Inhibition of Improgan Antinociception by the Cannabinoid (CB)₁ Antagonist N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A): Lack of Obligatory Role for Endocannabinoids Acting at CB₁ Receptors


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Received March 22, 2002; accepted June 11, 2002

ABSTRACT

Improgan, a nonopioid antinociceptive agent, acts desceding, pain-relieving mechanisms in the brain stem, but the receptor for this compound has not been identified. Because cannabinoids also activate nonopioid analgesia by a brain stem action, experiments were performed to assess the significance of cannabinoid mechanisms in improgan antinociception. The cannabinoid CB₁ antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) induced dose-dependent inhibition of improgan antinociception on the tail-flick test after i.c.v. administration in rats. The same treatments yielded comparable inhibition of cannabinoid R(+)-(2,3-dihydro-5-methyl-3-[4-(4-morpholinyl)methyl]pyrrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl)methanone monomethanesulfonate, WIN 55,212-2) analgesia. Inhibition of improgan and WIN 55,212-2 antinociception by SR141716A was also observed in Swiss-Webster mice. Radioligand binding studies showed no appreciable affinity of improgan on rat brain, mouse brain, and human recombinant CB₁ receptors, ruling out a direct action at these sites. To test the hypothesis that CB₁ receptors indirectly participate in improgan signaling, the effects of improgan were assessed in mice with a null mutation of the CB₁ gene with and without SR141716A pretreatment. Surprisingly, improgan induced complete antinociception in both CB₁(+/−) and wild-type control (CB₁ (+/+)) mice. Furthermore, SR141716A inhibited improgan antinociception in CB₁ (+/+), mice, but not in CB₁(−/−) mice. Taken together, the results show that SR141716A reduces improgan antinociception, but neither cannabinoids nor CB₁ receptors seem to play an obligatory role in improgan signaling. Present and previous studies suggest that Δ⁸-tetrahydrocannabinol may act at both CB₁ and other receptors to relieve pain, but no evidence was found indicating that improgan uses either of these mechanisms. SR141716A will facilitate the study of improgan-like analgesics.

Improgan [N-cyano-N’-[3-(imidazole-4-yl) propyl]-N’-methylguanidine] is a chemical congener of the histamine H₂ receptor antagonist cimetidine, which has pain-relieving properties when administered directly into the brain (Li et al., 1996; Hough et al., 2000a). Inactive on H₂ receptors (Li et al., 1996), improgan (then called SKF92374) was originally characterized as a chemical “control” for cimetidine action (Ganellin, 1982). A family of analgesics that is chemically related to improgan and burimamide has been described (Hough et al., 1997, 2000a). Improgan inhibits thermal and mechanical nociception in rodents after i.c.v. administration. At maximal antinociceptive doses, improgan does not alter locomotor or rotordor performance, suggesting that this drug has selective analgesic activity (Li et al., 1997a). In contrast to the effects of morphine, repeated daily injections of improgan showed no tolerance to the analgesic activity, suggesting a favorable clinical profile (Bannoura et al., 1998). Known receptors for histamine (Li et al., 1997b; Zhu et al., 2001) and opioids (Li et al., 1997b; Hough et al., 2000b) have been excluded as mediators of improgan.

Abbreviations.

CB, cannabinoid; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; WIN55,212-2, R(+)-(2,3-dihydro-5-methyl-3-[4-(4-morpholinyl)methyl] pyrrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl)methanone monomethanesulfonate; THC, Δ⁸-tetrahydrocannabinol; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.
antinociception. In vitro studies have excluded 50 other potential central nervous system sites (Hough et al., 2000a), but the mechanism of action of this drug remains unknown.

Although the improgan target remains unknown, progress has been made in mapping the neuronal pathways used by this compound. Several findings support the hypothesis that improgan activates descending analgesic mechanisms originating in the brain stem. Intracerebral mapping studies showed that improgan is active after microinjections into the periaqueductal gray (Li et al., 1996) and rostral ventral medulla (J. Nalwalk, K. Svokos, and L. B. Hough, unpublished data). Also, improgan antinociception is reversed by the GABA_A agonist muscimol (Hough et al., 2001). Because improgan lacks activity toward GABA_A receptors, this finding suggests that, like morphine, improgan may activate descending mechanisms by inhibiting GABAergic transmission (Hough et al., 2001). Also similar to morphine action, improgan antinociception was recently shown to be inhibited by intrathecal administration of yohimbine, the α₂-adrenergic antagonist, implying the involvement of descending noradrenergic mechanisms (Svokos et al., 2001). However, unlike morphine, improgan does not use known opioid receptors (either directly or indirectly), because its activity is not affected by opioid antagonists (Li et al., 1997b) or altered in opioid receptor mutant mice (Hough et al., 2000b).

