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Inhibition of Inflammatory Hyperalgesia by Activation of Peripheral CB_2 Cannabinoid Receptors

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Background: **Cannabinoid receptor agonists inhibit inflammatory hyperalgesia in animal models. Nonselective cannabinoid receptor agonists also produce central nervous system (CNS) side effects. Agonists selective for CB_2 cannabinoid receptors, which are not found in the CNS, do not produce the CNS effects typical of nonselective cannabinoid receptor agonists but do inhibit acute nociception. The authors used the CB_2 receptor–selective agonist AM1241 to test the hypothesis that selective activation of peripheral CB_2 receptors inhibits inflammatory hyperalgesia.**

Methods: **Rats were injected in the hind paw with carrageenan or capsaicin. Paw withdrawal latencies were measured using a focused thermal stimulus. The effects of peripheral CB_2 receptor activation were determined by using local injection of AM1241. CB_2 receptor mediation of the actions of AM1241 was shown by using the CB_2 receptor–selective antagonist AM630 and the CB_1 receptor–selective antagonist AM251.**

Results: **AM1241 fully reversed carrageenan-induced inflammatory thermal hyperalgesia when injected into the inflamed paw. In contrast, AM1241 injected into the contralateral paw had no effect, showing that its effects were local. AM1241 also reversed the local edema produced by hind paw carrageenan injection. The effects of AM1241 were reversed by the CB_2 receptor–selective antagonist AM630, but not by the CB_1 receptor–selective antagonist AM251. AM1241 also inhibited flinching and thermal hyperalgesia produced by hind paw capsaicin injection.**

Conclusions: **Local, peripheral CB_2 receptor activation inhibits inflammation and inflammatory hyperalgesia. These results suggest that peripheral CB_2 receptors may be an appropriate target for eliciting relief of inflammatory pain without the CNS effects of nonselective cannabinoid receptor agonists.**

THE identification of cannabinoid receptors in the periphery has led to the concept that it may be possible to develop cannabinoid receptor agonists that act selectively outside the central nervous system (CNS) to produce pain relief without undesirable CNS effects. CB_2 cannabinoid receptors are not found in the CNS,[1–4] but are primarily located on immune cells in the periphery.[1,5–7] It has recently been shown that CB_2 receptor–selective agonists produce peripheral antinociception, but do not cause CNS effects produced by nonselective cannabinoid receptor agonists,[8,9] suggesting that selective activation of CB_2 receptors may achieve the goal of peripheral pain relief without CNS effects.

Increased sensory sensitivity produced by peripheral inflammatory processes is an important component of many pain states. Cannabinoid receptor agonists inhibit inflammatory hyperalgesia in animal models.[10] Significantly, peripheral cannabinoid receptors may be capable of inhibiting inflammatory hyperalgesia, as shown by the observation that the endogenous cannabinoid receptor agonist anandamide exhibits antihyperalgesic actions when injected locally into the inflamed hind paw of the rat.[11] The effects of anandamide were reversed by the CB_1 receptor–selective antagonist SR141716A, suggesting that they were mediated, at least in part, by CB_1 receptors. Recently, the ability of CB_2 cannabinoid receptors to inhibit inflammatory pain responses has begun to be studied using newly developed CB_2 receptor–selective agonists. In this regard, systemic administration of the CB_2 receptor–selective agonist GW405833 partially prevented carrageenan-induced inflammatory mechanical hyperalgesia.[12]

The current studies test the hypothesis that selective activation of peripheral CB_2 receptors can inhibit inflammation and inflammatory hyperalgesia. We tested the ability of the CB_2 receptor–selective agonist AM1241 to reverse carrageenan-induced edema and inflammatory hyperalgesia. The ability of peripheral CB_2 receptors to reverse inflammatory hyperalgesia was tested by site-specific drug administration. Peripheral CB_2 receptors may be an appropriate target for eliciting relief of inflammatory pain without the CNS effects of nonselective cannabinoid receptor agonists.

Methods

Animals

All procedures were approved by The University of Arizona Animal Care and Use Committee and conformed to the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals and the guidelines of the International Association for the Study of Pain.[13] Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250–300 g at the time of testing, were maintained in a climate-controlled room on a 12-h light–dark cycle and allowed food and water *ad libitum*. For all studies, animals were randomly assigned to treatment groups, and measurements were made by a single observer. The observer was not masked to the treatment administered.

