Activation of Cannabinoid CB₂ Receptors Suppresses C-Fiber Responses and Windup in Spinal Wide Dynamic Range Neurons in the Absence and Presence of Inflammation

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Nackley, A. G., A. M. Zvonok, A. Makriyannis, and A. G. Hohmann. Activation of cannabinoid CB₂ receptors suppresses C-fiber responses and windup in spinal wide dynamic range neurons in the absence and presence of inflammation. J Neurophysiol 92: 3562–3574, 2004. First published August 18, 2004; doi:10.1152/jn.00886.2003. Effects of the CB₂-selective cannabinoid agonist AM1241 on activity evoked in spinal wide dynamic range (WDR) neurons by transcutaneous electrical stimulation were evaluated in urethane-anesthetized rats. Recordings were obtained in both the absence and the presence of carrageenan inflammation. AM1241, administered intravenously or locally in the paw, suppressed activity evoked by transcutaneous electrical stimulation during the development of inflammation. Decreases in WDR responses resulted from a suppression of C-fiber-mediated activity and windup. Aβ- and Aδ-fiber-mediated responses were not reliably altered. The AM1241-induced suppression of electrophysiologically active responses was blocked by the CB₂ antagonist SR144528 but not by the CB₁ antagonist SR141716A. AM1241 (33 μg/kg intraplantar [ipl]), administered to the carrageenan-injected paw, suppressed activity evoked in WDR neurons relative to groups receiving vehicle in the same paw or AM1241 in the opposite (noninflamed) paw. The electrophysiological effects of AM1241 (330 μg/kg intravenous [iv]) were greater in rats receiving ipl carrageenan compared with noninflamed rats receiving an ipl injection of vehicle. AM1241 failed to alter the activity of purely nonnociceptive neurons recorded in the lumbar dorsal horn. Additionally, AM1241 (330 μg/kg iv and ipl; 33 μg/kg ipl) reduced the diameter of the carrageenan-injected paw. The AM1241-induced decrease in peripheral edema was blocked by the CB₂ but not by the CB₁ antagonist. These data demonstrate that activation of cannabinoid CB₂ receptors is sufficient to suppress neuronal activity at central levels of processing in the spinal dorsal horn. Our findings are consistent with the ability of AM1241 to normalize nociceptive thresholds and produce antinociception in inflammatory pain states.

INTRODUCTION

Cannabinoids attenuate nociceptive responses in behavioral (Calignano et al. 1998; Farquhar-Smith and Rice 2001; Hanus et al. 1999; Ko and Woods 1999; Malan et al. 2001; Martin et al. 1998), neurochemical (Hohmann et al. 1999b; Nackley et al. 2003a,b; Tsou et al. 1998), and electrophysiological (Chapman 2001; Drew et al. 2000; Harris et al. 2000; Hohmann et al. 1995, 1998, 1999a; Martin et al. 1996) studies. Both CB₁ and CB₂ receptor subtypes are implicated in cannabinoid antinociception. CB₁ is expressed (Matsuda et al. 1990; Munro et al. 1993; Zimmer et al. 1999) in the CNS, whereas CB₂ is expressed mainly in immune cells (Lynn and Herkenham 1994; Munro et al. 1993) and is absent in CNS neurons (Buckley et al. 2000; Munro et al. 1993; Zimmer et al. 1999). Cannabinoid receptors have been identified on primary afferent fibers (Bridge et al. 2003; Hohmann and Herkenham 1999b). Recent pharmacological evidence also supports the presence of CB₂ in human and guinea-pig vagus nerve (Patel et al. 2003). CB₂ has also been identified in microglia (Walter et al. 2003) that migrate toward dying neurons during neuroinflammation. In immune tissues, levels of CB₂ mRNA are 10–100 times greater than that of CB₁ (Galiegue et al. 1995). Ligand binding to Gi/o-coupled CB₂ receptors modulates intracellular signaling cascades through inhibition of adenyl cyclase (Bayewitch et al. 1995) and activation of mitogen-activated protein kinase (Bouaboula et al. 1996). Unlike CB₁, CB₂ does not couple to calcium channels (Felder et al. 1995). Activation of CB₂ inhibits the proliferation of T cells (Patrini et al. 1997), B cells (Valk et al. 1997), and natural killer cells (Parlarlo et al. 1999), which act synergistically to produce a robust immune response. Furthermore, CB₂ agonists suppress the expression of proteins elicited by macrophage immunomodulators (Cabral et al. 1989). Thus activation of CB₂ on immune cells in inflamed tissue may prevent the release of inflammatory mediators (e.g., nerve growth factor, cytokines, and ATP) that result in nociceptor sensitization (Mazzari et al. 1996).