There has been considerable recent interest in the pharmacology of cannabinoids (Piomelli et al., 2002). Biological roles for the CB₁ receptor (the principal brain cannabinoid target) and for the endogenous ligands for this receptor (the endocannabinoids; Walker et al., 1996; DiMarzo et al., 2001) are being elucidated by the use of highly selective antagonists (e.g., SR141716A; Compton et al., 1996; Lichtman and Martin, 1997) and CB₁-deficient knockout mice (Ledent et al., 1999; Zimmer et al., 1999). The analgesic properties of cannabinoids are well known (Ledent et al., 1999; Walker et al., 1999b; Pertwee, 2001), but clinically useful analgesics have not been developed from this class because activation of brain CB₁ receptors produces undesirable side effects (Martin et al., 1991).

Neurophysiological and behavioral studies have shown that the brain stem contains multiple, descending, neuronal systems capable of attenuating nociceptive transmission by both opioid and nonopioid mechanisms (Terman et al., 1984; Beitz, 1992). Several experiments suggest that cannabinoids can activate and/or participate in the latter. Intracerebroventricular, intrathecal, or systemic administration of CB₁ agonists (including WIN 55,212-2) reduce nociceptive responses, including the tail-flick response (Martin et al., 1993; Welch et al., 1995; Walker et al., 1999b; Pertwee, 2001). The supraspinal component is mediated by actions in the periaqueductal gray and rostral ventral medulla (Lichtman et al., 1996; Martin et al., 1998; Meng et al., 1998; Vaughan et al., 1999), regions that contain CB₁ receptors and that are known to function in descending analgesic pathways. The cannabinoid antagonist SR141716A inhibits CB₁ agonist actions both in vitro (e.g., in biochemical and isolated tissue assays; Rinaldi-Carmona et al., 1994; Croci et al., 1998) and in vivo (e.g., analgesia, catalepsy, and hypothermia; Compton et al., 1996; Lichtman and Martin, 1997; Martin et al., 1998; Welch et al., 1998). In the rostral ventral medulla, CB₁ agonists activate these pain-relieving circuits by disinhibition of GABAergic transmission (Adams et al., 1998; Meng et al., 1998; Vaughan et al., 1999) and by stimulation of descending noradrenergic activity (Lichtman and Martin, 1991). SR141716A reduces some forms of stress-induced analgesia, supporting the suggested mediator role for endocannabinoids (Valverde et al., 2000).

There are strong similarities between the pain-relieving properties of improgan and those of cannabinoids. As discussed above, these include sites of action in the periaqueductal gray and rostral ventral medulla, antagonism by the GABA_A agonist muscimol, and inhibition by intrathecal administration of the adrenergic α₂-antagonist yohimbine. Most significantly, both improgan and cannabinoids induce nonopioid antinociception in a variety of tests (Li et al., 1997b; Meng et al., 1998; Hough et al., 2000b; Pertwee, 2001). Because cannabinoid receptors had not been previously studied as potential improgan targets, and because endocannabinoids may function in endogenous pain-relieving mechanisms, the present studies assessed the role of cannabinoid mechanisms in the antinociceptive actions of improgan.

Materials and Methods

Animals. Male Sprague-Dawley rats (175–350 g) and male Swiss-Webster mice (25–40 g; both from Taconic Farms, Germantown, NY) were maintained on a 12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM) and provided with food and water ad libitum. Rats were housed in groups of three or four until the time of surgery and individually thereafter. Mice were housed in groups of four to six. Male, homozygous mutant [CB₁(+/−), n = 22] and wild-type [CB₁(+/+), n = 20] mice (25–40 g; C57BL/6J background; Zimmer et al., 1999) were bred at the Kansas University Medical Center (Kansas City, KS) from heterozygotic parents, genotyped, and shipped to Albany Medical College (Albany, NY) for testing. Four additional C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used to supplement the CB₁(+/+) group (total n = 24). All animal experiments were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

Drugs and Solutions. SR141716A and THC (both bases) were kindly provided by the National Institute on Drug Abuse. WIN-55,212-2 (dosed as mesylate salt; RBI/Sigma, Natick, MA) and SR141716A were dissolved in 60 and 100% DMSO, respectively. Morphine sulfate (dosed as salt, dissolved in saline; RBI/Sigma) and improgan base (synthesized as described previously; Hough et al., 2000b), dissolved in dilute acid and neutralized) were diluted with 60% DMSO to serve as controls for WIN-55,212-2. THC was dissolved in 100% ethanol and diluted with ethanol/alkamuls EL-620 (Rhodia, Cranbury, NJ)/saline (1:1:18).

Surgery. For i.c.v. injections in rats, animals were anesthetized with Brevital (50 mg/kg i.p.) and supplemented with isofluorane. Chronic cannulas were stereotaxically implanted into the left lateral ventricle and anchored to the skull with three stainless steel screws and dental cement (Crane and Glick, 1979). Coordinates (in mm from bregma; Paxinos and Watson, 1986) were anterior-posterior, −0.8; medial-lateral, +1.5; and dorsoventral, −3.3. After surgery, the animals were individually housed with food and water available and were allowed to recover for at least 5 to 7 days before testing. Each animal was used for a single experiment.

