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Inhibition of nitric oxide production in RAW264.7 macrophages by cannabinoids and palmitoylethanolamide

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Abstract

We have investigated the inhibition of lipopolysaccharide stimulated nitric oxide production in RAW264.7 macrophages by the cannabinoids and the putative cannabinoid CB_2-like receptor ligand, palmitoylethanolamide. (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate ((+)-WIN55212) and, to a lesser extent (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxy-propyl)cyclohexan-1-ol (CP55940), significantly inhibited lipopolysaccharide stimulated nitric oxide production. The level of inhibition was found to be dependent on the concentration of lipopolysaccharide used to induce nitric oxide production. Palmitoylethanolamide significantly inhibited nitric oxide production induced by lipopolysaccharide. The inhibition of nitric oxide production by (+)-WIN55212 but not palmitoylethanolamide was significantly attenuated in the presence of the cannabinoid CB_2 receptor antagonist, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528). (+)-WIN55212 produced a pertussis toxin-sensitive parallel rightward shift in the log concentration–response curve for lipopolysaccharide, causing a fivefold increase in the EC_{50} value for lipopolysaccharide with no change in the E_{max} value. (−)-WIN55212 had no effect on the log concentration–response curve for lipopolysaccharide. Palmitoylethanolamide did not produce a rightward shift in the lipopolysaccharide concentration–response curve. However, it did produce a pertussis toxin-insensitive reduction in the E_{max} value. The results suggest that the inhibition of lipopolysaccharide mediated nitric oxide release by (+)-WIN55212 in murine macrophages is mediated by cannabinoid CB_2 receptors. In contrast, the inhibition by palmitoylethanolamide does not appear to be mediated by cannabinoid receptors.

Keywords: Cannabinoid CB_2 receptor; WIN55212; Palmitoylethanolamide; Nitric oxide (NO); RAW264.7; Macrophage; cAMP

1. Introduction

Cannabinoid CB_2 receptor expression has been identified in a range of immunological cells including B and T cells, monocytes and macrophages (Bouaboula et al., 1993; Galiègue et al., 1995). It has been suggested that cannabinoid CB_2 receptors may have an immunomodulatory role. These receptors have been shown to modulate cytokine production, humoral response, proliferative responses, microbiocidal activity and antigen processing in a number of immune cell types (Klein et al., 1998). Most of these in vitro studies have implied an immunosuppressive action of cannabinoids (Luo et al., 1992; Klein et al., 1991; Fischer-Stenger et al., 1993) however, in some studies, enhancing effects have been observed (Derocq et al., 1995; Zhu et al., 1994).

Nitric oxide has a dual role as (1) a proinflammatory mediator in the immune system with both antiviral (Lowenstein et al., 1996) and antibacterial (Nathan and Hibbs, 1991) actions and (2) a neurotransmitter in the central nervous system, affecting memory and learning (Dawson and Snyder, 1994). The murine macrophage cell line RAW264.7 expresses mRNA for CB_2 but not CB_1 cannabinoid receptors (Jeon et al., 1996; Waksman et al., 1999). Reports have shown that the psychoactive cannabinoid (−)-Δ^9-tetrahydrocannabinol inhibits nitric oxide production by murine macrophages and RAW264.7 macrophages while the inactive isomer, (+)-Δ^9-tetrahydro-

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cannabinol, only weakly inhibits nitric oxide release (Coffey et al., 1996). In addition, in these cells (−)-Δ^9-tetrahydrocannabinol appears to inhibit both forskolin-stimulated cyclic AMP production and inducible nitric oxide synthase (iNOS) transcription (Jeon et al., 1996). However, these studies do not directly demonstrate the involvement of cannabinoid CB_2 receptors, as they did not involve the use of cannabinoid receptor antagonists. Moreover, although (−)-Δ^9-tetrahydrocannabinol has affinity for both cannabinoid CB_1 and CB_2 receptors, it has particularly low efficacy at cannabinoid CB_2 receptors, to the extent that it can behave as an antagonist at these sites in some preparations (Bayewitch et al., 1996; Bouaboula et al., 1999).

The purpose of this investigation was three-fold. Firstly, to further investigate whether the effects of cannabinoids on lipopolysaccharide induced nitric oxide production in RAW264.7 macrophages could be directly linked with the cannabinoid CB_2 receptor. Secondly, to address the need for a sensitive assay for cannabinoid CB_2 receptor agonists and antagonists in a native system. Thirdly, to investigate any functional interaction of palmitoylethanolamide with cannabinoid CB_2 receptors. There is some degree of confusion as to the ability of this compound to interact with cannabinoid CB_2 receptors. Palmitoylethanolamide reduces inflammatory responses (Mazzari et al., 1996) and has potent analgesic actions, which are attenuated by the cannabinoid CB_2 receptor antagonist, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) (Jaggar et al., 1998; Calignano et al., 1998). However, although palmitoylethanolamide has been proposed as an endogenous cannabinoid CB_2 receptor ligand (Facci et al., 1995), it does not appear to displace the high affinity cannabinoid ligand [^{3}H]CP55940 from membranes of Chinese Hamster Ovary (CHO) cells transfected with human cannabinoid CB_2 receptors (Showalter et al., 1996) or from cannabinoid CB_2 receptors on spleen or mast cell membranes (Ross et al., 1999a). Thus, we felt it appropriate to investigate whether palmitoylethanolamide interacts with the cannabinoid CB_2 receptor in macrophages.

