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Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids

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Abstract

We have investigated the effects of cannabinoid agonists and antagonists on tumour necrosis factor-α (TNF-α)-induced secretion of interleukin-8 from the colonic epithelial cell line, HT-29. The cannabinoid receptor agonists {(−)-3-[2-hydroxy-4-(1,1-dimethyl-heptyl)-phenyl]4-[3-hydroxypropyl]cyclo-hexan-1-ol} (CP55,940); Δ-9-tetrahydrocannabinol; [*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl] pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthyl) methanone mesylate} (WIN55,212-2) and 1-propyl-2-methyl-3-naphthoyl-indole (JWH 015) inhibited TNF-α induced release of interleukin-8 in a concentration-dependent manner. The less active enantiomer of WIN55212-2, [*S*(−)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthyl) methanone mesylate (WIN55212-3), and the cannabinoid CB_1 receptor agonist arachidonoyl-2-chloroethylamide (ACEA) had no significant effect on TNF-α-induced release of interleukin-8. The cannabinoid CB_1 receptor antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1,4-pyrazole-3-carboxamide hydrochloride (SR141716A; 10^{-6} M) antagonised the inhibitory effect of CP55,940 ($pA_2 = 8.3 \pm 0.2$, $n = 6$) but did not antagonise the inhibitory effects of WIN55212-2 and JWH 015. The cannabinoid CB_2 receptor antagonist N-(1,S)-endo1,3,3-trimethylbicyclo(2,2,1)heptan-2-yl)-5(4-chloro-3-methyl-phenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528; 10^{-6} M) antagonised the inhibitory effects of CP55,940 ($pA_2 = 8.2 \pm 0.8$, $n = 6$), WIN55212-2 ($pA_2 = 7.1 \pm 0.3$, $n = 6$) and JWH 015 ($pA_2 = 7.6 \pm 0.3$, $n = 6$) , respectively. Western immunoblotting of HT-29 cell lysates revealed a protein with a size that is consistent with the presence of cannabinoid CB_2 receptors. We conclude that in HT-29 cells, TNF-α-induced interleukin-8 release is inhibited by cannabinoids through activation of cannabinoid CB_2 receptors.
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Keywords: Cannabinoid; Interleukin−8; TNF-α (tumour necrosis factor-α); HT-29 cell; Inflammatory bowel disease

1. Introduction

The colonic epithelium is a specialised tissue lining the luminal surface of the intestine. Once considered solely as an absorptive and secretory barrier for the luminal contents of the bowel, it is now also recognised to exert a major influence in the maintenance of gastric immune homeostasis (Jordan et al., 1999). Human colon epithelial cells may contribute to inflammatory responses in Crohn's disease and ulcerative colitis by secreting chemokines such as interleukin-8 (Schuerer-Maly et al., 1994). Given the importance of interleukin-8 in neutrophil recruitment and the importance of neutrophils to the pathogenesis of inflammatory conditions (Baggiolini et al., 1997), modulation of interleukin-8 expression may provide an attractive pharmacological target.

The immunomodulatory properties of cannabinoids are well established. Many reports suggest that cannabinoids have immunosuppressive effects through an action on a variety of inflammatory cells (for detailed review, see Berdyshev, 2000). For example, cannabinoids have been shown to inhibit lymphocyte proliferation (Luo et al., 1992; Schwartz et al., 1994). Cannabinoids inhibit cytokine production in a range of immune cells, including macrophage/monocytes, lymphocytes and rodent splenic lymphocytes (Klein et al., 1991). In our laboratory, cannabinoids have been shown to suppress nerve growth factor and substance P-induced release of reactive oxygen species from rat peritoneal mast cells (Brooks et al., 1999). However, in most instances, the concentrations of cannabinoids required to modulate immune cell function are greater than those

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used in cannabinoid receptor binding studies on neuronal tissue (Felder, 1998), thereby warranting further characterisation of these receptors.

To date, two cannabinoid receptors, CB_1 and CB_2 have been identified (Matsuda et al., 1990; Munro et al., 1993). Cannabinoid CB_1 receptors are localised mainly in the central nervous system (Matsuda et al., 1993), but are also present in peripheral tissues such as the spleen and peripheral blood leukocytes (Kaminski et al., 1992; Gerard et al., 1991; Bouaboula et al., 1993). Cannabinoid CB_2 receptors have been identified in a range of immune cells including B and T lymphocytes, monocytes/macrophages and rat splenic lymphocytes (Bouaboula et al., 1993; Galigue et al., 1995). Cannabinoid CB_1 receptors inhibit adenyl cyclase via a pertussis toxin sensitive guanosine triphosphate binding protein (Howlett and Fleming, 1984) and inhibit N-type calcium channels (Mackie and Hille, 1992). Like cannabinoid CB_1 receptors, cannabinoid CB_2 receptors are members of the G-protein coupled receptor family and upon activation cause inhibition of adenyl cyclase and activation of mitogen-activated protein kinases (Felder et al., 1995). However, the cannabinoid receptor modulating cytokine release from epithelial cells has yet to be characterised.