Rat i.c.v. Injections and Nociceptive Testing. Rats were tested with the tail-flick test (D’Amour and Smith, 1941). The ventral surface of the tail (a randomly selected location 2–5 cm from the tip) was exposed to radiant heat, and the latency for tail movement was recorded. The heat source was set so that baseline latencies were generally between 3 and 4 s with a 1.5-s cutoff. The heat source was not adjusted for individual animals. Subjects were tested with three tail-flick tests performed at 1-min intervals, and the third test used
as the baseline score. Animals were then gently secured by wrapping with a laboratory pad, the stylet was removed, and the i.c.v. injection cannula was inserted. This cannula extended 1 mm beyond the guide to penetrate the lateral ventricle. Intracerebroventricular injections were performed manually over a 1-min period with the volumes specified in each experiment. One minute after the end of the infusion, wire cutters were used to cut off and seal the injection cannula approximately 2 mm above the juncture with the guide cannula. After the interval specified, a single tail-flick test was performed, followed by a second i.c.v. injection. The cannula was resealed and single tail-flick latencies were recorded at the specified time intervals (Fig. 1). Successful i.c.v. injections were assured by following the movement of an air bubble in the tubing between the syringe and the cannula and by the absence of leakage. Subsequently animals received pentobarbital sodium (100 mg/kg i.p.) and India ink (5 μl i.c.v.). Proper distribution of the ink in the cerebroventricular system verified successful i.c.v. injections. Data from animals with poor placements or unsuccessful injections were excluded.

Mouse i.c.v. Injections and Nociceptive Testing. Mouse nociceptive testing was performed with the hot water tail immersion test (Li et al., 1997a) and, in some cases, the hot-plate test. For the former, animals were restrained in a conical polypropylene tube. The tail (2–3 cm) was immersed into a 55°C water bath and the latency to sudden movement (flick) or removal of the tail was recorded. Cutoff latencies were 10 s when mice were tested in a single experiment (Fig. 2), but 8 s when multiple experiments were performed (Figs. 3-4). For the mouse hot-plate test (Eddy and Leimbach, 1953), animals were placed on a 52°C surface and the latency to a hind paw lift, lick, or jump was recorded with a maximal exposure of 60 s. After baseline testing, animals were lightly anesthetized with ether. A microliter syringe was connected to a 26-gauge needle with polyethylene 20 tubing. The needle was inserted into the lateral ventricle through a stereotaxically drilled Plexiglas plate as described previously in detail (Glick et al., 1975). Drug solutions (see figure legends for volumes) were manually injected over a 1-min period, and the needle removed after an additional minute. Animals regained consciousness within 5 min after the injection. After the intervals specified, animals were retested, briefly anesthetized a second time, received a second i.c.v. injection, and were retested as described.

Fig. 1. Effects of the CB_1 antagonist SR141716A on the antinociception induced by improgan and WIN 55,212-2 in rats. Each subject was tested (ordinate, mean ± S.E.M. tail-flick latencies for the number of subjects in parentheses) for baseline nociceptive responses (PRE), received a single i.c.v. injection (2 μl) of either SR141716A (SR, 20 or 50 μg) or vehicle (100% DMSO), and was retested 10 min after this i.c.v. infusion (POST). This was immediately followed by a second i.c.v. injection (10 μl) of improgan (A, IMP, 40 or 80 μg), WIN (B, 10 or 20 μg), or vehicle (A and B, 60% DMSO). Retesting followed at the times shown after the end of the second i.c.v. infusion (abscissa). Latencies from SR20/DMSO and SR50/ DMSO groups were not different from each other and were pooled. The DMSO/DMSO and SR20 and 50/DMSO groups are the same in A and B. *P < 0.05; **P < 0.01 (least significant difference test) of SR141716A versus no SR141716A in the same analgesic group at the same time.

Fig. 2. Effects of SR141716A on the antinociception induced by improgan, WIN 55,212-2, and morphine in Swiss-Webster mice. Each subject was tested on the tail immersion test, and the data are shown as in Fig. 1. After baseline testing (PRE), each mouse received a single i.c.v. injection (2 μl) of either SR141716A (SR, 30 μg) or vehicle (VEH, 100% DMSO) and was retested 26 min after the end of this i.c.v. infusion (POST). This was followed by a second i.c.v. injection (5 μl) of improgan (A, 30 μg), WIN (B, 20 μg), morphine sulfate (C, 0.3 μg), or vehicle (A, B and C, 60% DMSO). Retesting followed at the times shown after the end of the second i.c.v. infusion (abscissa). The same VEH/VEH and SR30/VEH groups are given in A, B, and C. *P < 0.05 (least significant difference test) of SR141716A versus no SR141716A in the same analgesic group at the same time.
Improgan Antinociception and CB₁