In order to investigate whether inhibition of lipopolysaccharide induced nitric oxide release from macrophages is linked to the cannabinoid CB_2 receptor we used a number of approaches. A comparison was made of the

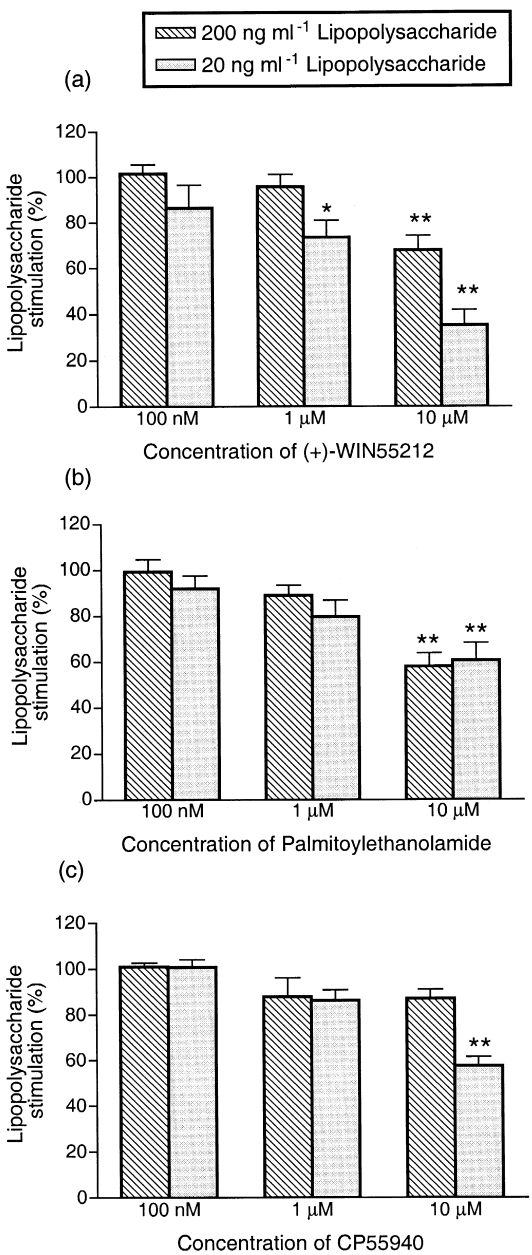

Fig. 1. The inhibition by (a) (+)-WIN55212 (b) palmitoylethanolamide and (c) CP55940 of nitric oxide production in RAW264.7 cells stimulated by 200 or 20 ng ml^{-1} lipopolysaccharide. For each datum point, the data are expressed as a percentage of a lipopolysaccharide control, which contained the vehicle, and has been normalised to 100%. Nitric oxide production induced by 200 ng ml^{-1} and 20 ng ml^{-1} lipopolysaccharide was 21.6±4.91 μM nitrite per 5×10^5 cells plated and 9.65±1.17 μM nitrite per 5×10^5 cells plated respectively. Drugs or vehicle and lipopolysaccharide were co-administered and incubated for 24 h at 37°C. For palmitoylethanolamide experiments 50 μM phenylmethylsulphonyl fluoride was included in the incubation. The data are the mean of separate experiments done in triplicate, and the error bars represent the S.E.M. Asterisks indicate significant difference from 100% ($^*P<0.05$ and $^{**}P<0.01$, one sample *t*-test). (+)-WIN55212 inhibited nitric oxide production induced by 200 ng ml^{-1} lipopolysaccharide by 31.9±6.4% at 10 μM and by 26.3±7.4% and 64.7±6.7% at 1 and 10 μM respectively for nitric oxide production induced by 20 ng ml^{-1} lipopolysaccharide. Palmitoylethanolamide significantly inhibited nitric oxide production by 41.9±4.11% (200 ng ml^{-1} lipopolysaccharide; $n=11$) and by 39.1±7.7% at 10 μM (20 ng ml^{-1} lipopolysaccharide; $n=6$). 10 μM CP55940 did not significantly inhibit nitric oxide production induced by 200 ng ml^{-1} lipopolysaccharide but produced a significant inhibition of 42.45±3.88% at 20 ng ml^{-1} lipopolysaccharide ($n=4$). None of the compounds tested had any effect on the basal levels of nitric oxide of 0.71±0.246 μM nitrite per 5×10^5 cells plated ($n=50$; data not shown).

actions of the cannabinoid receptor agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate ((+)-WIN55212), which has slightly higher affinity for the cannabinoid CB_2 receptor, with its inactive isomer (−)-WIN55212 (Pertwee, 1997). We have made use of the high affinity, cannabinoid CB_2 selective antagonist SR144528 (Rinaldi-Carmona et al., 1998). In addition, we investigated the effects of pretreatment of the cells with the $G_{\alpha i}/G_{\alpha o}$ receptor uncoupling agent, pertussis toxin on the action of these compounds.

2. Materials and methods

2.1. Drugs and chemicals

CP55940 [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexan-1-ol] was obtained from Pfizer, WIN55212 from Research Biochemicals International, palmitoylethanolamide from Tocris and SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] and SR144528 from Sanofi Recherche. Roswell Park Memorial Institute Medium (RPMI), Dulbecco's Modified Eagles Medium (DMEM)/f-12 Ham, penicillin/streptomycin, Greiss reagent, Bovine Serum Albumin, Phosphate Buffered Saline (PBS), geneticin (G418), hygromycin, forskolin, rolipram and phenylmethylsulphonyl fluoride were all obtained from Sigma. Lipopolysaccharide was from *Escherichia coli* 026:B6 (Sigma). RAW264.7 cells were obtained from the European Cell Culture Collection and human cannabinoid CB_2 transfected cells were a gift from GlaxoWellcome.

2.2. Cell culture

RAW264.7 cells were grown in RPMI 1640 supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 unit ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. CHO cells transfected with human cannabinoid CB_2 receptors (see Green et al., 1999) were grown in Dulbecco's Modified Eagles Medium (DMEM) f-12 Ham supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 300 μg ml^{-1} hygromycin, 600 μg ml^{-1} geneticin (G418), 100 unit ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Hygromycin and geneticin were included in the media to achieve selective pressure for the expression of the human cannabinoid CB_2 receptor.