In this study, we explore the pharmacological actions of a range of cannabinoid receptor ligands on TNF-α-induced interleukin-8 release from HT-29 cells in vitro. Part of this study has previously been published in abstract form (Ihenetu et al., 2001).

2. Materials and methods

2.1. Reagents and drugs

CP55,940{(−)-3-[2-hydroxy-4-(1,1-dimethyl-heptyl)-phenyl]4-[3-hydroxy propyl] cyclo-hexan-1-ol} was generously donated by Pfizer. SR144528 (N- (1, S)- endo1, 3, 3-trimethylbicyclo(2,2,1)heptan-2-yl)-5(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) and SR141716A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1,4-pyrazole-3-carboxamide hydrochloride) were gifts from the Chemistry department, Sanofi Recherche (Montpellier, France). Δ^9-Tetrahydrocannabinol, anandamide (arachidonoyl ethanolamide),WIN55212-2 mesylate {*R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl] pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthyl) methanone mesylate}, ACEA (arachidonoyl-2-chloroethylamide) and JWH 015 (1-propyl-2-methyl-3-naphthoyl-indole) were purchased from Tocris Cookson (Bristol, UK). MTT, 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide was purchased from Sigma-Aldrich (Dorset, UK). Cannabinoid CB_2 receptor antibody and fusion protein were gifts from Dr K Mackie (University of Washington, Seattle, WA, USA). Ethanol was used as the vehicle for CP55,940, SR141716A, SR144528, Δ-9-Tetrahydrocannabinol and ACEA whereas dimethyl sulfoxide (DMSO) was the vehicle for WIN55212-2 and JWH 015. Vehicle controls were included in all assays. All other drugs and chemicals were purchased from standard commercial sources.

2.2. Cell cultures

The HT-29 colon epithelial cell line was obtained from European collection of animal cell cultures (ECACC, Salisbury, Wiltshire, United Kingdom). The cells were grown at 37 °C in McCoy's 5A medium supplemented with 10% foetal calf serum, penicillin/streptomycin (50 U/ml and 50 μg/ml), respectively and amphotericin B (0.5 μg/ml). Cells were grown in 75 cm^2 culture flasks and were confluent after approximately 3 days. Cultures were subdivided every 7 days. Prior to each experiment, the culture medium was discarded and cells were washed once with warm (37 °C) sterile phosphate buffered saline (20 ml; pH 7.4). Monolayers were detached from the flasks with (0.25% trypsin/ethylene diamine tetracetic acid). The flask was then incubated at 37 °C for 10 min. Once the cells were detached, the action of trypsin was stopped by the addition of 20 ml McCoy's 5A medium supplemented with 10% foetal calf serum. Cells were resuspended at a density of 5×10^5 cells/ml in foetal calf serum-free McCoy's 5A medium and 1 ml aliquots placed in the wells of a 24-well plate for 2 h before experimentation.

2.3. Enzyme linked immunosorbent assay

Interleukin-8 release from HT-29 cells was measured by Enzyme linked immunosorbent assay (ELISA) of the culture supernatants according to the manufacturer's guidelines. In brief, anti-human interleukin-8 monoclonal capture antibody (Cat. No. 554716; Pharmingen BD, Oxford UK) was paired with biotinylated anti-human interleukin-8 monoclonal detection antibody (Cat. No. 554718). Ninety-six-well plates Nunc-immunoplates (maxisorp F96, Pharmingen BD) were coated with 1 μg/ml capture antibody at 4 °C for 24 h. Following washing, blocking and addition of standards and samples, a one-step detection comprising the use of biotinylated antibody/streptavidin linked peroxidase (0.5 and 0.5 μg/ml), respectively was carried out. Tetramethylammonium-benzidine was used as a substrate solution and reaction was stopped with 2 M H_2SO_4 solution. Absorbance was read at a wavelength of 450 nm.

2.4. Treatment of cells

To study the effects of TNF-α on interleukin-8 release, HT-29 cells were seeded in 24-well plates as described above. TNF-α (0–100 ng/ml) was added to the cells, and incubated for 24 h at 37 °C in a humidified incubator (5% CO_2/95% air). At the end of the incubation period, medium was removed and placed into 1.5 ml tubes and centrifuged at $250 \times g$ for 5 min. Cell-free supernatants were stored at − 70 °C until assayed for interleukin-8 release by ELISA.