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Drug Administration

AM1241 is a cannabinoid receptor agonist with 70-fold selectivity for the CB_2 receptor *in vitro* (K_i = 3.4 nM for binding to CB_2 receptors in mouse spleen tissue and K_i = 239 nM for binding to CB_1 receptors in rat brain tissue). AM630 is a CB_2 receptor–selective antagonist with 70- to 165-fold selectivity for binding to the CB_2 receptor *in vitro*.[14,15] AM251 is a 300-fold-selective CB_1 receptor antagonist.[16,17] All cannabinoid drugs were synthesized in the laboratory of one of the authors (Dr. Makriyannis). Cannabinoid drugs were dissolved in dimethyl sulfoxide and injected subcutaneously in the dorsal surface of the hind paw (intrapaw, 50 μl) or intraperitoneally (0.5 ml). Measurements were taken 25 min after intrapaw injection or 15 min after intraperitoneal injection. In preliminary experiments, these were the times of maximal drug effect. Carrageenan and capsaicin were purchased from Sigma (St. Louis, MO). Carrageenan was dissolved in water, and capsaicin was dissolved in 7% Tween 80.

Carageenan-induced Thermal Hyperalgesia and Paw Edema

Inflammation was induced by injection of 50 μl 2% carrageenan in the dorsal surface of the hind paw. Thermal paw withdrawal latency was measured as described in the Testing of Thermal Withdrawal Latency section. In separate experiments, hind paw edema was assessed by measuring paw volume using a plethysmometer (model 7140; Stoelting Company, Wood Dale, IL). Measurements were made before carrageenan administration and 3 h after carrageenan administration. Cannabinoid test drugs were then administered, and measurements were again made 15 min after intraperitoneal drug administration or 25 min after intrapaw drug administration.

Capsaicin-induced Flinching and Hyperalgesia

Capsaicin (20 μg in 20 μl) was injected into the dorsal surface of the hind paw. Flinches of the hind paw were counted for 5 min. In separate experiments, thermal responses were measured before capsaicin administration and 10 min after capsaicin administration. Cannabinoid test drugs were administered to separate groups of animals in the paw 15 min before capsaicin administration.

Testing of Thermal Withdrawal Latency

Withdrawal latency to thermal stimuli was tested, as described by Hargreaves *et al.*,[18] by using equipment purchased from the Department of Anesthesiology of the University of California, San Diego. Rats were allowed to acclimate within Plexiglas enclosures on a clear glass plate maintained at 30°C using a warming mechanism driven by a thermocouple. A radiant heat source, a projector bulb, was focused onto the plantar surface of the hind paw. The intensity of the heat source was adjusted to yield a baseline paw withdrawal latency of 20 s. The temperature of the paw at the time of withdrawal was not directly measured. Activation of the heat source activated a timer that stopped when withdrawal of the paw was detected with a photodetector. A maximal cutoff of 40 s was used to prevent tissue damage.

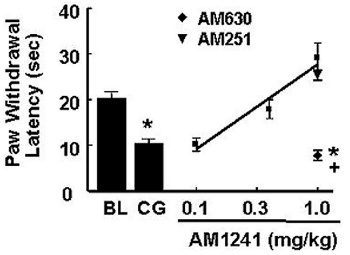

Fig. 1. Reversal by systemic (intraperitoneal) AM1241 of carrageenan-induced thermal hypersensitivity. Antagonism of the effects of AM1241 by the CB_2 receptor–selective antagonist AM630 (100 μg/kg, intraperitoneal). Lack of antagonism by the CB_1 receptor–selective antagonist AM251 (300 μg/kg, intraperitoneal). Data are expressed as mean ± SEM. Groups were compared using ANOVA followed by pair-wise comparisons using the Student *t* test with Bonferroni correction. * $P < 0.05$ compared with precarrageenan baseline; + $P < 0.05$ compared with AM1241 alone; n = 6 per group. BL = precarrageenan baseline; CG = postcarrageenan value.

Data Analysis

Groups were compared using ANOVA followed by pair-wise comparisons using the Student *t* test with Bonferroni correction. Repeated-measures ANOVA was used when repeated measurements were made on the same animals. Significance was defined as *P* less than 0.05.

Results

Carrageenan-induced inflammation decreased the paw withdrawal latency to thermal stimuli by 48% to 63%, when measured 3 h after carrageenan injection (figs. 1 and 2).

