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Modulation of cytokine responses in *Corynebacterium parvum*-primed endotoxemic mice by centrally administered cannabinoid ligands

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Abstract

The cannabinoid receptor agonists [(−)-11-hydoxy-Δ^8tetrahydrocannabinol-dimethylheptyl] (HU-210) and {(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl[pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthalenyl) methanone} (WIN 55212-2) were previously shown to downregulate inflammatory cytokines (tumor necrosis factor α and interleukin-12) and to upregulate antiinflammatory interleukin-10 when administered intraperitoneally (i.p.) to mice before an endotoxin challenge. Cytokine modulation coincided with the onset of behavioral changes that are associated with cannabinoid agonist activated central cannabinoid CB_1 receptors. Both effects were antagonized by [*N*-(piperdin-1-yl)-5-(4-chloropheny)-1-(2,4-dichloropheny)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] (SR141716A) a selective cannabinoid CB_1 receptor antagonist. In the present study, we have investigated further the apparent role of central CB_1 cannabinoid receptors in cytokine modulation by HU-210 and WIN 55212-2. When administered intracerebroventricularly (i.c.v.), the drugs modulated cytokine responses at doses that were threefold to fourfold lower than those found effective by the i.p. route. SR141716A blocked cytokine modulation when coadministered centrally with the agonists, while a selective cannabinoid CB_2 receptor antagonist, {*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3 methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide} (SR144528) had no effect. Surprisingly, SR144528 was found to modulate cytokines itself when injected i.c.v.

Keywords: HU-210; WIN 55212-2; SR141716A; SR144528; *C. parvum*; Cytokine; (Mouse)

1. Introduction

The biological effects of cannabinoid ligands are mediated through cannabinoid specific G-protein-coupled receptors (GPCR). The cannabinoid CB_1 receptor, which is highly expressed in tissues of the central nervous system (CNS) and conserved across species, has been cloned from CNS tissues of the rat (Matsuda et al., 1990), mouse (Chakrabarti et al., 1995; Abood et al., 1997) and human (Gerard et al., 1991). Although in earlier studies, cannabinoid CB_1 receptors were detected centrally in various areas of the brain and peripherally in the testis, they are now known to be more broadly distributed in the periphery (reviewed in Felder and Glass, 1998). The cannabinoid CB_2 receptor, which is more variable among species, has been cloned from human and mouse (Munro et al., 1993; Shire et al., 1996) and has been localized to cells in the periphery, particularly those of the immune system (Galiegue et al., 1995; Griffin et al., 1997; Carayon et al., 1998). It is still unclear as to whether cannabinoid CB_2 receptors are present in the CNS since studies in rats have yielded both positive and negative results (Skaper et al., 1996; Griffin et al., 1999; Lu et al., 2000).

Since the cannabinoid CB_2 receptor is present on the surface of immune cells, it has been widely assumed that the immunomodulatory and antiinflammatory activities of cannabinoid receptor agonists, such as Δ^9-tetrahydrocannabinol, are mediated through this receptor subtype (reviewed in Klein et al., 2000a). However, there is also a substantial body of evidence indicating a role for the cannabinoid CB_1 receptor in the antiinflammatory effects of cannabinoid receptor ligands (Jaggar et al., 1998; Richardson et al., 1998). The results obtained in a recent

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study of cytokine modulation by cannabinoid receptor agonists in *Corynebacterium parvum*-primed mice also led us to conclude that the antiinflammatory activities of these drugs can be mediated through the cannabinoid CB_1 receptor (Smith et al., 2000). When challenged with endotoxin, [(−)-11-hydoxy-Δ^8 tetrahydrocannabinol-dimethylheptyl] (HU-210)- and {(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl[pyrrolo[1,2,3-de]1,4- benzoxazin-6-yl] (1-naphthalenyl) methanone } (WIN 55212-2)-treated mice had reduced serum levels of tumor necrosis factor α (TNF-α) and interleukin-12 and elevated levels of interleukin-10 in comparison to those of control mice. The doses of HU-210 and WIN 55212-2 that caused cytokine modulation could not be separated from those that produced the characteristic CNS changes (hypomobility, catalepsy and hypothermia) that have been ascribed to cannabinomimetic agents. Both agonist-mediated effects were blocked by [*N*-(piperdin-1-yl)-5-(4-chloropheny)-1-(2,4-dichloropheny)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] (SR141716A), a selective cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1995; Compton et al., 1996), but not by {*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3 methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide} (SR144528), a selective cannabinoid CB_2 receptor antagonist (Rinaldi-Carmona et al., 1998). The doses of SR141716A that were required to antagonize the agonist effects on cytokines were identical to those that antagonized the central nervous system (CNS) changes caused by the agonists. Together, these observations suggested that cytokine modulation and the CNS effects that were caused by treatment with HU-210 and WIN 55212-2 occurred through activation of the central cannabinoid CB_1 receptor.

While results from this laboratory suggest a major role for central cannabinoid CB_1 receptors in cytokine modulation, there are indications that such responses can be modulated through the cannabinoid CB_2 receptor as well. Δ^9-Tetrahydrocannabinol was found to suppress interferon γ and interleukin-12 production in mice infected with *Legionella pneumophilia* (Klein et al., 2000a,b). These effects were reversed by SR141716A and SR144528, suggesting the involvement of both cannabininoid receptor subtypes in cytokine modulation by the cannabinoid receptor agonist. Since the antagonists were injected i.v. into mice, it was not clear whether the antagonism exhibited by SR141716A on cytokine modualtion by Δ^9-tetrahydrocannabinol was a result of binding to central, peripheral or both central and peripheral cannabinoid CB_1 receptors.

The present study was undertaken to further explore the potential role of central cannabinoid CB_1 receptors in cytokine modulation by cannabinoid receptor ligands. We describe the results of experiments in which HU-210 and WIN 55212-2 were examined for effects on endotoxin-induced cytokines when administered in low doses to mice by the intracerebroventricular (i.c.v.), intraperitoneal (i.p.) and intravenous (i.v.) routes. We also show that the selective cannabinoid CB_2 receptor antagonist, SR144528, is capable of modulating cytokine responses itself when administered centrally to mice.

2. Materials and methods

2.1. Mice

Specific pathogen-free BDF1 male mice, 8–12 weeks old (Jackson Laboratories, Bar Harbor, ME), were used in this study. Cannabinoid CB_1 receptor knockout mice (Zimmer et al., 1999) were obtained from Dr. Andreas Zimmer and bred at Charles River (Boston, MA).

