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In vivo and in vitro treatment with the synthetic cannabinoid CP55,940 decreases the in vitro migration of macrophages in the rat: involvement of both CB1 and CB2 receptors

Paola Sacerdote*, Paola Massi, Alberto E. Panerai, Daniela Parolaro

Department of Pharmacology, University of Milano, via Vanvitelli 32, 20129 Milano, Italy

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Abstract

Cannabinoids have been shown to affect immune responses, acting on different populations of immune cells. In the present paper we analyze the ability of in vivo and in vitro treatment with the potent synthetic cannabinoid CP55,940 to interfere with an important function of rat peritoneal macrophages, i.e. spontaneous migration and formyl-metionyl-leucine-phenylalanine (fMLP)-induced chemotaxis, that were assessed by the use of a Boyden-modified microchemotaxis chamber. When added in vitro, CP55,940 induced a significant and dose-dependent inhibition of both spontaneous migration and fMLP-induced chemotaxis. Both the Cannabinoid Receptor 1 (CB1) and the Cannabinoid Receptor 2 (CB2) antagonists were able to block the CP55,940-induced inhibition of spontaneous migration, although the CB2 antagonist was more potent and only the CB2 antagonist was able to reverse the effect of CP55,940 on fMLP-induced chemotaxis. Similarly, in the in vivo experiments, 1 h after the acute subcutaneous administration of 0.4 mg/kg of CP55,940, both spontaneous motility and chemotaxis were reduced. The pretreatment with the CB2 antagonist, but not with the CB1 antagonist, was able to prevent this effect. Our data confirm that cannabinoids can affect some macrophage functions, mainly throughout CB2 receptors, and suggest that the development of specific CB2 ligands may lead to an interesting new class of anti-inflammatory drugs.

Keywords: Macrophage; CP55, 940; Chemotaxis; Cannabinoid; CB1 receptor; CB2 receptor; Inflammation

1. Introduction

In recent years, a consensus has been reached that cannabinoids can influence immune function (Klein et al., 1998). Delta-9-tetrahydrocannabinol (THC), the major psychoactive component in marijuana extracts, as well as the potent synthetic cannabinoid ligands CP55,940 and R-WIN 55,212, are able to decrease multiple immune responses, interacting with most cells of the immune system both in the rodent and in the human. Cannabinoids can inhibit several T lymphocytes functions such as proliferation and cytotoxicity (Patrini et al., 1997; Cabral and Dove-Pettit, 1998; Klein et al., 1998; Massi et al., 1998, 2000), decrease antibody formation by B cells (Klein et al., 1998) and affect production of several cytokines, mostly decreasing the ThI cytokines IL-2, IFNγ and IL-12 and either increasing or not affecting Th2 cytokines IL-4 and IL-10 (Klein et al., 1995, 1998; Massi et al., 1998). Macrophage functions are also affected; several studies on mouse and rat peritoneal macrophages and cell lines in culture showed that various CBR ligands suppressed various functions including phagocytosis, protein expression, cytolysis and antigen presentation (Klein et al., 1998; Cabral and Dove-Pettit, 1998; Kaminsky, 1998). The macrophage plays a major role in both innate and acquired immunity to infections (Wahl, 1981). The ability of these cells to be chemotactically attracted to the site of initial microbial invasion or to an inflammatory focus is fundamental for the full activation of the immune response that follows (Schiffmann, 1982). In the present work we wanted therefore to analyze the effects of the potent cannabinoid compound CP55,940 on rat macrophage spontaneous migration and chemotaxis induced by the bacterial peptide fMLP, early step of the activation of these cells. Most of the studies showing that macrophage functions are regulated by cannabinoids have been conducted in vitro, and the transition from in vitro to in vivo treatment can be difficult. We therefore tested the ability of

*Corresponding author. Tel.: +003-902-719-750; fax: +003-902-730-470.

E-mail address: paola.sacerdote@unjmi.it (P. Sacerdote).

both in vitro and in vivo treatment with CP55,940 to interfere with macrophage locomotion.

Cannabinoids have been shown to induce their biological effects by binding to specific cannabinoid receptors (CBR) (Klein et al., 1998). To date, two CBRs have been identified, designated Cannabinoid Receptor 1 (CB1) and Cannabinoid Receptor 2 (CB2). Although CB1 is predominantly expressed in the brain, it has also been detected in testis, spleen cells and leukocytes, with levels in B cells >NK cells >PMNs >CD8 cells >monocytes >CD4cells (Kaminsky et al., 1992; Galiegue et al., 1995). The CB2 subtype is present principally in the immune system and its level of expression was found to be higher than that of CB1 in immune cells (Kaminsky et al., 1992; Galiegue et al., 1995, Massi et al., 1997). Recently, selective antagonists for the CB1 and CB2 receptors became available and provide a tool to better understand the role of the CBRs in cannabinoid immunomodulation (Rinaldi Carmona et al., 1994, 1998). We therefore tested the ability of the specific antagonists to interfere with the effects of CP55,940 on spontaneous migration and chemotaxis.