Systemic (intraperitoneal) administration of the CB_2 cannabinoid receptor–selective agonist AM1241 dose-dependently reversed the inflammation-induced decrease in thermal withdrawal latency and prolonged thermal withdrawal latency beyond preinflammation baseline values (fig. 1). The CB_2 receptor–selective antagonist AM630 (100 μg/kg, intraperitoneal) completely reversed the effect of AM1241 on thermal withdrawal latency, whereas the CB_1 receptor–selective antagonist AM251 (300 μg/kg, intraperitoneal) had no effect (fig. 1). This dose of AM630 blocked the antinociception produced by the CB_2 receptor–selective agonist AM1241, but not the CNS effects produced by the mixed CB_1–CB_2 receptor agonist WIN55,212–2, suggesting that this dose is selective for the CB_2 receptor.[9] This dose of AM251 blocked the CNS effects produced by the mixed CB_1–CB_2 receptor agonist WIN55,212–2, but not the antinociception produced by the CB_2 receptor–selective

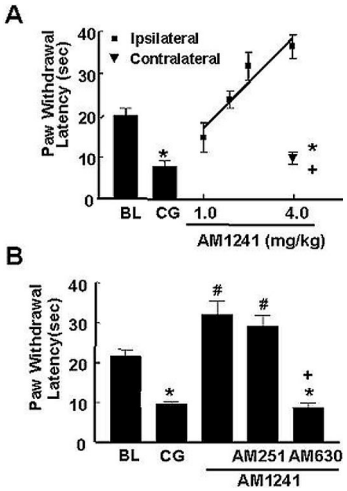

Fig. 2. (*A*) Reversal by peripheral (intrapaw) AM1241 of carrageenan-induced thermal hypersensitivity. (*B*) Antagonism of the effects of AM1241 by the CB_2 receptor–selective antagonist AM630 (100 μg/kg, intrapaw), but not by the CB_1 receptor–selective antagonist AM251 (300 μg/kg, intrapaw). Data are expressed as mean ± SEM. Groups were compared using ANOVA followed by pair-wise comparisons using the Student *t* test with Bonferroni correction. **P* < 0.05 compared with preinflammation baseline; #*P* < 0.05 compared with postcarrageenan value; +*P* < 0.05 compared with ipsilateral administration of AM1241 alone; n = 6 per group. BL = precarrageenan baseline; CG = postcarageenan value.

agonist AM1241, suggesting that this dose is selective for the CB_1 receptor.[9] These doses of AM630 and AM251 had no effect when administered alone. The paw withdrawal latency after carrageenan was 11.1 ± 1.3 s. After AM251, it was 10.5 ± 1.7 s, and after AM630, it was 9.7 ± 10.9 s.

AM1241 dose-dependently reversed the carrageenan-induced decrease in thermal withdrawal latency and prolonged thermal withdrawal latency beyond preinflammation baseline values when administered into the inflamed paw, but it had no effect when administered into the contralateral paw (fig. 2). The CB_2 receptor–selective antagonist AM630 (100 μg/kg, intrapaw) completely reversed the effect of intrapaw AM1241 (2 mg/kg), whereas the CB_1 receptor–selective antagonist AM251 (300 μg/kg, intrapaw) had no effect (fig. 2). These doses of AM630 and AM251 had no effect when administered alone. The paw withdrawal latency after carrageenan was 12.8 ± 1.1 s. After AM251, it was 11.6 ± 2.2 s, and after AM630, it was 9.2 ± 2.6 s.

Hind paw carrageenan injection increased hind paw volume to 230% of normal (fig. 3). At the dose used (2 mg/kg), intrapaw AM1241 reversed inflammation-induced paw edema by 63%. AM630 (100 μg/kg intrapaw) completely reversed the effect of AM1241, whereas AM251 (300 μg/kg intrapaw) had no effect.

Hind paw capsaicin injection decreased thermal paw withdrawal latency by 74% (fig. 4). Intraperitoneal injection of AM1241 dose-dependently reversed the capsaicin-induced decrease in paw withdrawal latency and

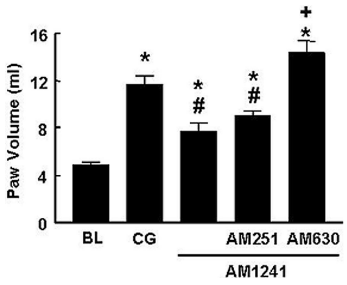

Fig. 3. Reversal by peripheral (intrapaw) AM1241 of carrageenan-induced hind paw edema. Antagonism of the effects of AM1241 by the CB_2 receptor–selective antagonist AM630 (100 μg/kg), but not by the CB_1 receptor–selective antagonist AM251 (300 μg/kg). Data are expressed as mean ± SEM. Groups were compared using ANOVA followed by pair-wise comparisons using the Student *t* test with Bonferroni correction. **P* < 0.05 compared with precarrageenan baseline; #*P* < 0.05 compared with postcarrageenan value; +*P* < 0.05 compared with AM1241 alone; n = 6 per group. BL = precarrageenan baseline; CG = postcarageenan value.