2.2. Priming with C. parvum and induction of lethal endotoxemia in primed mice

C. parvum (*Proprionibacterium acnes*) cells were obtained from the American Type Culture Collection (Rockville, MD). Normal BDF1 mice and CB1 receptor knockout mice and wild-type controls were primed with *C. parvum*, by an i.v. injection of 0.5 mg heat-killed cells of ATCC strain 11827 as previously described (Smith et al., 1996). An endotoxemic response was induced by challenging the mice with endotoxin (20 μg, lipopolysaccharide, *Escherichia coli* serotype 055:B5, Difco Laboratories, Detroit, MI), i.v. or i.p., 1 week after priming.

2.3. Determination of serum cytokine levels

TNF-α, interleukin-10 and interleukin-12 levels were measured by enzyme-linked immunosorbent assays (ELISAs) in which monoclonal anti-TNF-α (hamster clone TN3-19.12), anti-interleukin-10 (rat clone JES5-2A5) and anti-interleukin-12 (rat clone c15.6) antibodies from Pharmingen (San Diego, CA) were paired with biotinylated goat polyclonal immunoglobulin G (IgG) antibodies from R&D Systems (Minneapolis, MN). The goat polyclonal antibodies purchased from R&D Systems were anti-mouse TNF-α (cat # BAF410), anti-mouse interleukin-12 (cat # BAF419) and anti-mouse interleukin-10 (cat # BAF417). Standard curves were developed with recombinant mouse cytokines from Pharmingen; TNF-α (cat # 19321T), interleukin-12 (cat # 19401W) and interleukin-10 (cat # 19281V).

2.4. Drugs

WIN 55212-2 was purchased from Research Biochemicals International, (Natick, MA) and HU-210 and naloxone from BIOMOL Research Labs (Plymouth Meeting, PA). Nor-Binaltophimine (nor-BNI) was obtained from Tocris

Cookson (Ballwin, MO). SR 141716A and SR 144528 were synthesized according to methods published by Sanofi Recherche (France). HU-210, WIN 55212-2, SR141716A and SR144528 were dissolved in 1:1:18 emulphor/ethanol/saline for i.c.v. administration into conscious mice (Haley and McCormick, 1957). The same vehicle was used to prepare SR141716A and SR144528 for administration by the i.v. route. WIN 555212-2 was dissolved in 20% hydroxypropyl betacyclodextrin and HU-210 was prepared as a stock solution in dimethyl sulfoxide/Tween 80 (DMSO/Tween, 1:1 v/v) and further diluted in saline for i.v. injections. Pertussis toxin (List Biologicals, Campbell, CA) was dissolved in saline and injected into mice, i.c.v. or by the intrathecal (i.t.) route according to the method of Hylden and Wilcox (1980).

2.5. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test using the Graph PAD prism statistical program. P values < 0.05 were considered as statistically significant.

3. Results

3.1. Cytokine modulation following central and peripheral treatment with cannabinoid receptor agonists

The results obtained in a previous work (Smith et al., 2000) suggested a role for the central cannabinoid CB_1 receptor in the modulatory effects of cannabinoid receptor agonists on endotoxin-induced cytokine responses in mice. We reasoned that if cytokine responses were indeed modulated through the central cannabinoid CB_1 receptor, the cannabinoid receptor agonists should be effective at somewhat lower doses when injected directly into the brain (i.c.v.) than when administered i.p. Using this approach, identical doses of HU-210 or WIN 55212-2 were given to *C. parvum*-primed mice by the i.c.v. and i.p. routes, 1 h prior to an endotoxin challenge. The post-challenge levels of circulating cytokines were quantified by ELISAs. In the experiment shown in Fig. 1, HU-210 was found to suppress the endotoxin-induced circulating levels of TNF-α (Fig. 1A) and interleukin-12 (Fig. 1B) in a dose-dependent manner when injected into mice by the i.c.v. route. The levels of TNF-α and interleukin-12 detected in mice

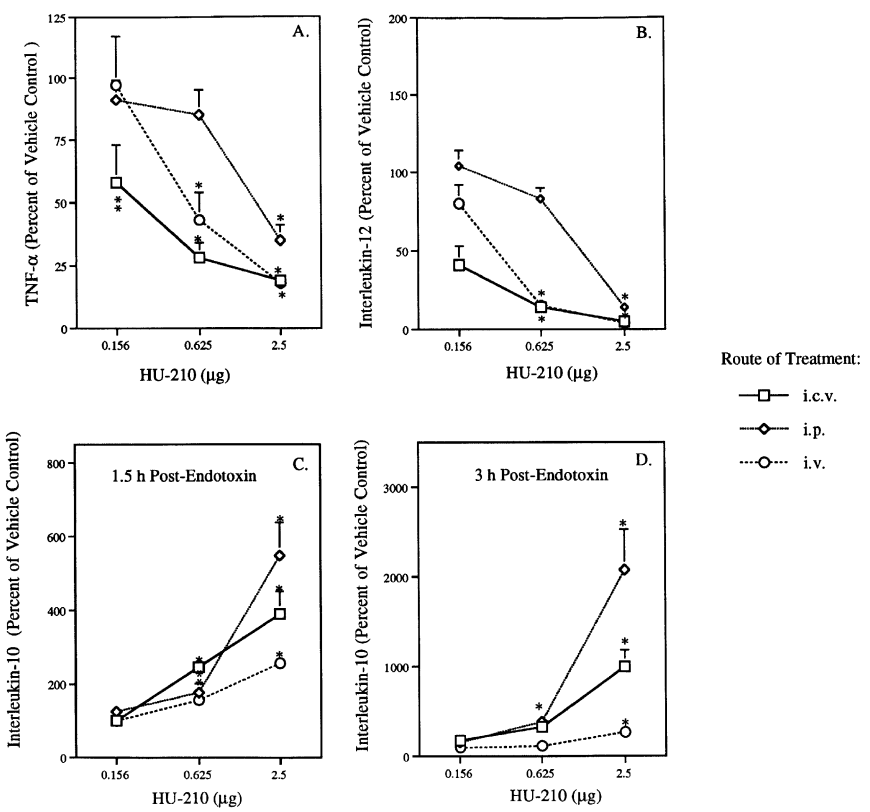

Fig. 1. Cytokine modulation by HU-210 when administered to *C. parvum*-primed mice i.c.v., i.p. or i.v., 1 h before challenge with endotoxin (20 μg, i.v.). The circulating levels of TNF-α (A) and interleukin-12 (B) were measured 1.5 h and 3 h after endotoxin, respectively. Interleukin-10 levels were measured at each timepoint (C) and (D). The data are expressed as the percentage ± S.E. of the mean cytokine levels in vehicle-treated control mice. Data are from one representative experiment of three in which the drugs were administered by the different routes. $N = 6$ for the i.c.v. and i.p. treatments and $N = 12$ for the i.v. treatment. Significantly different from the levels in vehicle-treated control mice, $^{*}P < 0.01$ and $^{**}P < 0.05$ by Bonferroni's test.