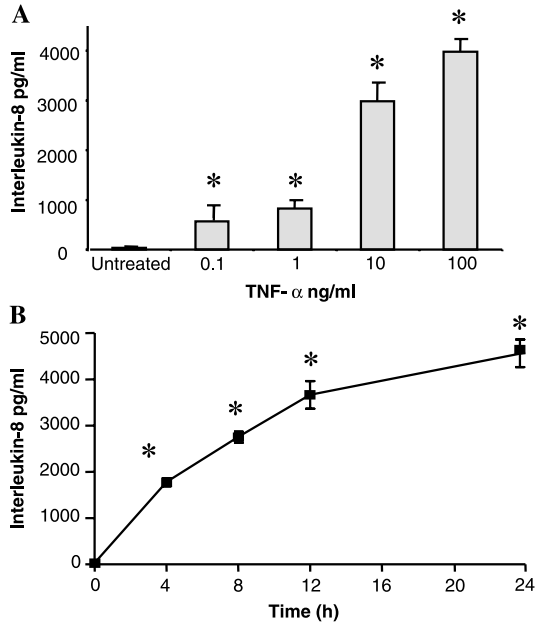

Fig. 1. TNF-α-induced release of interleukin-8 from HT-29 cells in vitro. (A) Confluent monolayers of HT-29 cells were stimulated with TNF-α (0.1–100 ng/ml) in foetal calf serum-free McCoy's 5A medium for 24 h. (B) Confluent monolayers of HT-29 cells were stimulated with TNF-α (100 ng/ml) in foetal calf serum free McCoy's 5A medium at the indicated time period. Cell-free supernatants were assayed for interleukin-8 release by ELISA as described in Materials and methods. Data are means and S.E.M. of at least five experiments. *Significant difference from control $P<0.05$.

For time course studies, TNF-α (100 ng/ml) was added to cell cultures and supernatants harvested for interleukin-8 assay 2, 4, 6, 12 and 24 h after addition of TNF-α.

To study the effect of cannabinoids on interleukin-8 release, cannabinoid receptor agonists (10^{-10}–10^{-4} M) or vehicle (0.1% ethanol or 0.1% DMSO) were added to cultures and incubated for 2 h at 37 °C in a humidified atmosphere (5% CO_2/95% air). At the end of the incubation period, cells were stimulated with TNF-α (100 ng/ml) for 24 h. In experiments involving the use of cannabinoid receptor antagonists, SR141716A (10^{-6} M), SR144528 (10^{-6} M), or vehicle were added to cultures 30 min prior to addition of the agonist, the culture supernatant was harvested and assayed for interleukin-8 as described above.

2.5. Western blotting

Western immunoblotting was carried out as described previously (Baydoun and Morgan, 1998) using antibodies raised against the amino terminus of the rat cannabinoid CB_2 receptor to the first transmembrane region using a method previously described for the cannabinoid CB_1 receptor (Tsou et al., 1998). This antibody was a gift from Dr K Makie and is now commercially available (Affinity Bioreagents, CO, U.S.A). Briefly, cell lysates (40 μg protein/lane) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto 0.2 μm nitrocellulose membranes (Andermann and Co, Kingston upon Thames, UK) and blocked for 1 h at room temperature with 100 mM NaCl, 10 mM Tris, 0.1%(v/v) Tween 20 (STT) buffer (pH 7.4) containing 5%(w/v) non-fat dried milk. Membranes were then incubated overnight with either the anti-cannabinoid CB_2 receptor antibody alone (1:1000 dilution in STT buffer containing 5%(w/v) non-fat dried milk) or with antibody pre-incubated with fusion protein (2 μg/well). Blots were washed with STT buffer (6 × 10 min) and incubated with 1:10,000 dilution of horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G for 1 h. Following further washing (6 × 10 min) with STT buffer, immunoreactive bands were visualised using an enhanced chemiluminescence detection system (Amersham, UK).

2.6. Cell viability assay

MTT tablets were dissolved in phosphate buffered saline (5 mg/ml) and filtered to remove any insoluble residue. Cells were cultured with drugs as described above. At the end of the incubation period, MTT reagent (100 μl/well) was added to all wells and incubated at 37 °C for 2 h. Cells were transferred onto 96-well plates and 100 μl/well DMSO was added to each well and mixed thoroughly to dissolve the dark crystals. Absorbance was read on a microtitre plate reader at a wavelength of 570 nm and results were expressed as percentage of the control value.