increased paw withdrawal latency beyond precapsaicin values (fig. 4). AM630 (100 μg/kg, intraperitoneal) completely reversed the effect of intraperitoneal AM1241 (300 μg/kg), whereas AM251 (300 μg/kg, intraperitoneal) had no effect. Hind paw capsaicin injection produced a flinching response of 17 ± 1 flinches/min. Intraperitoneal injection of AM1241 produced a dose-dependent decrease in capsaicin-induced hind paw flinches (fig. 4). AM630 (100 μg/kg, intraperitoneal) completely reversed the effect of intraperitoneal

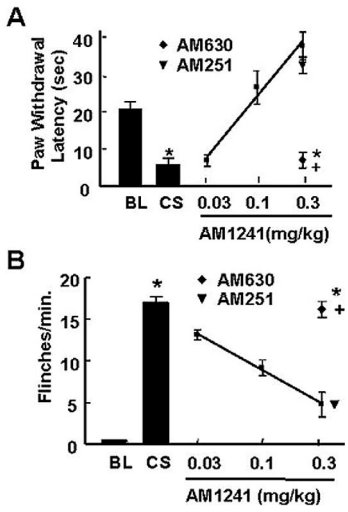

Fig. 4. (*A*) Reversal by systemic (intraperitoneal) AM1241 of capsaicin-induced thermal hypersensitivity. (*B*) Reversal by peripheral (intraperitoneal) AM1241 of capsaicin-induced flinches of the hind paw. Antagonism of the effects of AM1241 by the CB_2 receptor–selective antagonist AM630 (100 μg/kg, intraperitoneal), but not by the CB_1 receptor–selective antagonist AM251 (300 μg/kg, intraperitoneal). Data are expressed as mean ± SEM. Groups were compared using ANOVA followed by pair-wise comparisons using the Student *t* test with Bonferroni correction. **P* < 0.05 compared with precapsaicin baseline; +*P* < 0.05 compared with AM1241 alone; n = 6 per group. BL = precapsaicin baseline; CS = postcapsaicin value.

AM1241 (300 μg/kg), whereas AM251 (300 μg/kg, intraperitoneal) had no effect.

Discussion

These results show that selective activation of peripheral CB_2 cannabinoid receptors reverses inflammatory hyperalgesia. Local administration of AM1241 in the inflamed hind paw reversed the decrease in thermal withdrawal latency produced by carrageenan injection. The actions of intrapaw AM1241 were local to the paw and not the result of systemic uptake and spread to distant sites, as shown by the lack of effect of equivalent doses of AM1241 administered into the contralateral paw. Local peripheral effects of AM1241 are consistent with our finding that administration of AM1241 in the tested hind paw produced antinociception to thermal stimuli in otherwise untreated animals, whereas administration in the contralateral paw did not.[9]

The effects of AM1241 seem to be mediated by CB_2 receptors. They were fully reversed by coadministration of the CB_2 receptor–selective antagonist AM630, whereas coadministration of the CB_1 receptor–selective antagonist AM251 had no effect. These results are consistent with our previous demonstration that the antinociceptive effects of AM1241 are mediated by the CB_2 receptor.[9] In addition to reversing the inflammation-induced decrease in thermal paw withdrawal latency, CB_2 receptor activation prolonged thermal withdrawal latencies beyond preinflammation values, consistent with the thermal antinociception produced by CB_2 receptor activation.[9]