given 0.156 μg HU-210 were decreased to 58 ± 15% and 40 ± 12%, respectively, of levels present in vehicle-treated control mice. In mice given 0.625 μg HU-210, the inflammatory cytokine responses were < 30% of those in control mice. These same doses of HU-210 (0.156–0.625 μg) had virtually no effect on TNF-α and interleukin-12 when treatment was i.p., but there was marked suppression of inflammatory cytokines when the dose administered was 2.5 μg (Fig. 1A and B). Antiinflammatory interleukin-10 was significantly enhanced at both the 0.625 and 2.5 μg doses of HU-210 whether the drug was given i.c.v. or i.p. (Fig. 1C and D). Thus, the interleukin-10 response appeared to be slightly more sensitive than either TNF-α or interleukin-12 to modulation by HU-210 when the drug was given i.p., a phenomenon that was also observed in our earlier study. HU-210 (0.625 and 2.5 μg) caused CNS changes (hypomobility or catalepsy) within 10-20 min of treatment by the i.c.v. route. When treatment was i.p., the CNS symptoms were seen only in the 2.5 μg dose group and were more transient in nature than those triggered by the same dose injected i.c.v. These results are in accord with our previous findings of a close relationship between cytokine modulation and the CNS changes that were caused by the HU-210 treatment. Therefore, the present data serve to reinforce the concept that, as with changes in mobility, cytokine modulation by HU-210 is evoked through activation of central cannabinoid CB_1 receptors.

To probe further the apparent close association between cytokine modulation and the CNS changes caused by HU-210, the drug (0.156–2.5 μg) was administered to mice by the i.v. route. In principle, treatment by this latter route should make the drug more accessible to central cannabinoid CB_1 receptors than treatment i.p. That this appeared to be the case is also shown in the experiment presented in Fig. 1, where mice treated with HU-210 i.v. at a dose (0.625 μg) that was inactive when given i.p., had circulating levels of TNF-α (Fig. 1A) and interleukin-12 (Fig. 1B) that were decreased to 43 ± 11% and 15 ± 3%, respectively, of levels present in vehicle-treated control mice.

WIN 55212-2 is a cannabinoid receptor agonist that has been reported to have a higher selectivity for the cannabinoid CB_2 receptor than HU-210 (Felder et al., 1995). The drug exhibited a similar activity profile as HU-210 when evaluated for its effects on cytokine responses by the i.c.v., i.p. and i.v. routes (Fig. 2). The endotoxin-induced TNF-α (Fig. 2A) and interleukin-12 (Fig. 2B) responses were suppressed by WIN 55212-2 at the lowest dose adminis-

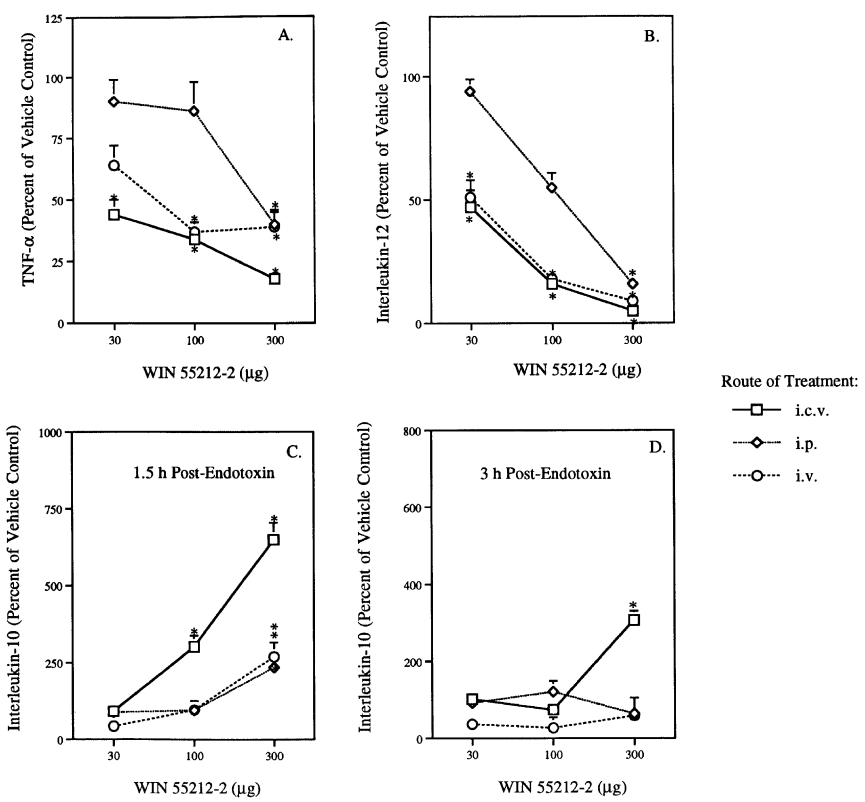

Fig. 2. Cytokine modulation by WIN 55212-2 when administered to *C. parvum*-primed mice by the i.c.v., i.p. and i.v. routes, 1 h before challenge with endotoxin (20 μg, i.v.). The circulating levels of cytokines were determined as described in the legend to Fig. 1. The data shown are expressed as the percentage ± S.E. of the mean cytokine levels in vehicle-treated control mice. $N = 12$ for each treatment group. Significantly different from the levels in vehicle-treated control mice, $^{*}P < 0.01$ and $^{**}P < 0.05$ by Bonferroni's test.

tered (30 μg) when the route was i.c.v. or i.v. Interleukin-10 levels were elevated at doses of 100 μg, i.c.v. and 300 μg, i.v. (Fig. 2C and D). The cytokine responses were only modulated at the high dose (300 μg) when treatment with WIN 55212-2 was by the i.p. route.