2. Methods

2.1. Drugs

The chemotactic peptide formyl-metionyl-leucine-phenylalanine (fMLP) (Sigma, St Louis, MO, USA) was stored as a stock solution of 10^{-2} M in dimethyl sulfoxide (DMSO, Sigma) at −80°C and diluted in Hanks solution (LPS free), just prior to assay.

CP55,940 (Tocris Cookson, Bristol, UK), SR141716A and SR144528 (generous gifts of Sanofi Recherche, Montpellier, France) were stored as stock solution of 10^{-3} M in DMSO, and prepared fresh for each experiment in Hanks solution at the desired concentrations. In the in vivo experiments, CP55,940 was dissolved in 1:1:18 cremophor, ethanol, saline. The CB1 receptor antagonist SR141716A, and the CB2 receptor antagonist SR144528 were dissolved in distilled water with 0.1% Tween 80 (Sigma) and DMSO.

2.2. Animals

Male Sprague–Dawley rats (200–250 g body wt., Charles River, Calco, Italy) were housed at 22±2°C with a light–dark cycle of 12:12 h and free access to water and food.

2.3. Collection of peritoneal macrophages

Each animal was decapitated, the abdomen was rinsed with 70% ethanol, the abdominal skin was carefully dissected without opening the peritoneum, and 15 ml of Hank's solution (Sigma) were injected intraperitoneally. Then the abdomen was massaged and approximately 10 ml of Hank's solution was removed. Resting macrophages, determined by morphology, were counted in a Neubauer chamber and were adjusted with the same medium to 3×10^5 cells per ml.

Cellular viability was routinely determined before and after each experiment using the trypan blue exclusion test.

The cells were not pooled, but chemotaxis was assayed on cells derived from the single animals. The experiments were repeated six times.

2.4. Chemotaxis assay

Chemotaxis was measured using a Boyden-modified 48-well microchemotaxis chamber, in which the upper and the lower compartments were separated by a polycarbonate filter (Biomap, Agrate Brianza, Italy), with a pore diameter of 5 μm. Cells (3×10^5 cells per ml, 15×10^3 macrophages per well) were placed in the upper chamber, and aliquots of either Hank's solution (in order to evaluate spontaneous mobility) or of the chemoattractant fMLP (in order to evaluate chemotaxis) were added in the lower chamber. The chambers were incubated for 3 h at 37°C, in an atmosphere of 5% CO_2 and then the migrated cells that adhered to the distal part of the filters were fixed and stained. Migrated cells were quantitated by counting three fields in triplicate with an optical image analyzer (Sacerdote et al., 1997; Bianchi et al., 1999).

2.5. In vitro effect of CBR ligands

When the effect of CP55,940 was determined in vitro, the substance at concentrations ranging from 10^{-5} to 10^{-9} M, diluted in LPS-free Hank's solution, was added to the lower chemotaxis chamber without (in order to assess spontaneous migration), or together with the fixed concentration of fMLP 10^{-7} M in Hank's solution, in order to evaluate chemotaxis. In a different experiment, macrophages obtained from untreated rats were preincubated in polypropylene tubes for 1 h at 37°C, in an atmosphere of 5% CO_2 in Hank's solution (3×10^5 cells per ml) in the presence or absence of CP55,940 at the concentrations of 10^{-6} and 10^{-7} M. At the end of preincubation the chemotactic activity of the cells was evaluated in the Boyden chamber in presence of 10^{-7} M fMLP.

In order to check the involvement of either CB1 or CB2 receptor, experiments with the CBR antagonists were performed.

In a first series of experiments the CBR antagonists were added in the lower chamber at concentrations ranging from 10^{-6} to 10^{-9} alone, or in combination with CP55,940 10^{-7} M in the absence or in the presence of fMLP10^{-7} M.

Moreover, the ability of the antagonists to shift the dose–response curve of CP55,940 was evaluated by adding a fixed dose of the antagonists (10^{-9}, 10^{-8} and 10^{-7} M) to the dose–response curve of CP55,940.

In all samples, DMSO was present in an amount ≤0.1%,

that was shown in previous work to have no effects on migration (Sacerdote and Panerai, 1989).

2.6. In vivo treatment

CP55,940 was administered intraperitoneally at the dose of 0.4 mg/kg. The animals were killed 1 h after treatment. This experimental protocol was chosen on the basis of results obtained in previous studies, showing that at this dose and at this time-point the psychoactive and the immune effects of CP55,940 were maximal (Patrini et al., 1997). The CB1 receptor antagonist SR141716A was injected i.p. at the dose of 2.5 and 5 mg/kg, 30 min before CP55,940. The CB2 receptor antagonist SR144528, was administered orally at the dose of 10 mg/kg, 90 min before CP55,940. The choice of the timing and of the route of administration was dictated by the efficacy, reported in the literature, of the antagonists given in a similar schedule (Rinaldi Carmona et al., 1998; Massi et al., 2000). Control animals were injected i.p. or treated p.o. with the same volume of vehicle. Each experimental group consisted of six rats.

