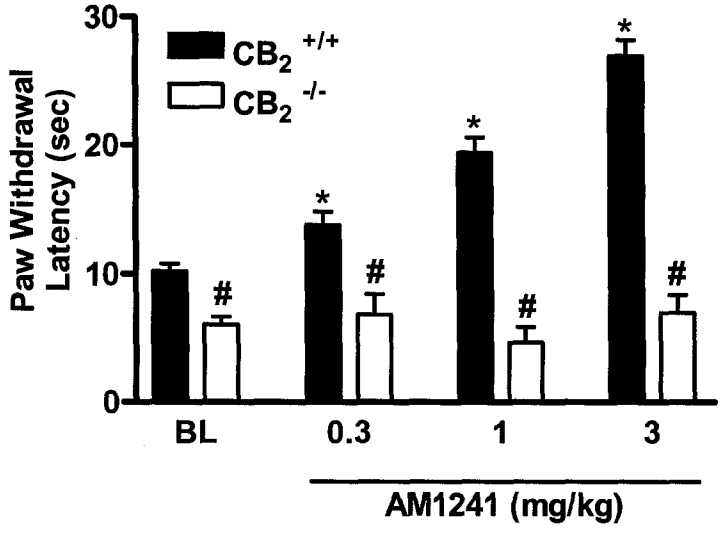

Figure 1. AM1241 (i.p) dose-dependently produced antinociception in $CB_2^{+/+}$ mice. *, $P < 0.05$ compared to baseline (BL). #, $P < 0.05$ compared to $CB_2^{+/+}$ mice.

Figure 2

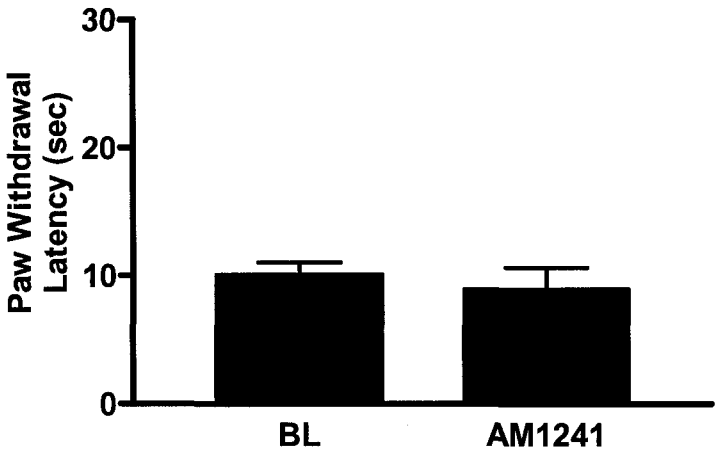

Figure 2. AM1241 (3mg/kg, i.p.) had no effect on $CB_2^{-/-}$ mice baseline (BL)

Figure 3

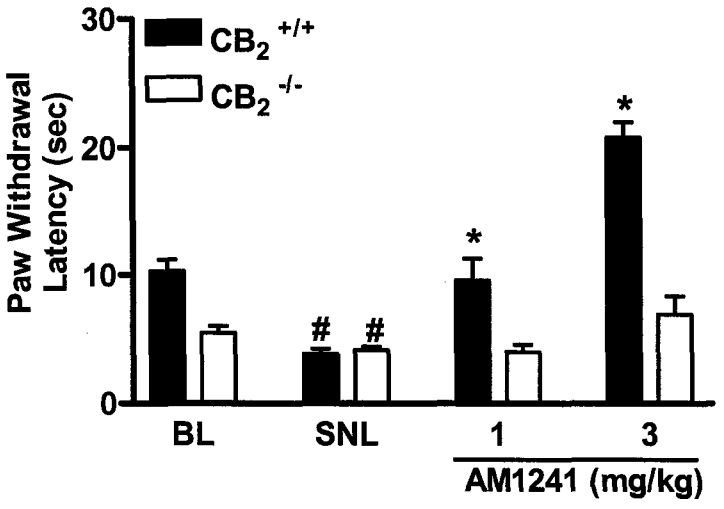

Figure 3. AM1241 (i.p.) dose-dependently reversed thermal hypersensitivity in $CB_2^{+/+}$ mice. *, $P < 0.05$ compared to spinal nerve ligation (SNL). #, $P < 0.05$ compared to baseline (BL).

Figure 4

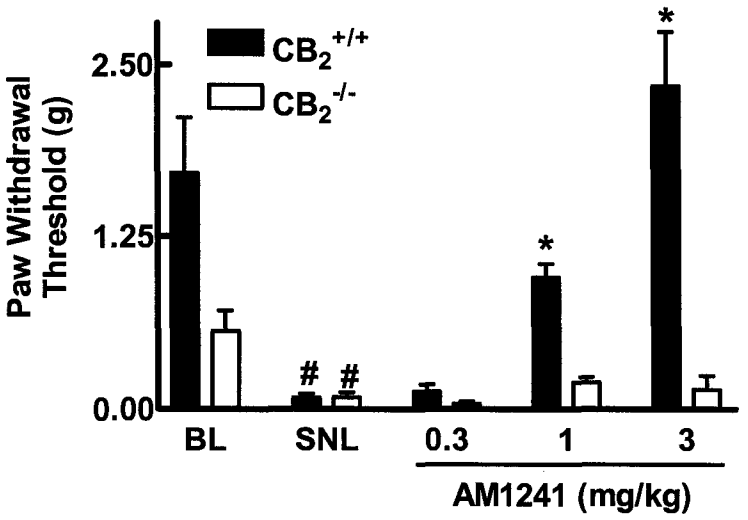

Figure 4. AM1241 (i.p.) dose-dependently reversed tactile hypersensitivity in $CB_2^{+/+}$ mice. *, $P < 0.05$ compared to spinal nerve ligation (SNL). #, $P < 0.05$ compared to baseline (BL).

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Chapter 5

LOCAL ADMINISTRATION OF CB_2 RECEPTOR-SELECTIVE AGONISTS INHIBITS NEUROPATHIC PAIN RESPONSE

Introduction

Studies using site-specific injections of the CB_2 cannabinoid receptor-selective agonist AM1241 have suggested that the pain modulating effects of AM1241 are produced locally in peripheral tissues (Malan et al. 2001 and Quartilho et al. 2003). AM1241 produces antinociception effects when administered in the paw ipsilateral to the site of testing, but not when administered in the contralateral paw. Had the effect of AM1241 been due to systemic absorption and spread to distant sites it should not have mattered in which paw it was administered, since systemic absorption should be equal from both sides. Further, systemically administered AM1241 failed to produce antinociception when CB_2 receptor antagonist AM630 was injected in the paw ipsilateral to the site of testing, but not when injected in the contralateral paw, suggesting again that the actions of systemically administered AM1241 are mediated locally at the site of nociceptive testing. Here, we test the hypothesis that AM1241 acts in peripheral tissue (outside the CNS) to

inhibit the sensory hypersensitivity produced in a model of neuropathic pain. In addition, we test the hypothesis that AM1241 which is postulated to act in peripheral tissues (e.g. skin and subcutaneous tissue) will act topically to inhibit pain responses. Finally we will test whether a second CB_2 receptor-selective agonist also inhibits pain responses to show that the inhibition of pain responses is not a function of only one CB_2 cannabinoid receptor-selective agonist.

Method

Animals: All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the guidelines of the International Association for the Study of Pain and the National Institutes of Health. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) were 250-350 g at the time of testing. Animals were maintained in a climate-controlled room on a 12-h light/dark cycle and allowed food and water *ad libitum*.

Spinal nerve ligation: L5/L6 spinal nerve ligation was performed as described by Kim and Chung. Animals were anesthetized with halothane. An incision was made lateral to the lumbar spine. The right L5 and L6 spinal nerves were isolated and tightly ligated with 4-0 silk sutures distal to the dorsal root ganglion. The incision was closed and animals were allowed to recover for 10 days. Sham-operated animals were prepared in an identical fashion except that the spinal nerves were not ligated.

Measurement of tactile withdrawal threshold: Tactile withdrawal threshold was determined as described by Chaplan et *al*. 1994. Animals were acclimated for 30 min in suspended cages with wire mesh bottoms. The hind paw ipsilateral to spinal nerve ligation was probed with

calibrated von Frey filaments (Stoelting, Wood Dale, IL) applied perpendicularly to the plantar surface of the paw. A positive response was indicated by a sharp withdrawal of the paw. The 50% paw withdrawal threshold was determined by the non-parametric method of Dixon, 1980, in which the stimulus is incrementally increased until a positive response is obtained, then decreased until a negative result is observed. The protocol was repeated until three changes in behavior were determined. A maximal cut-off value of 15 g was used for. The 50% paw withdrawal threshold was calculated as $(10^{[Xf+k\delta]})/10,000$, where Xf = the value of the last von Frey filament employed, k = Dixon value for the positive/negative pattern, and δ = the logarithmic difference between stimuli.

Measurement of thermal withdrawal latency: The method of Hargreaves *et al.* 1988 was used. Animals were acclimated within plexiglass enclosures on a clear glass plate maintained at 30°C. A radiant heat source (high-intensity projector lamp) was focused onto the plantar surface of the paw. When the paw was withdrawn, a motion detector halted the stimulus and a timer. A maximal cut-off of 40 sec was used to prevent tissue damage.

Drug Administration:

AM1241 is an aminoalkylindole analog substituted at the 1-position with a methylene group linked to an N-methylpiperidine ring at the 2-position The affinity of AM1241 (K_i) for the CB_2 receptor in a mouse spleen preparation was 3.4 ± 0.5 nM (mean ± SEM) and its affinity (K_i) for the CB_1 receptor in a rat brain preparation was 280 ± 41 nM (mean ± SEM) (Fig. 1*B*), indicating an 82-fold selectivity for the CB_2 receptor in rodent tissue.

AM630 is a CB_2 receptor-selective antagonist with 70- to 165-fold selectivity for binding to the CB_2 receptor *in vitro* (Ross et al., 1999 and Hosohata et al., 1997). AM251 (Ki=7.49 nM) is a 300-fold selective CB_1 receptor antagonist (Gatley *et al.*, 1996, 1997). All drugs were dissolved in 100% DMSO. For intra-peritoneal (i.p.) administration, drugs were injected in a total volume of 0.5 ml. AM1241 was injected at the injury site on the nerve in a volume of 250 µl. AM1241 was administered topically in a volume of 50 µl on the dorsal surface of the hind paw.

