CHARACTERISATION OF CANNABINOID RECEPTORS ON IMMUNE CELLS AND CELL LINES

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Dedicated to the loving memories of

Mr C.O Ihenetu Esq (Dad)

And

Mr C.N. Ihenetu (brother)

May your souls rest in peace, Amen

"Blessed are the meek, for they will inherit the earth"

Matthew 5: 5

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Abstract

Cannabinoids may inhibit immune cell function by modulating cytokine/chemokine release but the receptors mediating these events are poorly characterised. The aim of this thesis is to characterise cannabinoid receptors mediating cytokine/chemokine release from immune and inflammatory cells by measuring the effects of cannabinoids on cytokine release using ELISA technique. Apoptosis of inflammatory cells was also assessed by visual evaluation of cells treated with cannabinoids using a nuclear fluorochrome 4'6-diamidino-2 phenyl indole dihydrochloride (DAPI).

Non-selective cannabinoid receptor agonists CP55,940 (10^{-6} –10^{-4} M – 10^{-5} M), Δ^9-THC (10^{-10} M) and anandamide (10^{-6} M – 10^{-4} M) inhibited LPS-induced release of TNF-α from THP-1 cells, a monocytic cell line. The cannabinoid CB_2 receptor antagonist SR144528 (10^{-6} M) but not the cannabinoid CB_1 receptor antagonist SR141716A (10^{-6} M) antagonised the inhibitory effects of CP55,940 ($_pA_2$ = 6.1 ± 0.1, n=6) on THP-1 cells. Similarly, CP55,940 (10^{-6} –10^{-4} M – 10^{-5} M), Δ^9-THC (10^{-10} M -10^{-5} M) and anandamide (10^{-6} M – 10^{-4} M) inhibited PHA/PMA-induced IL-2 release from Jurkat cells, a lymphocytic cell line. However in contrast to THP-1 cells, neither SR141716A (10^{-6} M) nor SR144528 (10^{-6} M) antagonised the inhibitory effects of CP55, 940 on this cell line. In peripheral blood mononuclear cells a non-selective cannabinoid receptor agonist WIN55212-2 (10^{-10} M-10^{-5} M) and a selective cannabinoid CB_2 receptor agonist JWH 015 (10^{-10} M – 10^{-5} M) inhibited PHA-induced release of IL-2. These effects were antagonised by SR144528 (10^{-6} M) ($_pA_2$ = 6.3 ± 0.1; 6.5 ± 0.1, n=5 respectively) but not by SR141716A (10^{-6} M). CP55,940 (10^{-10} M –10^{-5} M) produced a small, non-significant (P> 0.05) inhibitory effect on IL-2 release. Δ^9-THC (10^{-10} M-10^{-6} M) and ACEA (10^{-10} M – 10^{-6} M) had no

significant inhibitory effect on the release of IL-2 from PBMC. CP55,940 (10^{-6} M) and Δ^9- THC (10^{-6} M) antagonised the inhibitory effects of WIN55212-2 ($pA_2 = 6.1 \pm 0.1$; 6.96 ± 0.16,n=5 respectively). In HT-29 cells, CP55,940 (10^{-10} –10^{-5} M – 10^{-5} M), Δ^9-THC (10^{-10} M -10^{-5} M), WIN55212-2 (10^{-10} M-10^{-5} M) and JWH 015 (10^{-10} M – 10^{-5} M) inhibited IL-8 release. SR141716A (10^{-6} M) antagonised the inhibitory effects of CP55,940 ($pA_2 = 8.3 \pm 0.2$ n=6) but did not antagonise the effects of WIN55212-2 and JWH 015. SR144528 (10^{-6} M) but not SR141716A (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), JWH 015 ($pA_2 = 7.6 \pm 0.4$, n=6) respectively.

A protein the size of cannabinoid CB_2 receptors was localised in this cell line by Western blotting. CP55,940 and WIN55212-2 inhibited basal and agonist-evoked increases in both intracellular cyclic AMP and intracellular calcium at the same concentration as that inhibiting TNF-α-induced release of IL-8. Furthermore anandamide (>1 μM) but not CP55,940 caused apoptosis in Jurkat and HT-29 cell.

These data suggest that activation of cannabinoid CB_2 receptors in THP-1 cells, PBMC and HT-29 cells could lead to inhibition of cytokine/chemokine release. Furthermore, cannabinoid-evoked inhibition of basal and agonist stimulated increases in HT-29 cells may be related to cannabinoid-evoked inhibition of IL-8 release. Thus data presented in this thesis suggest that cannabinoid CB_2 receptor agonists with high efficacy may have potential clinical utility in the treatment of inflammatory conditions such as inflammatory bowel disease (IBD) or chronic obstructive pulmonary disease (COPD) and other inflammatory disorders where epithelial cells have a major role.

Abbreviation

$[Ca^{2+}]_i$	Intracellular free calcium
AC	Adenylyl cyclase
ACh	Acetyl choline
ADP	adenosine diphosphate
AEA	Arachidonoyl ethanolamide
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AP-1	Activator protein 1
AP-2	Activator protein 2
APC	Antigen presenting cells
ATF	Activator transcription factor
BCA	Bicinchoninic acid
cAMP	cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribo nucleic acid
CHO	Chinese hamster ovary cells
CIA	Collagen induced arthritis
CL	Confidence limit
CMI	Cell mediated immunity
COPD	Chronic obstructive pulmonary disease
CREB	cAMP responsive binding protein
CTX	Cholera toxin
DAPI	(4'6-diamidino-2 phenyindole dihydrochloride
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
$EC_{1/2\ max}$	I/2 maximum effective concentration
ECACC	European collection of animal cell cultures
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal transduction kinase
FAAH	Fatty acid amide hydrolase
FCS	Foetal calf serum
GABA	Gamma amino butyric acid
GIRK G	protein inward rectifying potassium current
GPCR	G protein coupled receptor
GTP	Guanine triphosphate
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disdease
$IC_{1/2\ max}$	½ maximum inhibitory concentration
ICAM	Intracellular adhesion molecules
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-1β	interleukin 1 beta
IL-10	Interleukin 10

IL-12	Interleukin 12
IL-2	interleukin 2
IL-4	Interleukin 4
IL-8	Interleukin 8
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
Kb	Kilobytes
KDa	kilodaltons
LPS	Lipopolysaccharide
MAP	Mitogen activated protein kinase
MRNA	messenger ribonucleic acid
MTT	(3-[4,5-dimethyl thiazole-2yl]2,5-diphenyl tetrazolium bromide
NBTS	National blood transfusion service
NF κB	Nuclear factor kappa B
NF IL6	Nuclear factor interleukin 6
NK	Natural Killer cell
NO	Nitric oxide
PA_2	A logarithmic measure of antagonist potency
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PGE2	Prostaglandin E 2
PHA	Phytohaemagglutinin
PKA	Protein kinase A
PMA	Phorbol 14 myristate 13 acetate
PMSF	Paramethyl sulphonic acid
PT	Paclitaxel
PTX	Pertussis toxin
PVDF	Polyvinyldene difluoride
SDS-PAGE	Sodium dodecyl sulkphate-polyacrylamide gel electrophoresis
SRBC	Sheep red blood cells
TCR	T cell receptor
Th	T helper cell
TMB	Tetramethyl benzidine
TNBS	Trinitrop benzene sulphonic acid
TNF-α	Tumour necrosis factor α
VR	Vanilloid receptor

PUBLICATIONS ARISING FROM THIS THESIS

Abstracts

Ihenetu, K., Molleman, A., Parsons, M.E. & Whelan, C.J. (2001). Modulation of interleukin-8 (IL-8) release in human colon epithelial cell line (HT-29). *Br. J. Pharmacol.* **134**: P164.

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Andersson, H., Summers, A.E., **Ihenetu, K.** & Whelan, C.J. (2002). Induction of apoptosis in the epithelial HT29 by nicotine and tobacco smoke condensate. *Br. J. Pharmacol.* **136**: P39.

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Full Papers

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CONTENTS

Chapter 1; General Introduction

1.1 Background

Cannabinoids, by definition, are a diverse group of compounds that display some or all of the pharmacological properties of Δ^9-tetrahydrocannabinol (Δ^9-THC), originally identified as the main psychotropic constituent of marijuana (Gaoni and Mechuolam, 1964). Marijuana (cannabis) and its derivatives have been used for over 5000 years for both medicinal and recreational purposes (Nahas and Peters, 1999). However, their introduction as a therapeutic medicine has been hampered largely because of their psychoactive effects up until recently.

Following the discovery that cannabinoids act through transmembrane receptors, there was a surge of scientific interest in cannabinoid pharmacology (Howlett *et al*, 1986). To date, two cannabinoid receptors have been identified and cloned (cannabinoid CB_1 and cannabinoid CB_2) receptors respectively (Matsuda *et al*., 1990; Munro *et al*., 1993). Cannabinoid CB_1 receptors are localised mainly in the central nervous system and some peripheral neurons (Glass *et al*., 1997). In contrast, cannabinoid CB_2 receptors are localised mainly in the immune system (Munro *et al*., 1993; Schatz *et al*., 1997).

For the past twenty years, data from clinical and basic research on the effects of cannabinoids now suggest that they may have utility in a wide range of clinical indications including analgesia, control of nausea and appetite stimulation (for reviews, see Pertwee, 1997). However, only two therapeutic cannabinoids have been approved for medicinal use. Nabilone marketed as Cesamet®, is currently used in the United Kingdom as an adjunct therapy for suppression of nausea and vomiting induced by anti-cancer drugs (Pertwee, 1996) and Marinol ®, an oral preparation of

Δ^9-tetrahydrocannabinol, approved by the State of California is used for the treatment of cachexia in acquired immune deficiency syndrome (AIDS) patients (Beal, *et al.*, 1995; Grinspoon *et al.*, 1995).

Recent evidence from *in vivo* and *in vitro* studies show that cannabinoids can either increase or decrease immune cell functions but the mechanisms are poorly understood. Thus cannabinoids may impair cell-mediated immunity (Nahas *et al.* 1976; Klein *et al.*, 1985), humoral immunity (Baczynsky and Zimmermann, 1983) and cellular defences against a variety of infectious agents, including inhibition of cytokine release from immune cells (Klein *et al*, 1998; Berdyshev, 2000). The reported differences on the effects of cannabinoids in immune cells is now known to depend upon experimental factors such as the concentration of drugs used, drug delivery time, type of cells and systems studied (Dewey, 1986). Therefore, given the ability of cannabinoids to increase or decrease the immune cell function, they are now re-classified as "immunomodulators".

The immunomodulating effects of cannabinoids e.g. Δ^9-THC are observed at a relatively high drug concentration (>1 μM or 5 mg/kg), i.e. higher than concentrations required to evoking psycho-activity (Klein *et al.*, 1995; Kaminski *et al.*, 1994). However, cannabinoid receptors involved in the inhibition of immune cell functions are poorly characterised. The aim of this project is to characterise the cannabinoid receptors mediating inhibition of cytokine release from immune cells and cell lines *in vitro* with a view to identifying the likely profile of activity required for a cannabinoid to have utility as an immunomodulator.

1.2 Cannabinoid receptors

Before the discovery of the cannabinoid receptors, the pharmacological properties of the cannabinoids and their lipophilic nature were suggestive of non-receptor mediated action (Dewey, 1986). However, some indications that cannabinoids acted through receptors came from studies showing that Δ^9-THC displayed enantiometric specificity (Howlett *et al*, 1986). Further evidence supporting this hypothesis came from experiments showing that cannabinoids inhibited adenylate cyclase resulting in a decrease in intracellular cyclic AMP (Howlett, 1984). Confirmation of specific cannabinoid binding sites (receptors) in the brain awaited the discovery of a synthetic analogue of Δ^9-THC, CP55,940 (Johnson and Melvin, 1986). This compound was more polar in nature than Δ^9-THC and the tritiated compound ^{3}H CP55,940, was synthesized in order to identify cannabinoid binding sites in the rat brain (Howlett *et al*., 1986). Consequently, the first cannabinoid receptor (CB_1) was identified by Devane *et al*., (1988) and cloned by Matsuda *et al*. (1990). The second cannabinoid receptor (CB_2) was identified and cloned by screening a human cDNA library from the human promyelocytic cell line HL-60 (Munro *et al*., 1993).

1.2.1 Cannabinoid CB_1 receptors

The human cannabinoid CB_1 receptor was identified and cloned by Matsuda *et al.* (1990). The cDNA encoded 473 amino acid proteins with the features of a G-protein coupled receptor (GPCR) (see figure 1.2 a, for the molecular structures of cannabinoid CB_1 receptors). The cannabinoid CB_1 receptors were shown to inhibit adenylate cyclase activity in a stereo-selective and dose-dependent manner (Pertwee, 1997). These receptors are localised in the brain and the spinal cord and in some peripheral tissues (Pertwee, 1997). The pattern of distribution of cannabinoid CB_1 receptors within the central nervous system is heterogenous and may account for

most, if not all, of their pharmacological properties, such as impairment of cognitive reasoning and memory e.t.c. In the peripheral tissues, cannabinoid CB_1 receptors are localised mainly in neurons residing within the nerve terminals (Tsou *et al.*, 1998), and they were shown to play key roles in inhibiting neurotransmitter release (Coutts and Pertwee, 1997). Cannabinoid CB_1 receptors have also been found in leukocytes but their role in the immune system has yet to be established (Shen *et al.*, 1996). The density of cannabinoid CB_1 receptors is less in peripheral tissues than in the central nervous system (Pertwee, 1997), with higher densities in nerve terminals (Tsou *et al.*, 1998). Figures 1.2 a and b below show the structural features of cannabinoid CB_1 and CB_2 receptors.