2.3. Nitrite quantification

RAW264.7 cells were plated in the above media at 5×10^5 cells ml^{-1} in 24 well plates and stimulated with lipopolysaccharide for 24 h at 37°C unless otherwise stated. For experiments where a single concentration of lipopolysaccharide was used, the cannabinoid agonists or vehicle were co-administered with lipopolysaccharide unless otherwise stated. In experiments with palmitoylethanolamide, 50 μM phenylmethylsulphonyl fluoride was included in the incubation. This compound inhibits fatty acid amide hydrolase, the enzyme which may have a role in terminating the biological activity of palmitoylethanolamide (Per-

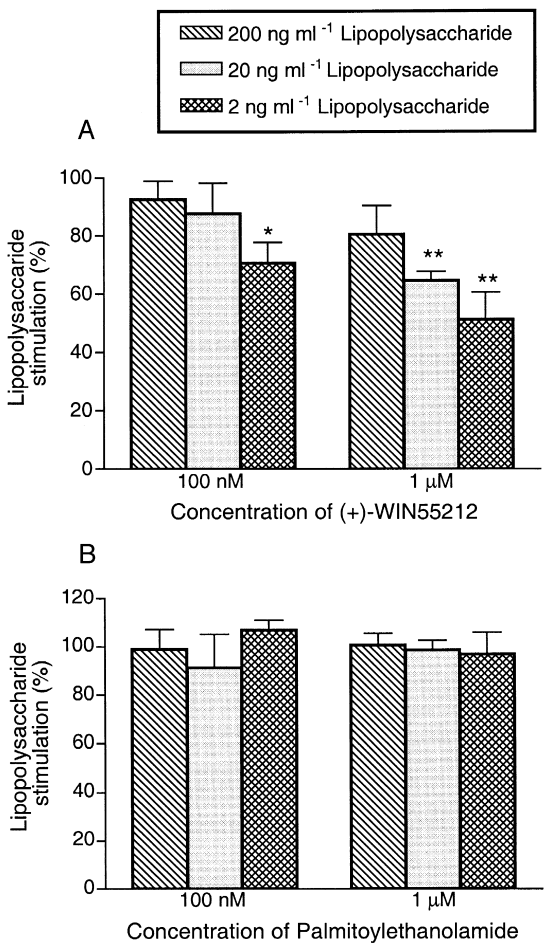

Fig. 2. The effect of (A) (+)-WIN55212 and (B) palmitoylethanolamide on the stimulation of nitric oxide production in RAW264.7 cells induced by lipopolysaccharide at concentrations of 200 ng ml^{-1} (27.5±3.9 μM nitrite per 5×10^5 cells plated), 20 ng ml^{-1} (13.35±2.06 μM nitrite per 5×10^5 cells plated) and 2 ng ml^{-1} (7.46±2.33 μM nitrite per 5×10^5 cells plated). The cells were incubated with compounds or vehicle for 7 h at 37°C prior to addition of lipopolysaccharide for a further 24 h. For each column, the data are expressed as a percentage of a lipopolysaccharide control, which contained the vehicle, and has been normalised to 100%. The data are the mean of separate experiments done in triplicate, and the error bars represent the S.E.M. Asterisks indicate significant difference from 100% ($^*P < 0.05$, $^{**}P < 0.01$; one sample t-test). 1 μM (+)-WIN55212 inhibited the nitric oxide production induced by lipopolysaccharide concentrations of 200, 20 and 2 ng ml^{-1} by 19.3±9.87%, 35.4±3.25% and 48.7±9.4% respectively. 100 nM (+)-WIN55212 did not significantly inhibit nitric oxide production induced by 200 or 20 ng ml^{-1} lipopolysaccharide, but inhibited the nitric oxide release induced with 2 ng ml^{-1} lipopolysaccharide by 29.4±7.1 % ($n=5$). Neither 100 nM nor 1 μM palmitoylethanolamide significantly inhibited the nitric oxide release at any of the lipopolysaccharide concentrations tested.

Table 1
Effect of cannabinoid CB_2 (SR144528) and cannabinoid CB_1 (SR141716A) receptor antagonists on the inhibition of lipopolysaccharide-mediated nitric oxide release from RAW264.7 cells by (+)-WIN55212 and palmitoylethanolamide

Compound	% inhibition in the presence of:		
	Vehicle	SR144528	SR141716A
(+)-WIN55212	84.4±2.0 (6)	62.5±3.3[a] (6)	74.3±2.6 (6)
Palmitoylethanolamide	56.7±6.1 (6)	67.8±4.3 (6)	60.6±3.7 (6)

The cells were incubated at 37°C for 20 min with 500 nM antagonist or antagonist vehicle, then for 30 min with 5 μM agonist prior to the addition of 20 ng ml^{-1} lipopolysaccharide for 24 h. The nitric oxide release by lipopolysaccharide with agonist vehicle alone was 7.44±0.2 μM nitrite per 5×10^5 cells plated. The data are the mean of separate experiments done in triplicate, the errors represent the S.E.M. and the number of experiments is shown in brackets.

[a] Inhibition by (+)-WIN55212 was significantly attenuated following pretreatment with 500 nM SR144528 ($P<0.01$, one-way ANOVA, followed by Tukey's multiple comparison).

twee, 1997). In experiments using cannabinoid receptor antagonists, the cells were incubated for 20 min with antagonist followed by 30 min with agonists at 37°C prior to the addition of lipopolysaccharide. For lipopolysaccharide concentration–response experiments, lipopolysaccharide was added at concentrations ranging from 0.2 ng to 2 μg ml^{-1} to determine the maximum response. The cells were preincubated with cannabinoid agonists or vehicle for 30 min at 37°C. Following a 24 h incubation at 37°C, supernatants were mixed with an equal volume of Greiss reagent and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an absorbance reading at 550 nM. Cannabinoids and palmitoylethanolamide were stored as stock solutions in ethanol, serially diluted in RPMI containing 1 mg ml^{-1} bovine serum albumin and 50 μl added to the assay giving a final ethanol concentration of 0.4%. The total assay volume was 1 ml.