Data analysis: Differences between groups were tested using ANOVA followed by post-hoc testing with Students't-test with Bonferroni's correction. Significance was defined as $P < 0.05$.

Results

Ligation of the L5 and L6 spinal nerve roots decreased the tactile withdrawal threshold ten folds from 15 ± 0 to 1.5 ± 0.2 g. Injection of AM1241 in the ipsilateral paw produced a dose-dependent inhibition of tactile hypersensitivity (Figure 1). AM1241 injected in the contralateral paw had no effect when tested 25 minutes after administration. Similarly, after spinal nerve roots ligation, the thermal withdrawal latency decreased from 20 ± 1 sec to 12.4 ± 0.5 seconds. Injection of AM1241 in the ipsilateral, but not the contralateral paw, produced a dose-dependent inhibition of thermal hypersensitivity and prolongation of latency beyond pre-ligation values (Figure 2). The pain modulating effects of AM1241 were reversed by the CB_2 receptor-selective antagonist AM630 (0.1 mg/kg, i.paw), but not by the CB_1 receptor-selective antagonist AM251 (0.3mg/kg, i.paw) (Figures 3 and 4).

AM1241 reversed tactile hypersensitivity when injected at the site of nerve injury, but had no effect when AM1241 was injected on the contralateral side (Figure 5). The effects of AM1241 injected at the site of injury on spinal nerve ligation-induced thermal hypersensitivity were not tested. AM630 (0.1 mg/kg) injected at the site of injury

blocked the effects of AM1241, while AM251 (0.3 mg/kg at the site of injury) had no effect (Figure 6). Topical administration of AM1241 on the paw ipsilateral to nerve injury and sensory testing reversed tactile (Figure 7) and thermal (Figure 8) hypersensitivity. Contralateral topical administration produced no effect. Topical AM630 (0.1 mg/kg) (Figure 9), but not AM251 (0.3 mg/kg) (Figure 10) blocked the actions of topical AM1241. The pain modulating effects is not limited to AM1241. Topically administered AM1714 dose dependently inhibited spinal nerve ligation-induced tactile hypersensitivity (Figure 11). The ability of AM1714 to inhibit thermal hypersensitivity has not been tested.

Discussion

Studies using site-specific injection of the CB_2 receptor-selective agonist AM1241 and the reversal of inflammatory hyperalgesia by AM1241 are mediated by local peripheral actions of the drug at the site of sensory testing (and of inflammation in a model of peripheral inflammation). The present data suggest that the inhibition of nerve injury-induced sensory hypersensitivity by AM1241 may be mediated by local actions at the site of nerve injury and at the site sensory testing in the paw. Locally mediated actions of AM1241 are suggested by the findings that ipsilateral administration of AM1241 at either site inhibit sensory hypersensitivity, supporting our hypothesis. These data argue against the effects of AM1241 being due to systemic uptake and spread to distant sites because systemic absorption should be equivalent from both sides.

The blockade of nerve injury-induced sensory hypersensitivity by AM1241 appears to be mediated by CB_2 receptors, because the CB_2 receptor-selective antagonist AM630, but not the CB_1 receptor-selective antagonist AM251 blocked the actions of AM1241 when administered at the same site. These data are consistent with our findings that AM1241 inhibited sensory hypersensitivity in wild type and

in CB_1 receptor-knockout (chapter 2) but not in CB_2 receptor knockout (See chapter 3) mice.

The lipophilic nature of AM1241 suggested to us that it may be suitable for topical application because it was predicted to penetrate the skin. As hypothesized, AM1241 applied topically to the hind paws ipsilateral relative to the site of nerve injury and sensory testing inhibited both thermal and tactile hypersensitivity. Again, this is probably due to local actions because topical administration of AM1241 on the paws contralateral to the side of nerve injury and sensory testing had no effect. The actions of topical are mediated by the CB_2 receptors as demonstrated by the fact that topical administration of the CB_2 receptor-selective antagonist AM630, but not the CB_1 receptor-selective antagonist AM251 blocked the effects of AM1241.

To further test whether CB_2 receptor-activation is sufficient to reverse spinal nerve injury-induced sensory hypersensitivity, we examined the effects of the CB_2 receptor-selective agonist AM1714. Topical application of AM1714 reversed tactile hypersensitivity in spinal nerve-ligated rats.

The potential medical applications of CB_2 receptor activation are clear. Activation of CB_2 receptors has been shown to inhibit tactile and thermal hypersensitivity in a neuropathic pain model in rats. Locally-mediated actions of AM1241 and its effectiveness by topical application make it a promising candidate for peripherally mediated pain relief in humans.

List of Illustrations

Figure 1

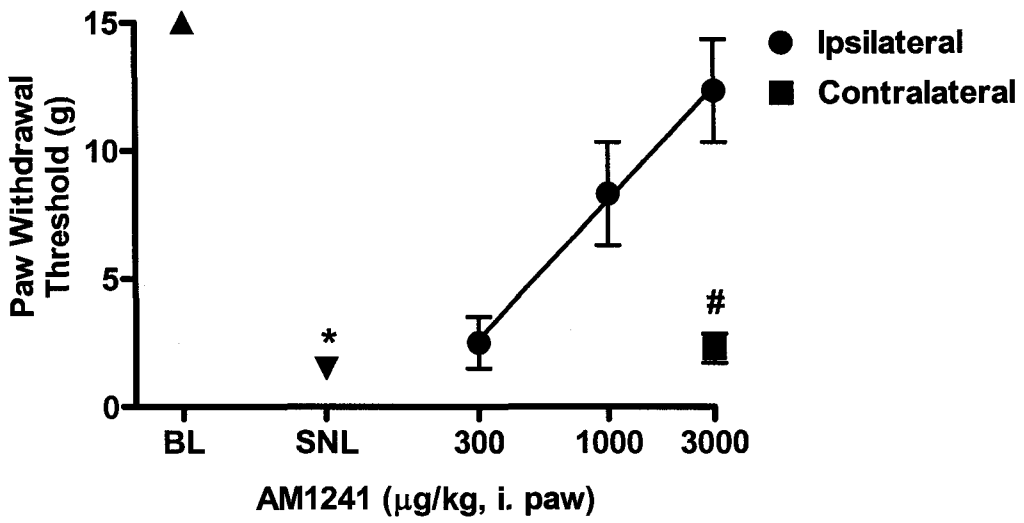

Figure 1. Spinal nerve ligation (SNL) decreased the paw withdrawal threshold baseline (BL). AM1241 dose-dependently blocked the tactile hypersensitivity. *, $P < 0.05$ compared BL. #, $P < 0.05$ compared to AM1241 alone.

Figure 2

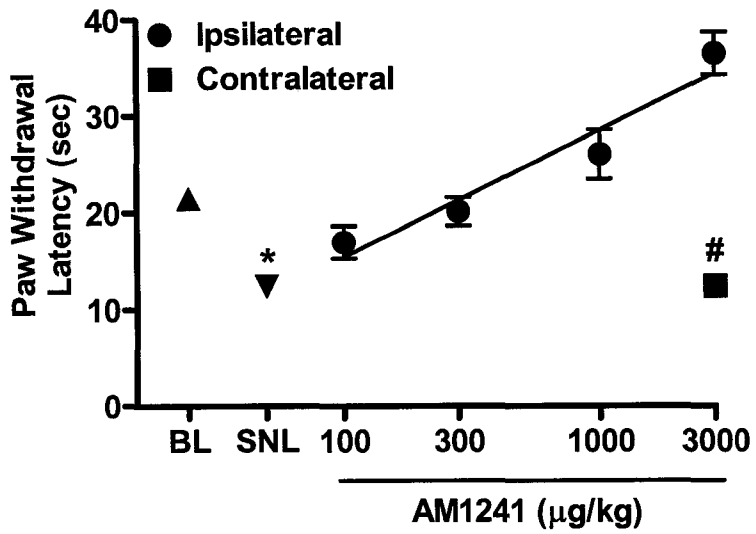

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Figure 3

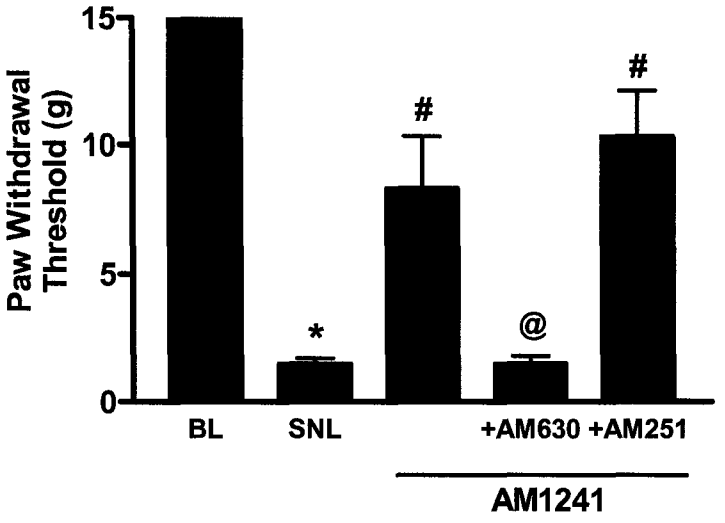

Figure 3. antitactile hypersensitivity effect of AM1241 is blocked by AM630, not AM251. *, P < 0.05 compared to baseline (BL). #, P < 0.05 compared to spinal nerve ligation (SNL). @, P < 0.05 compared to AM1241 alone.