Fig. 3. Effect of SR141716A on improgan antinociception in CB₁ (+/+ ) control (A) and CB₁ (−/− ) (B) mice. Subjects were treated and tested exactly as in Fig. 2, except that an 8-s cutoff latency was used because of repeated testing in these mice. After baseline testing (PRE), each mouse received a single i.c.v. injection (2 μL) of either SR141716A (SR, 30 μg) or vehicle (VEH, 100% DMSO). They were retested 26 min after the end of this infusion (POST), followed by a second i.c.v. injection (5 μL) of improgan (30 μg) or vehicle (60% DMSO). Retesting followed at the times shown after the end of the second i.c.v. infusion (abscissa). One week later, the experiment was repeated on the same subjects with crossover treatments (see Materials and Methods). Data from both weeks were combined and the values in parentheses are total numbers of observations for each group. Forty-two (21 and 21 for weeks 1 and 2, respectively) and 39 (21 and 18) observations are represented for CB₁ (+/+ ) and CB₁ (−/− ) groups, respectively. *, P < 0.05 (two-tailed t test) between SR30/IMP30 versus VEH/IMP30 in the same group.

above. When hot-plate testing was combined with tail immersion, the former was performed only once during baseline testing; in all cases, the hot-plate test was performed just after the tail immersion test.

Experimental Design of Mouse Studies. Swiss-Webster mice were used only once for each experiment. Data from one mouse (of five) who showed no antinociception after WIN-55,212-2 treatment were omitted (Fig. 2). However, due to the extremely short supply of CB₁ (−/− ) mice, these subjects were treated three times over weekly periods with combinations of improgan, SR141716A and THC. In the first week, mice of both genotypes received all four combinations of improgan, SR141716A and vehicles, and were tested for hot-plate and tail immersion as described above (Fig. 3). Seven days later, the experiment was repeated with the same animals, but the treatment groups were crossed such that each mouse only received improgan once and SR141716A once over the 2-week period. Statistical analysis ensured that the repeated testing did not affect conclusions from the experiments (see Results). One week after the second test, mice were treated with THC and SR141716A as described above (Fig. 4). After all testing, mice received pentobarbital, and an i.c.v. injection of dilute India ink (1 μL) to verify the accuracy of injections into the lateral ventricle. Whole brains were removed and processed for radioligand binding. During the 3-week study of 47 mice, a total of eight died (six mutant and two wild type). Four of these deaths were anesthesia-related; the others occurred between experiments for unknown reasons. In addition, data from three wild-type subjects were omitted: one (of eight) vehicle-treated subjects who gave all cutoff scores, and two (of 14) improgan-treated subjects who gave no antinociception. The resulting sample sizes from all 3 weeks are given in the figure legends for both genotypes.
Analysis of Antinociceptive Data. Results are expressed as latencies (s, mean ± S.E.M.). One-, two-, or three-way ANOVAs with one or two levels of repeated measures were used as appropriate. If indicated, specific post hoc tests (given in the figure legends) were performed to determine significant differences between groups (Statistica; CSS, Inc., Tulsa, OK).

Radioligand Binding. CB1 specific binding was performed with minor modifications of published methods (Compton et al., 1993; DiMarzo et al., 2000). Mice were anesthetized with pentobarbital and decapitated. Whole brains were removed, weighed, and homogenized in 5 ml of ice-cold binding buffer (50 mM Tris-HCl, 3 mM MgCl2, 0.2 mM EDTA, and 100 mM NaCl, pH 7.4) containing phenylmethylsulfonyl fluoride (50 µM), and the homogenates centrifuged (40,000g, 10 min). Supernatant fractions were discarded and the pellets rehomogenized in the same volume of buffer and centrifuged; this procedure was repeated to wash the pellet a second time. The final pellet was resuspended in 2.5 ml of ice-cold homogenizing buffer and either used fresh or stored at −80°C. Under some circumstances, crude homogenates were frozen at −20°C for 2 to 5 days. These homogenates were thawed on ice the day of the binding assay, and the rest of the tissue homogenate preparation was completed. Separate experiments confirmed that storing the brain homogenates in this manner did not affect the specific binding signal. Separate experiments were also performed with Swiss-Webster mice to confirm that the THC and SR141716A treatments of Fig. 4 did not alter control CB1 binding levels 48 h later (data not shown). Protein content was determined using the bicinchoninic acid method (Pierce Chemical, Rockford, IL). Radioligand binding of [3H](11a,29)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenyl (CP-55,940) (120 Ci/mmol, 0.4 nM unless specified otherwise; PerkinElmer Life Sciences, Boston, MA) was performed in silanized borosilicate glass test tubes in a total volume of 1 ml of binding buffer containing 1 mg/ml bovine serum albumin, 30 to 60 µg of protein, and 0.9% ethanol at 30°C for 1 h. Stock solutions of radioligand were pipetted from silanized borosilicate tubes containing 30% ethanol to prevent ligand binding to pipette tips and glass. Samples were placed on ice, received 2 ml of ice-cold 50 mM Tris-HCl, pH 7.4, containing 1 mg/ml bovine serum albumin, and were filtered through GF/F glass fiber filters (Whatman, Maidstone, UK) presoaked with 0.1% polyethyleneimine. Tubes and filters were rinsed twice with 4 ml of the same buffer. Filters were placed in polyethylene scintillation vials, shaken with 5 ml of scintillation fluid for 1 h, and counted in a scintillation counter (Beckman Coulter, Inc, Fullerton, CA). Nonspecific binding was evaluated with [3H](11a,29)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenyl (CP-55,940) (120 Ci/mmol, 0.4 nM unless specified otherwise; PerkinElmer Life Sciences, Boston, MA). Nonspecific binding was evaluated with [3H](11a,29)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenyl (CP-55,940) (120 Ci/mmol, 0.4 nM unless specified otherwise; PerkinElmer Life Sciences, Boston, MA). Nonspecific binding was evaluated with [3H](11a,29)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenyl (CP-55,940) (120 Ci/mmol, 0.4 nM unless specified otherwise; PerkinElmer Life Sciences, Boston, MA).