Peritoneal macrophages obtained from vehicle- or drug-treated rats were tested in vitro for their ability to migrate toward three concentrations of fMLP (10^{-6}, 10^{-7}, 10^{-8} M).

2.7. Statistical analysis

The statistical analysis of results was obtained by analysis of variance followed by the Bonferroni's *t*-test for multiple comparisons.

3. Results

3.1. In vitro drug treatment

When added in vitro in the chemotaxis chamber, CP55,940 inhibited in a concentration-dependent manner both peritoneal macrophages spontaneous mobility and fMLP-induced chemotaxis. As shown in Fig. 1, spontaneous migration was significantly reduced at doses as low as 10^{-8} M of CP55,940, with a 70% inhibition reached at the highest concentration of 10^{-6} M.

Also chemotaxis induced by 10^{-7}M fMLP was inhibited in a dose–response fashion by the cannabinoid agonist (Fig. 1). The chemotaxis reduction is significant starting from the CP55,940 concentration of 10^{-8} M, and a 68% inhibition was reached at 10^{-6} M. Similar results were observed when macrophages had been preincubated for 1 h in the presence of 10^{-6} and 10^{-7} M CP55,940 and thereafter their motility tested in the Boyden chamber in the presence or in the absence of fMLP (Table 1).

At the concentration of 10^{-5} M the compound started to be toxic, since a significant 10% mortality was observed by the trypan blue exclusion test (data not shown).

In order to test the involvement of the two cannabinoid receptors in the inhibition of macrophage migration induced by CP55,940, different concentrations of the CB1 and CB2 antagonists SR141716A and SR144528 respectively were added in the chemotaxis chamber. The antagonists had no effect by themselves either on spontaneous migration or fMLP chemotaxis (Table 2). As shown in Fig. 2 upper panel, the effect of 10^{-7} M CP55,940 on spontaneous migration (60±8% of inhibition) was blocked by increased concentrations of the CB2 antagonist SR144528 and only slightly by the CB1 antagonist SR141716A. In

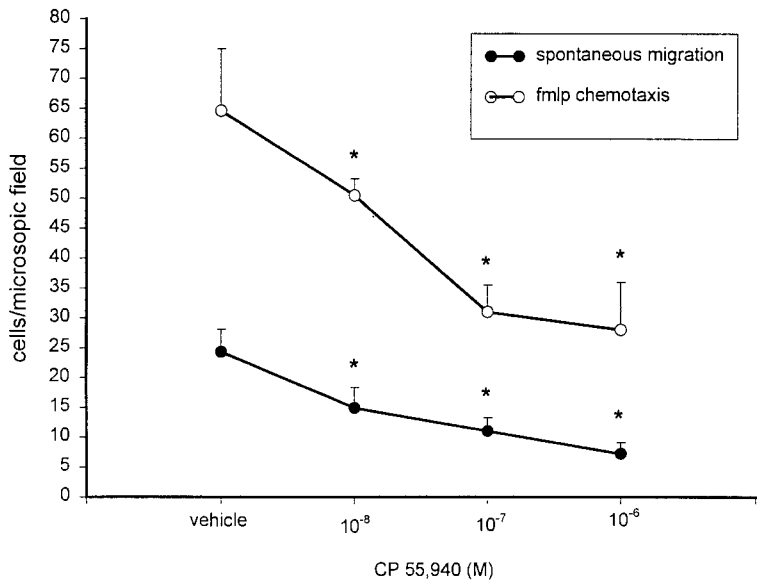

Fig. 1. Effects of CP55-940, added in vitro, on spontaneous migration and on fMLP-induced chemotaxis of rat peritoneal macrophages. fMLP was used at the concentration of 10^{-7} M. The results are the means±S.D. of six experiments. * $P<0.01$ vs. control values (vehicle).

Table 1
Spontaneous migration and fMLP (10^{-7} M)-induced chemotaxis of peritoneal macrophage after 1 h incubation with CP55,940

Preincubation (1 h)	Spontaneous migration (cells per microscopic field)	FMLP-chemotaxis (cells per microscopic field)
Medium	23.2± 2.7[a]	65.4±8.3
CP 10^{-6} M	12.7± 1.4[b]	25.4±3.6[b]
CP 10^{-7} M	8.1± 1.5[b]	26.0±3.4[b]

[a] Values are means±S.D.
[b] $P<0.01$ vs. medium.

fact, SR144528 induced a significant block at concentrations as low as 10^{-9} M, and the reversal of CP55,940 inhibition was almost complete at 10^{-8} M SR144528 while the CB1 receptor antagonist induced a significant effect only starting from 10^{-7} M, and a complete inhibition was never achieved.