Figure 4

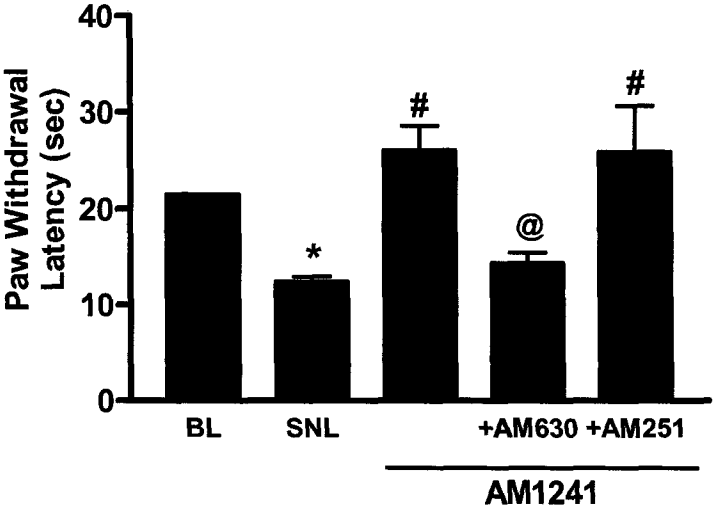

Figure 4. antithermal hypersensitivity effect of AM1241 is blocked by AM630, not AM251. *, $P < 0.05$ compared to baseline (BL). #, $P < 0.05$ compared to spinal nerve ligation (SNL). @, $P < 0.05$ compared to AM1241 alone.

Figure 5

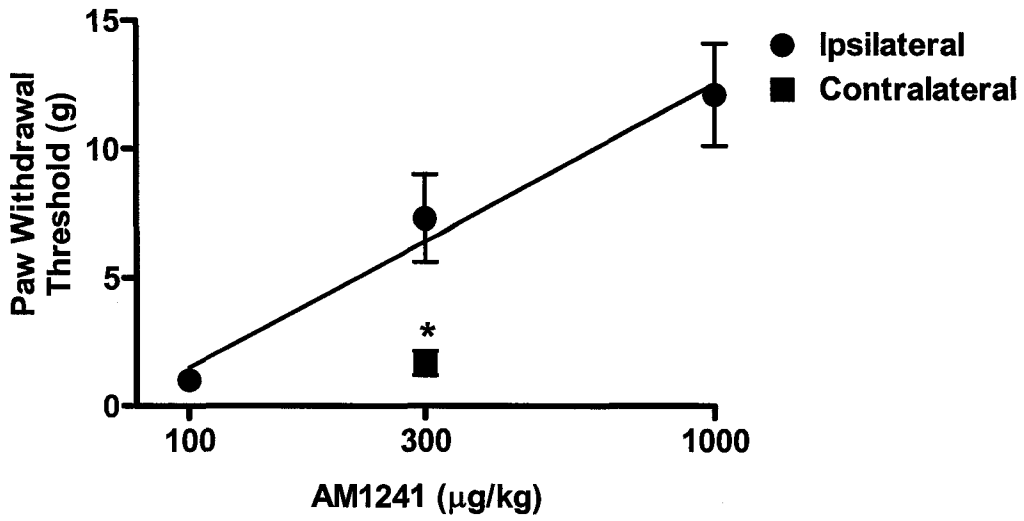

Figure 5. Ipsilateral AM1241 at the site of nerve injury dose-dependently reversed tactile hypersensitivity in spinal nerve ligation rats. *, $P < 0.05$ compared to ipsilateral values.

Figure 6

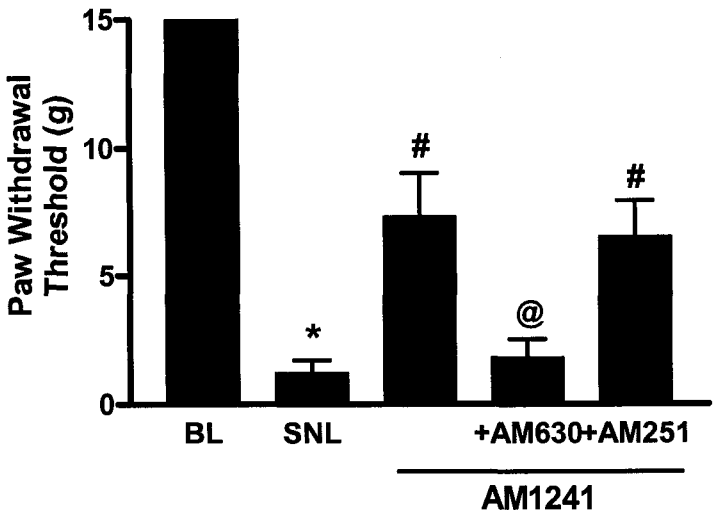

Figure 6. Reversal of tactile hypersensitivity by nerve injury-site injection of AM1241 in spinal nerve ligation rats is blocked by AM630, not AM251 at site of nerve injury. *, $P < 0.05$ compared to baseline (BL). #, $P < 0.05$ compared to spinal nerve ligation (SNL). @, $P < 0.05$ compared to AM1241 alone.

Figure 7

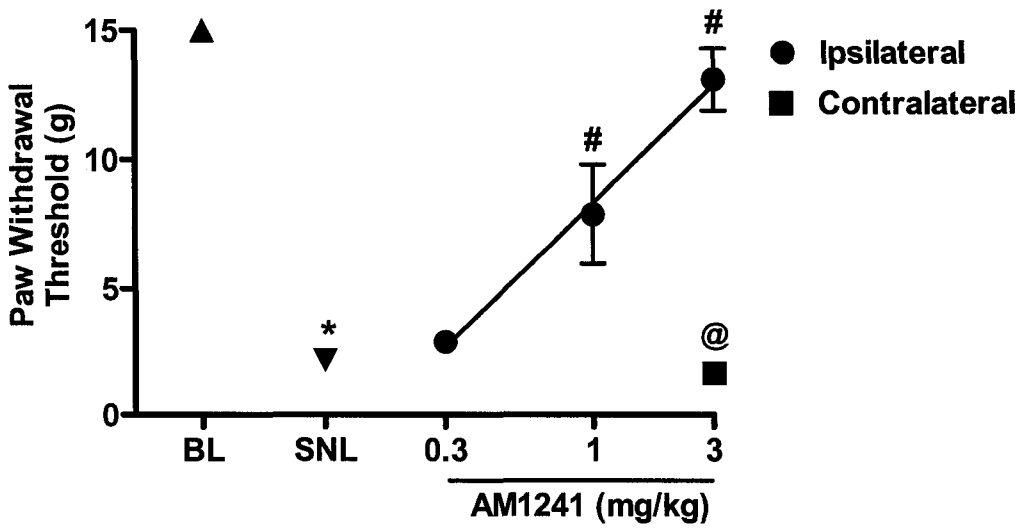

Figure 7. Ipsilateral topical application of AM1241 dose-dependently blocked tactile hypersensitivity produced by spinal nerve ligation (SNL). *, $P < 0.05$ compared to Baseline (BL). #, $P < 0.05$ compared to SNL. @, $P < 0.05$ compared to ipsilateral values.

Figure 8

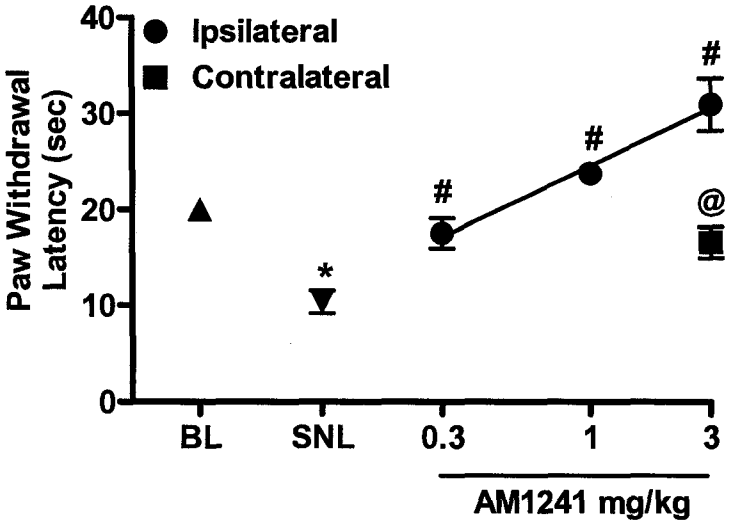

Figure 8. Ipsilateral topical application of AM1241 dose-dependently blocked thermal hypersensitivity produced by spinal nerve ligation (SNL). *, $P < 0.05$ compared to Baseline (BL). #, $P < 0.05$ compared to SNL. @, $P < 0.05$ compared to ipsilateral values.

Figure 9

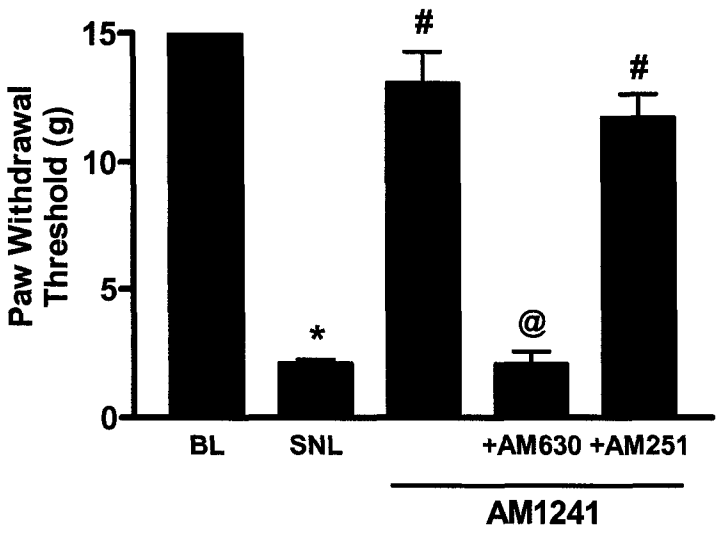

Figure 9. Topically applied AM1241 reversal of tactile hypersensitivity in spinal nerve ligation rats is blocked by topically applied AM630, not AM251. *, $P < 0.05$ compared to baseline (BL). #, $P < 0.05$ compared to spinal nerve ligation (SNL). @, $P < 0.05$ compared to AM1241 alone.