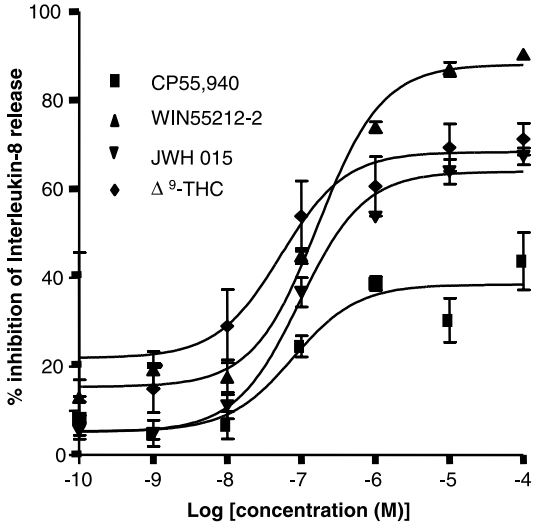

Fig. 2. Inhibition of TNF-α-induced interleukin-8 release by cannabinoids. Confluent monolayers of HT-29 cells were treated with CP55,940 (10^{-4}–10^{-10} M), WIN55,212-2 (10^{-10}–10^{-4} M), Δ^9-Tetrahydrocannabinol (10^{-10}–10^{-4} M) and JWH 015 (10^{-10}–10^{-4} M) for 2 h before stimulation with TNF-α (100 ng/ml). Incubation was continued for 24 h. Supernatants were assayed for interleukin-8 release by ELISA as described in Materials and methods. Data are presented as percentage inhibition from control (TNF-α treated cells alone). Error bars represent S.E.M. of six separate experiments.

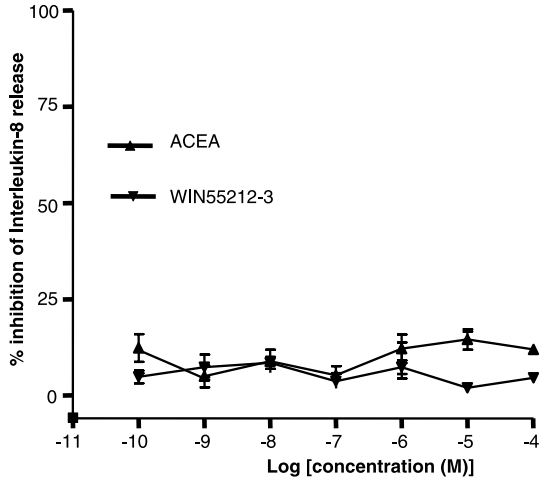

Fig. 3. The effect of ACEA and WIN55212-3 on the release of interleukin-8 from HT-29 cells. Confluent monolayers of HT-29 cells were treated with ACEA (10^{-10}–10^{-4} M) or WIN55212-3 (10^{-10}–10^{-4} M) for 2 h before stimulation with TNF-α (100 ng/ml). Incubation was continued for 24 h. Supernatants were assayed for interleukin-8 release by ELISA as described in Materials and methods. Data are presented as percentage inhibition from control (TNF-α treated cells alone). Error bars represent S.E.M. of six separate experiments.

2.7. Data analysis

Concentration–response curves were analysed by Prism (GraphPad, San Diego, CA, 92121, U.S.A.). Other results are shown as bar graphs. In some experiments, the results were expressed as percentage inhibition of interleukin-8 release from TNF-α treated control. $EC_{1/2\ max}$ values were calculated by Prism and pA_2 values calculated from single agonist concentration-ratio values by the Schild equation assuming a slope of unity (Kenakin, 1993). All values are expressed as arithmetic (pA_2 values) or geometric mean ($EC_{1/2\ max}$ values) ± S.E.M. (standard error of the mean) or 95% confidence limits as appropriate. Statistical significance was determined using a one sample *t*-test or analysis of variance (ANOVA) followed by a post hoc test. Statistical significance was assumed if the *P* value was ≤ 0.05.

3. Results

3.1. The effect of TNF-α and the kinetics of interleukin-8 secretion in HT-29 cells

HT-29 cells constitutively expressed low levels of interleukin-8 (33.8 ± 3.8 pg/ml, $n = 6$) after 24 h incubation at 37 °C. Following stimulation with TNF-α (0.1–100 ng/ml), there was a concentration-dependent increase in the release of interleukin-8 from HT-29 cells (Fig. 1A).

Fig. 1B shows the time course of interleukin-8 release from HT-29 cells after stimulation with TNF-α (100 ng/ml). Initially, there was a steep rise in interleukin-8 release within 4 h of stimulation of HT-29 cells with TNF-α (100 ng/ml), followed by a slower rise over the next 8 h and an even slower increase for the rest of the 24 h incubation period. Overall, the cumulative release of interleukin-8 was (4578 ± 378 pg/ml, $n = 6$) after the 24 h incubation period.