2.4. Cyclic AMP production

The method used was previously described in Ross et al. (1999b). Briefly, cells (6×10^5 cells ml^{-1}) were preincubated in phosphate buffered saline containing 1 mg ml^{-1} bovine serum albumin at 37°C with 10 μM rolipram for 10 min prior to the addition of cannabinoids or vehicle for 20

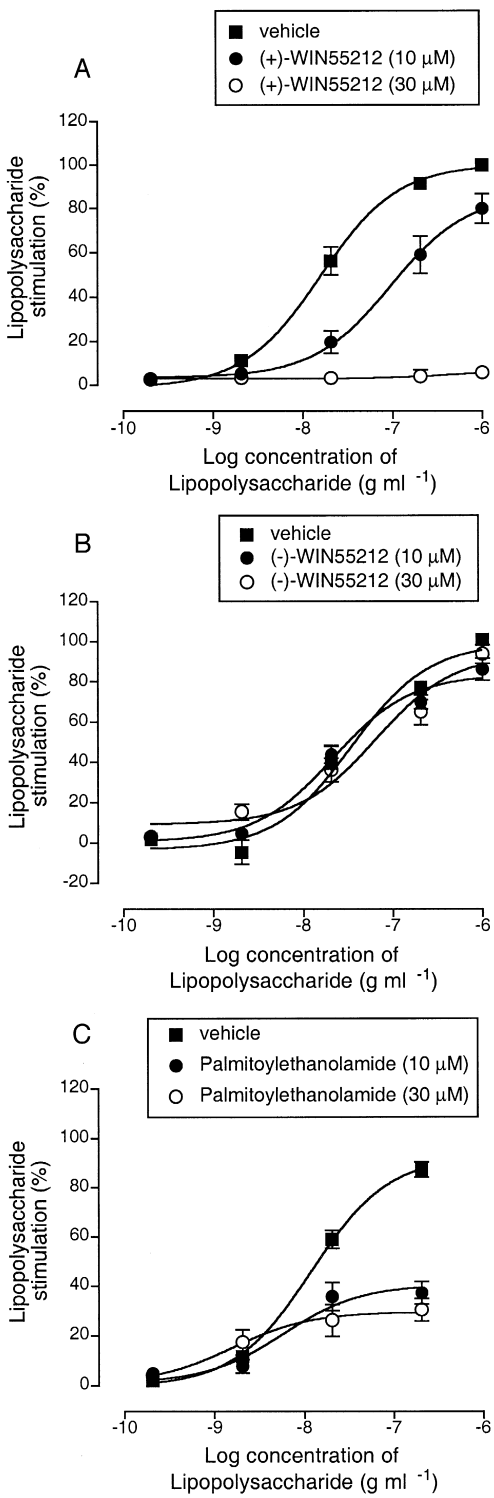

Fig. 3. The effect of 10 and 30 μM (A) (+)-WIN55212 (B) (−)-WIN55212 and (C) palmitoylethanolamide on the log concentration–response curve for lipopolysaccharide induced nitric oxide production in RAW264.7. The data are expressed as a percentage of the maximum response to lipopolysaccharide as determined from a lipopolysaccharide log concentration–response curve constructed in the absence of vehicle for each set of experiments: (A) 24.49±2.8 μM nitrite per 5×10^5 cells plated (B) 22.53±2.9 μM nitrite per 5×10^5 cells plated (C) 25.18±2.7 μM nitrite per 5×10^5 cells plated. The vehicle curve (0.4% ethanol) did not differ significantly from the concentration–response curve for lipopolysaccharide in the absence of cannabinoid vehicle. Drugs/vehicle were incubated for 30 min prior to the addition of lipopolysaccharide for 24 h at 37°C. The data are the mean of 8 or 10 separate experiments done in triplicate, and the error bars represent the S.E.M. For the log concentration–response curves of lipopolysaccharide the E_{max} and EC_{50} values and their 95% confidence limits were calculated using GraphPad Prism (see Table 2).

min, followed by a further 20 min incubation with forskolin in a total volume of 500 μl. Experiments with palmitoylethanolamide were done in the presence of 50 μM phenylmethylsulphonyl fluoride. The reaction was terminated by the addition of 0.1 M HCl and centrifugation performed to remove cell debris. The pH was brought to 8–9 using 1 M NaOH and cyclic AMP content measured using a radioimmunoassay kit (Biotrak, AP Biotech).

2.5. Data analysis

In experiments with a single concentration of lipopolysaccharide, nitric oxide release for each lipopolysaccharide concentration in the presence of vehicle was normalised to 100%, and the nitric oxide release in the presence of drug expressed as a percentage of this control. For lipopolysaccharide log concentration–response curve experiments, nitric oxide release was calculated as a percentage of the maximum, as determined from a full lipopolysaccharide log concentration–response curve constructed for each series of experiments. Calculations of the EC_{50} and E_{max} values for lipopolysaccharide or forskolin concentration–response curves were made using GraphPad Prism. Values are expressed as mean and variability as standard error of the mean (S.E.M.) or as 95% confidence limits. Statistical significance was determined using a one-sample t-test or an Analysis of Variance (ANOVA) followed by Tukey's multiple comparison (GraphPad Prism).