Figure 10

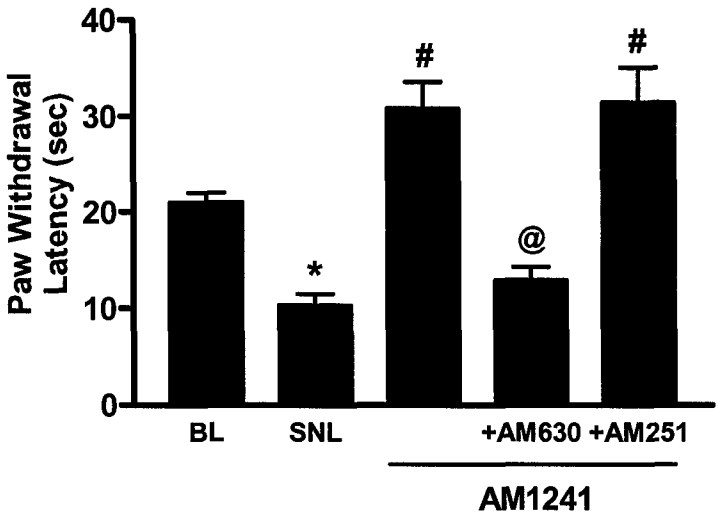

Figure 10. Topically applied AM1241 reversal of thermal hypersensitivity in spinal nerve ligation rats is blocked by topically applied AM630, not AM251. *, $P < 0.05$ compared to baseline (BL). #, $P < 0.05$ compared to spinal nerve ligation (SNL). @, $P < 0.05$ compared to AM1241 alone.

Figure 11

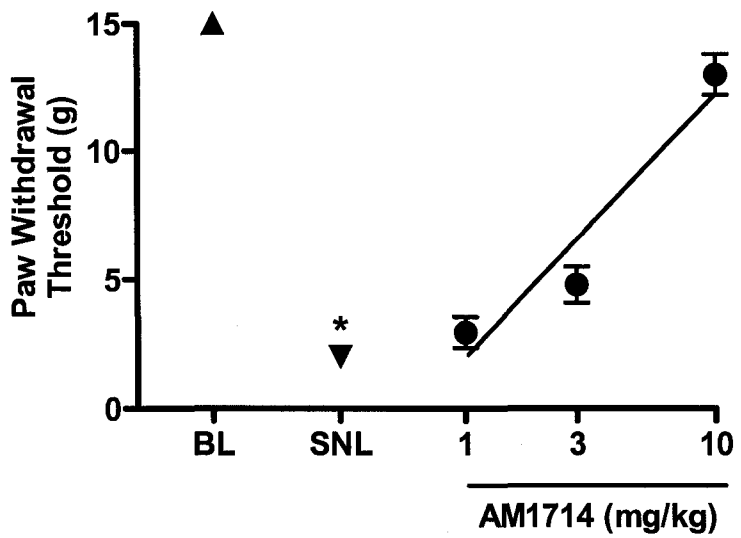

Figure 11. Ipsilateral topical application of AM1714 dose-dependently blocked tactile hypersensitivity produced by spinal nerve ligation (SNL). *, $P < 0.05$ compared to Baseline (BL).

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Chapter 6

POSSIBOLE MECHANISMS FOR PAIN-MODULATING EFFECTS OF THE CB_2 RECEPTOR

Introduction

We have previously shown that the activation of CB_2 receptors in peripheral tissue inhibits nociception to thermal stimuli (Malan et al., 2001), thermal hypersensitivity produced by peripheral inflammation (Quartilho et al., 2003) and tactile and thermal hypersensitivity produced in a neuropathic pain model (Ibrahim et al., 2003). However, the mechanism by which CB_2 receptor activation inhibits pain responses is not known. CB_2 receptor have not been found in the central nerves system (CNS) or on primary afferent neurons, suggesting that CB_2 receptor activation produces antinociception indirectly by mediating the release from neighboring cells of mediators that alter the responsiveness of primary afferent neurons to noxious stimuli. Experimental support for the peripheral nature of antinociception mediated by CB_2 receptor-selective agonist comes from studies using site-specific injections of CB_2 receptor-selective agonist and antagonists (Malan et al., 2001, and Quartilho et al., 2003). One class of cells that might mediate the actions

of CB_2 receptor agonists is immune cells. Immune cells are found in close proximity to the peripheral nerves, a variety of which contain antinociception mediators that might alter the responsiveness of primary efferent neurons and contain CB_2 receptors. Another local cell types are Keratinocytes, which have also been reported to express CB_2 receptors (Casanova et al., 2003). Since immune cells and Keratinocytes contain endogenous opioid peptides (Cabot et al., 1997, Rittner et al., 2001, and Kauser et al., 2003), we tested the hypothesis that activation of CB_2 receptors results in the release of the endogenous opioid peptide β-endorphin in skin tissue, which act on primary afferent neurons to inhibit nociception.

Methods:

Animals: All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the guidelines of the International Association for the Study of Pain and the National Institutes of Health. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) were 250-350 g at the time of testing. Mice were maintained in a climate-controlled room on a 12-h light/dark cycle and allowed food and water *ad libitum*.

Drugs and Chemicals:

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO.). Beta-endorphin, β-endorphin antiserum, and rabbit serum were purchased from Peninsula Laboratories (San Carlos, CA). AM1241 and AM630 were the gift of Alexandros Makriyannis of the University of Connecticut. AM1241 is a CB_2 agonist with 70-fold selectivity for rodent CB_2 receptors (Ibrahim et al., 2003). AM630 is a CB_2 receptor-selective antagonist with 70- to 165-fold selectivity for binding to the CB_2 receptor *in vitro* (Ross et al., 1999 and Hosohata et al., 1997).

Drug Administration: AM1241 was dissolved in dimethyl sulfoxide and administered intraperitoneally (i.P.) 20 minutes before nociceptive testing. All other drugs were

dissolved in normal saline and administered sub cutaneously in the dorsal surface of the hind paw, (i.paw), in a volume of 50 µl. Testing took place 20 minutes after administration. Beta-endorphin antiserum was dissolved in normal saline at a final concentration of 40ug/ml. Non-immune control serum was dissolved in normal saline at a final concentration of 200ug/ml. Naloxone was dissolved at a final concentration of 6mg/ml. in normal saline.

Measurement of thermal withdrawal latency: The method of Hargreaves *et al.* 1988 was used. Animals were acclimated within plexiglass enclosures on a clear glass plate maintained at 30°C. A radiant heat source (high-intensity projector lamp) was focused onto the plantar surface of the paw. When the paw was withdrawn, a motion detector halted the stimulus and a timer. A maximal cut-off of 40 sec was used to prevent tissue damage.

Beta-Endorphin Release From Skin Tissue:

Drug Preparation:

Hank's balanced salt solution, HBSS, <$CaCl_2$ 1.26mM, KCl 5.33mM, KH_2PO_4 0.44mM, $MgCl_2$-$6H_2O$ 0.5mM, $MgSO_4$ 0.41mM, NaCl 138mM, $NaHCO_3$ 4mM, Na_2HPO_4 0.3mM, Glucose 5.6mM; PH 7.4> was used for tissue release assays. AM1241 was dissolved dimethyl sulfoxide at a concentration of 2.5µg/ul. One

hundred µl of AM1241 solution was then dissolved into 1ml of HBSS containing 1%BSA. Subsequent dilutions were made in HBSS to achieve the desired final concentrations of AM1241. DMSO was added so that each diluted sample contained an equivalent concentration of DMSO. The same method was employed to prepare AM630.

Tissue Preparation:

Animals were euthanized using 4% halothane. Skin from the dorsal surface of the hind paw was quickly collected and placed in HBSS at 37°C. A punch of 8 mm in diameter was used to prepare skin samples of equivalent surface area. Each skin sample was cut in half and equilibrated in HBSS 30 minutes at 37°C.

Release Preparation:

Each skin sample was placed in a 1.5 ml plastic tube containing 120µl HBSS + 30µl of HBSS containing DMSO or AM1241. AM1241 was present at a final concentration of 10, 3.3, or 1 µM. DMSO was present at a final concentration of 0.2%. Tubes containing both 10µM AM1241 + 10µM AM630 were prepared in an analogous manner. Tissue was placed in 90 µl of HBSS + 30µl 50µM AM630. Five min later, 30µl of 50 µM AM1241 was added to the tubes, to reach final concentration of 10 µM AM630 and 10 µM AM1241. The tubes

containing the tissue were incubated at 37°C for 30 minutes with periodic gentle agitation to improve oxygenation. The supernatant was collected and placed on ice. Beta-endorphin content was measured immediately by enzyme immunoassay (Peninsula Laboratories, San Carlos, CA.)

Beat-Endorphin Release from Cultured Keratinocytes:

HaCat cells were grown in 12-well plates in a Iscove's Modified Dulbecco's Medium supplemented with 10% Fetal Bovine Serum and Penicillin-Streptomycin (Invitrogen, Carlsbad, CA.) at 37°C. Each well contained a final volume of 350 µl for release assay. AM1241 and AM630 were dissolved in DMSO, and subsequently diluted in culture medium. Following addition of AM1241, AM630, or vehicle, plates were incubated for 30 min. The media was then collected. Beta-endorphin was measured by enzyme immunoassay.

Agonist-Induced CB_2 Receptor Desensitization:

HaCat cells were grown in 12-well plate in medium at 37°C. For testing of agonist induced desensitization they were treated in final volume of 350 µl at 37°C with vehicle or 1µM AM1241. In the vehicle group, cells were incubated with vehicle for 30 min. In the control group, HaCat cells were incubated for 30 minutes in 1 µM AM1241 before the

media was collected and β-endorphin was measured. In the desensitized group, cells were incubated with 1 μM AM1241 for 2 hours. Cells were then washed and the wells were replaced with fresh media containing 1 μM AM1241. After 30 minutes, the samples of medium were collected and β-endorphin was measured by enzyme immunoassay.

RT-PCR Detection of CB_2 Receptor mRNA:

Total RNA was isolated from homogenized tissue and from cells using RNAqueuos ™ (Ambion, Austin, TX.) following the manufacturer's instructions.

Total RNA was reverse-transcribed using oligo dT and random decamers methods of priming. These reactions were carried out using the following conditions: initial incubation of the reaction cocktail at 44°C for 1 hour followed by an incubation at 92°C for 10 min.