WIN 55212-2 caused behavioral changes at all dose levels when administered to mice i.c.v. The animals became cataleptic within 5 min of the treatment, but mice in the 30 and 100 μg dose groups appeared to partially recover from the CNS effects of the drug, exhibiting some increase in mobility at the time of the endotoxin challenge (1 h after drug treatment). Mice in the 300 μg i.c.v. dose group remained in a cataleptic state up to the time of the endotoxin challenge. When the drug was administered by other routes, there was also a strong correlation between the doses that modulated cytokines and those that caused changes in behavior in the mice. The strong correlation that was observed between cytokine modulation and the behavioral changes caused by the HU-210 and WIN 55212-2 treatments is summarized in Table 1.

Table 1
Relationship between CNS changes and cytokine modulation in mice treated with cannabinoid agonists and antagonists

Treatment	Dose(s) (μg/mouse)	Route	Hypomobility/ Catalepsy	Cytokine modulation
HU-210	0.156	i.c.v.	±	±
	0.625		+	+
	2.5		+	+
	0.156	i.v.	−	−
	0.625		+	+
	2.5		+	+
	0.156	i.p.	−	−
	0.625		−	±
	2.5		+	+
WIN 55212-2	30	i.c.v.	+	±
	100		+	+
	300		+	+
	30	i.v.	±	±
	100		+	+
	300		+	+
	30	i.p.	−	−
	100		−	−
	300		+	+
SR141716A	100	i.c.v.	−	±
SR141716A + HU-210	100 + 0.625	i.c.v.	−	± (antagonist activity)
SR141716A + WIN 55212-2	100 + 100	i.c.v.	−	± (antagonist activity)
SR144528	100	i.c.v.	−	+
SR144528 + HU-210	100 + 0.625	i.c.v.	+	+
SR144528 + WIN 55212-2	100 + 100	i.c.v.	+	+

Hypomobility: +, prolonged; ±, transient; −, absent.
Cytokine modulation: +, statistically significant changes in TNF-α, interleukin-12 and interleukin-10; ±, slight, but statistically significant, change in at least one cytokine; −, no modulation.

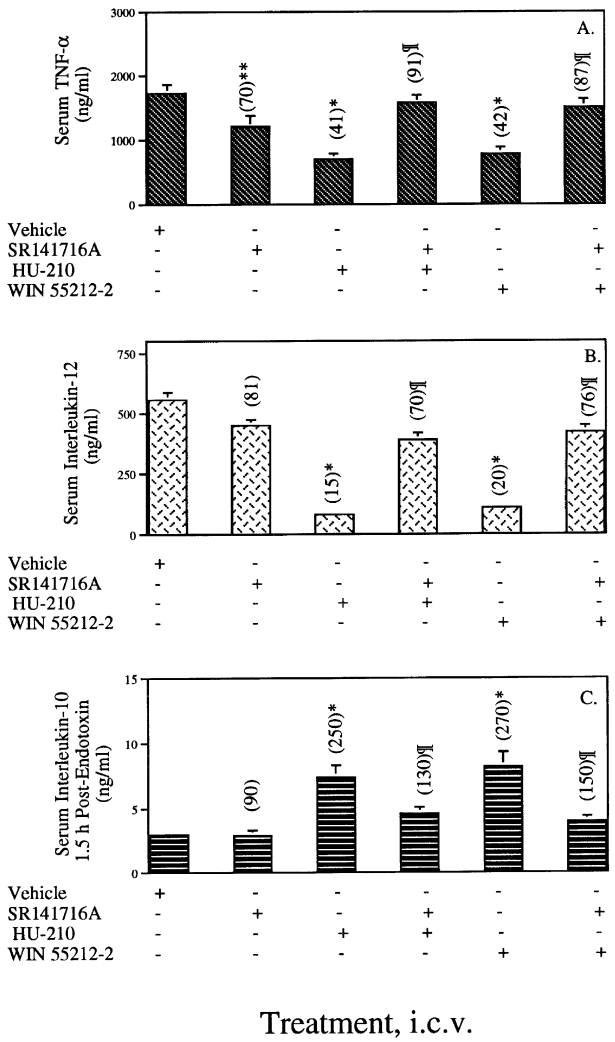

Fig. 3. Effect of a selective cannabinoid CB1 receptor antagonist, SR141716A on cytokine modulation by HU-210 and WIN 55212-2. The cannabinoid receptor agonists were administered to mice by the i.c.v. route alone and in combination with SR141716A. Treatment was 1 h before challenge with endotoxin (20 μg, i.v.). Data were pooled from three independent experiments and the values shown represent the mean ± S.E.M. of 18 mice per treatment group. TNF-α (A) and interleukin-10 (C) were measured 1.5 h after the challenge and interleukin-12 (B) 3 h after challenge. Significantly different from the levels in vehicle-treated control mice, $^{*}P < 0.01$ and $^{**}P < 0.05$ by Bonferroni's test. Significantly different from the levels in mice treated with HU-210 or WIN 55212-2 alone, $^{¶}P < 0.01$.

3.2. Cytokine modulation in mice cotreated centrally with cannabinoid receptor agonists and SR141716A, a cannabinoid CB_1 receptor antagonist

In our previous work, we described the ability of SR141716A, a selective cannabinoid CB_1 receptor antagonist, to block the modulatory activities of HU-210 and WIN 55212-2 when the cannabinoid receptor agonists were given i.p. and the cannabinoid receptor antagonist i.p. or i.c.v. The selective cannabinoid CB_1 receptor antagonist