Fig. 2 lower panel shows the effect of the antagonists on the inhibition induced by CP55,940 on fMLP chemotaxis (50±6% of inhibition). It is evident that only the CB2 antagonist SR144528 was able to block the CP55,940 effect in a dose–response fashion, while the CB1 antagonist did never modulate the CP55,940 effect on chemotaxis. In addition, as shown in Fig. 3, SR144528 produced a concentration-dependent shift of the dose–response-curve for CP55,940 inhibition of spontaneous macrophage migration (Fig. 3, panel A) and fMLP-induced chemotaxis (Fig. 3, panel B).

The CB1 receptor antagonist SR141716A was also able to induce a rightward shift of the concentration–response curve for CP55,940-induced inhibition of spontaneous migration (Fig. 4, panel A). In contrast, SR141716A was unable to modify the concentration–response curve for CP55,940-induced inhibition of fMLP-induced chemotaxis (Fig. 4, panel B).

3.2. In vivo drug treatment

Rats were treated either with 0.4 mg/kg CP55,940 or vehicle, and 1 h later peritoneal macrophages were tested in vitro for their ability to migrate in the Boyden chamber in the absence or the presence of three concentrations of fMLP. As shown in Fig. 5 panel A, macrophages obtained from CP55,940-treated animals showed a reduced spontaneous migration and fMLP-induced chemotaxis in comparison to macrophages obtained from vehicle-treated animals. In order to test the CBRs involvement in the observed macrophage locomotion inhibition, animals were pretreated with either the CB1 receptor antagonist SR141716A or the CB2 receptor antagonist SR144528. At the 2.5 mg/kg dose, the CB1 receptor antagonist did not modify the inhibition induced by CP55,940 (panel B, Fig. 5): macrophage migration was in fact lowered to the same extent in CP55,940-treated rats as in SR141716A+ CP55,940-treated animals. Not even at the highest dose (5 mg/kg) did the antagonist reverse CP55,940-induced inhibition of chemotaxis. However at this dose the antagonist by itself affected both spontaneous migration and chemotaxis, that were significantly reduced.

In contrast, as shown in panel C of Fig. 5, the CB2 receptor antagonist SR144528 was devoid of any intrinsic effect, but it was able to partially antagonize the inhibition of both spontaneous migration and fMLP chemotaxis induced by CP55,940.

4. Discussion

It is well known that the recruitment of macrophages and monocytes to sites of inflammation, injury and infection is a crucial step in inflammation and antimicrobial immune responses. The ability to migrate towards chemoattractant substances is a first and relevant event in macrophage physiology. This work reports for the first time that CP55,940, a potent cannabinoid receptor agonist, is able to interfere with this basic macrophage function. CP55,940 is a potent in vitro inhibitor of both macrophage

Table 2
In vitro effect on the CB1 receptor antagonist SR141716A and the CB2 receptor antagonist SR144528 on spontaneous migration and fMLP-induced chemotaxis of macrophages

Concentration	SR141716A		SR144528	
	Spontaneous migration (cells per microscopic field)	fMLP (10^{-7} M) chemotaxis (cells per microscopic field)	Spontaneous migration (cells per microscopic field)	fMLP (10^{-7} M) chemotaxis (cells per microscopic field)
0	23.2±3.4[a]	62.6±5.8	21.7±2.0	61.0±7.0
10^{-9} M	24.1±2.4	56.0±5.8	23.3±2.3	58.4±8.2
10^{-8} M	21.5±3.1	64.0±3.4	21.6±2.6	64.0±2.1
10^{-7} M	20.3±2.4	58.0±5.0	22.0±1.8	56.4±1.7
10^{-6} M	20.5±2.5	66.0±5.4	23.4±1.2	62.2±2.4

[a] Values are means±S.D. of six experiments.