DNA amplification was carried using commercially available kit (RETROscript ™, Ambion, Austin, TX.). The conditions for DNA amplification were as follows: Initial incubation at 92°C for 2 minutes followed by 40 cycles of 94°C incubation for 30 sec, 60°C incubation for 30 sec, and 72°C incubation for 1 min. After the completion of the 40 cycles, the reaction cocktail was incubated at 72°C for 7

min and held at a 4°C until samples are frozen in the -20°C.

For detection of CB_2 receptor mRNA in rats tissue, we used the primers designed by Porcella et al., 1998. The sense primer was 5'-TTTCCCACTGATCCCTAACG-3'. The antisense primer was 5'-AGTTAACAAGGCACAGCATG-3'. The expected amplicon size is 328 bp. For detection of CB_2 receptor mRNA from human cells, we used those primers designed for human tissue designed by Emmanuel et al., 1999. The sense primer was 5'-GCATCATGTGGGTCCTCTC-3'. The antisense primer was 5'-TCTCCACTCCGTAGAGCATAG-3'. The expected amplicon size is 451 bp. A negative control consisted of the RT-PCR reaction in the absence of homogenized tissue

Data analysis: Differences between groups was tested using ANOVA followed by post-hoc testing with Students't-test with Bonferroni's correction. Significance was defined as $P < 0.05$.

Results:

AM1241 (100µg/kg i.p.) increased paw withdrawal latency (PWL) from 20 ± 1 sec to 31 ± 3 sec (Figure 1)($P < 0.05$ compared to baseline values), demonstrating the production of antinociception to thermal stimuli. Naloxone (1mg/kg i. paw) reversed the antinociceptive effects of AM1241 and returned the withdrawal latency to 22 ± 2 sec ($P < 0.05$ compared to AM1241). Antiserum to β-endorphin (2µg i.paw) prevented AM1241-induced antinociception, and the paw withdrawal latency remained at 17 ± 2 sec. Non-immune serum had no effect.

Intrapaw injection of β-endorphin peptide also produced antinociception. Twenty and 40 µg increased paw withdrawal latencies to 33.7 ± 0.5 and 39.1 ± 0.7 sec, respectively ($P < 0.05$ compared to baseline) (Figure 2). The effects of β-endorphin were prevented by naloxone (1mg/kg i. paw) and by antiserum to β-endorphin (2 µg i.paw) (Figure 3). Withdrawal latencies were 20 ± 3 and 19 ± 2 sec, respectively, after these treatments. Non-immune control serum had no effect. Control serum, β-endorphin antiserum, and naloxone produced paw withdrawal latencies in the absence of AM1241 that were not different from baseline; 20 ± 1, 20. ± 1, 19 ± 1 sec, respectively.

AM1241 produced antinociception in wild-type (μ-opioid receptor $^{+/+}$) mice. Three, 6, and 10 mg/kg of AM1241 produced paw withdrawal latencies of 15 ± 1, 18 ± 1, and 24 ± 2 seconds ($P < 0.05$ compared to baseline of 11 ± 1 sec) (Figure 8). In μ-opioid receptor -/- mice AM1241 produced no effect (Figure 8).

In the absence of AM1241, 15 ± 2 pg of β-endorphin was released from paw skin into the buffer. AM1241 dose dependently stimulated release of β-endorphin. 1, 3.3, and 10uM AM1241 increased β-endorphin release by 19 ± 2, 24 ± 3, and 29 ± 3 pg, respectively (Figure 4). 10μM AM630 completely prevented the increase in release of β-endorphin seen produced by 10 μM AM1241 ($P < 0.05$ compared to AM1241 alone). 10 μM of AM630 alone had no effect on β-endorphin release.

Beta-endorphin release stimulated by 10 μM AM1241 was time dependent. Ten, 20, 40, and 60 minutes incubation with AM1241 resulted in the release of 9 ± 1, 11 ± 3, 16 ± 4, and 20 ± 4 pg β-endorphin, respectively. Incubation with HBSS resulted in the release of 6 ± 1, 7 ± 1, 7 ± 1, and 13 ± 2 pg β-Endorphin, respectively.

AM1241 stimulated β-endorphin release from paw skin obtained from wild-type ($CB_2^{+/+}$) mice (Figure 7). The release increased from 4.5 ± 0.1 to 7 ± 1 pg. In contrast, skin from CB_2 receptor-knockout mice ($CB_2^{-/-}$) did not display AM1241 stimulated β-endorphin release.

Incubation of HaCat cells with AM1241 also resulted in a dose dependent stimulation of β-endorphin release. AM1241 (0.3, 0.65, and 1 μM) stimulated β-endorphin release by 175 ± 24, 221 ± 17, and 246 ± 19 % release when compared to vehicle alone ($P < 0.05$). One μM AM630 did not effect the release of β-endorphin, but inhibited AM1241-stimulated β-endorphin release (Figure 5).

G-protein coupled-receptors desensitize typically with prolonged agonist exposure. When HaCat cells were incubated with 1μM AM1241 for 2 hours 5 ± 2 pg β-endorphin was released during a subsequent 30 min exposure to AM1241 (Figure 6). In contrast, after 30 min agonist pretreatment 10 ± 1 pg was released. Total β-endorphin content in HaCat cells did not differ between groups after agonist pretreatment (Figure 6).

Reverse transcriptase-polymerase chain reaction analysis was performed to verify that HaCat cells and skin

tissue contain CB_2 receptor mRNA. For the rat, the expected size of the amplicon was calculated to be 328 bp. This analysis yielded strong bands of the correct size for samples from skin and spleen tissue (positive control) (Figure 10). The expected amplicon size was 451 bp for the human transcript. The human leukemia cell line, HL-60, was used as a positive control. This analysis yielded bands of the expected size for samples from both HaCat and HL-60 cell lines (Figure 11). In neither analysis was a band detected in reactions to which cells or homogenized tissue were not added.

Discussion:

The antinociception produced by the activation of CB_2 receptors was not easily explained since CB_2 receptors are not normally present in the central or peripheral nervous system (Munro et al., 1993, Schatz et al., 1997, Facci et al., 1995, and Galiegue et al., 1995). Therefore, we hypothesized that CB_2 receptor-selective agonist produced antinociception indirectly, by modulating the release from local cells of substances that affect the responsiveness of primary afferent neurons to noxious stimuli. Two types of cells located near the peripheral nerve endings have been reported to express CB_2 receptors, Keratinocytes and immune/inflammatory cells (e.g. mast cells). Using western blots and immunohistochemistry, Casanova et al., 2003 showed that skin cells expressed both type of cannabinoids receptors, CB_1 and CB_2. Additionally, we have shown using RT-PCR that rat skin express CB_2 receptor mRNA. CB_2 receptors are expressed on immune and inflammatory cells (Munro et al., 1993 and Galiegue et al., 1995). Therefore, we hypothesized that CB_2 receptor activation produces antinociception by stimulating the release of β-endorphin from inflammatory cells and/or keratinocytes. Recent evidence suggests the existence of an interaction between

the skin and the nervous system through neuropeptides (Ansel et al., 1996). Skin cells were shown to constitutively express pro-opiomelanocortin (POMC) mRNA and product protein, which is the precursor for a number of peptides including β-endorphin (Wintzen et al., 1996). Recent studies have shown that immune cells contain β-endorphin and Met-enkephalin. Both of these endogenous opioid peptides are capable of being released from immune cells and interacting with the peripheral terminals of primary afferent neurons leading to antinociception (Rittner et al., 2001 and Stein et al., 1993).

The antinociceptive actions of the CB_2 receptor-selective agonist AM1241 were reversed by the opioid receptor antagonist naloxone. AM1241 dose not bind to opioid receptors (Alexanderos Makriyannis, unpublished data). Therefore, the actions of AM1241 are unlikely to be mediated by direct interaction with opioid receptors. Blockade of the effects of AM1241 by naloxone would, however, be explained if AM1241 stimulated the release of endorphin opioids and their effects were inhibited by naloxone. To test this possibility we examined the ability of antiserum to β-endorphin to inhibit the antinociception produced by AM1241. Beta-endorphin sequestering antiserum

blocked antinociception, suggesting that β-endorphin plays an essential role in CB_2 receptor-mediated antinociception.

Non-immune control serum had no effect, suggesting that the effects of β-endorphin antiserum are produced by antibodies directly against β-endorphin. To further examine the importance of β-endorphin in mediating the antinociception produced by AM1241, we administered AM1241 to mice lacking the gene for the μ-opioid receptor. Beta-endorphin is a high-affinity agonist of the μ-opioid receptor. AM1241 did not produce antinociception in $\mu^{-/-}$ mice, but produced typical antinociception in $\mu^{+/+}$ mice, suggesting that endogenous opioid activity at the μ-opioid receptor are necessary for CB_2 receptor agonist-induced antinociception.

If endogenous β-endorphin mediates the antinociceptive effects of AM1241, then the administration of β-endorphin should have similar effects to those produced by AM1241. Indeed, administration of exogenous β-endorphin resulted in antinociception that was reversed by β-endorphin antiserum and by naloxone.

Finally, AM1241 stimulated β-endorphin release in vitro from skin tissue and from cultured keratonicytes. The CB_2

receptor antagonist, AM630, blocked AM1241 induced β-endorphin release. In addition, β-endorphin release was not observed in skin tissue from $CB_2^{-/-}$ mice but was observed in skin tissue from $CB_2^{+/+}$ mice, demonstrating that these effects are mediated by CB_2 receptors. Taken together, these data strongly suggest that CB_2 receptor activation produces antinociception by stimulating release of the endogenous μ-opioid receptor agonist, β-endorphin.

The intracellular-signaling mechanism that couples CB_2 receptor activation to endogenous opioid release in not known. One of the most important characteristics of G-protein coupled receptors is desensitization resulting from prolonged exposure to agonist. This phenomenon leads to a decreased response to a previously effective dose of an agonist. When cultured keratinocytes were exposed to AM1241 for 2 hours the release of β-endorphin in response to subsequent exposure to AM1241 was reduced compared to cells not exposed to AM1241. Agonist pretreatment did not change the total β-endorphin content of the cells, suggesting that the decrease in β-endorphin release was not due to depletion of intracellular β-endorphin stores.

Agonist-induced receptor desensitization suggests that β-endorphin release is mediated by G-protein.