CB₂ agonists are antinociceptive in models of acute (Malan et al. 2001) and persistent pain (Clayton et al. 2002; Hanus et al. 1999; Ibrhim et al. 2003; Nackley et al. 2003a; for review, see Hohmann 2002; Malan et al. 2002). Activation of CB₂ also reduces substance P–induced plasma extravasation (Mazzari et al. 1996) and arachidonic acid–induced ear edema (Hanus et al. 1999). AM1241 (Goutopoulos et al. 2002) is a CB₂-selective agonist that exhibits 340-fold selectivity for CB₂ over CB₁. AM1241 suppresses thermal nociception in naive rats after systemic and local hind paw injections, while failing to elicit centrally mediated side effects such as hypothermia, catalepsy, and hypoactivity (Malan et al. 2001). AM1241 also suppresses carrageenan and capsaicin-evoked thermal and mechanical hyperalgesia and allodynia (Hohmann et al. 2004; Nackley et al. 2003a; Quartilho et al. 2003) and carrageenan-evoked Fos protein expression (Nackley et al. 2003a) through a CB₂–specific mechanism.
In untreated animals, cannabinoids suppress responses evoked in spinal nonnociceptive neurons (Hohmann et al. 1995, 1998, 1999a; Strangman and Walker 1999) by attenuating activity of Aβ- and C-fiber primary afferents (Chapman 2001; Drew et al. 2000; Kelly and Chapman 2001; Strangman and Walker 1999). Repeated activation of C-fibers by electrical stimulation of the cutaneous receptive field leads to a phenomenon known as windup, in which the number of neuronal responses progressively increases with subsequent stimulation (Mendell 1966). Windup is generally attributed to C-fiber afterdischarge-evoked responses of spinal dorsal horn neurons and is involved in the maintenance of inflammatory and neuropathic pain states (Dubner 1986; Gracely et al. 1992). In the present study, electrophysiological methods were used to directly examine the effects of the CB2-selective agonist AM1241 on nociceptive neurons in the lumbar dorsal horn in the absence and presence of inflammation.

Peripheral sensitization of Aβ- and C-fiber terminals occurs at the site of inflammation (Hedo et al. 1998; Reeh 1994). Central sensitization, observed at the spinal level, is characterized by a decrease in threshold (Neugebauer and Schäible 1990; Simone et al. 1991), increase in firing rate (Dougherty et al. 1999; Guilbaud et al. 1986), enlargement of the receptive field (McMahon and Wall 1984; Ren et al. 1992), and recruitment of low threshold Aβ-fibers (Nakatsuka et al. 1999; Neumann et al. 1996). Opioids and cannabinoids known to suppress inflammatory hyperalgesia also suppress neuronal activity under inflammatory conditions (Drew et al. 2000; Haley et al. 1990; Harris et al. 2000; Hylden et al. 1991; Stanfa et al. 1992). Recently, local administration of anandamide has been shown to produce a CB2-mediated suppression of mechanically evoked responses in spinal dorsal horn neurons in the carrageenan model of inflammation (Sokal et al. 2003).

The present study was conducted to assess the role of CB2 in modulating the activity of spinal dorsal horn neurons evoked by transcutaneous electrical stimulation in the absence and presence of carrageenan inflammation. We hypothesized that AM1241 would suppress responses evoked in WDR neurons by transcutaneous electrical stimulation of the peripheral receptive field through a CB2-specific mechanism. The ability of AM1241 to selectively inhibit the transmission of nociceptive information was evaluated by examining its effects on purely nonnociceptive (low threshold) neurons. Pharmacological specificity was evaluated through the use of competitive antagonists for CB1 and CB2.

METHODS

Subjects

Eighty-three adult male Sprague–Dawley rats (245–345 g; Harlan, Indianapolis, IN and Charles River Laboratories, Wilmington, MA) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann 1983).

Drugs and chemicals

Lambda carrageenan was obtained from Sigma Aldrich (St. Louis, MO). AM1241 was synthesized (by A. M. Zvonok) in the Departments of Pharmaceutical Sciences and Molecular and Cell Biology at The University of Connecticut. SR144528, a CB2-selective antagonist/inverse agonist (Rinaldi-Carmona et al. 1994), and SR144528, a CB2-selective antagonist/inverse agonist (Rinaldi-Carmona et al. 1998), were provided by NIDA. Carrageenan (3%) was dissolved in saline and administered in a volume of 100 µl. Drugs were dissolved in dimethylsulfoxide (DMSO) for intraplantar (ipl) administration (50 µl) and in emulphur, ethanol, and saline (1:1:3) for intravenous (iv) administration (2 ml/kg body weight).

Surgical preparation

Rats were anesthetized with urethane (25%, 1.2 g/kg ip). Core body temperature was maintained at 37°C throughout surgical and experimental procedures using a feedback-controlled heating pad. A laminectomy was performed for electrophysiological recording as described previously (Hohmann et al. 1995, 1999). The tail vein was cannulated for iv drug administration. Animals breathed spontaneously and were not artificially ventilated. Respiration was monitored through close observation and color of ear and extremity was observed to monitor peripheral circulatory (Pertovaara et al. 1998). Absence of corneal reflexes and paw withdrawal responses to pinch indicated adequate anesthesia (Svedsen et al. 1999).