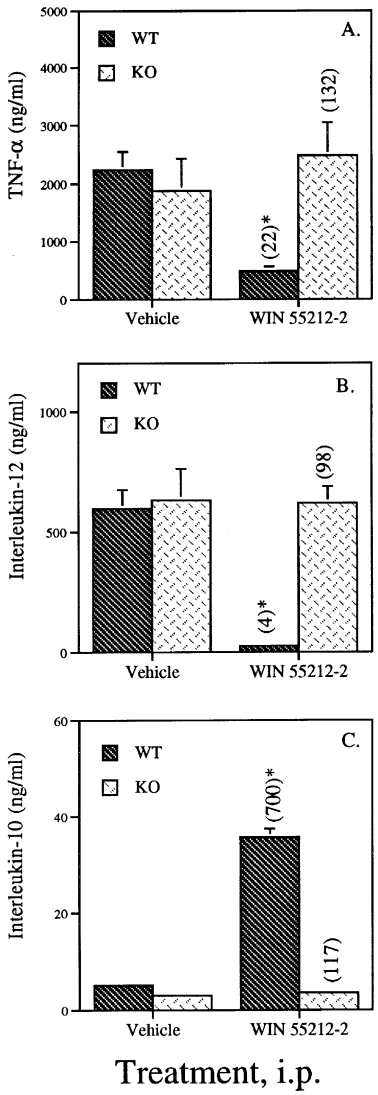

Fig. 4. Cytokine modulation by WIN 55212-2 when administered to *C. parvum*-primed wild-type and cannabinoid CB_1 receptor knockout mice by the i.p. route, 1 h before challenge with endotoxin (20 μg, i.v.). TNF-α (A) and interleukin-10 (C) were measured 1.5 h after the challenge and interleukin-12 (B) 3 h after challenge. There were six mice per treatment group. Significantly different from the levels in wild-type controls, $^{*}P < 0.01$ by Bonferroni's test.

was also found to modulate cytokine responses itself (partial agonism), particularly when administered alone to mice by the i.p. route. The ability of SR141716A to antagonize cytokine modulation by the cannabinoid receptor agonists was also observed in the present experiments when the drug was injected i.c.v. together with HU-210 or WIN 55212-2. Thus, as seen in the experiment presented in Fig. 3, the circulating levels of TNF-α (Fig. 3A) and interleukin-12 (Fig. 3B) were markedly reduced by treatment with the cannabinoid receptor agonists. The cytokine levels in mice that were treated with a cannabinoid receptor agonist and the cannabinoid CB_1 receptor antagonist did not differ significantly from those in vehicle-treated control mice. SR141716A also antagonized the stimulatory effects of the cannabinoid receptor agonists on interleukin-10 (Fig. 3C) and blocked completely their ability to evoke changes in the CNS (hypomobility/catalepsy).

The role of cannabinoid CB_1 receptors in the cytokine modulatory effects of cannabinoid receptor agonists was confirmed in experiments performed in *C. parvum*-primed cannabinoid CB_1 receptor knockout mice. In these experiments, the cannabinoid receptor agonists were administered to mice by the i.p. route in order to discern any

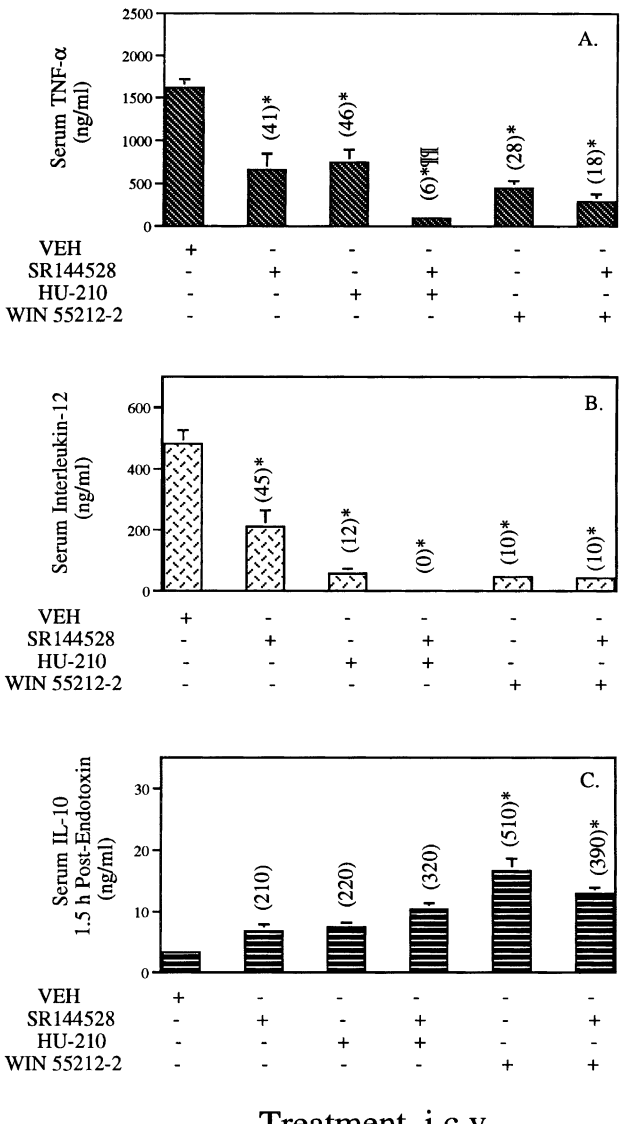

Fig. 5. Cytokine responses in mice treated with a selective cannabinoid CB_2 receptor antagonist, SR144528, alone and in combination with HU-210 or WIN 55212-2. The drugs were administered to mice by the i.c.v. route, 1 h before challenge with endotoxin (20 μg, i.v.). Data were pooled from three independent experiments and the values represent the mean ± S.E.M. of 18 mice per treatment group. TNF-α responses are shown in (A), interleukin-12 responses in (B) and interleukin-10 responses at 1.5 h post-endotoxin in (C). Significantly different from the levels in vehicle-treated control mice, $^{*}P < 0.01$ by Bonferroni's test. Significantly different from the levels in mice treated with HU-210 alone, $^{¶¶}P < 0.05$.

involvement of peripheral cannabinoid CB_2 receptors in cytokine modulation by the agonists. As shown in Fig. 4, WIN 55212-2 modulated endotoxin-induced cytokine responses in wild-type mice, but not in cannabinoid CB_1 receptor knockout mice. The pattern of cytokine modulation in wild-type mice was identical to that observed in primed BDF1 mice; decreased TNF-α and interleukin-12 (Fig. 4A and B) and increased interleukin-10. WIN 55212-2 also failed to evoke CNS changes in the knockout mice. Like WIN 55212-2, HU-210 failed to modulate cytokines or cause behavioral changes when given to knockout mice by the i.p. route (data not shown).