Activation of CB_2 cannabinoid receptors results in inhibition of adenyl cyclase activity by a Gi/o protein. CB_2 receptors have also been reported to stimulate mitogen-activated protein kinase coupled to G-protein-coupled inwardly rectifying potassium channels (Ho et al., 1999).

The activation of a Gi protein is typically predicted to inhibit exocytosis. However, there are reports of Gi linked receptors inhibitory release processes. Activation of a variety receptors results in release processes that are pertussis toxin-sensitive, suggesting that they are mediated by Gi or Gi/o proteins (Shefler et al., 1999, Conant et al., 2202, Ito et al., 2002, and Ferry et al., 2001).

Another possible explanation for release of mediators following activation of receptors thought to be coupled to Gi protein is the fact some G-protein-coupled receptors are coupled to both Gi and Gs proteins, simultaneously. For example, Felder et al. (1998) have shown that CB_1 cannabinoid receptors are coupled to both Gi and Gs protein subunits. Gi subunits are activated under normal conditions. However, when the Gi subunits are activated by

other Gi-linked receptors, CB_1 receptor activation results in increased activity of adenylyl cyclase activity and subsequent cAMP accumulation. The finding that some receptors are coupled to both Gi and Gs protein is not just limited to CB_1 receptors (Hampson et al., 2000, and Vasquez and Lewis, 2003).

Whether release of β-endorphin upon activation of CB_2 receptors is mediated through Gi or the Gs subunits remains to be seen. In preliminary studies AM1241-stimulated β-endorphin release was not sensitive to pertussis toxin, suggesting β-endorphin release was not mediated by Gi/o. Other intracellular mechanisms are also possible. Further studies will be required to elucidate the intracellular mechanisms responsible for the release of β-endorphin upon the activation of CB_2 receptors.

It is possible that other mediators, including other endogenous opioid could also be released from local cells following activation of CB_2 receptors. This possibility is currently being investigated.

In this chapter we have demonstrated that skin tissue and cultured Keratinocytes release β-endorphin. However, we do not mean to imply that Keratinocytes are the only components of skin that might release β-endorphin in

response to CB_2 receptor activation. Immune and inflammatory cells manufacture and release endorphins and they express CB_2 receptors. It is possible that resident immune and inflammatory cells (mast cells) in skin and subcutaneous tissue may augment the β-endorphin released by Keratinocytes. In this regard, we have shown that AM1241 also stimulates β-endorphin release from lymph tissue, presumably from lymphocytes. This fact merits further investigations.

CB_2 receptor-selective agonists may provide a novel way to relieve pain. They appear to act in the periphery to inhibit pain, but not to have NCS effects, consistent with their peripheral distribution.

In summary, we have demonstrated that the antinociception may be mediated, at least in part, by stimulation of β-endorphin release from local cells.

List of Illustrations

Figure 1

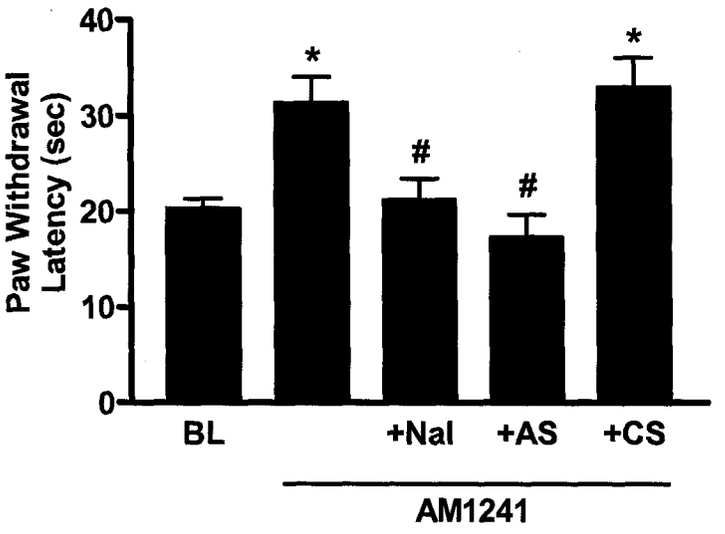

Figure 1. The CB_2 receptor-selective agonist, AM1241 (100 µg/kg i.p.), results in antinociception to thermal stimuli. Naloxone (Nal)(1mg/kg, i.paw) reversed the antinociceptive effects of AM1241. Beta-endorphin antiserum (AS)(2 µg, i.paw). Non-immune control serum (CS) had no effect. Data expressed as mean ± S.E.M. n = 6 per group. *, $P < 0.05$ compared to baseline (BL). #, $P < 0.05$ compared to AM1241 alone.

Figure 2

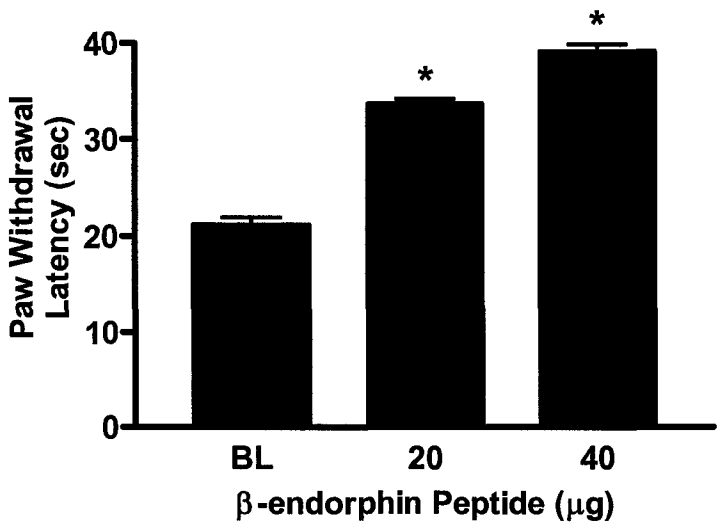

Figure 2. Beta-endorphin peptide (i.paw) produced dose dependent antinociception. Data expressed as mean ± S.E.M. n = 6 per group. *, $P < 0.05$ compared to baseline (BL).

Figure 3

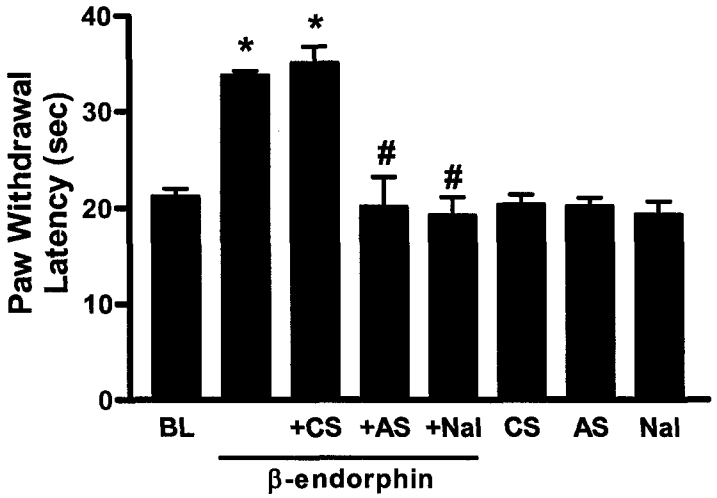

Figure 3. Naloxone (Nal)(1mg/kg, i.paw) and β-endorphin antiserum (AS)(2μg i.paw) reversed the antinociceptive actions of β-endorphin peptide (20 μg i.paw). Control serum (CS) had no effect. Beta-endorphin antiserum, and control serum had no effect in the absence of AM1241.

Data expressed as mean ± S.E.M. n = 6 per group. *, $P < 0.05$ compared to baseline (BL). #, $P < 0.05$ compared to β-endorphin alone.

Figure 4

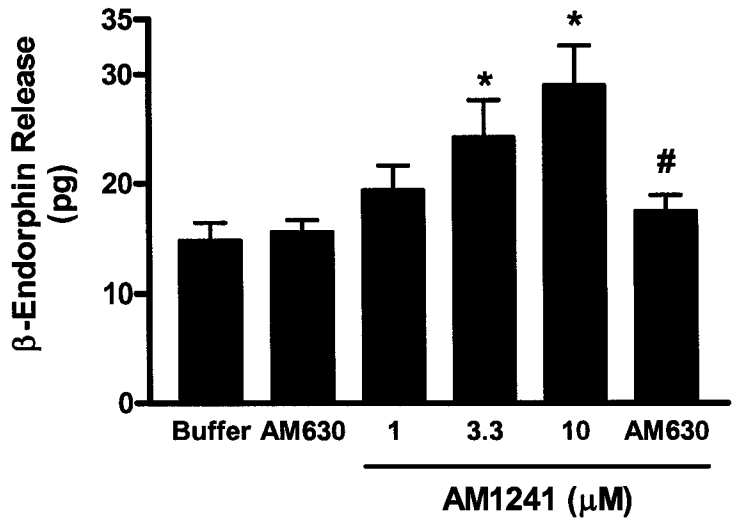

Figure 4. The CB_2 receptor-selective agonist, AM1241, increased β-endorphin release from paw skin. The CB_2 receptor antagonist, AM630 (10 μM), reversed the effects of AM1241. AM630 had no effect on β-endorphin release in the absence of AM1241. Data expressed as mean ± S.E.M. n = 12 per group. *, $P < 0.05$ compared to buffer (HBSS). #, $P < 0.05$ compared to 10 μM AM1241.