3. Results

3.1. Effect of cannabinoids and palmitoylethanolamide on single concentrations of lipopolysaccharide

Fig. 1 shows the actions of various compounds incubated simultaneously with either 200 or 20 ng ml^{-1} lipopolysaccharide. (+)-WIN55212 (Fig. 1a) significantly inhibited nitric oxide production induced by 200 ng ml^{-1} lipopolysaccharide at 10 μM and by 20 ng ml^{-1} lipopolysaccharide at 1 and 10 μM. The % inhibition by (+)-WIN55212 at both 1 and 10 μM was significantly greater in the presence of the lower concentration of lipopolysaccharide ($P < 0.01$, one way ANOVA followed by Tukey's multiple comparison). The EC_{50} for inhibition of nitric oxide production induced by 20 ng ml^{-1} lipopolysaccharide was 6.46 μM (+)-WIN55212 ($n = 6$; 95% confidence limits: 0.13–13.3). 1 μM palmitoylethanolamide (Fig. 1b) did not significantly inhibit nitric oxide release induced by either concentration of lipopolysaccharide. 10 μM palmitoylethanolamide significantly inhibited nitric oxide production induced by 200 ng ml^{-1} lipopolysaccharide and by 20 ng ml^{-1} lipopolysaccharide. The level of inhibition by 10 μM palmitoylethanolamide of nitric oxide production induced by 200 ng ml^{-1} was not significantly different from the level of inhibition of nitric oxide production induced by 20 ng ml^{-1} lipopolysaccharide (one-way ANOVA followed by Tukey's multiple comparison). The effect of palmitoylethanolamide was not significantly different in the absence of the fatty acid amide hydrolase inhibitor, phenylmethylsulphonyl fluoride than in its presence (data not shown). This indicates that enzymatic inactivation of palmitoylethanolamide does not influence the action of this compound in RAW264.7 macrophages. CP55940 (Fig. 1c) did not significantly inhibit nitric oxide production induced by 200 ng ml^{-1} lipopolysaccharide but 10 μM of this cannabinoid produced a significant inhibition when 20 ng ml^{-1} lipopolysaccharide was used.

3.2. Time course and low serum experiments

Derocq et al. (1995) have demonstrated that cannabinoids have a much enhanced effect on B-cell proliferation

Table 2
Effects of (+)-WIN55212, (−)-WIN55212 and palmitoylethanolamide on the EC_{50} and E_{max} values for lipopolysaccharide stimulation of nitric oxide production in RAW264.7 cells

Compound	Concentration	Lipopolysaccharide stimulation of NO production			
		EC_{50} (ng ml^{-1})		E_{max} (%)	
		Vehicle	Drug	Vehicle	Drug
(+)-WIN55212	10 μM	18.6 (12.6–27.5)	95.1 (38.2–236.9)	109 (99.4–118.7)	86.8 (70.7–102.5)
	30 μM		[a]		[b]
(−)-WIN55212	10 μM	25.8 (12.4–33.9)	18.8 (9.5–25.4)	97.6 (85.9–109.4)	83.2 (75.4–90.9)
	30 μM		64.8 (28.2–148.7)		93.9 (80.5–107.3)
Palmitoylethanolamide	10 μM	12.0 (8.3–17.4)	5.8 (1.17–28.9)	100 (95.5–106.5)	40.7 (30.9–50.6)
	30 μM		1.6 (0.37–11.8)		29.7 (21.3–38.1)

The data were normalised as a percentage of the maximum response to lipopolysaccharide in the absence of vehicle as determined from a full concentration–response curve for lipopolysaccharide constructed for each set of experiments. EC_{50} (the concentration of lipopolysaccharide producing 50% of the maximum nitric oxide production induced by lipopolysaccharide) values and E_{max} values were calculated using GraphPad Prism. The 95% confidence limits shown in brackets.

[a] An EC_{50} and E_{max} value could not be calculated (see Fig. 3A).

[b] An EC_{50} and E_{max} value could not be calculated (see Fig. 3A).

when the incubation time is increased or serum concentrations in the assay lowered. Thus, we investigated whether (+)-WIN55212 or palmitoylethanolamide would have an inhibitory effect on nitric oxide release at a lower concentration when co-administered with lipopolysaccharide for 48 or 72 h. 100 nM of these compounds had no significant effect on nitric oxide production by 20 ng ml^{-1} lipopolysaccharide at either of the time points (data not shown). We also investigated the effect of preincubating the cells with cannabinoid for 7 h prior to the addition of lipopolysaccharide. Under these conditions a concentration of 1 μM (+)-WIN55212 (Fig. 2A) significantly inhibited the nitric oxide release induced by lipopolysaccharide at concentrations of 20 and 2 ng ml^{-1}, but not that induced by 200 ng ml^{-1}. 100 nM (+)-WIN55212 did not significantly inhibit nitric oxide release induced by 200 or 20 ng ml^{-1} lipopolysaccharide, but significantly inhibited the nitric oxide release when a concentration of 2 ng ml^{-1} lipopolysaccharide was used. Neither 100 nM nor 1 μM palmitoylethanolamide (Fig. 2B) significantly inhibited the nitric oxide release at any of the lipopolysaccharide concentrations used. Decreasing the concentration of serum from 10% to 1% had no significant effect on the level of inhibition produced by either 100 nM or 1 μM (+)-WIN55212 or palmitoylethanolamide (data not shown) in the presence of 20 ng ml^{-1} lipopolysaccharide.

3.3. Effect of cannabinoid receptor antagonists

In this series of experiments the cells were preincubated with either cannabinoid receptor antagonist or vehicle for 20 min followed by agonist for 30 min before exposure to 20 ng ml^{-1} lipopolysaccharide. Under these conditions, inhibition of nitric oxide release by 5 μM (+)-WIN55212 (Table 1) was significantly attenuated by preincubation with 500 nM of the cannabinoid CB_2-selective receptor antagonist, SR144528. The same concentration of the cannabinoid CB_1-selective receptor antagonist, SR141716A, had no significant effect on the inhibition of nitric oxide release by (+)-WIN55212 (Table 1). Neither antagonist had a significant effect on the inhibition of nitric oxide release by 5 μM palmitoylethanolamide (Table 1). Nor, when administered alone, did 500 nM SR141716A or SR144528 have a significant effect on lipopolysaccharide-induced nitric oxide production (data not shown).