Identification of wdr neurons, nonnociceptive neurons, and their primary afferent inputs

Wide dynamic range (WDR) neurons were qualitatively identified by responses to mechanical stimulation (innocuous brush and noxious pinch) of the receptive field (Coghill et al. 1993; Hohmann et al. 1999). An innocuous brush stimulus was applied using an artist’s paintbrush. Noxious pinch was applied using teethed hemostats at an intensity deemed noxious by the experimenter. After isolating a single WDR neuron and mapping its receptive field on the plantar surface of the hind paw, each mechanical stimulus was presented for 10 s with a 10-s interstimulus interval. The oscilloscope and data-acquisition system were not in view of the experimenter during application of mechanical stimuli (brush, pinch). This procedure served to minimize possible feedback that could otherwise contribute to differences in manual presentation of stimuli and evoked responses. WDR neurons responded with greater frequency to noxious pinch as opposed to nonnoxious brush stimulation, demonstrating that they encode stimulus intensity. Purely nonnociceptive neurons were qualitatively identified based on responses to mechanical stimuli; innocuous brush elicited maximal responding, whereas noxious pinch failed to evoke neuronal activity (Hohmann et al. 1995; Martin et al. 1996).

Aβ-, Aδ-, and C-fiber–mediated responses evoked by transcutaneous electrical stimulation of the peripheral receptive field were characterized using the method of Chapman and Dickenson (1997). Aβ-, Aδ-, and early C-fiber–mediated responses occurred 0–20, 20–90, and 90–300 ms poststimulation, respectively. Evoked responses occurring 300–800 ms poststimulation were attributed to C-fiber sensitization (afterdischarge). Spontaneous activity was assessed over 100 ms immediately preceding each electrical stimulation within a given train.

Electrophysiological recording and stimulation

Extracellular recordings were obtained from isolated neurons using 3-MΩ tungsten microelectrodes (FHC, Brunswick, ME). One cell was recorded per rat. Two stimulating electrodes were placed transcutaneously within the center of the receptive field of isolated WDR neurons. Neuronal activity was evoked by a train of 16 electrical stimuli (2-ms pulses, 2 s apart) applied every 10 min. Stimuli were delivered at a voltage level near threshold (Strangman and Walker 1999). Stable baseline responsiveness to electrical stimulation of the receptive field was quantified over a 30-min period before drug or vehicle administration. Responsiveness to successive trains of transcutaneous electrical stimulation was subsequently assessed over 90
min after local administration of carrageenan or saline. Our previous work demonstrated that the 90-min postcarrageenan time interval was sufficient for observing a CB2-mediated suppression of inflammatory nociception (Nackley et al. 2003a,b). On conclusion of recording, rats were killed by iv injection of pentobarbital. Recording depth was documented to the nearest 5 μM.

Pharmacological manipulations

After stable baseline responsiveness to electrical stimulation was established, drug or vehicle was administered either systemically (2 ml/kg iv) or locally (50 μl ipl) in the plantar surface of the hind paw just before intraplantar administration of carrageenan or saline. Doses of AM1241 used here suppressed the development of carrageenan-evoked Fos protein expression and pain behavior through a CB2-specific mechanism (Nackley et al. 2003a). Doses of SR141716A and SR144528 were chosen based on their ability to block the effects of WIN55,212–2 (Hohmann et al. 1999) and AM1241 (Nackley et al. 2003a), respectively, after systemic administration. Rats received a unilateral intraplantar injection of carrageenan (3%, 100 μl) or saline together with drug or vehicle.

EXPERIMENT 1: EFFECTS OF AM1241 ON WDR NEURONS IN THE ABSENCE OF CARRAGEENAN INFLAMMATION. Effects of iv administration of AM1241 on activity evoked by transcutaneous electrical stimulation (electrically evoked activity) in spinal WDR neurons were evaluated. Separate groups of rats received iv injections of AM1241 (330 μg/kg; n = 6) or vehicle (n = 6) just before intraplantar administration of saline (100 μl). Saline was administered in the plantar surface of the hind paw to control for the volume of carrageenan injected at the same site in experiment 2. Effects of the intraplantar injection alone on electrically evoked activity in WDR neurons were evaluated in a separate control group (n = 6).

EXPERIMENT 2: EFFECTS OF AM1241 ON WDR NEURONS DURING THE DEVELOPMENT OF CARRAGEENAN INFLAMMATION. Effects of systemically administered AM1241 on electrically evoked responses were assessed in WDR neurons during the development of inflammation. Separate groups of rats received iv injections of AM1241 (330 μg/kg; n = 6) or vehicle (n = 6) just before intraplantar administration of saline (100 μl). Saline was administered in the plantar surface of the hind paw to control for the volume of carrageenan injected at the same site in experiment 2. Effects of the intraplantar injection alone on electrically evoked activity in WDR neurons were evaluated in a separate control group (n = 6).

EXPERIMENT 3: SITE OF ACTION OF AM1241. To evaluate the site of action of AM1241 in modulating the activity of spinal WDR neurons, AM1241 (33 or 330 μg/kg ipl; n = 6 per group) or vehicle (n = 6) was injected concurrently with carrageenan in the plantar surface of the hind paw. Separate groups of rats received AM1241 (33 μg/kg ipl; n = 6) in the contralateral (noninflamed) paw. A separate control group received intraplantar saline together with vehicle (n = 6) to verify that changes in electrically evoked neuronal activity could not be attributed to the injection alone.