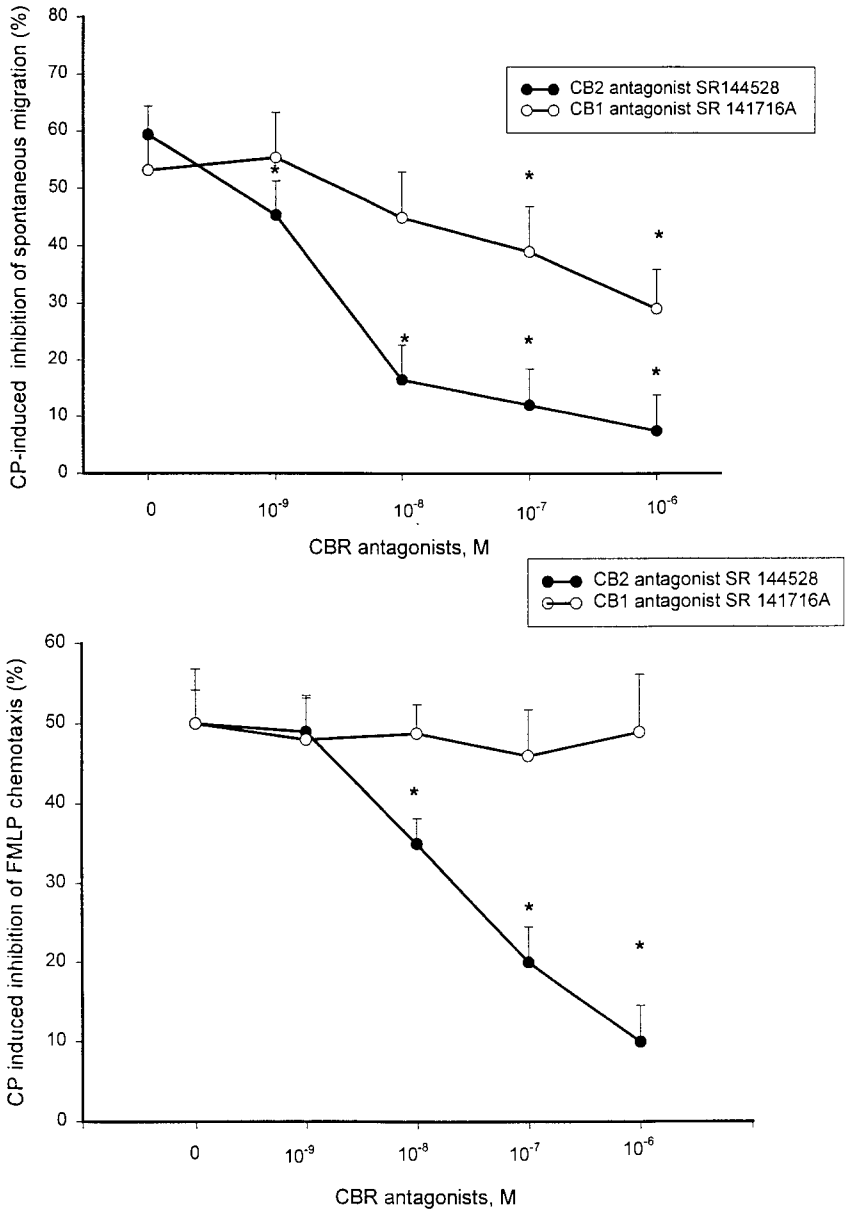

Fig. 2. Effect of increasing concentrations of SR141716A and of SR144528 on CP55,940-induced inhibition of spontaneous migration (upper panel) and fMLP-induced chemotaxis (lower panel) of rat macrophages. The CBR antagonists were added in vitro at the indicated concentrations in the lower chamber in combination with 10^{-7} M CP55,940 in the absence (upper panel) or in the presence of fMLP 10^{-7} M (lower panel). Values are means±S.D. of six experiments. * $P<0.01$ vs. CP55,940 alone (0 antagonist).

spontaneous migration and fMLP-induced chemotaxis, since it induced a significant reduction of these parameters (40–30%) already at the low concentration of 10 nM, while at μmolar concentrations the inhibition reached 70%. A relevant and significant decrease of both spontaneous migration and fMLP-induced chemotaxis is present also after in vivo administration of CP55,940. The dose of CP55,940 that we utilized was shown by us and by other groups to affect other immune responses, such as T lymphocyte proliferation and NK activity (Klein et al., 1998, 1995; Cabral and Dove-Pettit, 1998; Patrini et al., 1997; Massi et al., 1998). Moreover, at this dosage all the central effects typical of cannabinoids such as antinociception, depression of motor activity, hypothermia etc. are present (Massi et al., 1998).

To date, two CBRs have been identified: CB1 is the primary type within the CNS, but it is expressed at low levels also within the immune system; CB2 appears to be the predominant form of cannabinoid receptors within the immune system with no quantifiable expression within the brain (Kaminsky et al., 1992; Galiegue et al., 1995). The data that we obtained by adding the specific CB1 and CB2 receptor antagonists in vitro, seemed to confirm this distribution. The inhibition of spontaneous migration in-