3.4. Effect of cannabinoid agonists and palmitoylethanolamide on log concentration–response curves of lipopolysaccharide

Fig. 3 and Table 2 show the effects of a 30 min preincubation of (+)-WIN55212, (−)-WIN55212 or palmitoylethanolamide on the log concentration–response curves for lipopolysaccharide. 1 μM (+)-WIN55212 did not significantly shift the log concentration–response curve for lipopolysaccharide (data not shown). However, 10 μM (+)-WIN55212 (Fig. 3A; Table 2) produced a parallel rightward shift in the lipopolysaccharide log concentration–response curve causing a 5 fold increase in the EC_{50} value, with no change in the E_{max} value. At 30 μM

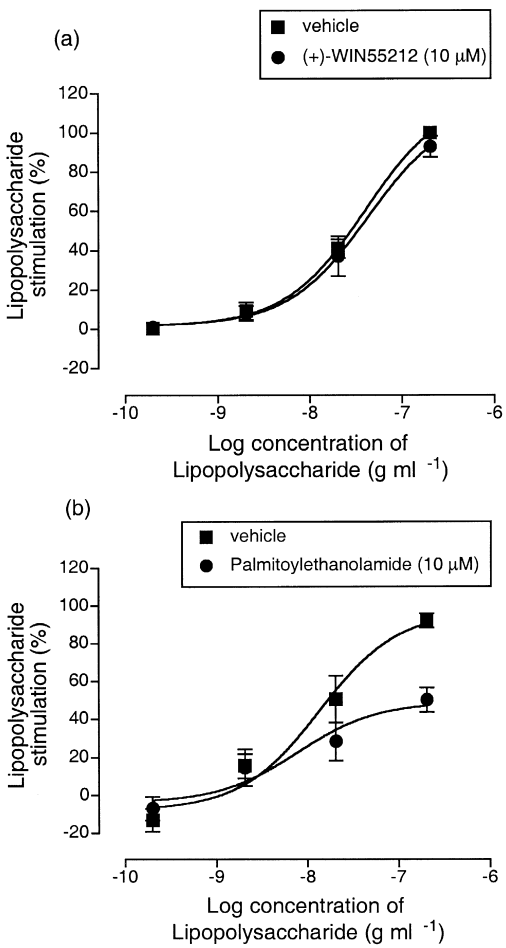

Fig. 4. The effect of 10 μM (a) (+)-WIN55212 and (b) palmitoylethanolamide on the log concentration–response curve for nitric oxide production induced by lipopolysaccharide in RAW264.7 cells pretreated with pertussis toxin (100 ng ml^{-1} for 12 h). The data are expressed as a percentage of the maximum response to lipopolysaccharide as determined from a lipopolysaccharide log concentration–response curve constructed for each set of experiments. The vehicle curve (0.4% ethanol) did not differ significantly from the concentration–response curve for lipopolysaccharide in the absence of cannabinoid vehicle. Drugs or vehicle and lipopolysaccharide were incubated for 24 h at 37°C. Data are the mean of separate experiments done in triplicate, and the error bars represent the S.E.M. For the log concentration–response curve of lipopolysaccharide the E_{max} and EC_{50} values and their 95% confidence limits were calculated using GraphPad Prism. The EC_{50} values and their 95% confidence limits for lipopolysaccharide were 40.0 ng ml^{-1} (25.1–62.7) with vehicle and 42.7 ng ml^{-1} (27.6–83.2) with 10 μM (+)-WIN55212 ($n = 3$). Palmitoylethanolamide (10 μM) inhibited the nitric oxide release after pertussis toxin pretreatment, the E_{max} and its 95% confidence limits of lipopolysaccharide after pertussis toxin being 108.1% (81.8–134.3) for vehicle and 48.3% (27.0–69.7) for 10 μM palmitoylethanolamide ($n = 3$).

(+)-WIN55212 abolished the response to the concentrations of lipopolysaccharide tested. (−)-WIN55212 (Fig. 3B; Table 2) had no significant effect on the lipopolysaccharide concentration–response curve at 10 or 30 μM. Although palmitoylethanolamide (Fig. 3C; Table 2) did not produce a parallel rightward shift in the lipopolysaccharide concentration–response curve; however, it did significantly reduce the E_{max} at both 10 and 30 μM.

3.5. Pertussis toxin pretreatment experiments

Pertussis toxin pretreatment (100 ng ml^{-1} for 24 h) abolished the inhibitory effect of (+)-WIN55212 (Fig. 4a). Thus, EC_{50} and E_{max} values for stimulation of nitric oxide release by lipopolysaccharide in the presence of the cannabinoid vehicle were not significantly different from those with 10 μM (+)-WIN55212. In pertussis toxin pretreated cells, 10 μM palmitoylethanolamide retained the ability to significantly attenuate the E_{max} of lipopolysaccharide stimulated nitric oxide release (Fig. 4b).

3.6. Effect of WIN55212 and palmitoylethanolamide on forskolin-stimulated cyclic AMP production in human cannabinoid CB_2 transfected cells

In order to further investigate the action of palmitoylethanolamide at cannabinoid CB_2 receptors we extended our studies to human cannabinoid CB_2 transfected CHO cells. In these cells, treatment with 10 nM (+)-WIN55212 produced a parallel rightward shift in the log concentration–response curve for forskolin, causing a 4.7 fold increase in the EC_{50} value, with no change in the maximum (Fig. 5A). At a concentration of 2 μM forskolin, which is just below the EC_{50} value and routinely used by our laboratory and others for the cyclic AMP assay (Ross et al., 1999b; Rinaldi-Carmona et al., 1998), the EC_{50} for inhibition by (+)-WIN55212 was 2.26 nM ± 0.084 (Fig. 5B). At a supramaximal concentration of 100 μM forskolin, the EC_{50} for inhibition by (+)-WIN55212 was 10-fold higher ($P < 0.01$, unpaired t-test) at 22.84 ± 3.74 nM (Fig. 5B). At a concentration ranging from 0.1 nM to 10 μM, palmitoylethanolamide had no significant effect on the log concentration–response curve for forskolin ($n = 2$, data not shown), and did not significantly inhibit the stimulation of cyclic AMP production by either 100 or 2 μM forskolin in human cannabinoid CB_2 transfected cells ($n = 5$, Fig. 5C).