EFFECTS OF AM1241 ON PERIPHERAL EDEMA. In experiments 2 and 3, paw diameter, defined as the region extending from the midplantar surface to the middorsal surface of the paw, was measured in duplicate (to the nearest 0.1 mm) using a caliper square (Honore et al. 1996; Nackley et al. 2003a,b). Measurements were obtained before carrageenan administration and on termination of the recording period, about 2 h subsequent to the induction of inflammation.

EXPERIMENT 4: EFFECTS OF AM1241 ON LOW-THRESHOLD NEURONS. Effects of AM1241 on electrically evoked responses elicited in purely nonnociceptive lumbar dorsal horn were assessed by administering AM1241 (330 μg/kg; n = 3) or vehicle (n = 3) intravenously just before intraplantar carrageenan.

Data analysis

Data were acquired using a CED 1401 interface (Cambridge Electronic Design, Cambridge, UK) and a Pentium III computer with Spike-2 software. Spike shapes were templated and monitored throughout the recording interval to confirm that action potentials from the original cell of interest were recorded. Electrophysiological data were analyzed by ANOVA for repeated-measures and Fisher’s protected least-significant difference (PLSD) post hoc tests. ANOVA was used to assess the statistical significance of experimental differences in the number of spontaneous and stimulation-evoked action potentials attributed to Aβ-, Aδ-, and C-fiber-mediated responses. C-fiber-mediated action potentials were analyzed in terms of early, afterdischarge, and total responses.

Degree of windup was determined using the method of Chapman (2001)

\[ \text{Degree of windup} = \frac{\text{total number of observer responses in train X} - \text{(Number of neuronal responses elicited by stimulation 1 in train X)}}{\text{(Degree of post-inflammation windup in train X)}} \times 100 \]

Data were expressed as percentage of preinflammation windup, using the formula

\[ \% \text{ Pre-inflammation windup} = \frac{\text{Degree of post-inflammation windup in train X}}{100} \]

Degree of baseline windup responses Percentage of preinflammation/preinjection windup was calculated for each stimulation train. For each neurophysiological parameter, neuronal responsiveness over 3 successive trains of 16 stimulations (delivered at 10-min intervals) was averaged for data analysis. P < 0.05 was considered significant.

RESULTS

General characteristics of sampled neuronal population

Recordings were obtained from 77 WDR neurons and 6 nonnociceptive neurons. One cell was recorded per animal. WDR neurons were sampled from the lumbar dorsal horn in all experiments, as indicated by depths of recording sites. All cells responded to brush and pinch and encoded stimulus intensity as a function of inflammation. Before intraplantar administration of carrageenan or saline, % Pre-inflammation windup was calculated for each stimulation train. For each neurophysiological parameter, neuronal responsiveness over 3 successive trains of 16 stimulations (delivered at 10-min intervals) was averaged for data analysis. P < 0.05 was considered significant.

Intraplantar administration of saline together with the vehicle for cannabinoid administration failed to reliably alter Aβ2-, Aδ2-, or C-fiber–mediated neuronal responses or windup. By contrast, time-dependent changes in total C-fiber–mediated responses were reliably induced by carrageenan in experiments 2 and 3 (P < 0.02 for all comparisons). Aβ2- and Aδ2-fiber–mediated responses did not differ reliably between groups before or after carrageenan administration and did not increase as a function of inflammation.

Effects of AM1241 on spontaneous activity

Before intraplantar administration of carrageenan or saline, levels of spontaneous firing were low and did not differ between groups in any experiment. No differences in spontaneous firing were observed over time or between groups in
TABLE 1. Characterization of wide dynamic range and nonnociceptive neurons

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Recording Depth, μM</th>
<th>Range, μM</th>
<th>Brush, Hz</th>
<th>Pinch, Hz</th>
<th>Current, mA</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>166.53 ± 22.49</td>
<td>0–500</td>
<td>10.87 ± 0.83</td>
<td>39.10 ± 3.73**</td>
<td>4.52 ± 0.15</td>
</tr>
<tr>
<td>2</td>
<td>208.50 ± 36.93</td>
<td>0–750</td>
<td>9.26 ± 0.87</td>
<td>25.94 ± 2.91**</td>
<td>4.55 ± 0.18</td>
</tr>
<tr>
<td>3</td>
<td>180.83 ± 43.79</td>
<td>0–440</td>
<td>5.34 ± 0.44</td>
<td>21.98 ± 3.19**</td>
<td>4.52 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>155.83 ± 67.26</td>
<td>0–430</td>
<td>7.7 ± 0.84</td>
<td>0.75 ± 0.37*</td>
<td>3.88 ± 0.39</td>
</tr>
</tbody>
</table>

Values are means ± SE. Depths and ranges of recording sites are shown together with responses evoked by cutaneous stimulation (nonnoxious brush, noxious pinch) in wide dynamic range (experiment 1–3) and low threshold (experiment 4) neurons. Current used to activate neurons by transcutaneous electrical stimulation of the receptive field did not differ between groups. **P < 0.0005, *P < 0.002, significantly different from responses to brush.