AM1241 was also active when administered systemically (*i.e.*, intraperitoneally). The effects of systemic AM1241 seem to be mediated at peripheral CB_2 receptors. We have previously shown that the antinociceptive effects of systemic AM1241 were reversed by injection of the CB_2 receptor–selective antagonist AM630 in the tested hind paw.[9] AM1241 was more potent when administered systemically than when injected into the dorsal surface of the ipsilateral paw. We have hypothesized that intraperitoneal AM1241 potency may be high as a result of efficient systemic absorption through the large surface area of the peritoneum, leading to distribution through the circulation to the peripheral site of action.[9] We have also hypothesized that the potency of intrapaw AM1241 may be low because AM1241 does not efficiently penetrate the tissue of the paw from the dorsal surface, where the drug was injected, to the plantar surface, where the test stimulus was applied.[9] Drug administration in the dorsal hind paw was necessary because injection of the vehicle (*i.e.*, dimethyl sulfoxide) into the plantar surface prolonged withdrawal latency. We have also hypothesized that after subcutaneous injection into the paw, systemic absorption of the vehicle (dimethyl sulfoxide) in excess of AM1241 may cause AM1241 to precipitate, thereby resulting in local deposition of the drug and diminished availability of the drug to receptors at the site of action.[9] This hypothesis is supported by the qualitative observation that at necropsy, a white precipitate was frequently observed subcutaneously at the site of injection. The observation that AM1241 is more potent when administered intraperitoneally than when administered locally seems to argue against a local, peripheral effect of AM1241. However, comparison of peripheral and intraperitoneal administration of drugs is complicated by issues of drug distribution, such as those discussed in this paragraph. Therefore, comparison of ipsilateral and contralateral administration of drugs is a much more direct test of a local site of action than is local *versus* systemic administration. The observation that AM1241 is active when administered in the ipsilateral paw, but has no effect when administered contralaterally, argues strongly for a local site of action.

Previous investigators have observed hyperalgesia after administration of the CB_1 receptor–selective antagonist SR141716A or antisense oligodeoxynucleotide-mediated knockdown of spinal CB_1 receptor expression, suggesting the presence of endogenous cannabinoid tone leading to constitutive activation of CB_1 receptors.[19,20] In contrast, in our study, intraperitoneal administration of the CB_1 receptor–selective antagonist AM251 alone did not result in hyperalgesia. This difference may have been caused by differences in the route of administration or in the drug used. Similarly, administration of the CB_2 receptor–selective antagonist AM630 alone did not result in hyperalgesia, suggesting the absence of an endogenous CB_2 receptor–mediated cannabinoid tone.

The hypothesis that activation of CB_2 receptors inhibits inflammatory hyperalgesia came from studies of the endogenous fatty acid ethanolamide, palmitoylethanolamide. Among other actions, palmitoylethanolamide inhibited inflammation-induced edema, carrageenan-induced hyperalgesia of the hind paw, and a referred hyperalgesia caused by inflammation of the bladder.[21,22] The antinociceptive effects of palmitoylethanolamide were blocked by the CB_2 receptor–selective antagonist SR144528, but not by the CB_1 receptor–selective antagonist SR141716A, suggesting that the actions of palmitoylethanolamide were mediated by the CB_2 receptor. However, palmitoylethanolamide has no significant affinity for CB_1 or CB_2 receptors expressed in cultured cells or for CB_2 receptors in rat spleen slices.[23,24] Therefore, it has been proposed that palmitoylethanolamide may indirectly activate CB_2 receptors, perhaps by inhibiting the inactivation of other endocannabinoids that are direct CB_2 receptor ligands.[25] Alternatively, it has been suggested that palmitoylethanolamide may act at an as-yet uncharacterized, possibly cannabinoid-like, receptor.[26] Because of these uncertainties, direct testing of the

effects of CB_2 receptor activation awaited synthesis of direct CB_2 receptor–selective ligands.

In a preliminary report, the CB_2 receptor–selective agonist, 1-(2-(4-morpholinyl)ethyl)-2-methyl-3-(4-bromo-1-naphylcarbonyl)-7-methoxyindole suppressed Freund's adjuvant-induced hypersensitivity of the flexor reflex to mechanical, touch, and pinch stimuli.[27] In a published report, the CB_2 receptor–selective agonist GW405833 prevented carrageenan-induced mechanical hyperalgesia by as much as 50%.[12] This effect was not increased when higher doses were used. Our results significantly extend these findings in two ways. First, we observed a complete reversal of inflammatory thermal hyperalgesia by AM1241, whereas Clayton *et al.*[12] observed only a partial inhibition of inflammatory mechanical hyperalgesia by GW405833. It is not clear whether the differences in maximal effect are the result of differences in the drugs tested, of the different sensory modalities tested, or of the use of a prevention protocol when testing GW405833 and a reversal protocol when testing AM1241. Second, we used site-specific drug injection to show that activation of CB_2 receptors in the periphery is sufficient to reverse inflammatory hyperalgesia.