Figure 5

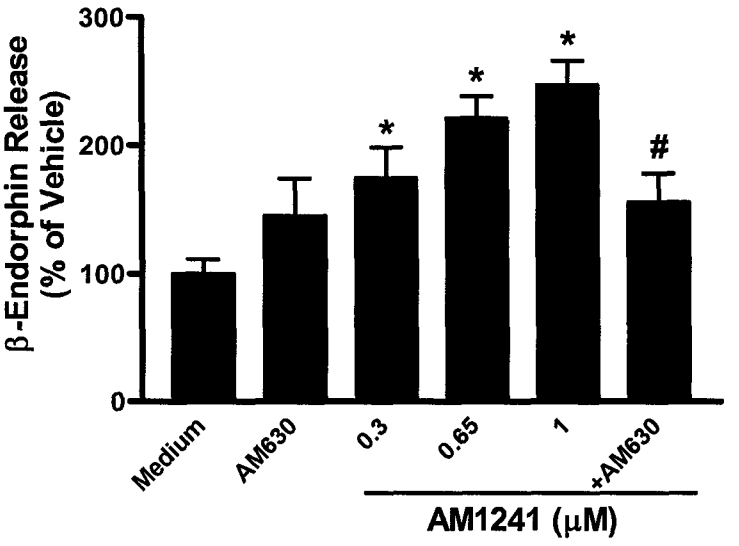

Figure 5. AM1241 increased β-endorphin release from cultured human keratinocytes (HACat cells). AM630 (1 μM) inhibited the effects of AM1241. AM630 had no in the absence of AM1241. Data are expressed as percent of release in medium alone and presented as mean ± S.E.M. n = 12 per group. *, $P < 0.05$ compared to medium alone. #, $P < 0.05$ compared to 1 μM AM1241.

Figure 6

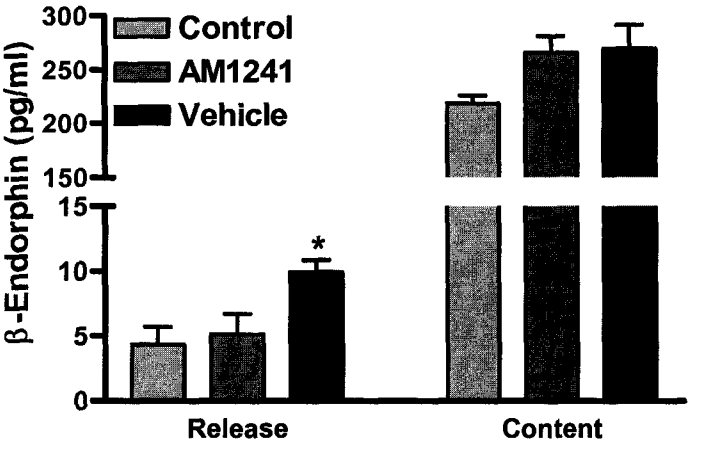

Figure 6. Cultured Keratinocytes (HaCat cells) pretreated with AM1241 released less β-endorphin in response to subsequent agonist exposure when compared to cells pretreated with vehicle and subsequently exposed to AM1241. The control group was not pretreated with AM1241 and AM1241 was not added during the release assay. Total β-endorphin content of the cells was not affected by pretreatment. Data expressed as mean ± S.E.M. n = 5 per group. *, $P < 0.05$ compared to the control group.

Figure 7

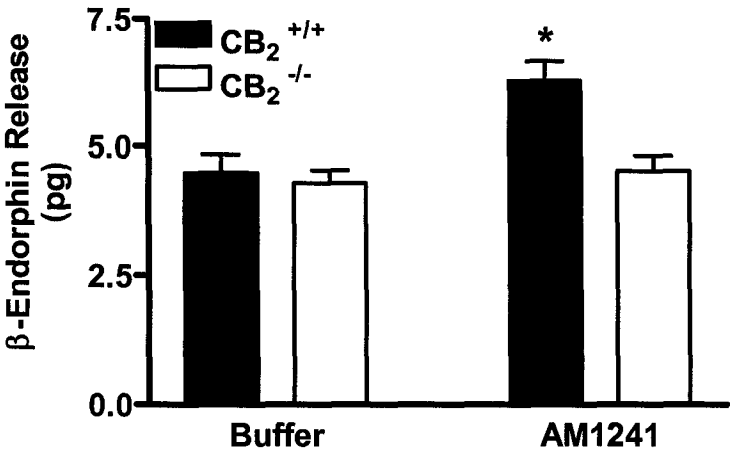

Figure 7. AM1241 (10 μM) increased β-endorphin peptide release from skin of wild-type, but not from CB_2 receptor-knockout mice. Data expressed as mean ± S.E.M. n = 8 per group. *, $P < 0.05$ compared to buffer (HBSS).

Figure 8

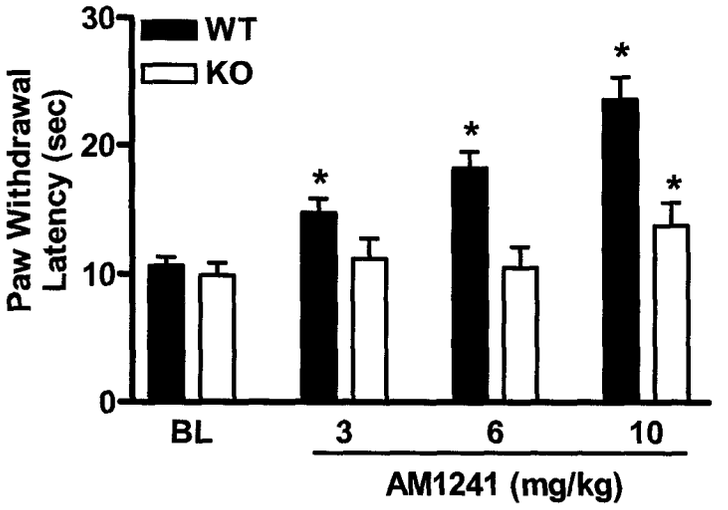

Figure 8. AM1241 (i.p.) produced a dose-dependant antinociception response in wild type mice, but not in μ-receptor knockout mice. Data expressed as mean ± S.E.M. n = 6 per group. *, $P < 0.05$.

Figure 9

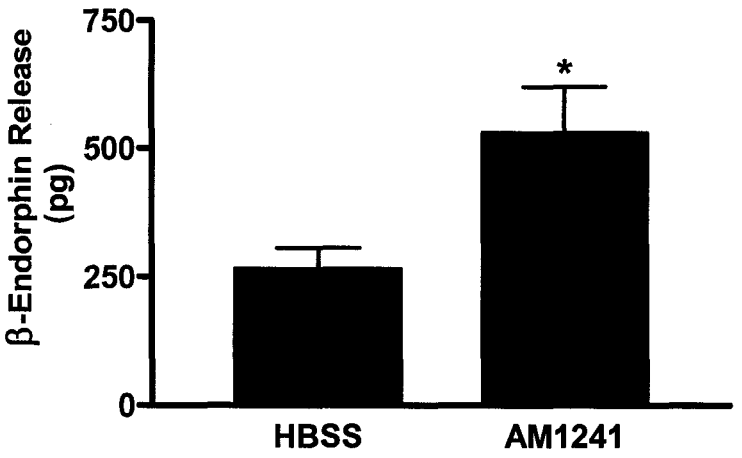

Figure 9. AM1241 (1 μM) stimulated release of β-endorphin from disrupted lymph node tissue. Data expressed as mean ± S.E.M. n = 6 per group. *, $P < 0.05$.

Figure 10

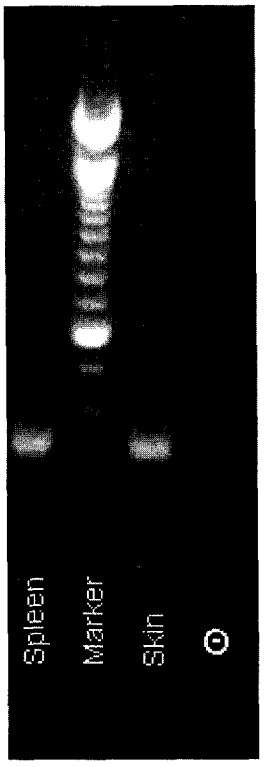

Figure 10. Skin and express CB_2 mRNA was detected using RT-PCR. The negative control (□) consisted of the RT-PCR reaction in the absence of homogenized tissue. The size expected is 328 bp. The size observed is 328 bp.

Figure 11

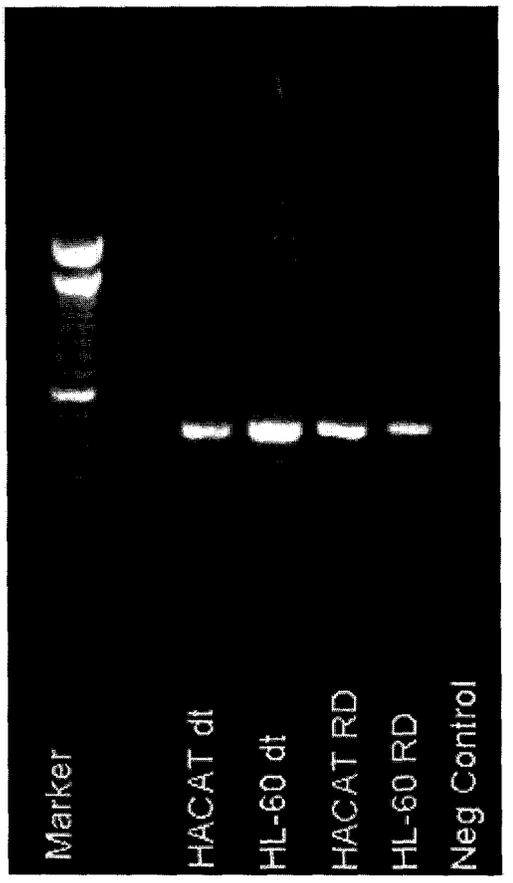

Figure 11. HaCat cultured human keratinocytes and HL-60 cultured human leukemia cells express CB_2 receptor mRNA. The negative control consisted of the RT-PCR reaction in the absence of homogenized tissue. The size expected is 451 bp. The size observed is 451 bp.