Intraplantar carrageenan increased spontaneous firing in controls in experiment 2 [F(1,32) = 14.45, P < 0.0007; Fig. 1A]. A trend toward an AM1241-induced suppression of spontaneous activity was observed (P = 0.06; Fig. 1A). In experiment 3, spontaneous activity was lower in groups receiving intraplantar (ipl) injections of AM1241 (33 or 330 μg/kg ipsilateral (ipsi) or 33 μg/kg contralateral (contra) to the site of carrageenan administration) relative to vehicle [F(1,20) = 11.46, P < 0.0002; P < 0.002 for all comparisons]. Time-dependent changes in spontaneous activity were induced by the experimental treatment in control animals [F(1,22) = 9.32, P < 0.006]. Activation of CB2 by ipl administration of AM1241 (33 or 330 μg/kg ipsi ipl) suppressed inflammation-evoked increases in spontaneous activity relative to controls at 70–90 min postcarrageenan (P < 0.05 for all comparisons; Fig. 1B). Spontaneous firing in groups treated with AM1241 (33 or 330 μg/kg ipsi ipl or 33 μg/kg contra ipl) was also lower than that observed in groups treated with vehicle from 10 to 60 min postcarrageenan.

Experiment 1: Effects of AM1241 on WDR neurons in the absence of carrageenan inflammation

Administration of AM1241 (330 μg/kg iv) suppressed total C-fiber–mediated activity [F(1,10) = 5.74, P < 0.04; Fig. 2A], early C-fiber–mediated activity [F(1,10) = 5.06, P < 0.05; Fig. 2B], C-fiber–mediated afterdischarge [F(1,10) = 5.74, P < 0.04; Fig. 2C], and windup [F(1,10) = 7.07, P < 0.03; Fig. 2D] relative to vehicle in rats receiving ipl saline in lieu of carrageenan. No differences in Aβ- or Aδ-fiber–mediated responses were observed between groups receiving either AM1241 or vehicle in the absence of carrageenan. Time-dependent changes in neuronal activity were absent for every neurophysiological parameter evaluated.

Experiment 2: Effects of AM1241 on WDR neurons during the development of carrageenan inflammation

Total C-fiber–mediated activity was lower in groups receiving AM1241 relative to vehicle [F(3,20) = 6.02, P < 0.005; Fig. 3A]. Intravenous administration of AM1241 maximally suppressed total C-fiber–mediated responses relative to controls at 70–90 min after carrageenan administration [F(9,60) = 5.29, P < 0.0003; P < 0.002 for all comparisons]. The CB2 antagonist SR144528 (P < 0.008) blocked the AM1241–induced suppression of total C-fiber–mediated responses, but the CB1 antagonist SR141716A failed to do so.

AM1241 (330 μg/kg, iv) suppressed early C-fiber–mediated activity relative to vehicle [F(3,20) = 3.29, P < 0.05; Fig. 3B]. The suppressive effects of AM1241 (P < 0.007 relative to vehicle) were blocked by the CB2 antagonist. Early C-fiber–evoked responses observed in rats receiving the CB2 antagonist together with AM1241 did not differ from vehicle. The suppressive effects of AM1241 on early C-fiber–mediated responses were time dependent [F(9,60) = 3.16, P < 0.008]. At 70–90 min postcarrageenan, AM1241 suppressed early C-

Fig. 1. Effects of AM1241 on spontaneous activity during the development of inflammation. A: levels of spontaneous firing did not differ between groups receiving intravenous (iv) administration of AM1241 (330 μg/kg) or vehicle before carrageenan administration. A trend toward an AM1241-induced suppression of spontaneous activity was observed. B: intraplantar (ipl) administration of AM1241 ipsilateral to the carrageenan-injected paw suppressed carrageenan-evoked increases in spontaneous activity relative to vehicle administration in the ipsilateral (ipsi) (carrageenan-injected) paw (ipsi ipl) or AM1241 administration in the contralateral (contra) noninflamed (contra ipl) paw. Data (means ± SE) represent the mean response observed in 3 successive stimulation trains delivered at 10-min intervals and are plotted at the midpoint. *P < 0.05 different from vehicle and AM1241 (33 μg/kg contra, ipl). **P < 0.01 different from AM1241 (33 μg/kg ipsi, contra; 330 μg/kg ipsi) by ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc test; n = 6 per group (1 cell/rat).
fiber–mediated activity relative to vehicle ($P < 0.02$). This suppression was blocked by the CB$_2$ antagonist SR144528 ($P < 0.02$) at 70–90 min after inflammation, but not by the CB$_1$ antagonist SR141716A.

C-fiber–mediated afterdischarge differed between groups receiving AM1241 and control conditions [$F_{(3,20)} = 5.56$, $P < 0.007$; Fig. 3C]. The AM1241-induced suppression of C-fiber–mediated afterdischarge was blocked by SR144528 ($P < 0.02$),
but not by SR141716A. The AM1241-induced suppression of C-fiber–mediated afterdischarge became more pronounced as inflammation developed over time \( F_{9,60} = 3.90, P < 0.02 \). Preemptive administration of AM1241 attenuated C-fiber–mediated afterdischarge beginning 10 min after the induction of inflammation \( P < 0.01 \) for all comparisons). The suppressive effect of AM1241 on C-fiber–mediated afterdischarge was blocked by SR144528 beginning 40 min postcarrageenan \( P < 0.02 \) for all comparisons).