Our data confirm previous findings that CB_2 receptor activation inhibits tissue inflammation and extend them by showing that CB_2 receptor activation not only prevents but also reverses inflammation-induced edema. Peripheral (intrapaw) AM1241 reversed the increase in paw volume produced by injection of carrageenan in the hind paw. The inhibitory effect of AM1241 was completely blocked by the CB_2 receptor–selective antagonist AM630, whereas the CB_1 receptor–selective antagonist AM251 had no effect. Potential antiinflammatory effects of CB_2 receptor activation were initially suggested by the presence of CB_2 receptors on inflammatory cells.[1,5–7] Antiinflammatory effects of CB_2 receptors were shown when the CB_2 receptor–selective agonist HU-308 inhibited arachidonic acid–induced ear edema[8] and when the CB_2 receptor–selective agonist GW405833 prevented carrageenan-induced paw edema.[12] GW405833 administered 30 min before carrageenan produced approximately 45% inhibition of paw edema that did not increase with further increases in dosage.[12] HU-308 administered 60 min before arachidonic acid[8] or AM1241 administered 3 h after carrageenan also produced only partial inhibition of ear or paw edema. Because only a single dose of each was used, however, it is not known whether these are the maximal effects possible.

It is not known how activation of local, peripheral CB_2 receptors inhibits inflammatory hyperalgesia. One possibility is that CB_2 receptors on the peripheral terminals of primary afferent neurons inhibit transduction or conduction of the pain signal. However, evidence regarding the expression of CB_2 receptors on primary afferent neurons is conflicting. A recent preliminary report showed labeling of medium-diameter cells in trigeminal ganglia by an antibody directed against the CB_2 receptor.[28] However, CB_2 receptor mRNA was not detected in dorsal root ganglia or trigeminal ganglia by *in situ* hybridization, although the presence of CB_1 receptor mRNA was clearly shown.[28,29]

Alternatively, CB_2 receptor agonists could inhibit pain responses by an indirect mechanism. The CB_2 receptor is present on immune and mast cells.[1,5–7] Activation of CB_2 receptors on mast or immune cells could inhibit the release of molecules that sensitize the peripheral nociceptor. In particular, nerve growth factor (NGF) seems to play an essential role in the production of inflammatory hyperalgesia.[30,31] Other mediators, such as interleukin-1β and tumor necrosis factor-α, are also essential for the production of inflammatory hyperalgesia, but seem to contribute to the hyperalgesia primarily by increasing tissue NGF content.[32,33] NGF seems to have a direct action on primary afferent neurons,[34,35] and these actions have been proposed to be mediated by the VR1 vanilloid receptor.[36] NGF and bradykinin have recently been shown to remove phosphatidylinositol-4,5-bisphosphate–mediated inhibition of the VR1 receptor.[37] Similarly, we have hypothesized that AM1241 may produce acute antinociception by inhibiting constitutive release of NGF or other sensitizing substances from mast or immune cells.[9]

Because VR1 vanilloid receptors have been proposed to mediate inflammatory thermal hyperalgesia,[30] we tested the ability of AM1241 to inhibit sensory responses to capsaicin, an agonist of the VR1 receptor. AM1241 inhibited capsaicin-induced inflammatory hyperalgesia and flinching. Because capsaicin acts directly on vanilloid receptors of primary afferent neurons, AM1241 could be exerting its effects by activating CB_2 receptors on primary afferent neurons. Alternatively, as described earlier, CB_2 receptor activation could indirectly inhibit neuronal responses to capsaicin, by inhibiting the release of pronociceptive substances, such as NGF, from inflammatory and immune cells. Although cannabinoid compounds structurally related to anandamide can exert effects at VR1 receptors, it is unlikely that AM1241 does so, because it is structurally dissimilar from anandamide-like compounds. In addition, its actions are blocked by the CB_2 receptor–selective antagonist AM630, a compound unlikely to act at VR1 receptors because it is not structurally similar to anandamide.

The findings presented in this article show that activation of peripheral CB_2 receptors is sufficient to reverse inflammatory thermal hyperalgesia. Because CB_2 receptors are not found in the CNS[1–4] and CB_2 receptor–selective agonists do not produce the CNS effects typical of nonselective cannabinoid receptor agonists,[8,9] CB_2 receptor–selective agonists may have a significant clinical advantage over nonselective cannabinoid compounds. Although it has not been shown that CB_2 recep-

tor–selective agonists are free of CNS effects in the presence of peripheral inflammation, it is likely that they will be. CB_2 receptor agonists are predicted to be effective in treating inflammatory pain without the central side effects of cannabinoids retaining activity at the CB_1 receptor.

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