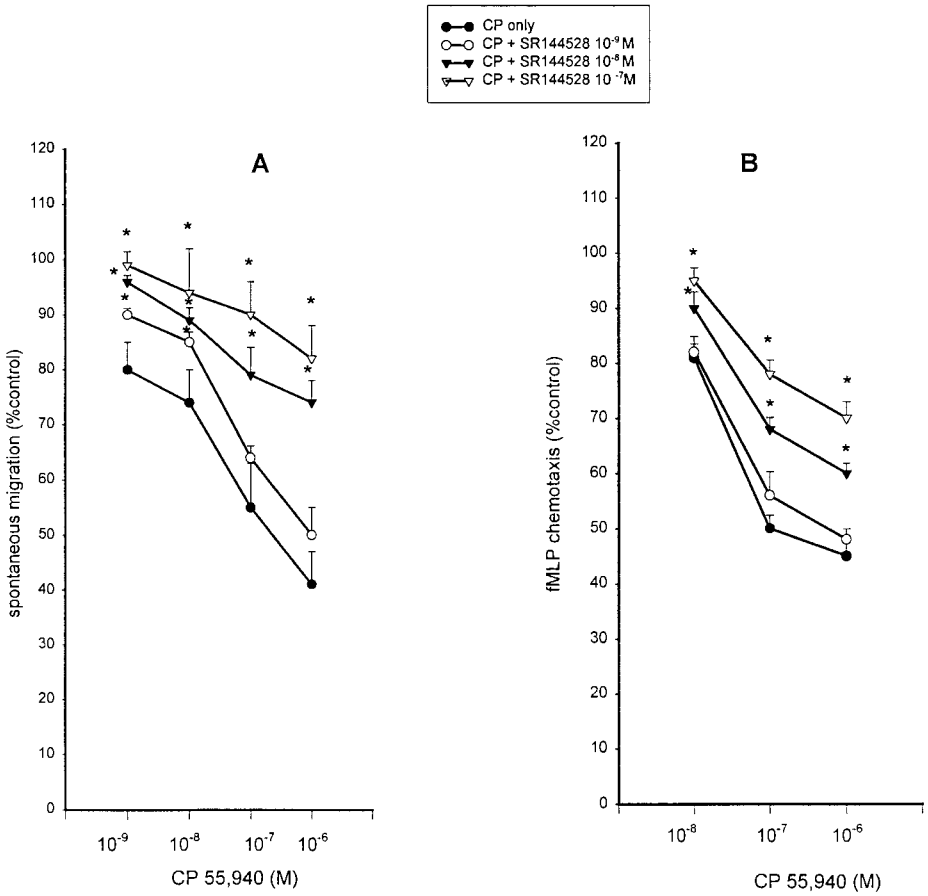

Fig. 3. Concentration–response curves for CP55,940 inhibition of spontaneous motility (panel A) and fMLP-induced chemotaxis (panel B) in the absence and in the presence of different concentrations of SR144528. Data are expressed as the percentage of control migration (i.e. observed without CP55,940). * $P<0.01$ vs. CP55,940.

duced by CP55,940 is in fact significantly blocked by increasing concentrations of the CB2 antagonist SR144528. At 10^{-7} M, SR144528 completely prevented the inhibition of locomotion induced by equimolar concentrations of CP55,940. The CP55,940-induced inhibition of spontaneous migration is prevented in vitro by adding also the CB1 antagonist SR141716A. This antagonist is however less effective than SR144518, since μmolar concentrations are needed in order to induce a relevant block of CP55,940-induced inhibition. Interestingly, while the CB2 antagonist was able to significantly block the inhibition induced by CP55,940 of both spontaneous migration and of fMLP-chemotaxis, the CB1 antagonist SR141716A was able to partially reverse only the inhibition of spontaneous migration. It has been suggested that CBR expression in different immune subsets could be related to the stage of cellular differentiation and activation (Klein et al., 1995, 1998; Daaka et al., 1996). From our observation it can be speculated that the level of cell surface CBRs changes following activation with fMLP, and the CB2 effects become predominant. This can be due either to a decrease of the number of CB1 receptors or to an increase of the expression of CB2 binding sites. Further binding studies and/or experiments evaluating receptor message levels are needed in order to fully elucidate this possibility. However, these antagonists are not CBR specific, but CBR selective, and there is about 60–1000 fold difference in selectivity depending on the system used (Pertwee, 1999). It is therefore possible that the inhibition observed with 10^{-6} M SR141716A should be attributed to the block of the CB2 receptor, considering that SR144528 was already effective at concentrations as low as 10^{-8} M.

Both CB1 and CB2 receptors are negatively coupled to adenylate cyclase through a pertussis-sensitive G protein (Klein et al., 1998). However the question whether the immunosuppressive effects of cannabinoids, including those on chemotaxis, could be due to cAMP inhibition remains to be elucidated (Kaminsky, 1998). Interestingly it has recently been described that opioids, which also bind to a G protein-receptor negatively coupled to adenylate cyclase, can decrease chemokine-induced chemotaxis of monocytes and neutrophils by heterologous desensitization through phosphorylation of chemokine receptor (Grimm et al., 1998). It can be hypothesized that a similar mechanism could be involved also in the cannabinoid effects.