The percentage of preinflammation windup differed in groups receiving AM1241 and vehicle \( F_{9,60} = 4.52, P < 0.02 \); Fig. 3D). AM1241 suppressed windup 10–90 min after the induction of inflammation \( F_{9,60} = 3.48, P < 0.02 \) for all comparisons]. Administration of SR141716A failed to block the suppressive effects of AM1241 on windup \( P < 0.02 \) relative to vehicle), whereas animals receiving SR144528 together with AM1241 showed levels of windup comparable to those receiving vehicle.

**FIG. 3.** Preemptive administration of the CB\(_2\) agonist AM1241 (330 μg/kg, iv) suppressed total C-fiber–mediated activity (A), early C-fiber–mediated activity (B), C-fiber–mediated afterdischarge (C), and windup (D) during the development of inflammation. No reliable differences were observed in inflammation-evoked (E) Aβ- or (F) Aδ-fiber–mediated responses. Data (means ± SE) represent the mean response observed in 3 successive stimulation trains delivered at 10-min intervals and are plotted at the midpoint. **\( P < 0.01 \), *\( P < 0.05 \) different from vehicle and AM1241 + SR144528, **\( P < 0.01 \), *\( P < 0.05 \) different from vehicle, *\( P < 0.05 \) different from vehicle and AM1241 + SR141716A by ANOVA and Fisher’s PLSD post hoc test; \( n = 5–6 \) per group (1 cell/rat).
Aβ- and Aδ-fiber–mediated responses did not differ reliably between groups before or after carrageenan administration (Fig. 3, E and F). Moreover, no increases in Aβ-fiber–mediated activity were observed as a function of inflammation at 70–90 min postinjection. Effects of SR144528 or SR141716A administration on Aβ-, Aδ-, and C-fiber–mediated responses and windup did not differ from vehicle.

The suppressive effects of AM1241 on C-fiber activation and windup were enhanced in the presence of inflammation (Fig. 4). The suppression of C-fiber–mediated afterdischarge was greater in inflamed rats relative to noninflamed rats 70–90 min postinjection \( [F_{(3,30)} = 3.75, P < 0.05; \text{Fig. 4C}] \). A trend toward a greater AM1241-induced attenuation of total C-fiber–mediated activity was observed in rats receiving carrageenan \( (P < 0.07; \text{Fig. 4A}) \). AM1241 also produced a greater suppression of windup 40–90 min after intraplantar administration of carrageenan relative to intraplantar administration of saline \( [F_{(3,30)} = 4.86, P < 0.03; \text{Fig. 4D}] \).

Experiment 3: site of action of AM1241

Total C-fiber–mediated activity differed between groups receiving AM1241 (33 or 330 μg/kg) in the carrageenan-injected paw and controls \( [F_{(3,20)} = 4.71, P < 0.02; \text{Fig. 5A}] \). AM1241 (33 or 330 μg/kg ipsi ipl) suppressed total C-fiber–mediated responses at 40–90 min after carrageenan \( [F_{(9,60)} = 5.48, P < 0.0004; P < 0.05 \text{ for all comparisons}] \).

Early C-fiber–mediated responses were suppressed in groups receiving AM1241 (33 or 330 μg/kg) locally in the carrageenan-injected (ipsilateral) paw relative to controls \( [F_{(9,60)} = 6.08, P < 0.0003; P < 0.05 \text{ for all comparisons}] \). AM1241 (33 μg/kg contra ipl) induced a transient suppression of early C-fiber–mediated activity relative to vehicle at 10–30 min postcarrageenan. Local administration of either the low or high dose of AM1241 reduced early C-fiber–mediated responses relative to AM1241 administration to the contralateral (contra) noninflamed paw \( (P < 0.0003 \text{ for all comparisons}) \).

Time-dependent changes in windup, relative to baseline, were also induced by locally administered AM1241 during the development of inflammation \( [F_{(9,60)} = 2.36, P < 0.04; \text{Fig. 5D}] \). A trend toward an AM1241-induced attenuation of the percentage of preinflammation windup was observed \( (P = 0.065) \). At 70–90 min postcarrageenan, the high dose (330 μg/kg ipl) of AM1241 suppressed windup relative to controls \( (P < 0.005) \), whereas the low dose (33 μg/kg ipl) failed to do so.

The effects of AM1241 were mediated, at least in part, locally in the paw; AM1241 administered to the ipsilateral (carrageenan-injected) paw suppressed early and total C-fiber–mediated activity.
mediated neuronal responses and windup relative to the same dose applied to the contralateral (noninflamed) paw (Fig. 5). Example records show the effects of AM1241 and vehicle on neuronal responses evoked by transcutaneous electrical stimulation in spinal WDR neurons before and after concurrent administration of carrageenan (Fig. 6).