3.3. Effects of pretreatment with pertussis toxin on cytokine modulation by WIN 55212-2 and HU-210

The antinociceptive effects of i.c.v. or intrathecally (i.t.) administered cannabinoid receptor agonists were shown to be blocked by an i.t. injection of pertussis toxin (Welch et al., 1995). Thus, it appeared that cannabinoid-induced antinociception was mediated by pertussis toxin-sensitive G_i/G_o proteins. In the present study, mice were pretreated with pertussis toxin using different experimental protocols where the route of administration (i.c.v. or i.t.), the dose (0.5–2.0 μg/mouse) and the time of pretreatment (1–7 days) relative to the injection of WIN 55212-2 and HU-210 were varied. Cyokine modulation by WIN 55212-2 and HU-210 was not affected by pertussis toxin under any of the conditions of pretreatment that were employed (data not shown). The doses of pertussis toxin that failed to affect cannabinoid receptor agonist-mediated cytokine modulation were identical to those used in other studies to attenuate cannabinoid- and opioid-induced antinociception (Sanchez-Blazquez and Garzon, 1991; Welch et al., 1995; Tseng and Collins, 1996).

3.4. Cytokine modulation by centrally administered SR144528

SR144528, a selective cannabinoid CB_2 receptor antagonist, did not antagonize the modulatory effects of HU-210 and WIN 55212-2 on cytokine responses when coinjected i.c.v. with the cannabinoid receptor agonists (Fig. 5). In fact, the circulating levels of TNF-α (Fig. 5A) and interleukin-12 (Fig. 5B) induced by endotoxin in mice treated with the cannabinoid CB_2 receptor antagonist alone were significantly lower than the levels in control mice (41% and 45% of control, respectively). There was no evidence of antagonism by SR144528 of the modulated cytokine responses that resulted from treatment with HU-210 or WIN 55212-2. The cannabinoid CB_2 receptor antagonist was also ineffective in blocking the CNS changes caused by the cannabinoid receptor agonists.

Cytokine modulation by central SR144528 was examined more extensively in subsequent experiments. The cannabinoid CB_2 receptor antagonist was found to reproducibly suppress the production of TNF-α and interleukin-12 in response to endotoxin, but was somewhat variable in its stimulation of interleukin-10. To better delineate the effects of SR144528 on interleukin-10 production, mice treated centrally with the drug were given an i.p. challenge with endotoxin. The i.p. challenge was used because peritoneal macrophages and liver Kupffer cells are excellent sources of interleukin-10 (Knolle et al., 1997; Seki et al., 1998) and are more directly exposed to endotoxin when the agent is injected into the peritoneal cavity. Data pooled from a relatively large number of experiments are summarized in Table 2. There were similar levels of circulating TNF-α, interleukin-12 and interleukin-10 in vehicle-treated control mice whether the challenge was given by the i.v. or i.p. route. Moreover, the modulatory effects of SR144528 on cytokine responses could be observed following an endotoxin challenge by either route. However, the drug did appear to have a slightly stronger modulating effect on interleukin-12 and interleukin-10 when the endotoxin challenge was i.p. (Table 2).

While SR141716A was shown to act as a partial agonist on cytokine responses when injected into mice alone by the i.p. route, this property was much less apparent when the drug was injected i.c.v. An experiment in which the cannabinoid receptor antagonists were compared for dose-dependent effects on cytokine responses when adminis-

Table 2
Summary of the modulatory effects of SR144528 on cytokine responses when administered to mice by the i.c.v. route

Number of Experiments	Treatment i.c.v.	Route of endotoxin challenge	Serum cytokine Levels (ng/ml)			
			TNF-α	Interleukin-12	Interleukin-10 (1.5 h)	Interleukin-10 (3 h)
10	VEH	i.v.	1553 ± 100	404 ± 36	2.7 ± 0.3	3.3 ± 0.4
	SR144528	i.v.	866 ± 75 (56)[a]	171 ± 17 (42)[a]	5.1 ± 0.9 (190)[b]	3.6 ± 0.7 (110)
17	VEH	i.p.	2127 ± 156	369 ± 25	2.6 ± 0.2	3.8 ± 0.4
	SR144528	i.p.	1066 ± 155 (50)[a]	101 ± 14 (27)[a]	7.9 ± 1.4 (300)[b]	5.7 ± 0.8 (150)[a]

The cannabinoid CB_2 receptor antagonist, SR144528 (100 μg), was injected into *C. parvum*-primed mice by the i.c.v. route, 1 h prior to challenge with endotoxin. Serum levels of TNF-α and interleukin-12 were measured by ELISA, 1.5 and 3 h, respectively, after the challenge. Interleukin-10 levels were measured at both timepoints. Data (means ± S.E.M.) were pooled from multiple experiments as shown, with six mice per treatment group in each experiment.

The value in the parenthesis is the percent of the vehicle control response.

[a] $P < 0.0001$ vs. controls.

[b] $P < 0.001$ vs. controls.

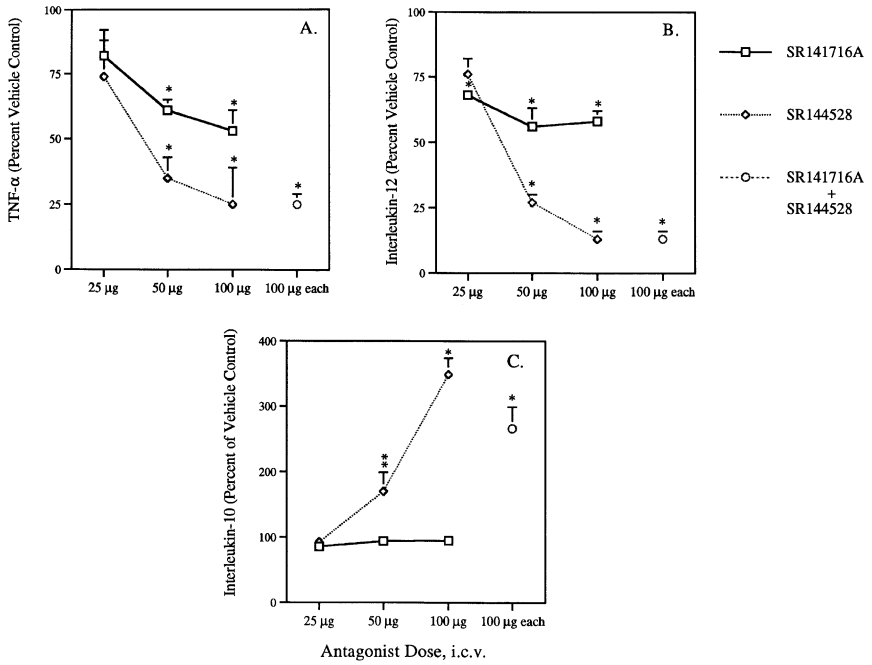

Fig. 6. Dose-dependent inhibition of TNF-α (A) and interleukin-12 (B) and stimulation of interleukin-10 (C) in mice treated with SR141716A and SR144528 by the i.c.v. route 1 h before challenge with endotoxin (20 μg, i.p.). Cytokine responses were also assessed in mice that were co-injected with the cannabinoid receptor antagonists (100 μg, each). TNF-α and interleukin-10 were measured 1.5 h after endotoxin and interleukin-12, 3 h after endotoxin. Data are from one representative experiment of four in which the antagonists were evaluated for dose-dependent effects on cytokine responses. The data are expressed as the percentage ± S.E. of the mean cytokine levels in vehicle-treated control mice. $N = 6$ for each treatment group. Significantly different from the levels in vehicle-treated control mice, * $P < 0.01$ and ** $P < 0.05$ by Bonferroni's test.