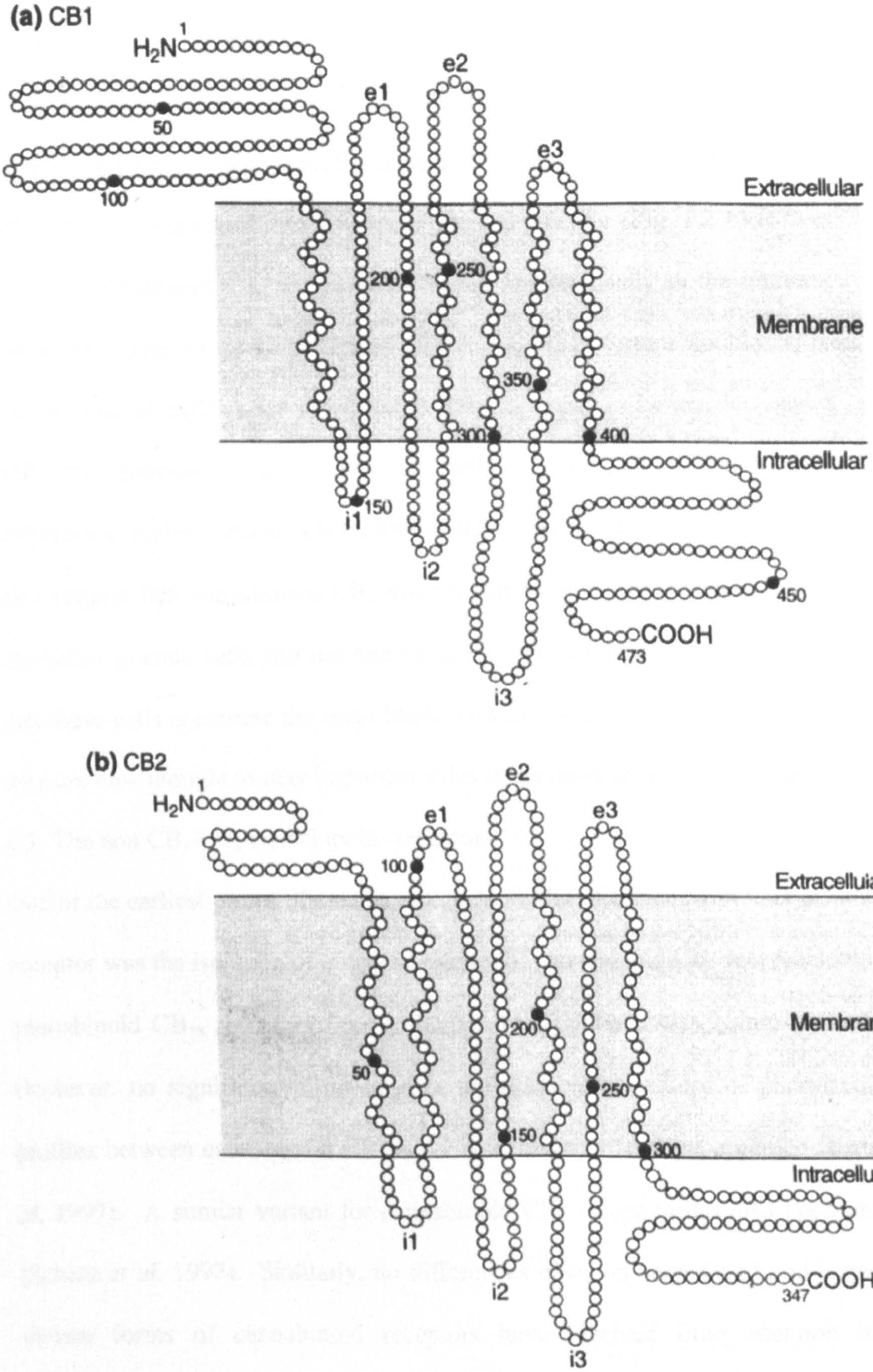

Figure 1.2 (a) The structural features of a cannabinoid CB_1 and (b) CB_2 receptors based on protein sequences U22948 and X864405 from GenBank sequences respectively. Adapted from Klein *et al*., 1998.

1.2.2 Cannabinoid CB_2 receptors

Cannabinoid CB_2 receptors were cloned from the human promyelocytic cell line (HL-60) by screening a human cDNA library (Munro *et al.*, 1993). Like cannabinoid CB_1 receptors, the primary amino acid sequence of cannabinoid CB_2 receptors is consistent with that of a transmembrane G-protein coupled receptor (Fig. 1.2 b) (Munro *et al.*, 1993). Cannabinoid CB_2 receptors are found predominantly in the immune system where the expression is 10-100 times greater than that of cannabinoid CB_1 receptors. The amount of mRNA for cannabinoid CB_2 receptors in human leucocytes occurs with the following rank order: B cell> natural killer cells> monocytes> polymorphonuclear neutrophils> T8> T4 cells (Galigue *et al.*, 1995). Some studies also suggest that cannabinoid CB_2 mRNA can be found in the brain, mainly in the cerebellar granule cells and the microglia e.g. (Skaper *et al.*, 1996). Given the fact that these cells constitute the main brain immune "scavengers" (Skaper *et al.*, 1996), they are now thought to play important roles in the brain immune response.

1.3 The non CB_1/CB_2 cannabinoid- receptors

One of the earliest pieces of evidence suggesting the existence of another cannabinoid receptor was the isolation of a spliced variant of cannabinoid CB_1 receptors known as cannabinoid CB_{1a} receptors from a human lung cDNA library (Shire *et al.*, 1995). However, no significant differences in the distribution pattern or pharmacological profiles between cannabinoid CB_1 and CB_1a receptors have yet emerged (Pertwee *et al*, 1997). A similar variant for cannabinoid CB_2 receptors has also been proposed (Schatz *et al.* 1997). Similarly, no differences have yet been found and hence these variant forms of cannabinoid receptors have received little attention to date. However, with the discovery of the endogenous cannabinoids, there is accumulating

pharmacological and biochemical evidence to support the existence of non-CB_1/non-CB_2 cannabinoid receptors.

1.3.1 Cannabinoid CB_1-like receptors

Several examples of cannabinoid CB_1 receptor-like mediated responses have been described. For example, anandamide was shown to induce vasodilation of rat mesenteric arteries, which was attenuated by SR141716A, the CB_1 receptor antagonist, suggesting an action via cannabinoid CB_1 receptors. However, this effect was still present in knock out mice lacking the gene for cannabinoid CB_1/CB_2 receptors, indicating a target for anandamide on endothelial cells that is distinct from existing cannabinoid CB_1 or CB_2 receptors (Jarai *et al.*, 1999). Other studies on cannabinoid receptor signal transduction mechanisms have also shown that anandamide and WIN55212-2, but not the classical e.g. Δ^9-THC or non-classical cannabinoids, e.g. CP55,940 can stimulate [^{35}S] GTPγS binding in the cannabinoid CB_1 knockout mice indicating the presence of another G-protein coupled receptor for anandamide and WIN55212-2 that may be different from those activated by other cannabinoid agonists (Breivogel *et al.*, 2001). Taken together, these examples could be interpreted as indicating a low level of expression of the already characterised CB_1 cannabinoid receptors or an unidentified cannabinoid CB_1-like receptors, at which some cannabinoid receptor agonists show a low level of efficacy.

1.3.2 Cannabinoid CB_2-like receptors

In cannabinoid CB_2-like receptor-mediated responses, palmitoylethanolamide was shown to exhibit a poor affinity for cannabinoid CB_1 and CB_2 receptors (Devane *et al.*, 1992), yet this agonist induced antinociceptive responses in the mouse formalin paw test as well as in the mouse abdominal stretch test (Calignano *et al.*, 1998). These actions were attenuated by the cannabinoid CB_2 receptor antagonist SR144528

but not by the cannabinoid CB_1 receptor antagonist SR141716A, indicating that these effects were mediated by cannabinoid CB_2. receptors (Calignano *et al.*, 1998). In contrast, lipopolysaccharide-induced-inducible nitric oxide (iNOS) production in RAW 2647 cells was inhibited by palmitoylethanolamide, an effect that could be mimicked by other cannabinoid receptor agonists, suggesting that this action was mediated via a distinct receptor from the known cannabinoid receptors (Gross, 2000). The presence of cannabinoid CB_2-like receptors has also been demonstrated in peripheral nerve terminals in the mouse vas deferens and myenteric longitudinal muscle preparations (Griffin *et al.*, 1997). Pharmacological studies suggest that sufficient differences have already been established to support the existence of CB_2-like receptors distinct from cannabinoid receptors found in immune tissues (Griffin *et al.*, 1997). Other evidence for cannabinoid CB_2-like receptors includes the finding, that anandamide not only binds to cannabinoid CB_1 receptors and CB_2 receptors but can also bind to vanilloid type 1 (VR1) receptors, a ligand-gated, non selective cation channel (Zygmunt *et al.*, 1999; Smart *et al.*, 2000). The nature of this binding to vanilloid receptors is different from the binding to cannabinoid receptors (Di Marzo *et al.*, 2001). While anandamide has been shown to bind to the intracellular domain of the cannabinoid CB_1 receptors (Di Marzo *et al.*, 2001), it binds to the extracellular domain in the vanilloid receptors suggesting that an unidentified transport factor might regulate its distribution and its action via these two receptors (Di Marzo *et al.*, 2001). Taken together, these observations raise the possibility for the existence of cannabinoid receptors distinct from the established receptors (CB_1 and CB_2).

1.4 Cannabinoid receptor agonists

Cannabinoid agonists are classified into four major groups according to their chemical structures namely: (a) classical e.g. (-)-Δ^9-6a, 10a-trans-tetrahydro cannabinol (Δ^9-THC), (b) non-classical, e.g. (-)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxy propyl] cyclo hexan-1ol (CP55,940), (c) aminoalkylindoles, e.g., (+)-[2,3-dihydro-5- methyl -3- [(4-morpholinyl) methyl]pyrrolo[1,2,3-de] -4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate (WIN55,212-2) and (d) the endogenous cannabinoids or eicosanoids e.g. arachidonoyl ethanolamide (anandamide). This section describes various cannabinoid receptor ligands including those employed in this study.

1.4.1 The classical cannabinoids

These are dibenzopyrane derivatives of which Δ^9-THC is a typical example (fig. 1.4 a) and are the main psychoactive components of the herbal plant, *Cannabis sativa* (Gaoni and Mechuolam, 1964). Another plant-derived cannabinoid Δ^8-THC is also a component of cannabis sativa (Gaoni and Mechuolam, 1964). The synthetic analogues belonging to this group are Δ^8-THC-dimethylheptyl (HU 210) and 3-(5'-cyano-1',1'-dimethyl-pentyl)-1(4-N-morpholinobutyryloxy)-Δ^8-THC hydrochloride (O-1057). Compound O-1057 deserves special attention by the virtue of the fact that it is a water-soluble cannabinoid. The availability of a water-soluble cannabinoid has implications both as a useful tool in laboratory studies and in the clinic, where they may be administered as injections or aerosols.

1.4.2 The non-classical cannabinoids

The non-classical cannabinoids are bi or tricyclic analogues of Δ^9-THC in which the central pyran ring of Δ^9-THC has been removed. A typical example in this group is CP55,940, (fig. 1.4 b), a synthetic product from Pfizer (Johnson and Melvin, 1986).

CP55,940 shows a high degree of correlation with its classical congener Δ^9-THC in its *in vivo* activity and other pharmacological characteristics typical of Δ^9-THC (Compton *et al*, 1993).