Intracerebroventricular administration of improgan to rats induced a dose- and time-dependent antinociceptive effect in the tail-flick test (Fig. 1A), as seen in previous studies. Intracerebroventricular pretreatment with the CB1 blocker SR141716A produced a dose-dependent antagonism of improgan antinociception (Fig. 1A). At both 5 and 10 min after i.c.v. improgan, the inhibition was 100% by the 50-µg dose of SR141716A. Interestingly, SR141716A did not reliably inhibit the effects of doses of improgan less than 80 µg (data not shown). Under conditions identical with those used in Fig. 1A, additional experiments verified that SR141716A induced a dose-dependent inhibition of cannabinoid antinociception (Fig. 1B). The cannabinoid agonist WIN55,212-2 caused dose- and time-dependent analgesia that was completely inhibited by SR141716A pretreatment. The potency of this inhibition (Fig. 1B) was similar to that found when the compound was tested against improgan antinociception (Fig. 1A). It is interesting to note that SR141716A did not reliably inhibit the activity against doses of WIN 55,212-2 that were less than 20 µg (data not shown).

Because improgan is effective in the mouse (Li et al., 1997a) and because studies with knockout mice were planned, it was of interest to determine the effects of SR141716A on improgan antinociception in Swiss-Webster mice. Figure 2A confirms the antinociceptive activity of i.c.v. improgan on the tail immersion test. Intracerebroventricular pretreatment with 30 µg of SR141716A inhibited improgan antinociception by about 70% at the 5- and 10-min points (Fig. 2A). Under identical experimental conditions, this pretreatment induced comparable inhibition of cannabinoid (WIN 55,212-2; Fig. 2B) but not opioid (morphine; Fig. 2C) analgesia.

The antagonism of improgan antinociception by appropriate doses of SR141716A in rats and mice suggested that this compound could be acting through a CB1 analgesic mechanism. Because the receptor for improgan is unknown, a detailed examination of improgan’s affinity for CB1 receptors was performed. Table 1 summarizes radioligand experiments showing that improgan (up to 30 µM) lacked measurable affinity for CB1 and CB2 receptors from various biological sources as assessed with several different radioligands.

Because improgan does not act directly at the CB1 receptor, yet a CB2 antagonist effectively inhibits improgan antinociception, it is conceivable that improgan initiates biolog-

### Table 1
Synopsis of in vitro radioligand competition experiments assessing actions of improgan on the CB1 receptor

<table>
<thead>
<tr>
<th>Receptor Source</th>
<th>Receptor</th>
<th>3H-Radioligand</th>
<th>Improgan</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW mouse whole brain</td>
<td>CB1</td>
<td>CP-55,940 [0.4]</td>
<td>0.03–30</td>
</tr>
<tr>
<td>SW mouse whole brain</td>
<td>CB1</td>
<td>SR141716A [1.0]</td>
<td>1–30</td>
</tr>
<tr>
<td>Rat whole brain</td>
<td>CB1</td>
<td>CP-55,940 [0.4]</td>
<td>0.03–30</td>
</tr>
<tr>
<td>CB1 (+/+ ) mice</td>
<td>CB1</td>
<td>SR141716A [1.0]</td>
<td>1–30</td>
</tr>
<tr>
<td>Human recombinant (HEK-293)</td>
<td>CB2</td>
<td>WIN-55,212-2 [0.8]</td>
<td>10</td>
</tr>
<tr>
<td>Human recombinant (CHO)</td>
<td>CB2</td>
<td>WIN-55,212-2 [2.4]</td>
<td>10</td>
</tr>
</tbody>
</table>