tered i.c.v. to mice is shown in Fig. 6. SR144528 was found to be a more effective modulator of cytokine responses than SR141716A. In addition, the cannabinoid CB_1 receptor antagonist had no effect on cytokine modulation by SR144528 when the two agents were coinjected i.c.v. It can be concluded from these observations that the central cannabinoid CB_1 receptor has no role in cytokine modulation by SR144528.

Since SR144528 did not appear to produce its effects through central cannabinoid CB_1 receptors and with the reported absence of cannabinoid CB_2 receptors in the brain, it was likely that noncannabinoid receptors were involved in cytokine modulation by the cannabinoid CB_2 receptor antagonist. Opioid receptors were considered to be one possibility, since such receptors were shown to be involved in the analgesic response of mice to an injection of Δ^9-tetrahydrocannabinol (Reche et al.,1996). The analgesia induced by Δ^9-tetrahydrocannabinol was partially blocked by the nonspecific opioid receptor antagonist naloxone and by the κ-opioid receptor antagonist nor-binaltorphimine. However, neither of these agents was found to have any effect on cytokine modulation by SR144528 when coadministered centrally or peripherally to mice at doses that were reported to be effective in inhibiting Δ^9-tetrahydrocannabinol-induced analgesia (data not shown).

3.5. Protective effects of SR144528 against lethal endotoxemia

Consistent with its ability to decrease the production of inflammatory cytokines in response to endotoxin, SR144528 (100 μg, i.c.v.) was also found to protect mice from a lethal endotoxin challenge. In the experiments shown in Fig. 7, approximately 80% of the SR144528-

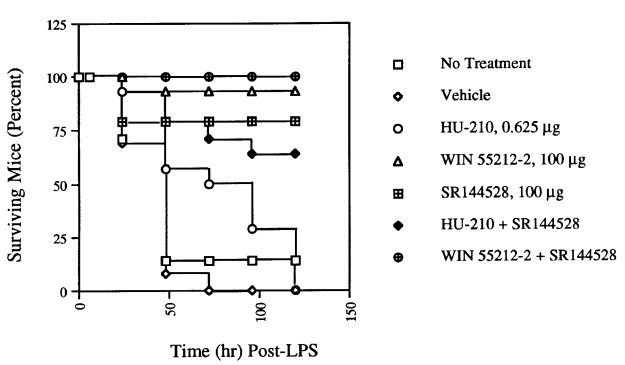

Fig. 7. Protective effects of HU210, WIN55212-2 and SR144528 on endotoxin-induced lethality when administered to *C. parvum* mice by the i.c.v. route. The cannabinoid receptor agonists were also administered to groups of mice together with the cannabinoid CB_2 receptor antagonist. Data were pooled from two identical experiments each containing seven mice per treatment group.

treated mice survived a lethal dose of endotoxin, while none of the vehicle-treated mice and only 14% of the untreated mice survived the challenge. WIN 55212-2 (100 μg, i.c.v.) afforded complete protection to the mice, while HU-210 (0.625, μg, i.c.v.) only delayed the onset of death despite its ability to strongly modulate cytokine responses at that dose. HU-210 was completely protective when administered to mice i.c.v. at a dose of 2.5 μg (data not shown).

4. Discussion

The present study provides additional evidence in support of the hypothesis that central cannabinoid CB_1 receptors are involved in cytokine modulation by HU-210 and WIN 55212-2. TNF-α and interleukin-12 were the inflammatory cytokines most frequently measured in the experiments presented because TNF-α was found to be least sensitive to modulation by cannabinoid receptor agonists, while interleukin-12 (along with interferon γ) was most sensitive. TNF-α and interferon γ appear to stimulate the production of interleukin-12 in *C. parvum* mice and are the direct cytokine mediators of lethal endotoxemia in this model (Smith et al., 1994, 1999). As previously observed, when HU-210 and WIN 55212-2 were administered peripherally to *C. parvum* mice prior to an endotoxin challenge, treatment with the cannabinoid receptor agonists by the i.c.v. route also resulted in reduced circulating levels of TNF-α and interleukin-12. The decrease in inflammatory cytokines was accompanied by an increase in the circulating levels of antiinflammatory interleukin-10. HU-210 and WIN 555212-2 modulated cytokines by the i.c.v. route at doses that were fourfold to sixfold lower than those needed to produce such effects when treatment was i.p. Cytokine modulation, whether caused by central or peripheral cannabinoid receptor agonist treatment, was antagonized when SR141716A, the selective cannabinoid CB_1 receptor antagonist, was given centrally. Mice in which cytokine responses were modulated by treatment with the cannabinoid receptor agonists also exhibited hypomobility or catalepsy indicative of agonist activity at the central cannabinoid CB_1 receptor. Such behavioral changes were never observed when cytokine modulation by the cannabinoid receptor agonists was effectively antagonized by SR141716A. These observations further implicate central cannabinoid CB_1 receptors in cytokine modulation by HU-210 and WIN 55212-2.

The ability of HU-210 and WIN 55212-2 to modulate cytokine responses at lower doses when administered i.c.v. as compared to the doses that were needed to show effects by the i.p. route, did not appear to be a matter of poor absorption into the circulation. The CNS changes caused by the cannabinoid receptor agonist treatment were rapid in onset at those doses that modulated cytokines by the i.p. route. Thus, when given i.p. at cytokine modulating doses, HU-210 and WIN 55212-2 appeared to be rapidly absorbed into the circulation and readily crossed the blood brain barrier. The requirement for higher i.p. doses of the cannabinoid receptor agonists was probably reflective of the blood levels that were needed to allow the drug to reach the CNS at concentrations that were sufficient to activate central cannabinoid CB_1 receptors.