1.4.3 The aminoalkylindole cannabinoids

Of all cannabinoid receptor ligands, the aminoalkylindoles are the most structurally dissimilar to the classical cannabinoids. A typical example in this group is WIN55212-2 (see Fig.1.4 c) an aminoalkylindole cannabinoid (Pacheco *et al*., 1991). WIN55212-2 inhibited electrically evoked contraction in the mouse vas deferens over a wide concentration range (0.1-100 nM) (Compton *et al*., 1993). WIN55212-2 is stereo specific in action with the (+) isomer being more active than the (-) isomer, WIN55212-3 (Compton *et al*., 1993). Developments using indole and pyrolle derivatives led to 1-propyl-2-methyl-3-(1-naphthoyl) indole (JWH 015), a compound that has a high affinity for the cannabinoid CB_2 receptors (Ki= 14 ± 5 nM) and a 12-fold selectivity for cannabinoid CB_2 receptors (see table 1) (Showalter *et al*., 1996). Further structural adjustments on WIN55212-2 involving iodination of C6 on the indole ring produced a compound, AM 630, which is an antagonist at cannabinoid CB_1 receptors (Hosohata *et al*., 1997)

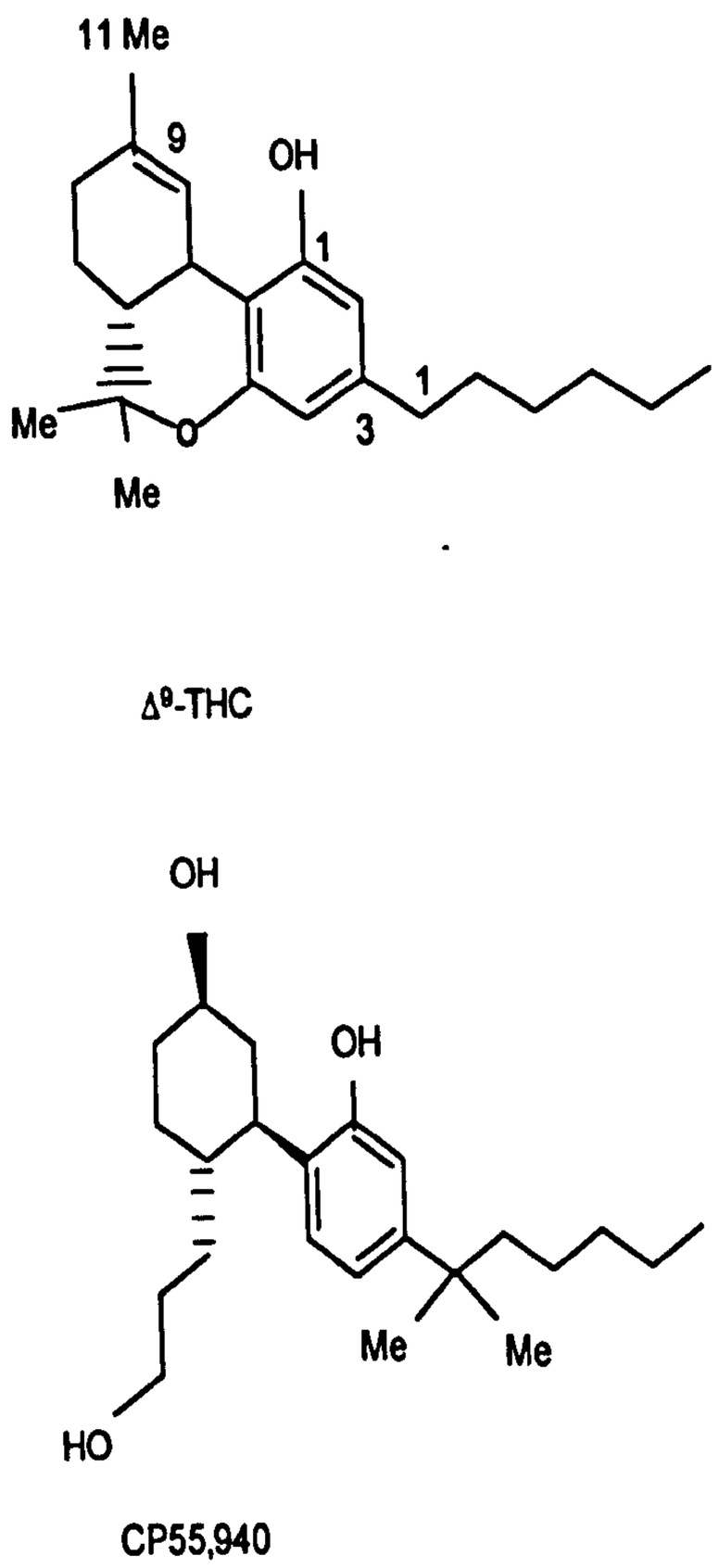

Figure 1.4 The chemical structures of cannabinoid receptor agonists

1.4.4 The endogenous or eicosanoid cannabinoids

Endogenous cannabinoids are unsaturated fatty acid derivatives of ethanolamide. Arachidonoyl ethanolamide, or anandamide, (fig. 1.4 d), the first endogenous cannabinoid identified, was isolated from porcine brain and found to possess pharmacological properties typical of a cannabinoid agonist (Devane *et al.*, 1992). Like other cannabinoid receptor agonists, anandamide evokes a "tetrad" of characteristic pharmacological effects; antinociception, hypothermia, sedation, and catalepsy, the combination of which has proved acceptable as a screening procedure for cannabimimetic compounds (Mechuolam and Fride, 1995). The effects of ananadamide are mediated via G-proteins (Felder *et al.*, 1995), modulation of calcium channels and activation of MAP kinases (all of which are properties of a typical cannabinoid receptor ligand) (Mackie *et al.*, 1993; Bouaboula *et al.*, 1995).

Despite the interest in endogenous cannabinoids, little is known about their physiological roles. However, recent evidence suggests that anandamide and other endogenous cannabinoids transmit neuronal signals and fulfil many criteria for atypical classical neurotransmitters including:

- Synthesis of transmitters and influx of calcium in response to depolarisation: A number of recent studies suggest, that anandamide is synthesised and released from neurons in response to neurotransmitters or depolarisation and or via calcium influx e.g. (Stella and Piomelli. 2001; Wilson and Nicoll, 2001).
- Mimicry in responses to neuronal stimuli: Neuronal stimulation or exogenous addition of endocannabinoids such as anandamide interacts with postsynaptic receptors and mimics the effects of the classical cannabinoids as shown by Di Marzo *et al.* (1998).

- Degradation of surplus transmitter: There are a number of studies showing that following the synthesis and release of the endocannabinoids from the presynaptic neurons, surplus endocannabinoids are rapidly removed from the extracellular space by a membrane transport process (Di Marzo, 1999; Di Marzo *et al.*, 1998; Hillard and Jarrahian, 2000, Piomelli and Beltramo, 1999; Deutsch and Chin, 1993).
- Re-uptake of transmitter or degradation of products: There is also evidence that endocannabinoids such as anandamide are biotransformed via a microsomal enzyme known as fatty acid amide hydrolase (FAAH) (Di Marzo, 1999; Di Marzo *et al*, 1998).

Taken together, the endogenous or eicosanoid cannabinoids seem to fulfil the criteria for a classical neurotransmitter. Unlike classical neurotransmitters, the endogenous cannabinoids can function as "retrograde synaptic messengers". Hence they are released from postsynaptic neurons and travel backward across synapses activating cannabinoid CB_1 receptors on presynaptic axons and suppressing neurotransmitter release as shown by Wilson and Nicoll (2001). Endocannabinoid release occurs via a calcium-activated mechanism that requires phospholipase D-catalysed hydrolysis of the phospholipase D prescursor, N-arachidonoyl-phosphatidylethanolamine (Di Marzo *et al,* 1994). Thus, despite having a rapid onset of action, the duration of action of anandamide is relatively short, perhaps because of its rapid hydrolysis by FAAH. This amidase activity is sensitive to serine protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF) (Deutsch and Chin, 1993; Koutek *et al.*, 1994). This compound is now frequently employed in studies with endocannabinoids.

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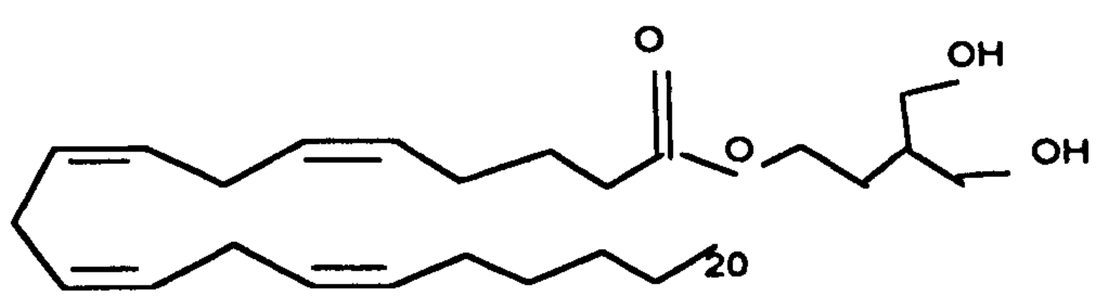
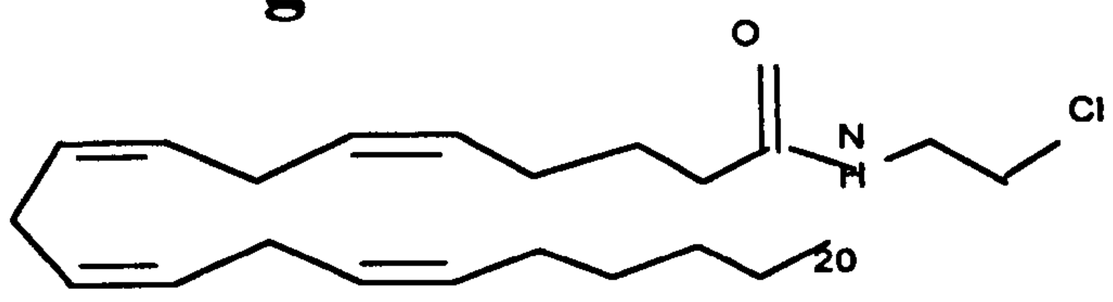


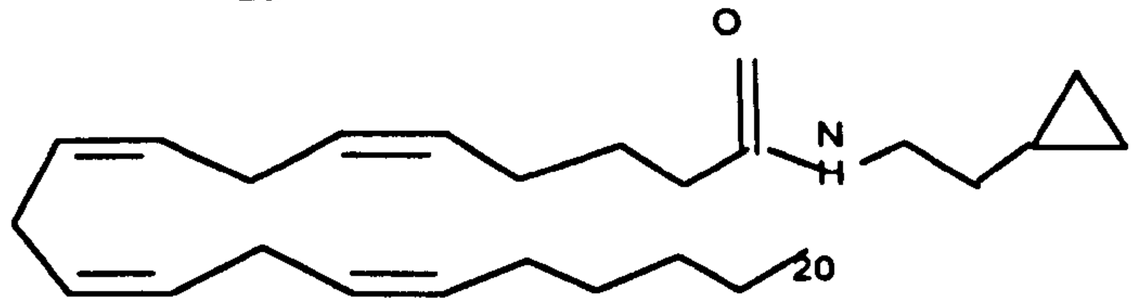
2-Arachidonoyl glycerol

g



ACEA

h



ACPA

Figure 1.4. Examples of the other eicosanoid cannabinoids. ACEA = arachidonoyl 2-chloroethylamide, ACPA = arachidonoyl cyclopropamide.

Some interactions between endogenous cannabinoids e.g. anandamide and other receptors such as opioids, vanilloids and $GABA_B$ receptors have been reported (Di Marzo *et al.*, 1994). The exact significance of these observations is not known but may reflect the physiological roles of the endogenous cannabinoid system in the inhibition of neurotransmitter release in the brain and the peripheral nervous systems (Pertwee, 1997).

1.4.5 Other endogenous (eicosanoid) cannabinoids and their actions

Other endogenous cannabinoids such as sn-2-arachidonoyl glycerol (2-AG) and -arachidonoylglycerol ether (nolandin ether) have been identified. These compounds were isolated from intestinal tissues and both bind to cannabinoid, CB_1 and CB_2 receptors (Howlett, 2002). 2-AG has 3 fold greater selectivity for cannabinoid CB_1 receptors over cannabinoid CB_2 receptors (refer to Table 1.1) and is present in the brain at concentrations about 170 times greater than anandamide (Stella *et al.*, 1997).

Recent evidence suggests, that endocannabinoids such as 2-AG may display "entourage effect". The entourage effect of 2-AG can best be illustrated in a study where 2-AG was accompanied by several 2-acyl-glycerol esters e.g. 2-Linoleoyl-glycerol (2-Lino-GI) and 2-palmitoyl (2-Palm GI) (Ben-Shabat, *et al*, 1998). These compounds do not on their own bind to cannabinoid receptors nor do they inhibit adenylate cyclase activity. However they potentiate the binding of 2-AG and contribute to the potentiation of the effects of 2-AG as measured by inhibition of adenylate cyclase and the resultant decrease in intracellular cAMP (Ben-Shabat *et al.*, 1998). The exact physiological significance of this effect remains unclear but may be important in the future and during characterisation of the effects of these compounds on the cannabinoid receptors.