Recently it has been reported that the CBR can sequester

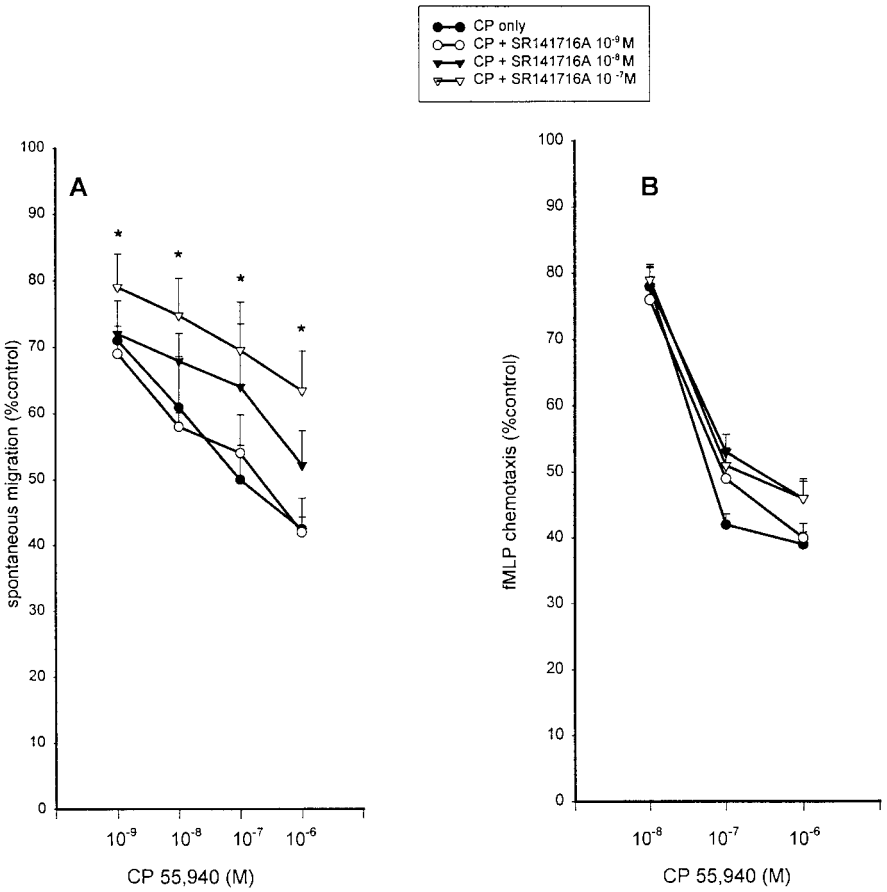

Fig. 4. Concentration response curves for CP55,940 inhibition of spontaneous motility (panel A) and fMLP-induced chemotaxis (panel B) in the absence and in the presence of different concentrations of SR141716A. Data are expressed as the percentage of control migration (i.e. observed without CP55,940). * $P<0.01$ vs. CP55,940.

$G_{i/o}$ proteins from a common pool and prevent other receptors from signaling (Vasquez and Lewis, 1999). Since the fMLP receptor couples to pertuxis toxin sensitive G_i -proteins to activate chemotaxis and other macrophage functions (Prossnitz and Ye, 1997; Wenzel-Seifert et al., 1998), an interaction between the two receptors at the level of G-proteins could be suggested. A relevance of this work is the fact that, as previously discussed, a significant modulation of macrophage motility is exerted also by in vivo treatment with the cannabinoid. Also, after in vivo administration of CP55,940, the effect on macrophage locomotion is prevented by pretreatment with the CB2 antagonist SR144528, while, in vivo, the CB1 receptor does not seem to be involved, as demonstrated by the inefficacy of SR141716A to inhibit either spontaneous motility or chemotaxis. When injected in vivo, also, the slight effect on CP-induced inhibition of spontaneous migration is lost. At the dose of 2.5 mg/kg, SR141716A completely inhibits all the central effects of CP55,940 (Patrini et al., 1997), and therefore the lack of effect on CP55,940-induced decreased of macrophage locomotion seems to rule out a centrally mediated effect of cannabinoid on this immune response. Also the higher dose of SR141716A did not affect the CP55,940-induced inhibition. However at this dose the antagonist acquired an intrinsic inhibitory activity on the immune system, that we and other groups have already observed on other different immune parameters, and for which a satisfactory explanation is not yet available (Patrini et al., 1997; Compton et al., 1996). The effects on locomotion seem therefore to be mediated mainly throughout the interaction with the CB2 receptors present on the cells of the immune system.

Immune cells, especially macrophages, are known to release arachidonic acid as well as arachidonic metabolites. Interestingly one of these metabolites released by macrophages is anandamide, the endogenous ligand of the CBRs (Hunter and Burstein, 1997). The observation that both in vivo and in vitro the CB2 antagonist was devoid of any intrinsic effect, seemed to indicate that anandamide did not play a tonic role in the regulation of cell migration. However, since the activation of the CBRs is a potent stimulus to the production of anandamide by macrophages, (Burstein et al., 1994) there is the possibility that anandamide released by macrophages could be involved in the modulation of cell migration induced by CP50,9915.