Cytokine modulation by the selective cannabinoid CB_2 receptor antagonist SR144528 when injected i.c.v. was particularly surprising in view of the reported paucity of cannabinoid CB_2 receptors in the CNS (Griffin et al., 1999) and the failure in this study to observe modulation when the drug was injected into the periphery of mice where cannabinoid CB_2 receptors are in relative abundance. Therefore, it is likely that SR144528 exerts its effects on cytokines by acting at a receptor other than a cannabinoid receptor. The receptors involved do not appear to be central opioid receptors since naloxone, a nonspecific opioid receptor antagonist and nor-binaltorphimine a selective kappa opioid receptor antagonist failed to block its effects. These findings, are consistent with published reports of the inability of the drug to bind to opioid receptors in vitro (Rinaldi-Carmona et al., 1998). We are presently assessing the ability of selective mu- and delta-opioid receptor antagonists to reverse cytokine modulation by SR144528.

There are also in vitro data indicating that SR144528 has little or no affinity for other receptor types that are prominently expressed in the CNS (Rinaldi-Carmona et al., 1998). Its ability to selectively bind to cannabinoid CB_2 receptors, which was initially demonstrated in in vitro and ex vivo experiments (Rinaldi-Carmona et al., 1998), has now been confirmed in studies in vivo (Hanus et al., 1999). In the latter study, SR144528 was found to block (or partially block) several biological responses (hypotension, inhibited defecation, antiinflammatory activity and analgesia) that were elicited in mice by systemic treatment with HU-308, a highly selective cannabinoid CB_2 receptor agonist. These data indicate that SR144528 is a functional antagonist at peripheral cannabinoid CB_2 receptors in vivo, as well as in vitro a finding that is helpful in attempts to interpret the results obtained with the cannabinoid CB_2 receptor antagonist in the present experiments. Thus, it can be assumed that SR144528 was capable of binding to peripheral cannabinoid CB_2 receptors when injected into endotoxemic mice, by the i.p. or i.v. routes. Yet, there was no hint of modulatory effects on cytokines when the drug was administered to mice by these routes. This makes it unlikely that cytokine modulation by centrally administered SR144528 resulted from "leakage" of the drug into the circulation and activation of peripheral cannabinoid CB_2 receptors. The more likely possibilities are that the drug acts through noncannabinoid central receptors or through "CB_2-like" central receptors that have been difficult to detect.

It can be concluded from the results of this investigation and those obtained in an earlier study that cytokine modulation by some cannabinoid receptor agonists is mediated through central cannabinoid CB_1 receptors. However, the data generated in these studies do not mean that cytokine modulation by cannabinoid receptor ligands occurs exclusively through these receptors. The difficulty in defining the role of the cannabinoid receptor subtypes in the biological actions of cannabinoid compounds is underscored by the differences that have been observed when cannabinoid CB_1 and CB_2 receptor antagonists were used to block agonist-mediated antiinflammatory and hypotensive responses. Thus, the cannabinoid CB_1 receptor antagonist SR141716A did not antagonize the hypotensive and antiinflammatory responses that were induced with the selective cannabinoid CB_2 receptor agonist HU-308 (Hanus et al., 1999), but was shown to block such responses when evoked by endogenous and exogenous anandamide (Varga et al., 1995; Lake et al., 1997a,b; Richardson et al., 1998). Therefore, it appears that the hypotensive and antiinflammatory effects of cannabinoid compounds can occur through interactions with cannabinoid CB_1 or CB_2 receptors. There are published observations which suggest that this may also be the case with cytokine modulation by cannabinoid receptor agonists (Klein et al., 2000b) although in our studies, cannabinoid CB_2 receptors have not been implicated in such effects. It is particularly noteworthy that we were unable to modulate cytokine responses with WIN 55212-2 and HU-210 in cannabinoid CB_1 receptor knockout mice, which presumably have intact cannabinoid CB_2 receptors. This latter result strongly suggests that cytokine modulation is not evoked through cannabinoid CB_2 receptors at least in mouse models of endotoxemia. Thus, in addition to pharmacological agents that are highly selective for one or the other cannabinoid receptor subtype, mice with targeted disruptions of the cannabinoid CB_1 or CB_2 receptor gene will be immensely helpful in determining the role of cannabinoid CB_1, cannabinoid CB_2 or noncannabinoid receptors in the various biological activities that are being discovered in experiments with cannabinoid compounds.

We have also observed effects of centrally administered cannabinoid receptor agonists on more localized inflammatory responses in the periphery of mice (manuscript in preparation). Therefore, it is our hypothesis that cytokine modulation by cannabinoid receptor agonists is initiated through central cannabinoid CB_1 receptors and leads to the formation of a factor(s) that is the direct mediator of the pharmacological actions of the cannabinoid receptor agonists in the periphery. We have considered the possibility that cytokine modulation by cannabinoid receptor agonists is in some way linked to stimulation of the hypothalamic–pituitary–adrenal axis. However, this is also an unlikely possibility because HU-210 and WIN 55212-2 do not cause an elevation in serum corticosterone and their modulating activities are unaffected by corticotrophin-releasing factor (CRF) antagonists, such as α-helical corticotrophin-releasing factor 9-41 (α-helical CRF 9-41) and astressin (unpublished observations).

In summary, the results presented here provide additional evidence for the involvement of central cannabinoid CB_1 receptors in the modulatory effects of HU-210 and WIN 55212-2 on endotoxin-induced cytokine responses. These effects of the cannabinoid receptor agonists do not appear to be mediated by pertussis toxin-sensitive G_i/G_o proteins. Although there was no direct demonstration of the uncoupling of the cannabinoid CB_1 receptor following in vivo treatment with pertussis toxin, our experimental conditions were similar to those that have been used successfully to uncouple several CNS receptors from G_i protein-linked signaling pathways. Furthermore, there is also evidence that the cannabinoid CB_1 receptor can couple to a stimulatory G protein (G_s-protein, Glass and Felder,1997), raising the possibility that G_s-protein (and not G_i-protein)-dependent mechanisms may be involved in cytokine modulation by the cannabinoid receptor agonists. Thus, the mechanism by which WIN 55212-2 and HU-210 modulate cytokine responses remains to be determined.

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