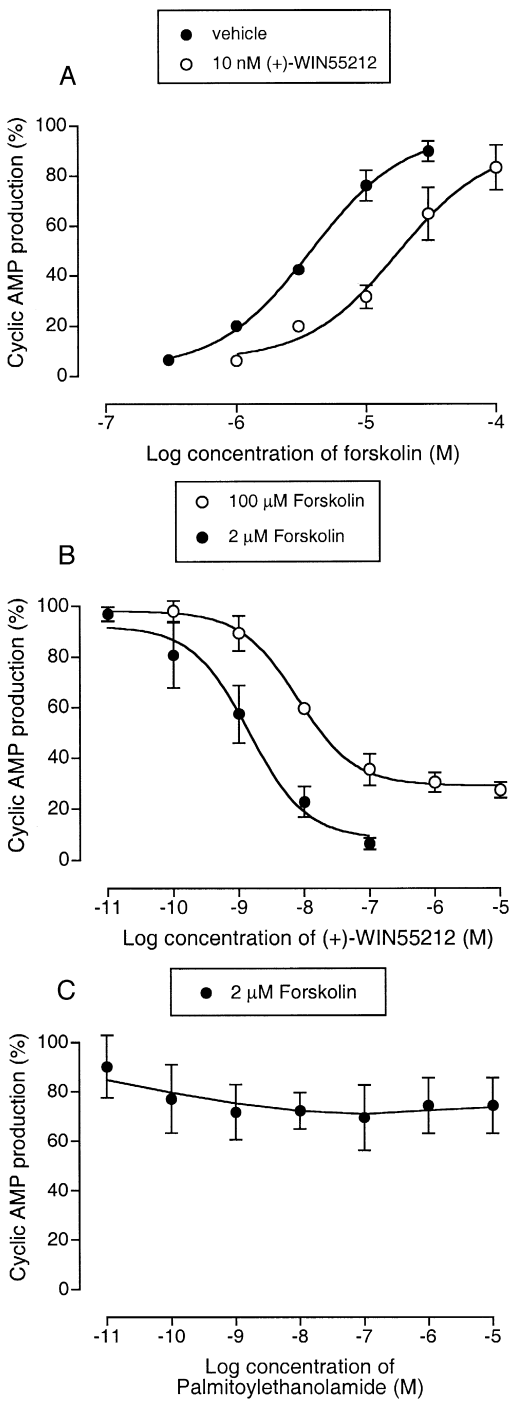

Fig. 5. Shows (A) the effect of 10 nM (+)-WIN55212 on the log concentration–response curve for stimulation of cyclic AMP production in human CB_2 transfected CHO cells by forskolin (B) the inhibition by (+)-WIN55212 of the stimulation of cyclic AMP production by 100 μM and 2 μM forskolin and (C) the effect of palmitoylethanolamide (in the presence of 50 μM phenylmethylsulphonyl fluoride) on the stimulation of cyclic AMP production by 2 μM forskolin. The cyclic AMP production (pmol $tube^{-1}$) was 13.4 ± 3.12, 41.8 ± 7.9, 89.6 ± 15.7, 178.0 ± 15.1 and 217.5 ± 16.5 at 0.3, 1, 3, 10 and 30 μM respectively. Drugs or vehicle were incubated for 20 min prior to exposure to forskolin for 20 min. Data are the mean of 3–5 separate experiments and the error bars represent the S.E.M. The EC_{50} and E_{max} values and their 95% confidence limits, calculated using GraphPad Prism, for forskolin were 3.78 μM (2.52–5.68) and 97.4% (80–114.7) respectively in the presence of vehicle and 17.67 μM (8.67–35.96) and 92.1% (68.9–115.2) respectively in the presence of 10 nM (+)-WIN55212.

4. Discussion

An inhibitory effect of (−)-Δ^9tetrahydrocannabinol on lipopolysaccharide mediated nitric oxide release in murine macrophage cell line RAW264.7 has been observed by others, however these experiments did not directly implicate cannabinoid CB_2 receptors (Jeon et al., 1996; Coffey et al., 1996). The data presented here demonstrate that this effect is also produced by the synthetic cannabinoid (+)-WIN55212. It was possible to attenuate the inhibition of lipopolysaccharide stimulated nitric oxide produced by (+)-WIN55212 by pretreatment of the cells with a sub-micromolar concentration of the cannabinoid CB_2 receptor antagonist, SR144528. These data suggest that the inhibition of nitric oxide release by (+)-WIN55212 is cannabinoid CB_2 receptor mediated. The cannabinoid CB_1 receptor antagonist, SR141716A had no effect on the inhibitory action of (+)-WIN55212, which is not surprising as these cells do not express cannabinoid CB_1 receptors (Jeon et al., 1996; Waksman et al., 1999).

Whilst CP55940 too significantly inhibited nitric oxide release it would appear to be less potent than (+)-WIN55212 in this system. (+)-WIN55212 and CP55940 have been shown to have high affinity for the cannabinoid CB_2 receptor (Pertwee, 1997). However, in line with our data, there is evidence that CP55940 is between 2 and 7 fold less potent than (+)-WIN55212 at this receptor type (Slipetz et al., 1995; Felder et al., 1995; Tao and Abood., 1998). However, the effects of (+)-WIN55212 and CP55940 are only observed at concentrations in the micromolar range, considerably higher than the nanomolar concentrations required for binding of these cannabinoids to the cannabinoid CB_2 receptor (Pertwee, 1997).

It is notable that (+)-WIN55212 and CP55940 exerted a significantly greater inhibition of nitric oxide release if this release was induced by lower concentrations of lipopolysaccharide, rather than the near maximal concentration of 200 ng ml^{-1} (Figs. 1a and 2a), indicating that the level of inhibition observed is affected by the absolute concentrations of nitric oxide released. Variations in the activity of lipopolysaccharide between batches means that the absolute amount of nitric oxide release at any one concentration of lipopolysaccharide may vary. This may explain why in some experiments (Table 1) it can be observed that the inhibition produced by (+)-WIN55212 and palmitoylethanolamide at 20 ng ml^{-1} lipopolysaccharide is greater than that produced in other experiments, (Figs. 1 and 2) in which the absolute level of stimulation at 20 ng ml^{-1} lipopolysaccharide was higher.