Moreover, the CB1 antagonist SR141716A and more

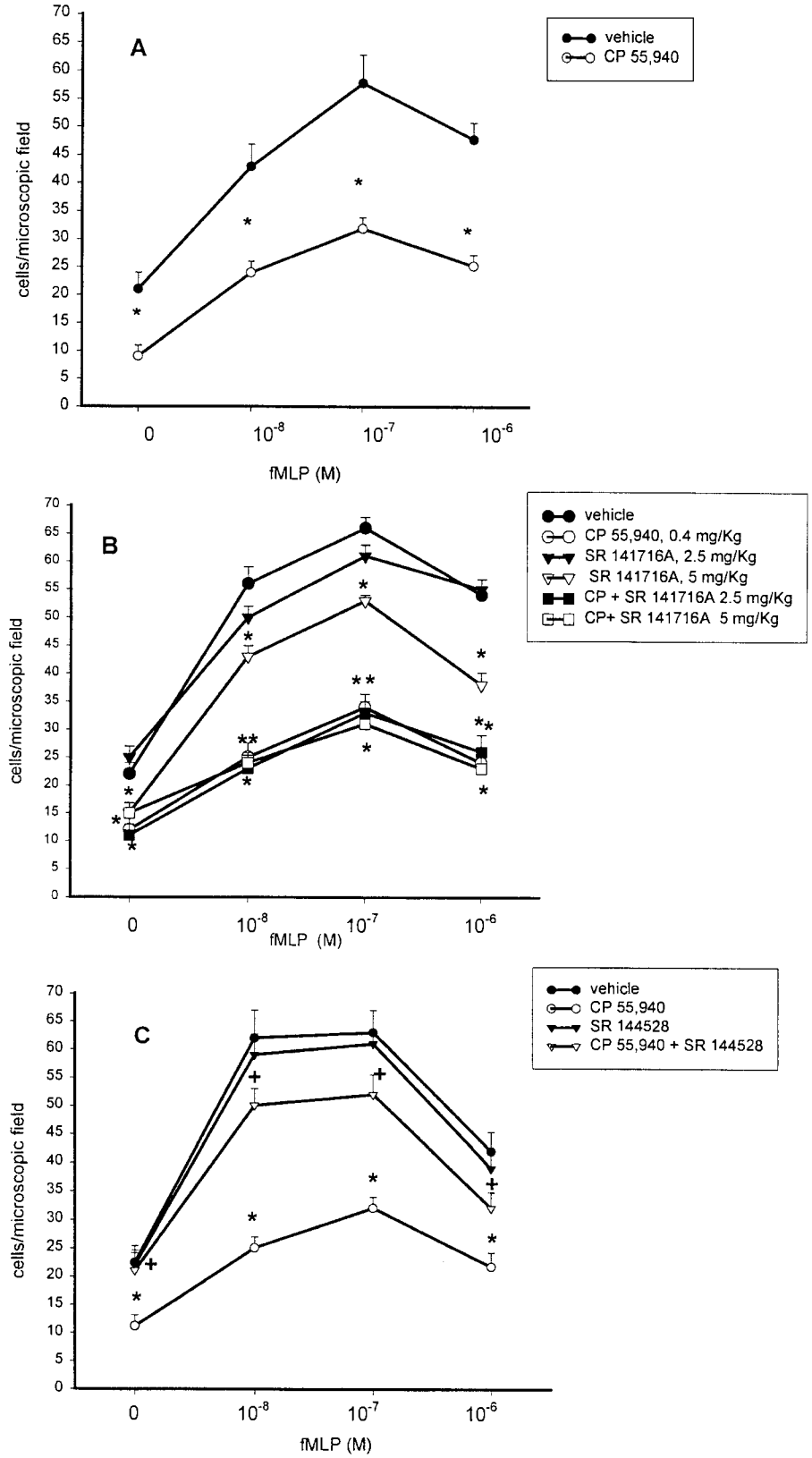

Fig. 5. Panel A: Effect of the in vivo administration of 0.4 mg/kg CP55,940 on spontaneous mobility (0 fMLP) and on fMLP (10^{-8}, 10^{-7} and 10^{-6} M) induced chemotaxis of peritoneal macrophages. Panel B: Effect of SR141716A pretreatment (2.5 and 5 mg/kg) on the reduction of spontaneous migration (0 fMLP) and fMLP-induced chemotaxis of macrophages obtained from rats treated in vivo with 0.4 mg/kg CP55,940. Panel C: Effect of SR144528 pretreatment (10 mg/kg) on the reduction of spontaneous migration (0 fMLP) and fMLP-induced chemotaxis of macrophages obtained from rats treated in vivo with 0.4 mg/kg CP55,940. The results are the means±S.D. for six animals. * $P<0.01$ vs. vehicle. + $P<0.05$ vs. CP55,940.

recently also the CB2 antagonist SR144528 have been described to behave, in particular conditions, as inverse agonists respectively on CB1 and CB2 receptors (Landsman et al., 1997; Portier et al., 1999). However, in our experimental conditions the data obtained do not support these observations, since the antagonists are devoid of any intrinsic effect on locomotion. In conclusion we show that the potent cannabinoid ligand CP55,940 can inhibit macrophage motility both in vitro and in vivo, mainly throughout involvement of CB2 receptors. The significance of these observations is double: from one side they further substantiate the immunosuppressive effects of cannabinoids, but on the other hand, they suggest that the development of specific CB2 ligands may lead to an interesting new class of anti-inflammatory drugs.

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