1.5 Cannabinoid receptor antagonists

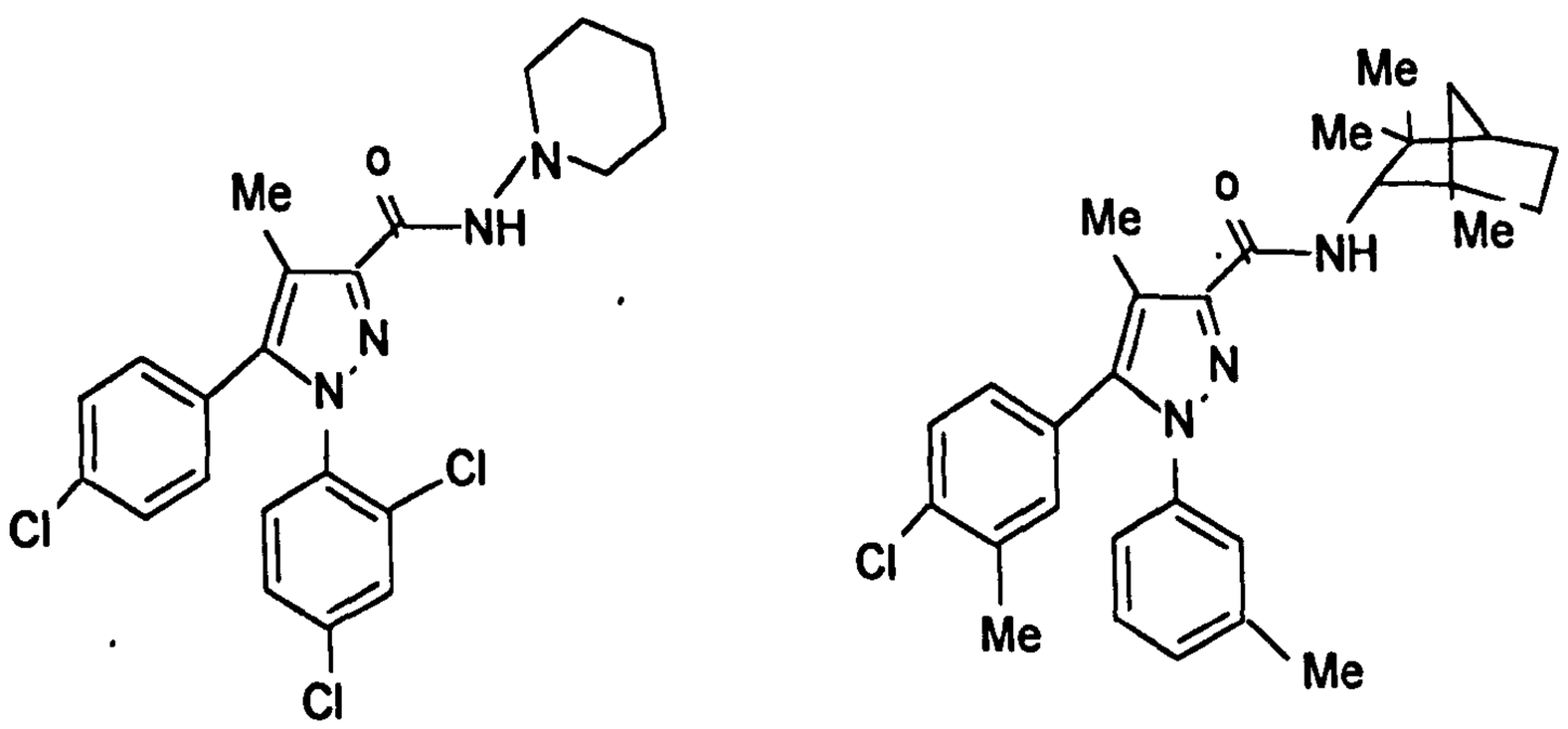
1.5.1 Cannabinoid CB_1 receptor antagonists

Studies at Sanofi Recherche led to the development of the first cannabinoid receptor antagonist, SR141716A (Fig. 1 5 a) (Rinaldi-Carmona *et al.*, 1994). This compound is a diarylpyrazole and displays nanomolar affinity for the cannabinoid CB_1 receptors with approximately 60 fold selectivity for cannabinoid CB_1 receptors over cannabinoid CB_2 receptors (Rinaldi-Carmona *et al.*, 1994 Table 1.1). SR141716 A, antagonises the inhibitory effects of cannabinoid agonists on mouse vas deferens and adenylate cyclase activity in rat brain membranes (Rinaldi-Carmona *et al.*, 1994). It does not affect cannabinoid CB_2 receptor-mediated effects such as inhibition of nitric oxide (NO) release from rat peritoneal macrophages (Ross *et al.*, 2000). SR141716 A evokes effects opposite to those produced by cannabinoid receptor agonists in some bioassays suggesting that this compound may be an inverse agonist (Pertwee *et al.*, 1997). Additional evidence for the inverse agonist activity of SR141716 A was demonstrated in Chinese hamster ovary cells CHO-cells transfected with cannabinoid CB_1 receptors (Bouaboula *et al.*, 1997). In these cells, guanine nucleotides decreased the binding of the cannabinoid agonist CP55,940, an effect usually observed with agonists, whereas it enhanced the binding of SR141716 A, a property of an inverse agonists. Whilst such "inverse cannabimimetic effects" may be attributed to a direct antagonism of responses evoked at cannabinoid CB_1 receptors by released endocannabinoids, there is evidence that this is not the only possible mechanism and that SR141716 A is in fact an inverse agonist (Bouaboula *et al.* 1997, Coutts *et al.*, 2000). Thus SR141716 A may produce inverse cannabimimetic effects in at least some tissues by reducing the activity of endogenous cannabinoids at the CB_1 cannabinoid receptors i.e. (the coupling of CB_1 receptors to their effector mechanisms

that is thought to occur in the absence of an exogenously added or endogenously produced CB_1 agonists). In addition to SR141716 A, other cannabinoid CB_1 receptor antagonists such as AM 630 and LY-320135 have been developed (Hosohata *et al.*, 1997). Like SR141716 A, these compounds also display inverse agonist activities at cannabinoid CB_1 receptors in some biological systems (Hosohata *et al.*, 1997).

1.5.2 Cannabinoid CB_2 receptor antagonists.

The first cannabinoid CB_2 receptor antagonist described was a diarylpyrazole, SR144528 (Fig. 1.5 b) (Rinaldi-Carmona *et al.*, 1998). SR144528 displays a high affinity for cannabinoid CB_2 receptors in rat spleen cells or in Chinese hamster ovary CHO-cells transfected with human cannabinoid CB_2 receptors (Table 1), where it has approximately 50 folds greater selectivity for cannabinoid CB_1 receptors over cannabinoid CB_1 receptors (Rinaldi-Carmona *et al.*, 1998). Like SR141716A, SR144528 also displays inverse agonist activity in CHO cells transfected with cannabinoid CB_2 (Bouaboula *et al.*, 1999).



SR141716A SR144528

Figure 1.5 The chemical structures of cannabinoid receptor antagonists

1.6 Cannabinoid sub-type selective ligands

The best characterised cannabinoid agonists e.g. CP55,940 and Δ^9-THC are essentially non-discriminatory since they bind to both CB_1 and CB_2 cannabinoid receptors with high affinity (Table 1) (Rinaldi-Carmona *et al.*, 1998). However, selective cannabinoid receptor agonists have been developed and these compounds have selectivity and affinity for each of the cannabinoid receptor subtypes described above. For example, two selective cannabinoid CB_1 receptor agonists have been developed. These are the N-arachidonoylethanolamine analogues namely: arachidonoyl cyclopropylamide (ACPA) and arachidonoyl 2-chloroethylamide (ACEA) (Hillard *et al.*, 1999). ACPA and ACEA show greater than 300 and 1000 selectivity respectively, for cannabinoid CB_1 receptors over cannabinoid CB_2 receptors (refer to Table 1). Other compounds showing some selectivity at cannabinoid CB_1 receptors include; anandamide and 2-arachidonoyl glycerol (Mechoulam *et al.*, 1995; Ben-Shabat *et al.*, 1998). Cannabinol (CBN) was reported to have 3.8 folds selectivity for cannabinoid CB_2 receptors over cannabinoid CB_1 receptors (Felder *et al.*, 1995). The substitution of an n-propyl group of the morpholino side chain of the aminoalkylindole (WIN55212-2) produced 1-propyl-2-methyl-3(1-naphthoyl) indole (JWH 015) which has 28 fold selectivity for cannabinoid CB_2 receptors than for the cannabinoid CB_1 receptor (Showalter, *et al.*, 1996; Table 1.1.

Table 1.1 Comparison of Ki values of Cannabinoid receptor Ligands

Receptor subtype	CB_1 (Ki) nM	CB_2 (Ki) nM	Ref.
<u>Classical</u>			
Δ^9-THC	40.7 ± 1.7	36.4 ± 10	Showalter *et al.*, (1996)
HU-210	0.06 ±0.01	0.52 ± 0.05	Felder (1998)
Cannabinol	308 ± 40	96.3± 14	Showalter *et al.*, (1996)
Cannabidiol	4350± 390	5150 ±4190	Showalter *et al.*, (1996)
<u>Non-Classical</u>			
CP55,940	0.58 ±0.07	0.69 ± 0.02	Showalter *et al*,(1996)
<u>Amino-alkylindole</u>			
WIN55,212-2	1.89 ±0.09	0.28 ±0.16	Showalter *et al*, (1996)
JWH-015	383 ±72	13.8 ±4.6	Showalter *et al*, (1996)
JWH-018	9.5 ±4.5	2.94 ±2.65	Showalter *et al*, (1996)
<u>Eicosanoids</u>			
Anandamide	89 ± 10	371 ±102	Showalter *et al*, (1996)
sn-arachidonylglycerol (2-AG)	472 ± 55	1400 ±172	Felder *et al*, (1998)
arachidonylcyclopropylamide (ACPA)	2.2 ± 0.4	715 ±14	Hillard *et al* ,(1999)
arachidonyl-2-chloroethylamide (ACEA)	1.4 ± 0.3	>2000	Hillard *et al*, (1999)
<u>Antagonists</u>			
SR141716A	12.3 ± 3.1	702±62	Shire *et al.*, (1996)
SR144528	33.0 ±5.09	0.67±30	Griffin *et al*, (1999)

Ki is defined as the inhibition constant for a drug, and is the concentration of competing ligand in a competition assay, which would occupy 50% of receptors if no radioligand were present.

1.7 Signal transduction mechanisms of cannabinoid receptors

There are many similarities in the signal transductions of both cannabinoid CB_1 and CB_2 receptors such as coupling to G_i/G_o GTP proteins and the activation of the MAP kinases (Felder *et al.*, 1995). There is also evidence that cannabinoid CB_1 but not cannabinoid CB_2 receptors are coupled to inhibition of N and P/Q-type calcium ion channels (Felder *et al.*, 1993; Howlett, 1995), and G_s protein (Glass and Felder, 1997). This section deals with the current knowledge and evidence in support of the proposed signal transduction mechanisms of cannabinoid receptors and therefore provides the basis for further studies on this topic as described in chapter 6 of this thesis.

1.7.1 Signal transduction mechanisms for cannabinoid CB_1 receptors

1.7.1.1 Inhibition of adenylyl cyclase

Perhaps one of the best-characterized functional properties of cannabinoid receptors is the negative regulation of adenylyl cycase with the resultant decrease in the intracellular cAMP (Felder *et al.*, 1995). The decrease in cAMP observed following treatment of guinea pig isolated tissues with cannabinoid receptor agonists is susceptible to reversal by pertussis toxin, a toxin, which induces ADP-ribosylation of G_i/G_o proteins and the eventual dissociation of its α, β and γ subunits (Pertwee, 1997). The concept that cannabinoid CB_1 receptors inhibit adenylate cyclase is supported by a number of studies employing a range of cannabinoid receptor ligands and cell lines. For example, Δ^9-THC and DALN inhibit cAMP production in N18GT2 cells via the pertussis toxin-sensitive G-protein, suggesting a coupling to G_i/G_o protein (Howlett *et al.*, 1986). This effect has been demonstrated with Δ^9-THC, CP55,940, levantradol and WIN55212-2 in other systems e.g. rat cultured cerebellar granule cells (Pacheco *et al.*, 1993), with CP55,940 in human U373 MG astrocytoma cells (Bouaboula *et al.*,

1995), with Δ^9-THC and anandamide in CHO cells transfected with human or rat cannabinoid CB_1 receptors (Felder *et al.*, 1993; Vogel *et al.*, 1993). Cannabinoid-induced inhibitions of cAMP production in preparations known to express cannabinoid CB_1 receptors are susceptible to inhibition by known cannabinoid CB_1 receptor antagonists such as SR141716A (Felder *et al.*, 1995), an effect that has been demonstrated in experiments with WIN55212-2 in synaptosomes obtained from rat *substantia nigra*, with CP55,940 in human U373 MG astrocytoma cells, with WIN55212-2 and CP55,940 in GH4C1 cells, or mouse X rat hybridoma NG108-15 cells which are transfected with both rat and mouse CB_1 receptors (Ho and Zhao 1996) by transfection. The ability of cannabinoids to inhibit adenylate cyclase activation correlates with their psychotropic potency and with their affinity for cannabinoid CB_1 receptors in radioligand binding studies (Felder *et al.*, 1992; 1995; Howlett, 1987). The rank order of agonist potency for inhibition of cAMP production corresponds to their displacement in radioligand binding studies and for eliciting cannabimimetic responses in various functional studies e.g HU 210>CP55,940>Δ^9-THC anandamide >cannabinol> cannabidiol (Felder *et al.*, 1992, 1995; Howlett, 1987). However, in some systems, cannabinoid receptor ligands did not always lead to inhibition of cAMP production; e.g. anandamide has been shown not to inhibit forskolin stimulated increases in cAMP in rat hippocampal membrane preparations (Childers *et al.*, 1994). Furthermore, chronic *in vivo* treatment of mice with CP55,940 is known to cause a 50 % reduction in the number of [^{3}H] CP55,940 binding sites without producing tolerance to the inhibitory effect of CP55,940 on cAMP production by cerebellar membranes (Fan *et al.*, 1996), suggesting that the inhibition of cAMP signalling system does not account for all of the effects evoked by cannabinoid receptor ligands.