3.2. The effect of cannabinoid receptor agonists on TNF-α induced interleukin-8 secretion from HT-29 cells

We examined the effect of the non-selective cannabinoid receptor agonists CP55,940, Δ^9-Tetrahydrocannabinol,

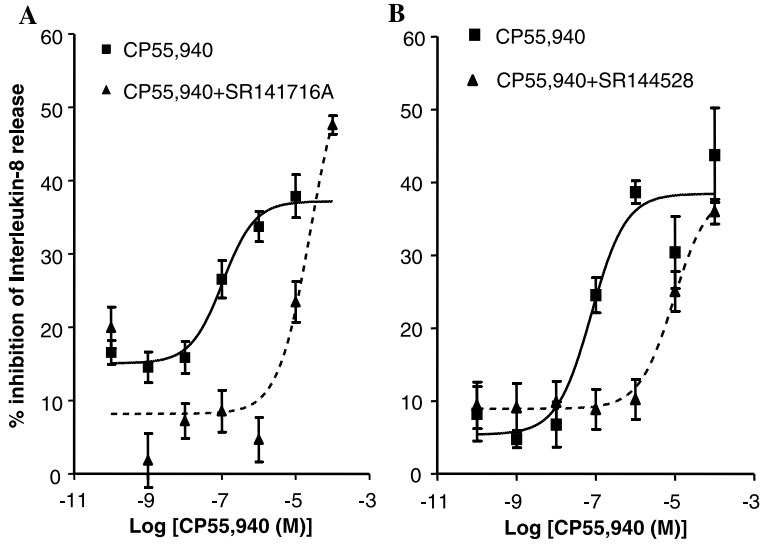

Fig. 4. The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by CP55,940. Confluent monolayers of HT-29 cells were incubated with SR141716A (10^{-6} M) (A) or SR144528 (10^{-6} M) (B) for 30 min before treatment with CP55,940 (10^{-10}–10^{-4} M) for 2 h. Cells were stimulated for further 24 h with TNF-α (100 ng/ml). Supernatants were assayed for interleukin-8 by ELISA as described in Materials and methods. Bars represent S.E.M. of six separate experiments.

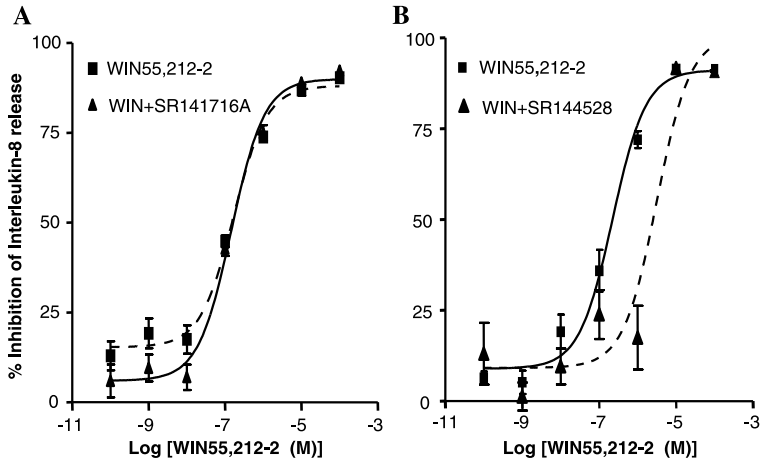

Fig. 5. The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by WIN55212-2. Confluent monolayers of HT-29 cells were incubated with SR141716A (10^{-6} M) (A) or SR144528 (10^{-6} M) (B) for 30 min before treatment with WIN55212-2 (10^{-10}–10^{-4} M) for 2 h. Cells were stimulated for further 24 h with TNF-α (100 ng/ml). Supernatants were assayed for interleukin-8 release by ELISA. Vertical bars represent S.E.M. of six separate experiments.

WIN55212-2 (10^{-10}–10^{-4} M) and a selective cannabinoid CB_2 receptor agonist, JWH 015, (10^{-10}–10^{-4} M) on TNF-α-induced secretion of interleukin-8 from HT-29 cells. All the agonists produced a concentration-related inhibition of interleukin-8 secretion and the following $EC_{1/2\ max}$ values were calculated; CP55,940 (1.2×10^{-7} M, 95% confidence limits (C.L.) = 3.8×10^{-8}–3.6×10^{-7} M, $n = 6$), Δ^9-Tetrahydrocannabinol (5.3×10^{-8} M, 95% C.L. = 9.71×10^{-9}–2.9×10^{-7} M, $n = 6$), WIN55212-2 (1.7×10^{-7} M, 95% C.L. = 1.2×10^{-7}–2.5×10^{-7} M, $n = 6$) and JWH 015 (9.8×10^{-8} M, 95% C.L. = 6.8×10^{-8}–1.3×10^{-7} M, $n = 6$). However, the cannabinoid agonists employed in this study produced different maximum effects (WIN55212-2 = 90.3 ± 1%, Δ^9-Tetrahydrocannabinol = 71.2 ± 9%, JWH 015 = 67.3 ± 4%, CP55,940 = 38.0 ± 10.0%, $n = 6$). Within the concentration ranges tested, CP55,940 (10^{-7} M–10^{-4} M), Δ^9-Tetrahydrocannabinol (10^{-8} M–10^{-4} M), WIN55212-2 (10^{-7} M–10^{-4} M) and JWH 015 (10^{-7} M–10^{-4} M) significantly ($P < 0.05$) inhibited TNF-α-induced interleukin-8 release from HT-29 cells (one-way ANOVA followed by Dunnett's post hoc test, $n = 6$). (Fig. 2).