We have further investigated the extent to which the level of lipopolysaccharide stimulation can affect the potency of (+)-WIN55212 and thus the sensitivity of the assay. To do this we have adopted the somewhat novel approach of studying the effects (+)-WIN55212 and palmitoylethanolamide on the full log concentration–response curve for lipopolysaccharide. This approach allows one to study the actions of the compound over a range of lipopolysaccharide concentrations, and is quite different to previous investigations in which a single supramaximal concentration of lipopolysaccharide has been used. Using this protocol we have demonstrated that 10 μM (+)-WIN55212 can cause a parallel rightward shift in the log concentration–response curve for lipopolysaccharide. The action of (+)-WIN55212 is stereoselective and its inhibitory effects are prevented by pretreatment of the cells with the $G_{\alpha i}/G_{\alpha o}$ receptor uncoupling agent, pertussis toxin. These data are further evidence for a cannabinoid receptor mediated action of (+)-WIN55212. Our observations emphasise the importance of the choice of stimulant concentration in such assays. Thus, if high concentrations of lipopolysaccharide are used to maximally stimulate nitric oxide release, compounds may fail to inhibit nitric oxide production. Indeed, using a high concentration of stimulant may have a more pronounced impact on the sensitivity of the assay to cannabinoids in native systems where cannabinoid CB_2 receptor expression levels may be low. Kenakin (1997) explains that the empiric nature of the IC_{50} stems from the fact that its magnitude depends on the concentration of stimulant drug used. Further, he states that an obvious approach to solving this problem is to track the inhibition of a submaximal concentration of stimulant drug, choosing 'as low a level of response as possible to block'. The sensitivity of the nitric oxide release assay to cannabinoids may be enhanced by using lower concentrations of lipopolysaccharide, however this would require a more sensitive method of nitric oxide measurement than that used in this study. Under these conditions, it may be possible to observe effects of cannabinoid receptor agonists at lower, more pharmacological concentrations.

It is notable that the sensitivity of assay systems to cannabinoids may also be increased under conditions in which the expression level of the cannabinoid CB_2 receptor is increased, perhaps due to inflammation or infection. To date very few studies have demonstrated a cannabinoid CB_2 receptor mediated effect of cannabinoids at sub-micromolar concentrations in native systems. Two such studies are those of Derocq et al. (1995) and Bouaboula et al. (1999) who have shown that at nanomolar concentrations cannabinoids significantly increased the DNA synthesis in human tonsilar B-cells, in which there is high cannabinoid CB_2 receptor expression (Galiègue et al., 1995). These effects were only observed in low serum conditions and were augmented when incubation times were increased from 24 h to 48 or 72 h. However, in the present investigations, neither lowering the serum concentrations from 10% to 1% foetal bovine serum nor increasing the incubation time significantly altered the sensitivity of the nitric oxide assay to (+)-WIN55212 or palmitoylethanolamide.

In contrast to (+)-WIN55212, the inhibition of nitric oxide release by palmitoylethanolamide is not sensitive to pertussis toxin pretreatment nor to inhibition by cannabi-

noid receptor antagonists. In addition, palmitoylethanolamide does not shift the lipopolysaccharide log concentration–response curve to the right; rather it reduces the maximal NO release inducible by lipopolysaccharide. These data suggest that palmitoylethanolamide is not mediating inhibition of nitric oxide release via an interaction with the cannabinoid CB_2 receptor. Although the involvement of an unidentified pertussis toxin-insensitive cannabinoid receptor cannot be totally excluded, it could well be that palmitoylethanolamide is having a non-specific, non-receptor mediated effect. Recently, Derocq et al. (1998) demonstrated that anandamide and palmitoylethanolamide potentiate growth of cytokine-dependent cell lines via a cannabinoid-receptor independent pathway. The possibility remains that the availability of palmitoylethanolamide is limited by the fact that this compound is a wax and may stick to plastic, or that this compound is subject to enzymatic degradation. We have attempted to address this problem by conducting the experiments in the presence of 1 mg ml^{-1} bovine serum albumin and 0.4% ethanol. There is no evidence that the lack of activity of palmitoylethanolamide is due to hydrolysis as the effect of palmitoylethanolamide unaffected by the inclusion of the fatty acid amide hydrolase inhibitor, phenylmethylsulphonyl fluoride.

In human cannabinoid CB_2 transfected CHO cells, 10 nM (+)-WIN55212 caused a parallel rightward shift in the log concentration–response curve for forskolin. The ability of (+)-WIN55212 to inhibit forskolin-stimulated cyclic AMP production is dependent on the concentration of forskolin used. Using a supra-maximal concentration of forskolin (100 μM), as opposed to a concentration around the EC_{50} (2 μM) led to a 10 fold reduction in the EC_{50} value for (+)-WIN55212. These data give another illustration of the effect of the choice of stimulant concentration on the sensitivity of the assay. In contrast, palmitoylethanolamide, at concentrations up to 10 μM, failed to significantly inhibit forskolin-stimulated cyclic AMP production at either 2 or 100 μM forskolin. This is further evidence that palmitoylethanolamide is not a cannabinoid CB_2 receptor agonist.

In summary, this study demonstrates for the first time that cannabinoid CB_2 receptors may be involved in mediating inhibition of nitric oxide release from macrophages by (+)-WIN55212. This is in contrast to a cannabinoid CB_1 receptor mediated inhibition of lipopolysaccharide stimulated nitric oxide release in rat microglial cells (Waksman et al., 1999). In addition, we have shown that the level of stimulation of nitric oxide release induced by lipopolysaccharide affects the sensitivity of the assay to cannabinoid receptor agonists. This study also demonstrates that palmitoylethanolamide inhibits nitric oxide release from macrophages but not via an interaction with cannabinoid CB_2 receptors. It is also unlikely that the action of palmitoylethanolamide is mediated by cannabinoid CB_2-like receptors (Calignano et al., 1998) as the inhibition of nitric oxide release by palmitoylethanolamide is not blocked by SR144528.

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