Peripheral edema

Before administration of carrageenan, paw diameter did not differ between groups (mean ± SE: 5.22 ± 0.03 mm and 5.11 ± 0.03 mm in experiments 2 and 3, respectively; Fig. 7). In both studies, intraplantar carrageenan increased hind paw diameter measured about 2 h after the induction of inflammation (P < 0.0002). Hind paw diameter was greater in rats receiving carrageenan relative to a control group receiving an equivalent volume of intraplantar saline together with vehicle (P < 0.0002 for all comparisons). Hind paw diameter was lower in carrageenan-injected groups receiving AM1241 (33 µg/kg ipl or 330 µg/kg iv or ipl) concurrently with carrageenan relative to groups receiving an equivalent volume of vehicle.
AM1241 (330 μg/kg iv or ipl or 33 μg/kg ipl) reduced hind paw diameter in the inflamed paw relative to control \( F(15,87) = 239, P < 0.03 \) and \( F(9,60) = 32.73, P < 0.0001 \) in experiments 2 and 3, respectively; \( P < 0.03 \) for all comparisons] conditions (Fig. 7). The antiinflammatory effects of intravenously administered AM1241 were blocked by the CB\(_2\) antagonist SR144528 (\( P < 0.02 \)) but not the CB\(_1\) antagonist SR141716A (Fig. 7). No group differences in paw diameter were observed in the noninflamed contralateral paw before or after carrageenan administration.

**Experiment 4: effects of AM1241 on nonnociceptive neurons during the development of carrageenan inflammation**

Nonnociceptive neurons were sampled from the dorsal horn and responded with increased frequency to nonnoxious brush relative to noxious pinch (Table 1). Before carrageenan and during the development of inflammation, levels of spontaneous firing were low and did not differ between groups. Current used to activate neurons ranged from 2.8 to 5.2 mA. Administration of AM1241 (330 μg/kg iv) failed to alter A\(\beta\)-fiber–
mediated responses in these purely nonnociceptive neurons (Fig. 8)

**Discussion**

In the present work, transcutaneous electrical stimulation of the peripheral receptive field was used to study the effects of the CB$_2$-selective cannabinoid agonist AM1241 on the excitability of spinal WDR neurons. Spike shapes were monitored throughout the recording interval and used to confirm that action potentials from the original cells of interest were recorded. Thus group differences cannot be attributed to changes in the cell population sampled after the induction of inflammation or injection procedures. The state of anesthesia is unlikely to complicate our findings because it failed to obscure detection of antiinflammatory actions of AM1241 in the present work.

Transcutaneous electrical stimulation directly depolarizes primary afferent axons, which in turn excite WDR neurons in the spinal dorsal horn. Action potentials thus generated bypass normal transduction mechanisms initiated by activation of nerve terminal receptors. Therefore transcutaneous electrical stimulation can be used to specifically study central changes in spinal dorsal horn neuronal excitability (Li et al. 1999). The present work demonstrates that local or systemic AM1241 administration produces a CB$_2$-mediated suppression of C-fiber responses and windup in spinal WDR neurons; this suppression was observed in both the absence and presence of carrageenan inflammation. These findings are in agreement with previous work showing that CB$_2$-selective agonists are antinociceptive in models of acute (Malan et al. 2001) and chronic pain (Clayton et al. 2002; Hanus et al. 1999; Hohmann et al. 2004; Ibrahim et al. 2003; Nackley et al. 2003a; Quartilho et al. 2003).

In the absence of inflammation or prior stimulation, primary afferent C-fibers lack spontaneous, ongoing discharges. However, noxious input can quickly enhance the sensitivity of spinal neurons for further C-fiber inputs. Spinal neuronal excitability may increase through several mechanisms including: 1) a cumulative depolarization that lowers the threshold for action potential initiation (induced by increased spontaneous or ongoing activity of primary afferents) (Sivliotti et al. 1993), 2) presynaptic facilitation (e.g., increased excitatory amino acid or tachkinin release evoked by prior primary afferent activation) (Gerber and Randic 1989; Urban and Randic 1984; Yoshimura and Jessell 1990), and 3) increased efficacy of postsynaptic receptors induced by prior activation of spinal neurons (e.g., because of facilitation of signal transduction/second messenger coupling and/or receptor upregulation) (Schmidt 1971; Sorkin and Puig 1996; Sorkin et al. 1998). In the present study, increased WDR neuronal excitability likely resulted from the summation of depolarizing postsynaptic potentials in WDR neurons and/or presynaptic facilitation because these changes can occur within minutes, whereas an increase in postsynaptic receptor number or efficacy takes much longer.

The effects of AM1241 on WDR neurons in the absence of inflammation

In noninflamed rats, spontaneous activity and C-fiber–mediated responses of WDR neurons were fairly stable across the recording interval, suggesting that there was little ongoing discharge in the primary afferents in the absence of inflamma-

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**Fig. 8.** AM1241 (330 µg/kg iv) fails to alter evoked Aβ-fiber–mediated responses in purely nonnociceptive neurons in the presence of inflammation; $n = 3$ per group (1 cell/rat).
tion. Nonetheless, AM1241 suppressed C-fiber–mediated responses and windup. This suppression likely involves a CB$_2$-mediated attenuation of presynaptic facilitation, rather than a change in ongoing primary afferent discharge. It is possible that repeated electrical stimulation, cutaneous pinch application, and/or intraplantar injection itself induced local inflammatory processes in the absence of carrageenan that led to increased neurotransmitter release by the same number of action potentials. This explanation requires that low levels of receptive field stimulation are sufficient to induce rapid, long-term changes by the above mechanisms. However, the relatively short time course for the observed changes likely precludes a role for modifications in postsynaptic receptor number or efficacy.