3.3. The effect of WIN55212-3 and ACEA and on TNF-α induced interleukin-8 release from HT-29 cells

The less active enantiomer of WIN55212-2, WIN55212-3 (10^{-10}–10^{-4} M) and the cannabinoid CB_1 receptor agonist, ACEA (10^{-10}–10^{-4} M) had no significant ($P > 0.05$, $n = 6$), inhibitory effect on TNF-α (100 ng/ml)-induced release of interleukin-8 from HT-29 cells (refer to

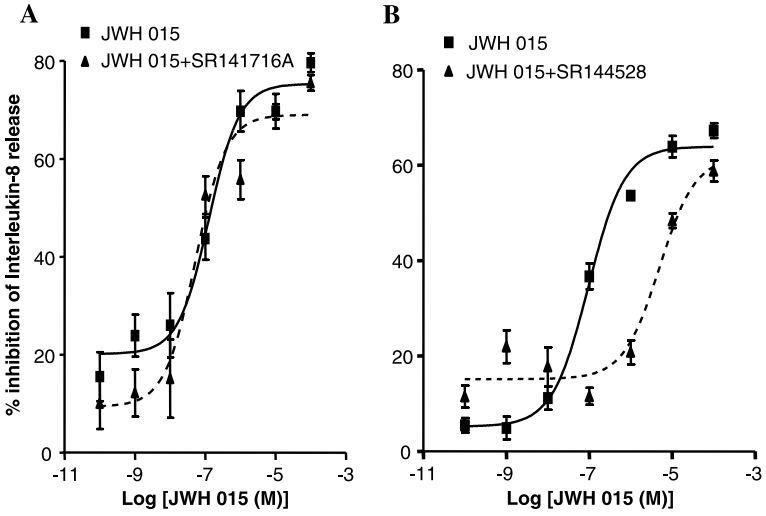

Fig. 6. The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by JWH 015. Confluent monolayers of HT-29 cells were incubated with SR141716A (10^{-6} M) or SR144529 (10^{-6} M) for 30 min before treatment with JWH 015 (10^{-10}–10^{-4} M) for 2 h. Cells were stimulated for further 24 h with TNFα (100 ng/ml). Supernatants were assayed for interleukin-8 release by ELISA. Bars represent S.E.M. of six separate experiments.

Fig. 3). Since ACEA is unstable and subject to degradation by amidases (Hillard et al., 1999), experiments were carried out in the presence or absence of the amidase inhibitor, phenylmethylsulfonyl fluoride (5.0×10^{-5} M). Under these conditions, ACEA (10^{-10}–10^{-4} M) still did not significantly alter interleukin-8 secretion (data not shown).

3.4. The effect of SR141716A and SR144528 on the inhibitory action of CP55,940, WIN55212-2 and JWH 015 on HT-29 cells

The cannabinoid CB_1 receptor antagonist, SR141716A (10^{-6} M) significantly ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test $n=6$) antagonised the inhibitory effects of CP55,940 ($pA_2=8.3\pm0.2$, $n=6$), but did not antagonise the effects of WIN55212-2 ($pA_2<6$) or JWH 015 ($pA_2<6$) (Figs. 4A, 5A and 6A). In contrast, the cannabinoid CB_2 receptor antagonist, SR144528 (10^{-6} M) significantly ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test $n=6$) antagonised the inhibitory effects of CP55,940 ($pA_2=8.2\pm0.8$, $n=6$), WIN55212-2 ($pA_2=7.1\pm0.3$, $n=6$) and JWH 015 ($pA_2=7.6\pm0.4$, $n=6$), respectively (Figs. 4B, 5B and 6B).

3.5. Immunolocalization of the cannabinoid receptor in HT-29 cells

To confirm the identity of the cannabinoid receptor mediating the functional responses in these cells, antibodies raised against the rat cannabinoid CB_2 receptor protein were used to visualise proteins on immunoblots obtained from whole cell lysates of HT-29 cells. Fusion protein against the cannabinoid CB_2 receptor was used as a negative control. The results showed clear immunoreactivity with a molecular weight of 40 kDa, along with other minor bands in the HT-29 cells (lanes 1–3, Fig. 7). In the lanes where this antibody was pre-incubated with fusion protein, these bands were completely absent (lanes 4–6, Fig. 7). Fig. 7 is a representative blot of six separate experiments, all of which gave similar results.

3.6. Effect of drugs on cell viability

The HT-29 cells were tested for viability by the MTT assay. Under our experimental conditions, the cell viability exceeded 95% at cannabinoid concentrations of 10^{-5} M and below. CP55,940, WIN55212-2 and Δ^9-Tetrahydrocannabinol induced mild cytotoxicity (35–40%), at a concentration of 10^{-4} M. However, maximum inhibition of interleukin-8 release was seen at 10^{-5} M (Fig. 2) a concentration where cell viability was >95%.