CHO, Chinese hamster ovary; HEK, human embryonic kidney; SW, Swiss-Webster.
rical activity through a different receptor linked to the release of endocannabinoids. Thus, it was hypothesized that the CB₁ receptor is essential for improgan antinociception, even though the drug does not act directly at this site. A test of this hypothesis was performed by assessing improgan antinociception in CB₁ mutant mice. Similar to the results in the Swiss-Webster mice (Fig. 2A), improgan induced antinociception in the CB₁ (+/+) (wild-type control) mice 10 and 15 min after i.v. administration (Fig. 3A). In further agreement, pretreatment with SR141716A inhibited this response by about 40% (Fig. 3A). In contrast to the proposed hypothesis, however, CB₁ (-/-) mice demonstrated improgan antinociception that was identical to that observed in Swiss-Webster and (+/+) mice (Fig. 3B). In the CB₁, -/- mice, however, SR141716A failed to modulate improgan antinociception (Fig. 3B).

Because the experiments of Fig. 3, A and B, were performed by testing each mouse twice over a 1-week period, the appropriate statistical analysis is a three-factor (genotype, improgan, and time) ANOVA of the raw latencies. Results of this analysis showed significant ($P < 0.01$) main effects of week, improgan, and time. Significant ($P < 0.01$) interaction terms were time by week and time by improgan; most importantly, an improgan by SR141716A interaction ($P = 0.017$) was noted, which confirms antagonism of improgan antinociception by SR141716A in this experiment. Although the latencies were slightly increased in the second week of testing (data not shown), there were no significant week-related interaction terms in the ANOVA ($P$ values were 0.30, 0.41, and 0.58 for week by improgan, week by SR141716A, and week by improgan by SR141716A, respectively), showing that the second week of testing did not affect conclusions about improgan or SR141716A effects. No terms related to genotype in the ANOVA reached the 0.05 value (data not shown); the four-way interaction term (genotype-improgan-SR141716A-time) had a $P$ value of 0.12.

Due to the complexity of the experiment and the results, the data were subjected to a second independent analysis, which consisted of summing analgesic difference scores for each subject at the postimprogan times tested (similar to Martin et al., 1998). In this second data analysis of the same experiment, improgan antinociception was significantly reduced by SR141716A in the CB₁ (+/+) but not in the CB₁ (-/-) mice (data not shown; $P < 0.04$ for the genotype-improgan-SR141716A interaction term), consistent with the results of Fig. 3.

CB₁ (+/+), and CB₁ (-/-) mice tested on the tail-flick test (Fig. 3) were also tested on the hot-plate test during the same experiment (data not shown). Improgan was fully effective in both genotypes, and SR141716A did not inhibit the improgan responses in any of the groups.

One week after the end of the improgan-SR141716A studies, the same CB₁ (+/+)) and (-/-) mice were treated with systemic combinations of THC and SR141716A (Fig. 4). As expected, THC induced analgesia on the tail immersion test in wild-type mice, and this effect was completely inhibited by SR141716A pretreatment (Fig. 4A). THC also induced slight, but significant analgesia in CB₁ (-/-) mice, and SR141716A tended to inhibit these responses as well. ANOVA of these results found significant ($P < 0.01$) main effects and interactions for SR141716A treatment and time, but no statistically significant genotype-related differences ($P$ values for genotype-related terms ranged from 0.13 to 0.75). Analysis of these data by difference scores (Fig. 4B) also found no significant genotype differences by ANOVA, but significant inhibition in all SR141716A-treated groups. Hot-plate data from the same experiment (data not shown) found virtually identical results: CB₁ (-/-) mice showed approximately 50% of the THC analgesia seen in CB₁ (+/+)) mice, but this tendency did not reach statistical significance. SR141716A completely inhibited all THC responses, effects that were highly statistically significant.

After these experiments, subjects were sacrificed and radioligand binding of brain membranes with [³H]CP-55,940 confirmed the absence of CB₁ binding in the CB₁ (-/-) brains (Fig. 4C). Specific binding to CB₁ receptors was also evaluated with [³H]SR141716A (1 nM) and 10 µM SR141716A to evaluate nonspecific binding, and found to be absent in CB₁ (-/-) brains (data not shown). Incubations with larger concentrations of [³H]SR141716A (up to 50 nM) for up to 3 h also failed to detect any specific binding in CB₁ (-/-) membranes (data not shown).

**Discussion**

Improgan antinociception has been the subject of intense scrutiny by our laboratory for several years because the compound exhibits morphine-like pain-relieving properties in a variety of tests, yet opioid receptors do not seem to participate in these responses (see Introduction). For example, both improgan and morphine attenuate high-temperature, spinal mediates thermal responses in the rat (e.g., tail-flick), whereas many analgesic compounds do not. Thus, it is important to discover the improgan receptor and also to characterize the neuronal pathways involved in the actions of this compound. Because of the anatomical and pharmacological overlap between cannabinoid and improgan antinociceptive mechanisms (see Introduction), we investigated the hypothesis that cannabinoid mechanisms might be important in improgan antinociception.