The effects of AM1241 on WDR neurons in the presence of inflammation

Peripheral carrageenan produces local C-fiber sensitization and subsequent spinal WDR neuronal hyperexcitability (Hedo et al. 1998; Woolf et al. 1994). In the presence of carrageenan inflammation, increases in WDR neuronal activity may involve increased ongoing discharge as well as presynaptic facilitation. This dual mechanism may account for the increase in spontaneous activity, C-fiber responsiveness, and windup observed during the development of inflammation as well as the more pronounced suppressive effect of AM1241 in the presence versus the absence of inflammation. These observations suggest a preferential effect of peripheral CB$_2$ activation in suppressing inflammation-evoked sensitization of C-fiber–mediated responses of WDR neurons (Svendsen et al. 1999; Zhang et al. 2001).

Peripheral edema

AM1241 also produced a CB$_2$-mediated antiinflammatory effect, consistent with previous findings (Nackley et al. 2003a; Quartilho et al. 2003). Systemic or local administration of AM1241 likely prevented the release of inflammatory mediators, thereby reducing inflammation-evoked ongoing discharge and presynaptic facilitation, mechanisms contributing to the enhanced spinal neuronal excitability we observed during the development of inflammation.

Effects of AM1241 on nonnociceptive neurons during the development of inflammation

AM1241 does not act as a local anesthetic. Local anesthetics typically reduce neuronal responses by blocking Na$^+$ channels, resulting in a suppression of electrically evoked A$\beta$ activity in nonnociceptive cells as well as C-fiber–mediated activity in WDR neurons (Chapman et al. 1997). In our study, low-threshold, purely nonnociceptive spinal neurons did not show sensitization during the development of inflammation and were not altered by AM1241 actions in the periphery. By contrast, high-threshold neurons in the spinal dorsal horn do relay nociceptive information to supraspinal sites and undergo sensitization (Woolf et al. 1994). Future studies should assess the effects of CB$_2$ activation on high-threshold cells to fully elucidate the consequences of CB$_2$ activation on nociceptive transmission.

Possible mechanisms of action of AM1241

Our findings are consistent with the possibility that AM1241 produces analgesia by suppressing peripheral nociceptor sensitization that may lead to central sensitization. However, the mechanism by which AM1241 suppresses WDR neuronal responses enhanced by ongoing primary afferent discharges and presynaptic facilitation remains to be determined. In the presence of inflammation, AM1241 may act locally on immune cells in the periphery to suppress C-fiber sensitization. Activation of CB$_2$ receptors localized to mast cells or other immune cells attenuates the release of inflammatory mediators, including nerve growth factor (Rice et al. 2002) and cytokines (Klegeris et al. 2003), that in turn sensitize nociceptors (Mazzari 1996). However, CB$_2$ modulation of immune responses does not readily account for the effects of AM1241 on windup and C-fiber responses in the absence of inflammation.

Direct actions at CB$_2$ receptors localized to primary afferent C-fibers (Patel et al. 2003) would provide a parsimonious explanation for the antinociceptive and electrophysiological actions of CB$_2$ agonists observed in the absence of inflammation. More work is necessary to identify the cellular elements that contain CB$_2$. CB$_2$ positive cells have been demonstrated in cultured dorsal root ganglion cells (Ross et al. 2001); however, other studies suggest that CB$_2$ mRNA in dorsal root (Hohmann and Herkenham 1999) and trigeminal (Price et al. 2003) ganglia is near background levels.

AM1241 may also indirectly suppress primary afferent activation by stimulating local release of $\beta$-endorphin in peripheral tissue; AM1241 stimulates $\beta$-endorphin release in both rat hindpaw skin and cultured keratinocytes (Malan et al. 2004). Furthermore, antinociception induced by AM1241 is blocked by either naloxone or antisera to $\beta$-endorphin and is absent in both $\mu$-opioid and CB$_2$ receptor knockout mice (Malan et al. 2004). CB$_2$ mRNA expression is also induced in the lumbar spinal cord coincident with the appearance of activated microglia (Zhang et al. 2003), suggesting that additional targets for CB$_2$ agonists may also be present in pathological pain states. It is therefore plausible that AM1241 produces antinociception by a combination of the aforementioned mechanisms.

In conclusion, CB$_2$ agonists offer considerable potential as a novel pharmacotherapy for pain because they fail to produce centrally mediated effects commonly associated with CB$_1$ (Hanus et al. 1999; Malan et al. 2001; Patel et al. 2003). Furthermore, such compounds are unlikely to be psychoactive or addictive. The present work provides evidence that activation of a peripheral CB$_2$ mechanism is sufficient to suppress C-fiber–evoked responses and windup at the level of the spinal dorsal horn. This suppression was observed in WDR neurons, which are known to modulate nociception and contribute to the ascending pain pathway, the spinalomotoric tract. These data collectively suggest that CB$_2$ agonists may be used preemptively to attenuate the development of persistent pain in the absence of unwanted central side effects.

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