4. Discussion

In the experiments described above, we have studied the effects of cannabinoid receptor ligands on the secretion of interleukin-8 from the human colon epithelial cell line HT-29. Epithelial cells are increasingly being recognised to play a pivotal role in host defense against microorganisms in the intestinal lumen, and in inflammatory responses (Panja et al., 1998). In addition to their functions as preventive and absorptive barriers, epithelial cells also express a variety of pro-inflammatory cytokines including interleukin-1, TNF-α and interferon-γ (Yang et al., 1997). These cytokines, in turn, induce the release of other inflammatory mediators from the epithelium including chemokines, such as interleukin-8 a key neutrophil chemoattractant (Schuerer-Maly et al., 1994), which are upregulated in inflammatory bowel disease (Warhurst et al., 1998).

In the present study, TNF-α induced release of interleukin-8 from HT-29 cells was measured in order to address whether or not cannabinoids altered the release of this chemokine. Preliminary experiments established optimal conditions for TNF-α-induced interleukin-8 release by these cells. Constitutive release of interleukin-8 from HT-29 cells was minimal after 24 h incubation whereas treatment with TNF-α (100 ng/ml) over 24 h evoked a marked increase in interleukin-8 release.

The cannabinoid agonists employed in this study (CP55,940, Δ^9-Tetrahydrocannabinol, WIN55212-2 and

Fig. 7. Western immunoblotting for cannabinoid CB_2 receptor protein in HT-29 cells. Cell lysates (40 μg protein/lane) obtained from HT-29 cells were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and probed with polyclonal anti-cannabinoid CB_2 receptor antibody and anti-cannabinoid CB_2 receptor antibody + fusion protein. A (lanes 1–3) when lysates were incubated with anti-cannabinoid CB_2 receptor antibody only and B (lanes 4–6) when anti-cannabinoid CB_2 receptor antibodies were pre-incubated with fusion protein.

JWH 015) induced concentration-related inhibition of interleukin-8 release from HT-29 cells. WIN55212-2 was a more effective inhibitor of interleukin-8 release from these cells than the other compounds since at a maximally effective concentration it evoked greater than 90% inhibition of interleukin-8 release whereas Δ^9-Tetrahydrocannabinol, CP55,940 or JWH 015 at maximally effective concentrations (10^{-5} M) evoked only 40–70% inhibition. No further inhibitory effect was seen at higher concentrations (10^{-4} M). Although this higher concentration of some compounds (CP55,940) was cytotoxic, the fact that a lower, non-toxic, concentration produced a similar effect suggests that the effect was not due to a cytotoxic action on the cells. The low maximal effect of compounds such as CP55,940 could indicate that these compounds are partial agonists at the cannabinoid CB_2 receptor and that HT-29 cells have a low number of cannabinoid CB_2 receptors compared to other cells. Thus, in common with other systems, compounds with high affinity, but low efficacy, produce a lower maximal effect than compounds with high efficacy (Kenakin, 1993). However, further experiments where attempts are made to antagonise WIN55212-2 with CP55940 may be necessary to confirm this hypothesis. WIN55212-2 has been reported to be between two and seven times more potent at cannabinoid CB_2 receptors than CP55,940 (Slipetz et al., 1995; Felder et al., 1995; Tao and Abood, 1998). In the present study, the potencies of WIN55212-2, JWH 015 and CP55,940 were almost identical although the former compound showed greater efficacy. However, these effects were still observed at concentrations well above their affinity constants as determined in binding studies on neuronal tissues (Pertwee, 1997). Whether these observations are due to the lipophilic nature of these compounds or their interaction with as yet an unidentified target is not known. Further experiments would be needed to understand these observed effects.

In contrast to the present study, Jbilo et al., (1999) showed that CP55,940 stimulated interleukin-8 release from HL-60 cells. While the reason for this difference is unclear, HL-60 cells are a human promyelocytic cell line (Sham et al., 1996) whereas the cells studied by us are a human colonic epithelial cell line and the observed difference could suggest that different tissues respond differently to cannabinoid receptor agonists. In addition, in non-transfected HL-60 cells, the characteristics of CP55,940-induced interleukin-8 release is different from that induced by TNF-α in our experiments. Of particular interest is the finding that interleukin-8 RNA expression induced by CP55,940 in HL-60 cells appeared to be short-lived in that there appeared to be less RNA in cells 6 h after CP55,940 than 3 h after CP55,940 (Jbilo et al., 1999). In HT-29 cells we did not measure any interleukin-8 release after 24-h incubation with cannabinoid receptor agonists (data not shown). Thus, it may be of interest to determine whether cannabinoid receptor agonists cause a small, transient release of interleukin-8 in epithelial cells. However, cannabinoid receptor agonists have been shown to inhibit cytokine release from many, but not all, immune cells (Berdyshev, 2000), suggesting that the effect seen in HL-60 cells may not be representative of the majority of cells.