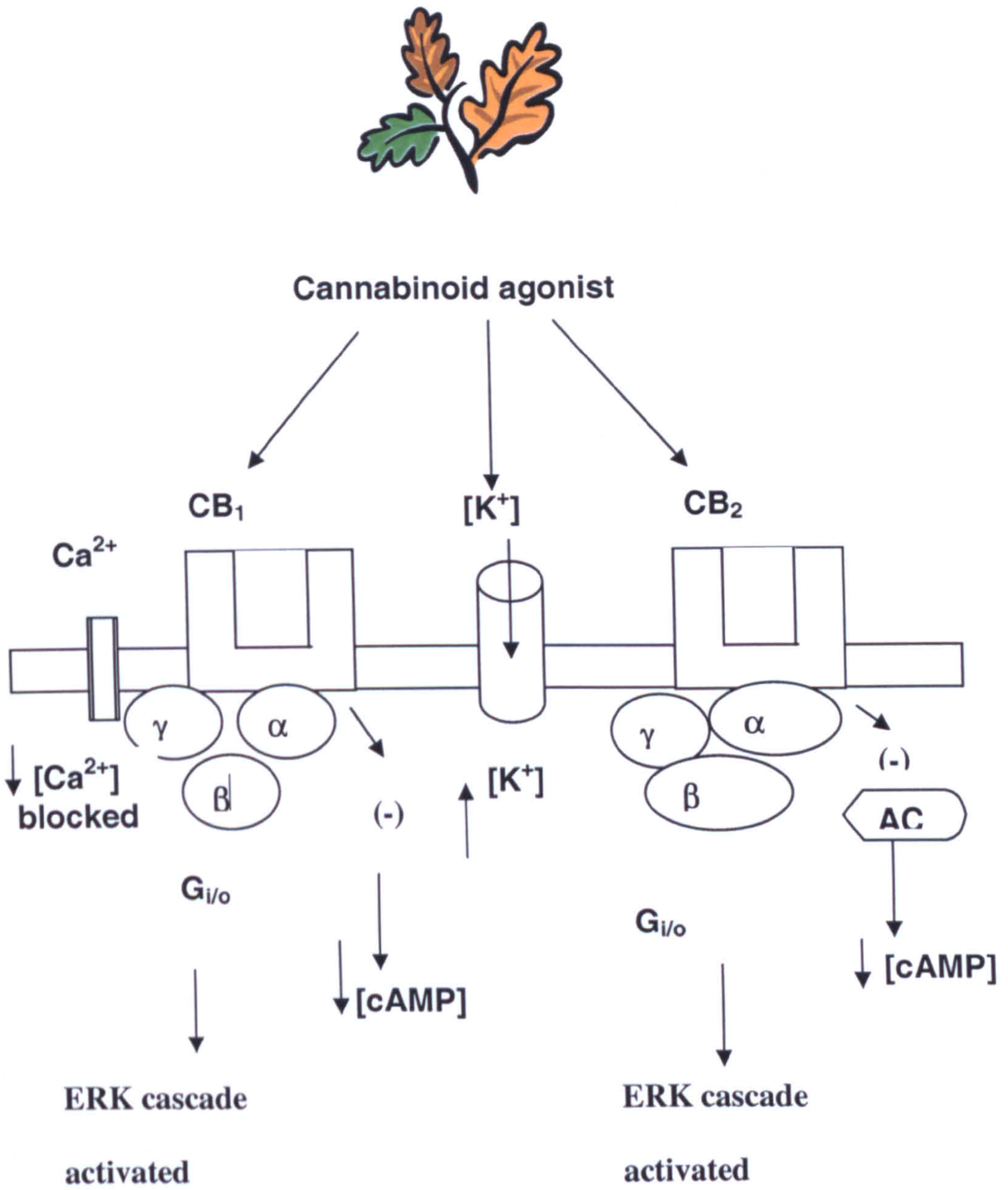


Figure 1.7. Signal transduction pathways activated by cannabinoid receptor agonists.

Cannabinoid receptor agonists activate cannabinoid CB_1 and or CB_2 receptors, both coupled to G_i/G_o proteins. This leads to inhibition of adenylate (AC) and activation of extracellular signal-transduction kinase (ERK) cascade. Furthermore, the CB_1 receptor can induce inhibition of N-type and P/Q-type sensitive Ca^{2+} channels and activate inward rectifying K^+ channels. This leads to membrane hyperpolarisation and inhibition of activity.

1.7.1.2 Modulation of ion channels

In addition to the negative regulation of adenylate cyclase, cannabinoid receptor ligands can also modulate ion channels via cannabinoid CB_1 receptors (Pertwee, 1997). Thus, several cannabinoid receptor agonists show concentration-related inhibition of voltage activated inward calcium currents in transfected and non-transfected cells (Pertwee, 1997). WIN55212-2 stereo-selectively inhibit calcium channels in N18 neuroblastoma cells and this effect is pertussis-toxin sensitive suggesting an action on G_i/G_o protein (Mackie and Hille, 1992; Caulfield and Brown, 1992). In NG108-15 and N18 neuroblastoma cells, the inhibition of calcium channels by Δ^9-THC was blocked by pre-treatment with ω- conotoxin GV1A, an N-type calcium channel blocker, but not by nitrendipine or nifedipine, the L-type calcium channel blockers (Mackie and Hille, 1992; Caulfields and Brown, 1992). These results suggest that the effect is mediated via N-type calcium channels, which inhibit calcium fluxes in these cells (Mackie and Hille, 1992; Caulfields and Brown, 1992). Experiments with cultured rat hippocampal neurons showed that WIN55212-2 acted mainly via N-type (ω-conotoxin GV1A-sensitive) and P/Q-type (ω-conotoxin MV11C-sensitive) calcium channels (Mackie and Hille, 1992). In studies on Xenopus oocyte and a transfected tumor cell line (AtT-20 cells), which expressed inward rectifying potassium channels, G-protein inward rectifying potassium currents, 1 (GIRK1) and cannabinoid CB_1 receptors, have shown that activation of these receptors by cannabinoid receptor ligands leads to inhibition of inward rectifying potassium channels (Henry and Chavkin, 1995; Mackie *et al.*, 1995).

1.7.1.3 Activation of mitogen-activated protein kinase

Another signalling event activated by cannabinoid CB_1 receptor stimulation is the mitogen-activated protein kinase (MAP) kinase pathway. Experiments in W1-38

human foetal lung cells showed that anandamide produced a concentration-related increase in the activity of MAP kinases (Pertwee, 1997). In support of this observation, CP55,940 also induced activation of MAP kinase phosphorylation in CHO cells transfected with human CB_1 receptor DNA and this effect was attenuated by nanomolar concentrations of SR141716A (Bouaboula *et al.*, 1995). However, activation of MAP kinase phosphorylation does not occur in non-transfected cells (Bouaboula *et al.*, 1995). The reason for this is still unclear, but it is known that the transfection process can alter the protein stoichiometry in cells, which may account for the effects described above (Berdyshev, 2000). Additionally, the stimulatory effect of CP55,940 on MAP kinase was found to be sensitive to pertussis toxin pre-treatment implicating G_i/G_o proteins in this pathway (Bouaboula *et al.*, 1995; Wartmann *et al.*, 1995). The other evidence for MAP kinase activation comes from experiments where the administration of CP55,940 into rat striatum stimulates expression of the *Krox-24* gene, thus suggesting a link between the production of this transcription factor and MAP kinase activation (Glass and Dragunow,1995).

1.7.2 Signal transduction mechanisms of cannabinoid CB_2 receptors

1.7.2.1 Inhibition of adenylate cyclase

Like cannabinoid CB_1 receptors, cannabinoid CB_2 receptors negatively regulate adenylate cyclase through a pertussis toxin-sensitive G binding protein (Pertwee, 1997). Several cannabinoid receptor ligands have been shown to inhibit forskolin-stimulated cAMP production from cells naturally expressing cannabinoid CB_2 receptors (Schatz *et al.*, 1997; Koh *et al.*, 1997; Herring *et al.*, 1998). Forskolin-stimulated cAMP accumulation in CHO and AtT-20 cells transfected with cannabinoid CB_2 receptors, an effect that was inhibited by cannabinoid receptor ligands (Felder *et al.*, 1995; Rinaldi-Carmona *et al.*, 1998). However Δ^9-THC and

anandamide evoked weak inhibitory effects on forskolin-stimulated production of cAMP in CHO- cells transfected with cannabinoid CB_2 receptors and antagonised the effects of other more potent cannabinoid agonists suggesting a weak partial agonist activity for these compounds at cannabinoid CB_2 receptors (Bayewitch *et al.*, 1995; 1996; Slipetz *et al.*, 1995). In line with its cannabinoid CB_1 receptor antagonist activity, SR141716A (Rinaldi–Carmona *et al.*, 1994), did not prevent the inhibition of cyclic AMP production mediated by cannabinoid CB_2 receptor activation (Slipetz *et al.*, 1995) in CHO cells transfected with CB_2 receptors. Inhibition of cAMP production mediated by cannabinoid CB_2 receptors in CHO cells was attenuated by pre-treatment with pertussis toxin suggesting a negative coupling to adenylate cyclase through G_i/G_o proteins (Bayewitch *et al.*, 1995; Felder *et al.*, 1995).

1.7.2.2 Mitogen activated protein kinase

In addition to negative regulation of adenylate cyclase, cannabinoid CB_2 receptors stimulate mitogen-activated protein kinase activity (Wartmann *et al.*, 1995; Bouaboula *et al.*, 1996). Activation of this mitogenic pathway by cannabinoid CB_2 receptors was linked to the regulation of *Krox 24* expression in the human promyelocytic cell line HL-60 (Bouaboula *et al.*, 1996). The endogenous cannabinoid 2-AG also induced a rapid phosphorylation of p42/44 MAPK in HL-60 cells and this effect was attenuated by prior treatment with SR144528 suggesting a cannabinoid CB_2 receptor mediated event (Kobayashi *et al.*, 2001). This response was also attenuated by pre-treatment of HL-60 cells with pertussis toxin suggesting that cannabinoid CB_2 receptors are G_i/G_o protein coupled receptors (Kobayashi *et al.*, 2001).

1.8 Effects of cannabinoids on immune cell function

Many *in vivo* and *in vitro* studies have shown that cannabinoids are immunosuppressive agents (Cabral and Dove Pettit, 1998; Klein *et al.*, 1998; Berdyshev, 2000). One possible explanation for the cannabinoid-induced immunosuppression could be due to the alteration/redistribution of leucocyte and lymphocyte subsets. Changes on immune cell functions in response to treatment with cannabinoids could also account for the observed effects. In this section, the effects of cannabinoid receptor ligands on immune cell function are discussed.

1.8.1 In vivo studies on whole animals

Most of the *in vivo* studies linking cannabis use to altered immune cell function have utilized rodent models largely because of the well-defined immune system in rats and mice and the availability of experimental reagents for use in these animals. To date, little *in vivo* data from man is available, however, in one study, Juel-Jensen (1972) documented a greater than normal increase in infection of *herpes simplex* virus among cannabis users. Epidemiologically, a link between the developments of acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus (HIV) infected individuals has been made among cannabis users suggesting that this drug truely suppresses the immune system (Tindall *et al.*, 1988). Further studies suggesting cannabinoid-induced immunosuppression involve an animal study in which 200mg/kg of Δ^9-THC was administered to mice over two consecutive days. A decreased resistance to *Listeria monocytogens* and *herpes simplex* virus infections were observed in these animals (Morahan *et al.*, 1979), thereby suggesting that cannabinoids decrease immune cell function. Although this study was criticized for the high drug concentration used, subsequent workers have replicated and extended these findings. Thus, several studies to date, have shown that Δ^9-THC and other

cannabinoids inhibit not only host resistance to infection by *herpes simplex* virus in guinea pigs and mice, but can cause a dose dependent down-regulation of immune response in these animals thereby suggesting a receptor-mediated mechanism (Mishkin and Cabral, 1985).

1.8.2 In vitro Studies.

Most *in vitro* studies using primary cells and cell lines have implicated cannabinoids as immunosuppressive agents. To date, unequivocal evidence *in vitro* is lacking primarily because the acquisition of such data have proved difficult. Furthermore, factors such as multiple drug use, environmental and ethical issues have rendered such studies even more difficult to interpret. In these studies, animal models have been used but isolated tissues, human immune cells and cell lines are preferred but because of the diverse nature and function of immune cells, such experiments are designed to study immune cell function using enriched preparations of specific cell populations *in vitro*. Data from such studies are reviewed below.

1.8.3 Macrophage/Monocyte

Macrophages/monocytes, play a major role in the innate and acquired immune responses. The innate functions include phagocytosis, ingestion of microbes and release of inflammatory mediators such as NO and arachidonic acid metabolites (Klein *et al.*, 1998). In acquired immunity, they act as antigen presenting cells (APCs) as well as the secretion of some inflammatory cytokines e.g. tumour necrosis factor-alpha (TNF-α) (Zheng and Specter, 1996). Studies with mouse peritoneal macrophages have consistently shown that cannabinoids suppressed a variety of macrophage functions albeit at micromolar concentrations (Klein *et al.*, 1998). Thus, various cannabinoid receptor agonists e.g. Δ^9-THC inhibited macrophage phagocytic activity and cell spreading *in vitro* (Lopez-Cepero *et al.*, 1986), protein expression

(Cabral and Mishkin, 1989), cytolysis of sheep red cells (Burnette-Curley, 1993) and their antigen presenting capacity (McCoy *et al.*, 1995). Modulations of cytokine release from a variety of immune cells *in vitro* by cannabinoids have also been reported (Berdyshev, 2000). The current state of knowledge on the effect of cannabinoids on macrophage/monocyte function suggests that cannabinoid receptor ligands may suppress many important macrophage functions but the effective drug concentration needed to do so is relatively high compared to that seen in the blood of cannabis users (<1 μM) under physiological concentrations e.g. (Azorlosa *et al.*, 1992). Whether this relates to the number of cannabinoid receptor expressed by these cells is presently unknown.