The findings that SR141716A inhibits improgan antinociception in rats and mice suggest that improgan might use a cannabinoid mechanism. Doses of the antagonist that inhibited improgan were also fully effective against WIN 55,212-5 analgesia, consistent with previous findings (Rinaldi-Carmona et al., 1994; Compton et al., 1996; Lichtman and Martin, 1997). The similarity in potencies of SR141716A against both analgesics also supports a cannabinoid mechanism of improgan action. However, careful screening of improgan in several receptor preparations showed no direct interactions between the drug and CB₁ receptors (Table 1). Improgan also does not seem to behave as a CB₁ agonist after administration into the spinal subarachnoid space, because CB₁ agonists (Welch et al., 1998), but not improgan (Svokos et al., 2001), induce antinociception when administered by this route. Taken together, data presented in Figs. 1 and 2 and Table 1 suggest the possibility that endocannabinoids acting at CB₁ receptors could mediate improgan antinociception.

The alternative explanation (less likely) is that although SR141716A may act at a CB₁ site to block cannabinoid analgesia, it may also act at another site (Jarai et al., 1999) to reduce improgan antinociception.

The development of CB₁ mutant mice (Ledent et al., 1999;
Zimmer et al., 1999) provides a powerful tool to test the hypothesis that CB1 receptors mediate improgan antinociception. Although there are pitfalls from this approach, demonstration of the absence of improgan antinociception in CB1 (-/-) mice would have been compelling (if incomplete) evidence for a role for endocannabinoids and CB1 receptors in improgan signaling. It was therefore a surprise that improgan antinociception was not reduced (Fig. 3) in mice lacking CB1 receptors (Fig. 4C).

Critical to understanding improgan’s actions are the effects of SR141716A in the CB1 (-/-) and CB1 (+/+). Because SR141716A blocked improgan antinociception in mice (Fig. 2) and rats (Fig. 3), it was predicted that this drug would reduce improgan antinociception in the CB1 (+/+), a finding verified by both ANOVA and the t test (Fig. 3). Once improgan antinociception was found in the CB1 (-/-) mice then the effects of SR141716A in these mice were expected to clarify the mechanism of antagonism: if SR141716A were blocking improvag by acting at a receptor other than CB1 then the SR141716A inhibition would persist in the CB1 (-/-) animals. However, if SR141716A blockade were occurring via the CB1 receptor (the most parsimonious explanation) then the SR141716A antagonism should have been present in the CB1 (+/+), but not in the CB1 (-/-) animals. The results (Fig. 3) support the latter conclusion.

Since the discovery of endogenous ligands for the CB1 receptor (Devane et al., 1992; Stella et al., 1997), the modulation of biological processes by CB1 antagonists has often been interpreted as support for the relevant biological roles for endocannabinoids (Valverde et al., 2000). Even though endocannabinoids do seem to have important neurobiological roles (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001), the present results show that modulation of a process by SR141716A per se does not necessarily prove such an obligatory role. Taken at face value, the present data suggest that pharmacological modulation of CB1 receptors can alter the antinociceptive actions of improgan. When these receptors are absent, however, the results suggest that improgan can still relieve pain, but the CB1 modulation is absent.

When the actions of a pharmacological antagonist implicate a biological role for an endogenous ligand, the assumption that must be made is that the blocker is behaving as a “neutral” antagonist (i.e., the drug must lack both agonist and inverse agonist actions; Kenakin et al., 1995). However, SR141716A may not be a neutral antagonist, but rather an inverse agonist (Bouaboula et al., 1997; Landsman et al., 1997), in which case this drug could act in vivo in the absence of exogenous or endogenous cannabinoids to reduce constitutive CB1 receptor activity. Conceivably, constitutive CB1 activity could be contributing to specific neural activity that is used for improgan action (accounting for the inhibition by SR141716A); studies on the CB1 mutant mice suggest that this activity is not critical, however. One explanation for the disparity in findings with the CB1 antagonist and those with the CB1 (-/-) mice could be that the improgan-dependent cellular activity in the CB1 (-/-) mice could have somehow been changed in addition to deletion of the CB1 receptor. Strain or species differences may also be relevant to the present data, because SR141716A reduced improgan antinociception completely in rats (Fig. 1), but only by about 70 and 40% in Swiss-Webster and C57BL/6 (+/+), respectively (Figs. 2 and 3).

A completely different idea that might explain the present results is suggested by work showing that SR141716A can inhibit the activation of noncannabinoid processes through a CB1-specific mechanism (Bouaboula et al., 1997). The inverse agonist action of the compound at the CB1 receptor was suggested to reduce available pools of G protein, thus attenuating receptor tyrosine kinase–induced activation of mitogen-activated protein kinase (Bouaboula et al., 1997). Although highly speculative, validation of this model could explain how SR141716A could inhibit improgan antinociception when CB1 receptors are present, but not when they are absent. Because SR141716A does not inhibit morphine analgesia (a process also initiated by Gi proteins), the hypothesis would require that CB1 receptors and opioid receptors be localized on different cells or not share the same intracellular Gi pools.