It is well established that cannabinoid receptors are linked to G_i/G_o protein and activation leads to inhibition of adenylate cyclase (Felder et al., 1995). In contrast to the idea that increases in intracellular cyclic adenosine monophosphate (cAMP) inhibit immune cell function (Haraguchi et al., 1995), it is surprising that activation of G_i protein would lead to inhibition of interleukin-8 release, however, recent evidence suggests that a decrease in cAMP, as seen with cannabinoids and opioids (Kaminski, 1998; Grimm et al., 1998), may also lead to inhibition of immune cell function suggesting that the role of cAMP in immune cells is likely to have been oversimplified (Kaminski, 1998). However, experiments in which second messenger concentrations are measured will be necessary to investigate the pathways mediating inhibition of cytokine release by cannabinoids.

To examine whether the cannabinoid-mediated inhibition of interleukin-8 release is linked to specific receptors, HT-29 cells were exposed to the less active enantiomer of WIN55212-2, WIN55212-3. WIN55212-3 produced no significant ($P<0.05$) inhibitory effect on TNF-α-induced release of interleukin-8 from HT-29 cells indicating that enantiomeric specificity is required for the effect, in turn, suggesting activity at specific receptors. Also experiments with ACEA, a cannabinoid CB_1 receptor selective agonist (Hillard et al., 1999) evoked no significant inhibitory effects on interleukin-8 expression. Taken together, these results suggest that the inhibition of stimulated interleukin-8 release by non-selective cannabinoid receptor agonists (CP55940, Δ^9-Tetrahydrocannabinol, WIN55212-2) and a cannabinoid CB_2 receptor selective agonist (JWH 015) (Chin et al., 1999), may be specifically linked to functional cannabinoid CB_2 receptors.

To confirm the identity of the cannabinoid receptor subtype involved in the inhibition of TNF-α-induced interleukin-8 release, the specific cannabinoid receptor antagonists SR141716A (CB_1) and SR144258 (CB_2) were used (Rinaldi-Carmona et al., 1994, 1998). When HT-29 cells were exposed to SR141716A, there was antagonism of the inhibitory effects of CP55,940 but not those of WIN55,212-2 or JWH 015. In contrast, treatment of HT-29 cells with the cannabinoid CB_2 receptor antagonist SR144528 reduced the inhibitory effects of CP55,940, WIN55212-2 and JWH 015. We do not know the reason for the unusual susceptibility of inhibition of CP55,940 to reversal by both classes of cannabinoid antagonists but it may be linked to the lower maximum inhibition seen with this compound. Clearly, additional work, such as binding studies would be necessary to answer whether or not HT-29 cells contain a small number of cannabinoid CB_1 receptors that contribute to the response to CP55940 but not to other more selective compounds. However, our functional observations suggest that cannabinoid CB_2 receptors mediate inhibition of TNF-α-induced interleukin-8 release from HT-29 cells. To confirm the existence of this receptor in HT-29 cells, we

employed a polyclonal antibody raised against the amino terminus of the cannabinoid CB_2 receptor to confirm the presence of cannabinoid CB_2 receptors on HT-29 cells by Western immunoblotting. We found an intense band of immunoreactivity at the 40 kDa position, which corresponds to the size of peripheral cannabinoid CB_2 receptor protein as reported by others, e.g. (Rhee et al., 2000). Furthermore, this band was ablated when the polyclonal antibody was pre-incubated for 10 min with fusion protein thus suggesting that this protein is the cannabinoid CB_2 receptor.

In summary, we have shown that cannabinoids exert an inhibitory effect on the expression of TNF-α-induced interleukin-8 release from HT-29 cells. Addition of the less active enantiomer of the cannabinoid receptor agonist, WIN55212-2, WIN55212-3 or a cannabinoid CB_1 receptor selective agonist had no inhibitory effect on interleukin-8 release. Cannabinoid-induced inhibition of interleukin-8 release was reversed by a cannabinoid CB_2 receptor antagonist, however, the cannabinoid CB_1 receptor antagonist was unable to reverse the effects of more selective cannabinoid CB_2 receptor agonists (WIN55212-2 and JWH 015) in this system suggesting a predominantly cannabinoid CB_2 receptor mediated event. Furthermore, Western immunoblotting revealed immunoreactive protein at a region with a size consistent with that of cannabinoid CB_2 receptor protein. We therefore conclude that HT-29 cells express functional cannabinoid CB_2 receptors and suggest that exploitation of this receptor could lead to a novel clinical approach in the treatment of inflammatory bowel disease.

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