1.8.4 T Lymphocytes.

T lymphocytes are important in protecting the host against microbes and viruses. Early investigators speculated that cannabinoids might suppress immune cell function by altering the number and the function of T cells (Cabral and Dove-Pettit, 1998). In an *in vitro* study, mitogen induced-proliferation of T cells was inhibited by cannabinoids at concentrations (>1μM) (Berdyshev, 2000). In other studies, the non-psychoactive cannabinoid, cannabidiol was marginally more potent than psychoactive cannabinoids such as Δ^9-THC on T-lymphocyte and B-lymphocyte mitogen responses (Klein *et al.*, 1985). These observations suggest an immunosuppressive action of the cannabinoid and also point to the fact that cannabinoids may be acting via a non receptor mechanism. In other studies, mitogen-induced proliferative responses of T and B cells were suppressed by Δ^9-THC at concentrations of (10 μM) with the B cell appearing more sensitive than the other cell types probably due to increased expression of peripheral cannabinoid receptors in these cells (Klein *et al.*, 1998). Other studies involving the T cell rosetting capacity of CD_4 and CD_8 T cell subsets

from marijuana users was impaired suggesting that cannabinoids are immunosuppressive agents (Klein *et al*, 1998). In studies on T cell subset numbers, the mean ratio CD_4/CD_8 of cannabis users was 1.95 as opposed to 1.27 in the controls (Klein *et al.*, 1998), indicating that cannabinoids can cause a shift in the Th_1/Th_2 cell ratio (Figure 1.9). Interestingly, similar shifts have been reported, in HIV infected subjects who eventually developed acquired immune deficiency syndrome.

1.8.5 B-Lymphocytes.

The B cells are a class of lymphocytes responsible for making antibodies (immunoglobulins), a function that is essential for humoral immunity. Several studies on human and animal subjects have examined changes in immunoglobulin levels following administration of natural and synthetic cannabinoids. In one study, no significant change in serum immunoglobulin levels in marijuana users was seen after two months usage when compared with the control group (Klein *et al.*, 1998). In another study, mice were given cannabinoid ligand, Δ^9-THC and antibody agglutination response to sheep red blood cell (SRBC) was suppressed even when injected into whole animals or splenocyte cultures (Baczynsky and Zimmermann, 1983; Kaminski *et al.*, 1992). When the synthetic cannabinoids HU-210 and HU-211 were used to assess anti-SRBC antibody response formation in mice, HU-210 significantly suppressed the haemagglutination titres and reduced the number of splenocytes and plaque forming cells (Titishov *et al.*, 1989). The non-psychoactive enantiomer, HU-211 only suppressed the plaque forming cell response indicating that HU-210 has a much greater cannabimimetic effect than HU-211. Whether the cellular mechanisms underlying this phase of antibody response are susceptible to both the cannabinoid receptor and non-receptor mechanisms is not known. However, anandamide, has been found to affect both the proliferative response in mouse

haemopoetic cell lines through a receptor independent mechanism (Derocq, *et al.*, 1995) and inhibit B cell antibody response in a cytokine-dependent cell line via a cannabinoid CB_2 receptor mechanism (Valk *et al.*, 1997).

Table 1.3. Summary of effects of Cannabinoids on human immune cells.

Studies	Cell type	Function	Effect	Refs
Human subjects	T cells	Proliferation Rossetting CD_4:CD_8 ratio	Decrease No effect Decrease Increase	Nahas *et al.*, 1974 Lau *et al.*, 1975 Nahas *et al.*, 1974 Wallace *et al.*, 1988
	B cells	IgE IgG	Increase Decrease	Rachelefsky *et al.*, 1976 Nahas and Osserman 1991
	Macrophages	Phagocytosis	No effect	Lopez-Cepero *et al.*, 1986.
	NK cells	Cytolysis	No effect	Specter *et al.*, 1986
Human cell culture	T cells	Proliferation	Decrease	Nahas *et al.*, 1977
	B cells	Proliferation	Increase	Derocq *et al.*, 1995
	Macrophages	Nitric oxide release Tumour necrosis factor-α	Increase Decrease	Stefano *et al.*, 1996 Zheng *et al.*, 1992
	Natural Killer cells	Cytolysis	Decrease	Specter *et al.*, 1986

1.8.6 Epithelial cells

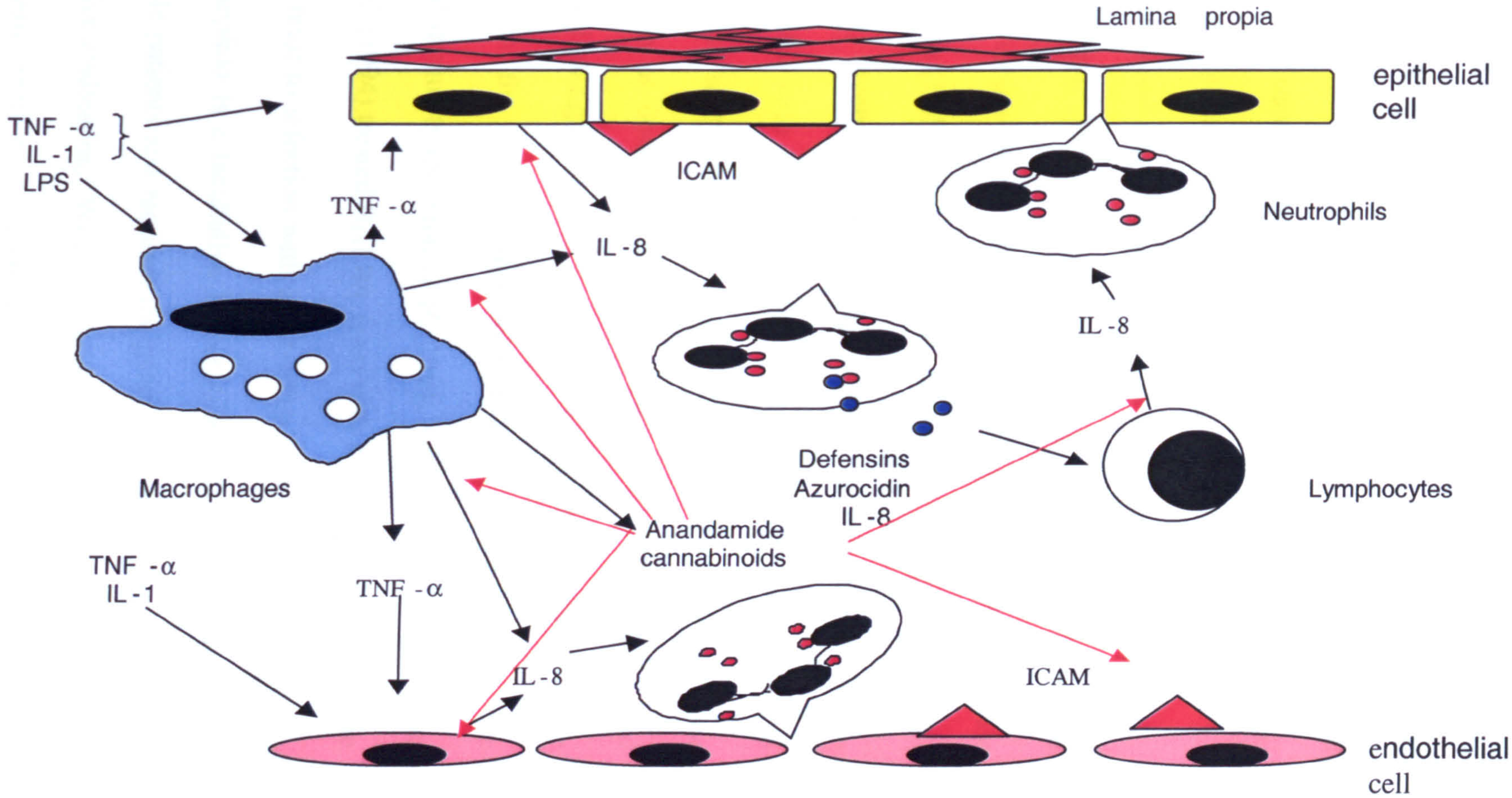
Epithelial cells form boundaries between different environments. For example, intestinal epithelial cells form single layer of cells that separates the host from the gut luminal environment. In addition to its role as absorptive and physical barriers, the intestinal epithelium is now known to play major roles in the gastric immune homeostasis (Schuerer-Maly *et al.*, 1994). The intestinal epithelial cells respond to a

wide array of agents commonly found in the normal gut, including bacteria products, by releasing pro-inflammatory cytokines such as interleukin-8. Human epithelial cells from different anatomical sites such as keratinocytes (Ansell *et al.*, 1990), bronchial epithelial (Nakamura *et al.*, 1991), and gastric carcinoma epithelial cells (Yasumoto *et al.*, 1992, Schuerer-Maly *et al.*, 1994) have all been shown to secrete IL-6, tumour necrosis factor-α and IL-8 and other pro-inflammatory markers.

Some published studies, and anecdotal evidence, suggest that cannabinoids may be effective in the treatment of inflammatory bowel disease and diabetic gastroparesis (Izzo *et al.*, 2001). mRNA for both the cannabinoid CB_1 and CB_2 receptors have been identified in the human gastrointestinal system (Shire *et al.*, 1995; Buckley *et al.*, 1998) and guinea pig whole gut (Griffin *et al.*, 1997). The exact cellular origin of this mRNA and their corresponding receptors/their physiological functions is presently unknown. However, these findings suggest that the cannabinoid system may be important in the maintenance of the gastric immune homeostasis. The gut epithelium is now considered a major source of IL-8 production, a potent chemoattractant for neutrophils and lymphocytes. These cells form the first line of defence in the gut and are thus expected to have major impact in the neighbouring intraepithelial and lamina propia cells. Thus cannabinoid receptor ligands, which have anti-inflammatory properties is now considered a potential clinical treatment for a variety of inflammatory disorders of the gut.

Figure 1.8. Possible mechanism of neutrophil recruitment during an inflammatory process and sites of action of cannabinoid receptor ligands.

Stimulation of epithelial cells, macrophages or vascular endothelium lead to secretion of IL-8 and an up-regulation of adhesion molecules e.g. intracellular adhesion molecule (ICAM). IL-8 acts as chemoattractant molecule to neutrophils and also to lymphocytes. Cannabinoids or anandamide , an endogenous cannabinoid inhibit IL-8 release from immune cells and potentially can resolve an inflammatory response.



1.9 Cannabinoids and Cytokine production.

Cytokines are important in the regulation of host resistance to infection. The production of acute phase cytokines such as IL-1, TNF-α and IL-6 from macrophages and other cells is important for the natural immune response. Cannabinoid-induced changes in the production of these cytokines are thought to account for the reduced anti-microbial immunity as reported by Klein *et al.* (1998).

1.9.1 The effects of cannabinoids on cytokine production

It has been shown that Δ^9-THC (10-30 μM) suppressed the release of IL-1 into the supernatant from cultured mouse peritoneal cells (Klein and Friedman, 1990) while the levels of other cytokines (TNF-α and IL-6) were raised by Δ^9-THC (Klein *et al.*, 1993). In some studies, levels of TNF-α was reduced in mouse and human macrophage cultures, treated with Δ^9-THC. The effect of Δ^9-THC was shown to involve mechanisms related to cytokine processing rather than an effect on gene transcription or translation as illustrated below (Zheng *et al.*, 1992; Zheng and Specter, 1996). Two well-designed mouse models have provided insight into the mechanism (s) by which the plant cannabinoid Δ^9-THC might regulate the development of acquired immunity (Newton *et al.*, 1994; Klein *et al.*, 2000b; Zhu *et al.*, 2000). Newton *et al.* (1994) treated BALB/c mice with a single intravenous dose of Δ^9-THC (4 mg ml^{-1}) prior to infection with a sub-lethal inoculation of *Legionella pneumophila*. *L.pneumophila* is a facultative intracellular bacterium that produces pneumonia in susceptible patients and requires the generation of an antigen-specific Th1 response for effective eradication. When challenged 3-4 weeks later with a sub-lethal bacterial load, control mice survived and demonstrated antigen-specific T cell proliferation associated with elaboration of IFN-γ. In contrast, a high percentage of mice pre-treated with Δ^9-THC during immunization phase died following re-