If one accepts that improgan acts at a single type of receptor to elevate both tail-flick and hot-plate latencies then the finding that SR141716A blocked the former but not the latter effects of improgan in CB1 (+/+), but not in the CB1 (-/-) mice further strengthens the conclusion that the cannabinoid antagonist is not an improgan receptor antagonist as well. Rather, the cannabinoid modulation of the tail-flick analgesia is likely to occur at a site beyond the improgan receptor. Antagonism of tail-flick responses is mostly likely to be the result of modulation of descending analgesic systems projecting to the spinal cord (e.g., in the rostral ventral medulla; Meng et al., 1998), whereas hot-plate analgesia can result from supraspinal (e.g., periaqueductal gray and thalamus) attenuation of ascending nociceptive transmission. Previous pharmacological and lesion studies have dissociated hot-plate and tail-flick analgesic responses (Hough and Nalwalk, 1992).

Many studies have established that THC activates CB1 receptors and that SR141716A is a THC antagonist by virtue of an action at the CB1 receptor (Compton et al., 1996). However, work with CB1 (-/-) mice suggests that THC may also be capable of pain modulation that is independent of CB1 receptors. Although THC produced catalepsy, hypothermia, and hot-plate analgesia in CB1 (+/+) and not in CB1 (-/-) mice, THC action on the tail-flick test was unaffected in latter (Zimmer et al., 1999). In a second report (Ledent et al., 1999), in which tail testing variables were considerably different than in the first, THC action on the tail immersion test was strongly reduced but still measurable in CB1 (-/-) mice. Although both the hot-plate and tail-flick tests both measure responses to thermal nociception, supraspinal and spinal reflexes subserves the respective responses in these tests. Our laboratory was also interested in assessing THC action on the spinal nociceptive reflex in CB1 (-/-) mice. Even more compelling, the effects of SR141716A on THC analgesia in CB1 (-/-) mice have not been reported. If SR141716A were found to antagonize THC analgesia in CB1 (-/-) mice then a case could be made for the existence of a receptor other than CB1 that might be relevant to pain relief. Such a receptor would certainly be of interest in searching for the improgan target. We therefore studied THC and SR141716A actions on hot-plate and tail-flick responses in CB1 (-/-) mice.
tion is based on comparisons of post-THC scores versus baseline scores, because the limited numbers of subjects prevented the use of vehicle groups. However, in other studies from our laboratory, i.e., vehicle-treated mice yielded latency equivalent to baseline scores 60 min later (data not shown). In contrast to one of the earlier reports showing that THC had equivalent tail-flick effects in CB1 (+/-) and CB2 (-/-) mice (Zimmer et al., 1999), the THC responses in the present knockout group showed approximately 50% lower mean responses compared with the former. These findings, which suggest both a CB1 and another component to this THC action, are not robust because of the failure of the ANOVA to detect statistically significant genotype differences. The equivocal nature of this result is likely to be due to the size of the tail-flick latencies and to variations in the response. The finding that is not equivocal is that SR141716A completely inhibited THC actions in all groups. Although further work is needed to confirm this, it seems likely that SR141716A may be capable of blocking THC analgesia by a mechanism distinct from the CB1 receptor. The results would also suggest that improgan is not acting by such a mechanism, because SR141716A did not inhibit improgan responses in the CB1 (-/-) mice. The search for additional SR141716A receptors in the brain should continue. Similar to findings reported by others (DiMarzo et al., 2000), we were unable to detect any specific binding of [3H]SR141716A (up to 50 nM) in whole brain homogenates from CB1 (-/-) mice with incubations up to 3 h (data not shown).

The present findings provide further distinctions between the mechanisms of pain relief produced by morphine and improgan. Like morphine, improgan's antinociceptive actions are inhibited by a supraspinally administered GABA_A agonist and by an intrathecally administered alpha-agonist (see Introduction). However, unlike that produced by morphine, improgan antinociception is resistant to opioid antagonists but reduced by CB1 blockade. The antagonism of improgan antinociception by SR141716A has not led to the discovery of the improgan receptor, but the results provide new insights into the pharmacological nature of nonopioid analgesia, and show that SR141716A is an important new tool for characterization of impogran-like compounds (Hough et al., 1997, 1999, 2000c).

Acknowledgments

We thank Dr. Douglas Cohn (Animal Resource Facility, Albany Medical College) for excellent assistance with animal care issues. We also thank Konstantina Svakos for technical assistance, and Dr. Mark Fleck (Albany Medical College) for comments on the manuscript. The National Institute on Drug Abuse Drug Supply Program provided several key test compounds. We thank MDS Pharma Services (Bothell, WA) for performing the recombinant CB1 and CB2 binding experiments.

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