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Chapter 7

Conclusion

Pain is a normal physiological response to a tissue-damage, or a potential tissue-damaging stimulus. It serves a protective role to prevent further injury and speed the recovery process. However, when pain is present in the absence of a tissue-damaging stimulus, it fails to serve any protective function. In fact, such pain would be detrimental to the life of the individual, the family, and the society (Vileikyte 1999). One such type of pain is neuropathic pain. Neuropathic pain is chronic in nature and results not from ongoing tissue injury, but rather from diseases of or injury to the nervous system. It is manifested in the absence of external nociceptive stimuli. Neuropathic pain may arise from conditions such as Varicella-zoster infection, diabetes, multiple sclerosis, cancer, and nerve injury. It is estimated that about 1-2% of the population may be suffering from neuropathic pain at any given time (Merskey & Bogduk, 1994 and Bowsher, 1991). Unfortunately, neuropathic pain is difficult to manage. Non-steroidal anti-inflammatory drugs are not effective in neuropathic pain. Most current treatments utilize

tricyclic antidepressants, anticonvulsants, α2-adreno receptor agonists, local anesthetics, or opioids (MacFarlane et al, 1997, Sindrup and Jensen, 1999). The clinical effectiveness of these drug classes is poor (Sindrup and Jensen, 1999. Lynch et al, 2003). This may be because the central nervous system (CNS) side effects of these medications may limit clinical effectiveness by limiting the doses that can be used. Such side effects may include sedation, lethargy, mental clouding, and respiratory depression (Henry, et al, 1995). Therefore, it is important to find a new approach for the treatment of neuropathic pain, particularly one without CNS side effects. In case reports, cannabis has been reported to relieve neuropathic pain. In the 1960's Δ^9-THC was isolated and identified as the active ingredient in cannabis (Gaoni Y, and Mechoulam, 1964 and Hively et al, 1966). In the early 1990's, the CB_1 cannabinoid receptor was first cloned (Matsuda et al, 1990). The CB_2 cannabinoid receptor was cloned in the mid 1990's (Munro et al, 1993). The identification of cannabinoid receptors renewed interest in cannabinoids research and for using cannabinoid to treat pain. Initially, most research focused on CB_1 receptors as a target for pain control because CB_1 receptors are present

in the central nervous system (CNS) (Matsuda et al, 1990, Romero et al, 1997). Cannabinoid receptor agonists were shown to be effective at controlling pain in a variety of animal models (Monhemius et al, 2001, Fox et al, 2001, Drew et al, 2000, Walker et al, 1999, Martin et al, 1999, Ko and Woods, 1999, Hohmann et al, 1999, Lichtman and Martin, 1997). However, the CNS side effects of cannabinoid medications may limit clinical effectiveness in humans. Sedation, anxiety, and increased appetite are examples of the side effects produced in humans by cannabinoids (Balint, 2001). Additionally, the addictive potential of cannabinoids overshadowed the potential use of treating pain. CB_2 cannabinoid receptors are not present in the normal CNS (Munro et al, 1993, Griffin et al, 1999, Buckley et al, 2000). We investigated whether they could be targeted to produce pain relief without CNS side effects. The CB_2 cannabinoid receptor-selective agonist, AM1241, inhibited acute and thermal nociception (Malan et al, 2001), the thermal hypersensitivity produced by carrageenan (Quartilho et al, 2003, Nackley et al, 2003) capsaicin (Quartilho et al, 2003), or complete Freund's adjuvant (unpublished observations). The expression of C-fos, usually upregulated in the spinal cord after hind paw

inflammation secondary to carrageenan, was suppressed by AM1241 administration (Nackley et al, 2003). The actions of AM1241 appeared to be mediated by CB_2, but not the CB_1 receptors as suggested by their inhibition by CB_2, but not CB_1 receptor antagonists. Equally important, when rats were given a fully analgesic dose of AM1241, they did not exhibit the CNS-mediated effects of hypothermia, catalepsy, motor coordination, or hypoactivity (Malan et al, 2001).

Therefore, in this dissertation I examined the effects of AM1241 in a model of neuropathic pain produced by ligation of the L5/L6 spinal nerves. AM1241 was equally effective in reversing nerve injury-induced thermal and mechanical hypersensitivity, both of which are also characteristic of human neuropathic pain (Rowbotham, 1995). When CB_1 receptor deficient mice were used, AM1241 was still active (Ibrahim et al, 2003), clearly demonstrating that CB_1 receptors do not mediate the effects seen with AM1241. However, when CB_2 receptors deficient mice were used, no change in pain response was observed, suggesting that AM1241 mediates its pain-modulating effects by activating the CB_2 receptors. Thus, the use of CB_2 receptor-selective agonists may be a promising strategy to treat neuropathic

pain without the CNS side effects of drugs currently used to treat neuropathic pain.

Using site-specific injection of AM1241, we investigated the drug's site of action. AM1241 was effective when injected locally in the paw on the side of nerve injury and sensory testing. Additionally, the lipophilic nature of AM1241 made it a candidate for external topical administration. AM1241 produced antinociception when applied topically to the ipsilateral paw. When AM1241 was injected in or applied topically to the paw contralateral to nerve injury and sensory side of testing, it produced no effect. Therefore, it can be argued that the effect of AM1241 is local, rather than systemic, since absorption should be equal from both paws. These findings are important because topical application is a convenient way to administer drugs to peripheral sites, such as the peripheral site of action of AM1241.

To further elucidate different sites of actions of the CB_2 receptor-selective agonist AM1241, we administered AM1241 at the site of nerve injury. Ipsilateral, but not contralateral injections of AM1241 modulated the pain response.

The mechanisms underlying pain relief produced by CB_2 receptor activation remains unclear. The mechanism by which CB_1 receptors modulate pain may be explained by the fact that they are present on neurons in the CNS and in the periphery. Therefore, activation of CB_1 receptors may diminish or prevent the transmission of the pain signal to the brain. Additionally, CB_1 receptor activation may interfere with processing of the pain signal within the brain. CB_2 receptors, in contrast, are not present in the CNS or on peripheral neurons. Instead, they are present on peripheral cells including immune cells and keratinocytes. Therefore, we investigated whether CB_2 receptor activation stimulates the release of mediators from these cells that modulate pain by inhibiting the responsiveness of primary afferent neurons. Since both keratinocytes and immune cells produce and store endorphins, we hypothesized that the activation of CB_2 receptors stimulates the release of endogenous opioids. This hypothesis was examined by testing the ability of the opioid antagonist, naloxone, to inhibit the effects of AM1241. Naloxone inhibited the antinociceptive effects of AM1241. Since AM1241 does not bind to the opioid receptors, these experiments suggest that the opioid systems play a significant role in CB_2

receptor-mediated antinociception. Further studies suggested that β-endorphin plays a central role in this process, when the effects of β-endorphin were blocked by a sequestering antiserum to β-endorphin and when AM1241 failed to produce antinociception in μ-opioid receptor deficient mice. Figure 1 illustrates a possible mechanism for CB_2 receptor-mediated antinociception.

Neuropathic pain has proven to be resistant to peripherally administered opioids. Additionally, the side effects of opioids limit the dose tolerated clinically. AM1241, in contrast, does not produces centrally mediated side effects in animals and there were no sign of drug resistance in animals model of neuropathic pain.

The effects of β-endorphin did not produce maximal possible effects in models of neuropathic pain, suggesting that other mediators may participate in the activation of CB_2 receptor by using AM1241. Further studies will be required to elucidate the roles played by other mediators.

An interesting issue that remains to be fully elucidated is the observation that G_i protein-coupled receptor activation stimulates release of β-endorphin. It is typically thought that G_i protein-coupled receptor activation results in inhibition of mediator release.

However, it has been reported that stimulated release can be stimulated by activation of G_i protein-coupled receptors. Such phenomena may be due to the presence of G_i/G_s dual-coupled receptors or other mechanisms that require further investigation.

CB_2 receptor agonists such as AM1241 offer a choice for treatment of acute, inflammatory, and neuropathic pain. AM1241 produces pain relief without CNS side effects, consistent with the fact that the effects of the CB_2 receptor agonists are local rather than systemic.

List of Illustrations

Figure 1

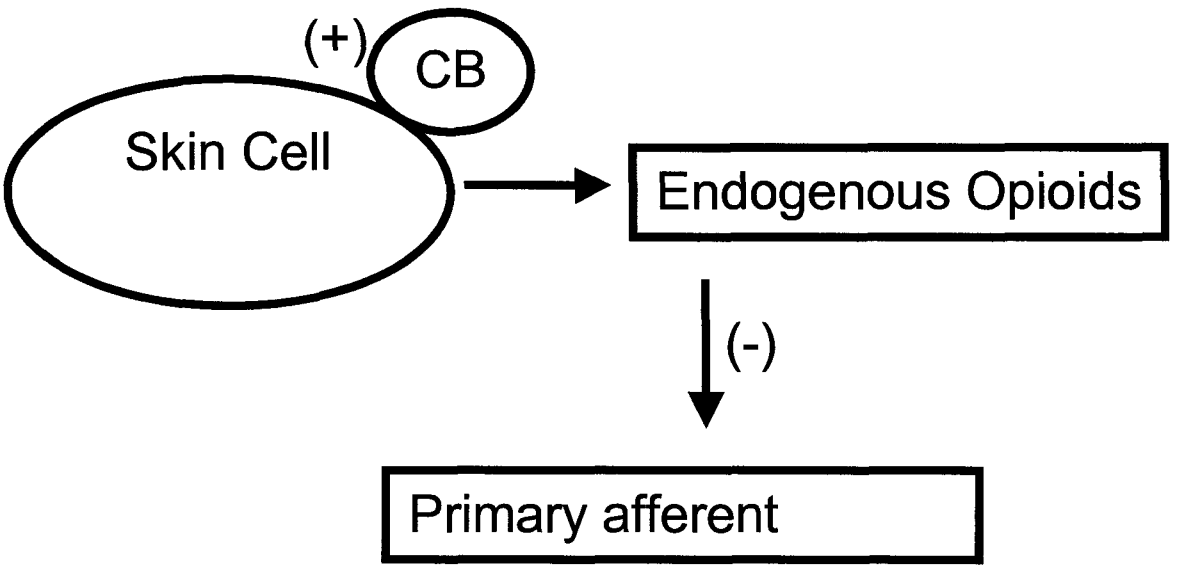

Figure 1. A cartoon representing a possible mechanism in which endogenous opioids may play a significant role in CB_2 receptor-mediated antinociception.

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Institutional Animal Care
and Use Committee

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Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 – USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#03-021 - "Cannabinoid Agonists for the Treatment of Pain"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Frank Porreca, PhD - Pharmacology

GRANTING AGENCY:

NIH

SUBMISSION DATE: **January 22, 2003**

APPROVAL DATE: **May 1, 2003** APPROVAL VALID THROUGH*: **April 30, 2006**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **May 2, 2003**

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell

Richard C. Powell, PhD, MS
Vice President for Research

DATE: May 2, 2003

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.