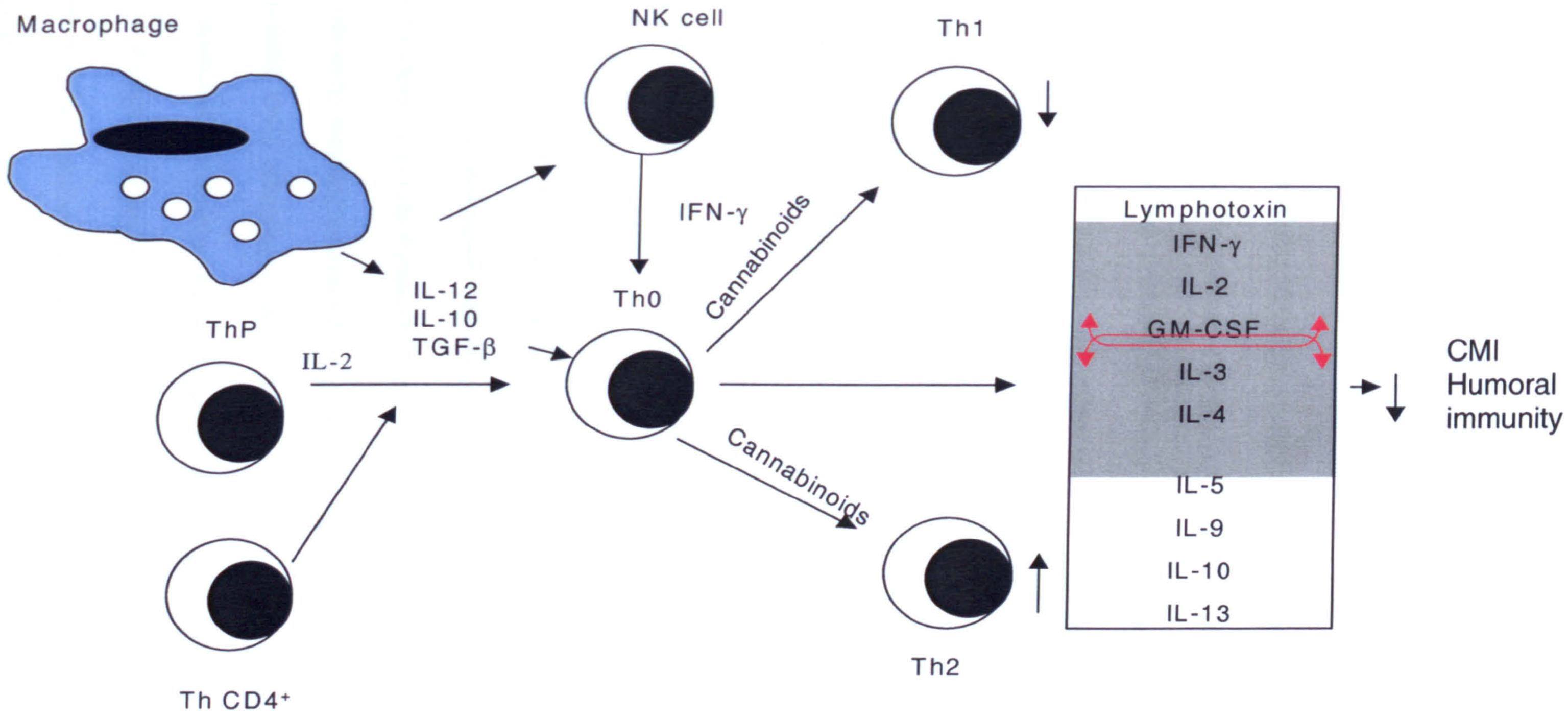
challenge, and their T-cell failed to proliferate in response to *L. pneumophila* antigen *in vitro*. In a similar way, it has been demonstrated that Δ^9-THC enhanced Th_2 cell responses and elevated production of IL-4 and IL-10 (Massi *et al.*, 1998). In summary, it is thought that cannabinoids directs the cytokine network away from cell mediated immunity while enhancing the shift towards Th_2 cell responses. (Refer to figure 1.13 below for the possible effects of cannabinoids on Th_1/Th_2 shifts).

Table 1.4. Selected cytokines, their cellular origin and their actions

Cytokines	Source	Site of action	Effects	References
Tumor necrosis factor α (TNF-α)	Macrophages, mast cells, epithelial cells	Tumor cells, inflammatory cells	Has cytotoxic effects, induces secretion of acute phase cytokines	Klein *et al.*, 1998
Interleukin 1 (IL-1α, β)	Monocytes, macrophages, B cells, dendritic cells, endothelial cells	Th cells, B cells, NK cells	Promotes maturation and clonal expansion, induces expression of adhesion molecules e.g. ICAM	Luo *et al.*, 1992
Interleukin 2 (IL-2)	Th1 cells	Haematopoietic cells, mast cells	Induces proliferation	Klein *et al.*, 1995
Interleukin 8 (IL-8)	Macrophages, endothelial cells, epithelial cells	Neutrophils, lymphocytes	Chemoattactants to neutrophils and leukocytes, induces adhesion molecule expression	Schuerer-Maly *et al.*, 1994

Figure 1.9. Cannabinoid treatment disrupts the balance between T helper 1 (Th1) and (Th2) cell activity suppressing the development of cell-mediated immunity (CMI).

Under normal conditions of antigen stimulation, various cell types contribute to the development of CMI and humoral immunity. For example, macrophages become stimulated to produce cytokines such as IL-12, IL-10, TGF-β supportive of CMI. Stimulated CD4+ T cells and other cell types e.g. NK cells produce IL-4 and IFN-γ supportive of Th2 cells and humoral immunity. Certain infections manipulate this balance and cause preferential development of one type of immunity over the other. Cannabinoids suppress Th1 arm of immunity while increasing Th2 arm. ThP=Precursor T helper cells, ThO= T helper null cells, NK= natural killer cells, CD4+=Cluster of differentiation 4 positive T cells.



1.10 Cannabinoids, apoptosis and regulation of cell fate

The regulation of cell growth, survival and death is known to play important roles in the pathogenesis and resolution of inflammatory processes. To date, two mechanisms by which cells die have been identified namely: necrosis and apoptosis (for reviews, see Cohen *et al.*, 1992; Steller, 1995). Necrotic cell death involves loss of membrane integrity, which leads to the release of potentially toxic intracellular materials into the surrounding environment and promotes inflammation (Cohen *et al.*, 1992; Steller, 1995). In contrast, apoptosis is a controlled process involving loss of membrane phopholipid asymmetry and condensation of nuclear chromatin and the activation of the internucleosomal cleavage commonly recognised as DNA ladders in agarose gel electrophoresis (Wyllie, 1980). Recent evidence suggests that cannabinoids may affect the immune system by regulating immune cell fate, which may involve the induction of apoptosis or proliferation of immune cells.

Recently, attention has focussed on the possible role of the endogenous cannabinoid, anandamide and other endocannabinoids in the regulation of cell growth and differentiation, which may account for some of the pathophysiological effects of these lipids. Anandamide at micromolar concentration was reported to cause inhibition of proliferation of human breast cancer cells (De Petrocellis *et al.*, 1998). In contrast, an enhancement of cell proliferation by anandamide at sub-micromolar concentration has been reported in haematopoietic cells (Derocq *et al.*, 1998). Further preliminary evidence suggesting that anandamide might be associated with inhibition of lymphocyte proliferation and induction of apoptosis has been reported (Schwartz *et al.*, 1994). Anandamide may also have pro-apoptotic activity, both *in vitro* (Sarker *et al.*, 2000) and *in vivo* (Galve Roperh *et al.*, 2000). Taken together, these observations

suggest that cannabinoids can offer promising leads to the development of the future anti-inflammatory and anti-cancer drug therapy.

1.10.1 Mechanisms of cannabinoid-induced regulation of cell fate

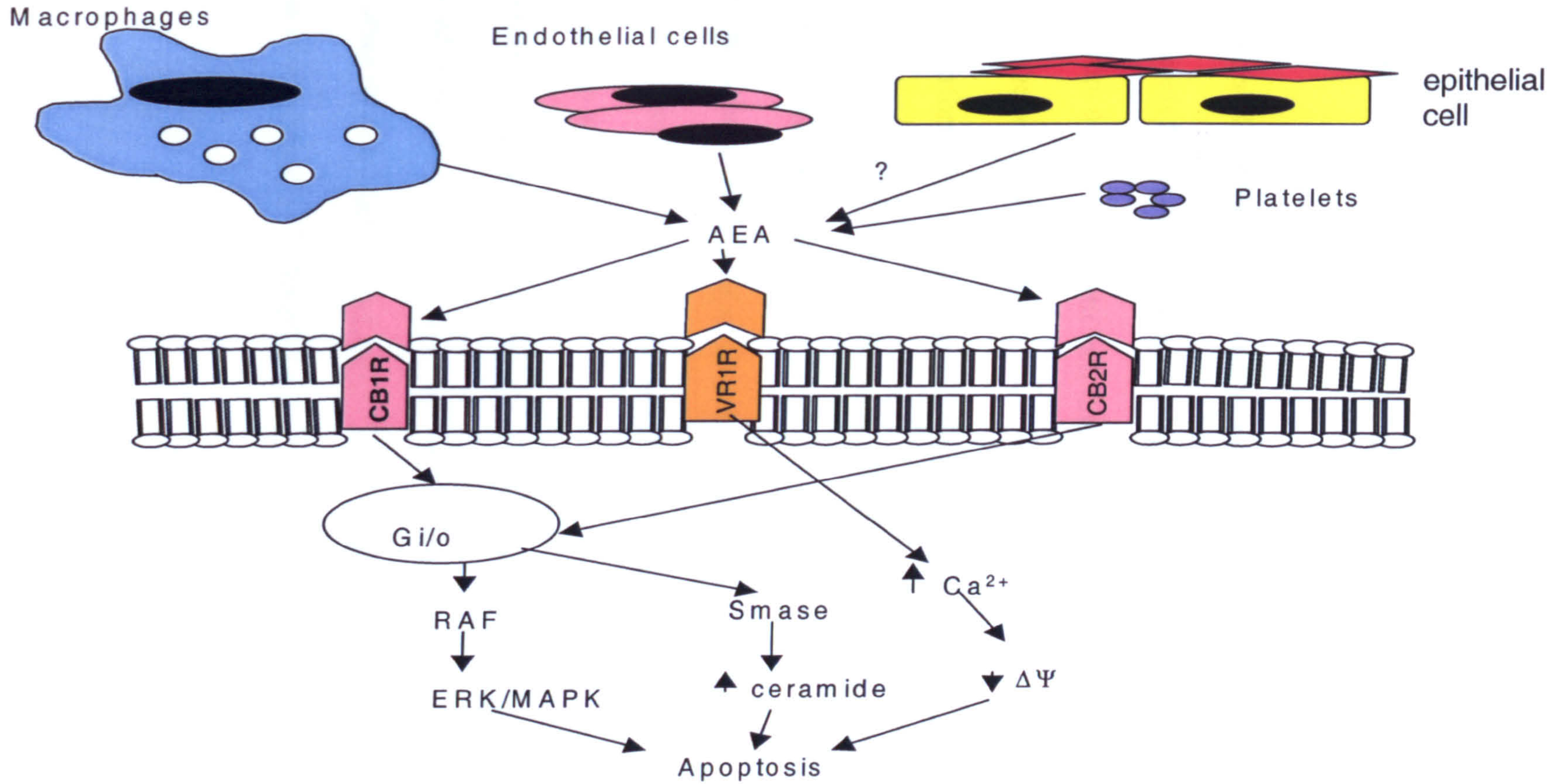
The exact mechanism by which cannabinoids induce apoptosis in immune cells remains unclear. Originally, it is believed that cannabinoids may act via two distinct mechanisms. Firstly, because of its lipophilic properties, cannabinoids may act through intercalation into the cell membrane (Dewey, 1986). However, it was soon realised that the activity of cannabinoids was highly stereospecific, suggesting a receptor-mediated effect. Some signals activated by cannabinoid receptor agonists identified as being relevant to apoptosis are described as follows.

Δ^9-THC-induced apoptosis of C6 glioma cells and breast cancer cells have been shown to cause accumulation of ceramide, a product of sphingomyelin hydrolysis in the cell membrane. This response was mediated via cannabinoid CB_1 and CB_2 receptor dependent pathways (Sanchez, *et al.*, 1998; Galve-Roperh *et al.*, 2000). These actions were shown to involve the activation of ERK and RAF1 downstream signalling pathways (Galve-Roperh *et al.*, 2000). Although, the c2-ceramide, an analogue of ceramide activates ERK pathway, it does not induce apoptosis in the breast cancer cells or C6 gloma cells, suggesting that the action of ceramide in these cells is stereo-specific and a receptor mediated event (Galve-Roperh *et al.*,2000). Anandamide had antiproliferative effects and induced apoptosis in a number of cell lines including breast cancer cells, C6 glioma cells and rat phaeochromocytoma cells (PC-12) via generation of superoxide anion (Sarker *et al.*, 2000). These effects were shown to be inhibited by an anti-oxidant N-acetyl cysteine suggesting that superoxide

anion may play an essential role as a signalling molecule in the induction of apoptosis (Sarker *et al.*, 2000)

The two cannabinoid agonists 2-AG and anandamide inhibited hormone-induced breast cancer cell proliferation by down-regulation of prolactin receptor (De Petrocellis *et al.*, 1998). These two agonists were also shown to reduce nerve growth factor (NGF)-induced breast cancer cell proliferation by down regulating the levels of trk NGF (Melck *et al.*, 2000). These events were shown to be cannabinoid CB_1 receptor mediated and the downstream signals related to inhibition of cAMP/PKA pathways as well as RAF1 translocation and consequently a stimulation of ERKs (Melck *et al.*, 1999). Anandamide was also shown to induce apoptosis in human neuroblastoma (CHP100) and lymphoma (U937) cells (Maccarone *et al.*, 2000). This effect occurred via cannabinoid receptors as well as the vanilloid receptors. However, in a more recent study, McKallip *et al.* (2002) truly demonstrated that cannabinoid receptor ligands caused a concentration-dependent increase in apoptosis of various immune cell lines *in vitro* via cannabinoid CB_2 receptors. *In vivo* they demonstrated that cannabinoids prolonged the survival of cancer bearing rats via cannabinoid CB_2 receptor-dependent mechanism. Collectively, these signals are thought to play important roles in the regulation of cell fate. Figure 1.10 below is a schematic representation of the signalling pathways that may lead to cannabinoid-induced apoptosis.

Figure 1.10. **Cannabinoid signalling pathways potentially involved in the induction of apoptosis**. Macrophages, endothelial cells, platelets and possibly epithelial cells are potential sources of the endogenous cannabinoid e.g. anandamide (AEA). Ligation of cannabinoid CB_1 and CB_2 receptors by cannabinoid receptor ligands leads to activation of $G_i/_o$ proteins as well as activation of mitogen and stress activated protein cascades e.g. extracellular signal-regulated protein kinases (ERK). Acute generation of ceramide may be a G-protein independent process involving adaptor proteins and Smase activation. The activation of vanilloid VR1 receptors by AEA may lead to rises in intracellular calcium, which inhibits mitochondrial oxidative metabolism. The above signals could ultimately lead to induction of apoptosis.



1.11 Aims and objectives

1.11.1 Aim

The aim of this study is to characterise cannabinoid receptors modulating cytokine and chemokine release from primary human immune cells and cell lines.

1.11.2 Objectives

- To investigate the effects of cannabinoids on the release of acute phase cytokines (tumour necrosis factor-α from a promonocytic cell line THP-1 and interleukin-2 release from a prolymphocytic cell line Jurkat E6.1 cells) with a view to characterising the receptors involved.
- Further studies were performed on the effect of cannabinoids on the release of IL-2 from human peripheral blood mononuclear cells (PBMC) and receptors mediating this event were characterised.
- The effects of cannabinoids on the release of interleukin-8 (IL-8) from a cell line distinct from immune cells of the lymphoid origin, the colon epithelial cell line (HT-29) was also studied.
- Studies to characterise the signal transduction pathways by the measurement of intracellular cAMP and the cytosolic free calcium in response to cannabinoids and other ligands were carried out in HT-29 cells.
- Cannabinoids induced apoptosis in T- lymphocytic was also studied.

Chapter 2; General materials and methods

2.1. Drugs and Suppliers

The cannabinoid receptor ligands used in this thesis are listed in Table 2.1. They were purchased from the sources listed below and dissolved in the vehicle as indicated. In all experiments the concentration of DMSO or ethanol vehicle in the final solutions did not exceed 0.1% v/v respectively.

Table 2.1. Shows the alphabetical list of the drugs used in this investigation. Drugs dissolved in ethanol or DMSO were stored at –20°C and protected from light. Drugs dissolved in distilled water were stored at 4°C

Drugs	**Actions**	**Vehicle**	**Source**
Δ^9-THC	CB agonist	Ethanol	RBI
ACEA	CB_1 agonist	Ethanol	Tocris
ACh	Muscarinic agonist	Distilled water	Sigma
anandamide	CB agonist	Soya oil/water emulsion (1:4)	Tocris
CP55,940	CB agonist	Ethanol	Gift from Pfizer/Tocris
dexamethasone	Glucorcorticoid agonist	Ethanol	Sigma
JWH 015	CB_2 agonist	DMSO	Tocris
SR141716A	CB_1 antagonist	Ethanol	Sanofi
SR144528	CB_2 antagonist	Ethanol	Sanofi
WIN55,212-2	CB agonist	DMSO	Tocris
WIN55,212-3	WIN55,212-2 enantiomer	DMSO	Tocris

2.2 Reagents and Supplies

The following reagents were purchased from the suppliers as shown below.

Amersham International PLC, Amersham UK
Biotrack cAMP detection EIA kit

BDH, UK
May and Grunwald stain
Giemsa stain

European collection of animal cell cultures (ECACC), Salisbury, Wiltshire, UK
HT-29 (human colonic epithelial adenocarcinoma cell line)
Jurkat E6.1 (human prolymphocytic cell line)
THP-1 (human promonocytic cell line)

Gibco BRL Life Technologies, Paisely, UK
Foetal calf serum
L-Glutamine
Penicillin/streptomycin
RPMI 1640
TMB (3,3',5,5'-tetramethyl benzidine-H_2O_2)

National Blood Transfusion Service, Colindale, London, UK
Buffy coat blood cells

Peprotech EC Ltd, London, UK
TNF-α standard
IL-2 standard
IL-8 standard

BD Pharmingen Plc, Oxford, UK
TNF-α capture antibody
TNF-α biotinylated detection antibody
IL-2 capture antibody
IL-2 biotinylated detection antibody
IL-8 capture antibody
IL-8 biotinylated detection antibody

Sigma Aldrich Co, Fancy Road, Poole, Dorset, UK
Cholera toxin
DAPI (4'6-diamidino 2-phenylindole dihydrochloride)
Digitonin
Ethylene glycol-bis (β-amino ethyl ether) N,N,N,N-tetra acetic acid) EGTA
Forskolin (7β-acetoxy-1α, 6β, 9α-trihydroxy-8-13-epoxy-labd-14-en-11-one)
HEPES (N-2-hydroxy ethyl piperazine-N'-2-ethane sulphonic acid)
Histopaque R-1077
LPS (lipopolysaccharides)
McCoys 5A medium
MTT (3-[4,5-dimethylthiazole-2-yl] 2,5-diphenyl tetrazolium bromide)

PBS (Phosphate buffered saline)
Pertussis toxin
PHA (phytohaemagglutinin)
PMA (phorbol-14-myristate-13-acetate)
Proteinase K
RNase
TRIS/EDTA

Gift from Dr Ken Mackie (University of Washington, Seattle, WA, USA)
Cannabinoid CB_2 antibody
Fusion protein for cannabinoid CB_2 receptor.
Peroxidase-conjugated goat anti-rabbit IgG

2.3. Cell cultures

2.3.1 THP-1 cells

THP-1 cells a human pro-monocytic cell line, were obtained from the European collection of animal cell cultures (ECACC) (CAMR, Porton Down, Salisbury, Wiltshire, UK). The cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10% foetal bovine serum, 50 $IU.ml^{-1}$ penicillin, 50 $\mu g.ml^{-1}$ streptomycin and 0.5 $\mu g.ml^{-1}$ amphotericin B. Cells were maintained seeded at a density of $1x10^6$ $cells.ml^{-1}$ at 95%/5% CO_2 atmosphere in a thermostatically maintained incubator (37°C) in $75cm^2$ standard cell culture flasks. Cell cultures were split every 2-3 days and the passage number noted.

2.3.2. Jurkat E6.1 cells

Jurkat E6.1 cells, a human T pro-lymphocytic cell line, was obtained from the European collection of animal cell cultures (ECACC) (CAMR, Porton Down, Salisbury, Wiltshire, UK). The cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10% foetal bovine serum, 50 $IU.ml^{-1}$ penicillin, 50 $\mu g.ml^{-1}$ streptomycin and 0.5 $\mu g.ml^{-1}$ amphotericin B. Cells were seeded at a density of 1×10^6 $cells.ml^{-1}$ at 95%/5% CO_2 atmosphere in a thermostatically maintained incubator (37 °C) in a 75 cm^2 standard cell culture flasks. Cell cultures were split every 2-3 days and the passage number noted.

2.3.4. HT-29 cells

The human colon epithelial cell line (HT-29) was obtained from the European collection of animal cell cultures (ECACC) (CAMR Salisbury, Wiltshire, United Kingdom). The cells were grown at 37 °C in McCoy's 5A medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 I $U.ml^{-1}$ penicillin, 50 $\mu g.ml^{-1}$ streptomycin and 0.5 $\mu g.ml^{-1}$ amphotericin B. Cultures were maintained 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 (Sigma-Aldrich Co, Poole, Dorset, UK). 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 of McCoy's 5A medium supplemented with 10% foetal calf serum. Detached cells were harvested and resuspended at a density of 5×10^5 cells.ml^{-1} in FCS-free McCoy's 5A medium and 1 ml aliquots placed in the wells of a 24 well plate for 2 h before experimentation.

2.3.4. Isolation and culture of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats purchased from the National Blood Transfusion Service (NBTS) (Brentwood, Essex, UK). Separation of blood mononuclear cells from erythrocytes was achieved by density gradient centrifugation using Histopaque R-1077 (Sigma-Aldrich Co. Dorset, Poole, UK), based on the modification of the original method described by Boyum (1968). In brief, buffy coat cells were diluted (1:2) in sterile PBS, layered over histopaque and PBMC isolated following centrifugation (800 x g for 25 min) in an Accuspin tube (Sigma-Aldrich, Dorset, Poole, UK). Cells, recovered from the interface between the plasma and Histopaque solution, were washed twice in Ca^{2+} and Mg^{2+} free PBS (250 x g for 10 min). Peripheral blood mononuclear cells were resuspended in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (50 U.ml^{-1}) and streptomycin (50 μg.ml^{-1}) and 10 % heat inactivated foetal calf serum. Aliquots of the cells was removed for cell counting in a Neubauer counting chamber and also assayed for viability by trypan blue dye exclusion method and by the 3-4

(4,5-dimethylthiazole-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay. Slides of the cell suspension were made and stained by Romanowsky stain (May Grunwald-Giemsa) and a differential cell count was obtained.

2.3.5. Isolation and culture of neutrophils

Human blood neutrophils were isolated from buffy coats purchased from the National Blood Transfusion Service (NBTS) (Brentwood, Essex, UK). Separation of human blood neutrophils was done by density gradient centrifugation using Histopaque R-1077 (Sigma-Aldrich Co, Dorset, Poole, UK), based on the modification of an original method described by Boyum (1968). Briefly, human buffy coats were diluted 1:2 (v/v) in sterile PBS and neutrophils separated by density gradient centrifugation (800 x g for 25 min) in an Accuspin tube (Sigma-Aldrich, Dorset, Poole, UK). Following removal of plasma and mononuclear cell layer, neutrophils were isolated from the sediment at the bottom of the tube that also contained the red blood cells. Red blood cells were removed by lysis in ammonium chloride buffer. The ammonium chloride buffer consisted of (NH_4Cl 155 mM; KCO_3 10 mM; Na/EDTA 100 mM). Neutrophils and erythrocytes were diluted 1:3 (v/v) with ammonium chloride solution, incubated for 5 min at room temperature, and centrifuged for 15 min at 250 x g. The supernatant was removed and if there were signs of residual red blood cells, the lysis procedure was repeated to remove the remaining red blood cells. Neutrophils were washed twice in Ca^{2+} and Mg^{2+} free PBS by centrifugation at 250 x g for 5 min. Cells were resuspended in RPMI 1640 supplemented with 10 % FCS, 2 mM L-glutamine, 50 I $U.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin. Neutrophil viability was assessed by the trypan blue dye exclusion method as described in section 2.8.2 and purity by May and Grunwald-Giemsa staining as described in section 2.7.1 followed by a manual differential leukocyte count in which 500 cells were examined

using an Olympus microscope under oil immersion (100 x objective). Neutrophils were finally suspended in RPMI 1640 medium at a final concentration of 1 x 10^6 cells.ml^{-1} in 75 cm^2 standard cell culture flask, Falcon, Becton-Dickinson (Oxford, UK).

2.4. General ELISA Protocols

2.4.1. Objective

The aim of this procedure was to use Enzyme linked immunosorbent assays (ELISA) to measure the amount of TNF-α, IL-2 or IL-8 released into the cell-free supernatant by cell cultures after stimulation with an appropriate mitogen e.g. LPS, PHA or TNF-α as the case may be.

2.4.2. Preparation of reagents

Coating buffer (0.1M carbonate, pH 9.5):

$NaHCO_3$ 8.40 g

Na_2CO_3 3.56g

The following salts were dissolved in distilled water and adjusted to a final volume of 1L. The pH was adjusted to 9.5 using a pH meter. (Buffer was stored at 2-8 °C and used within 30 days of preparation).

Capture antibody (Anti-human cytokine monoclonal antibody):

The capture antibody was supplied as a 1 $mg.ml^{-1}$ solution (Pharmingen, BD UK). The required concentration was 2.0 $\mu g.ml^{-1}$ and the required volume for 96 wells: 100 µl x 96 was 9.6 ml. 20 µl of a 1 $mg.ml^{-1}$ solution was removed and diluted to 10 ml with coating buffer.

Wash buffer (0.01 M PBS + 0.05% Tween 20):

NaCl	0.138 M
KCl	0.0027 M
Tween 20	0.05 %

The following salts were dissolved and pH was adjusted to 7.4 at 25 °C and adjusted to a final volume of 1 L. Alternatively, 1 sachet of PBS + 0.05 % Tween 20 (Sigma-

Aldrich Co) was added together and made up to 1L of distilled water and the pH adjusted to 7.4 at 25 °C.

Assay diluents (10% Foetal Bovine serum in PBS) PH 7.0/standards/samples

This solution was prepared by adding 10 ml of FCS to 90 ml PBS and used within 7 days. Standards and sample dilutions were prepared in RPMI 1640 medium

Working detection antibody:

Anti-human monoclonal biotin antibody was supplied as a 5 mg ml^{-1} solution (BD, Pharmingen PLC, UK). The required concentration was a 1 μg ml^{-1} solution. Therefore, 20 μl of anti-human monoclonal biotin antibody was added to a 10 ml of assay diluents. Streptavidin was also supplied as a 1.0 ml. The required working concentration of streptavidin was a 1 in 1000 dilution of the stock solution. Therefore, 10 μl of streptavidin was added to 10 ml of assay diluents 15 min prior to use.

2.4.3. Plate coating

Micro titre plate wells were coated with 100 μl per well of capture antibody diluted in coating buffer. Plates were sealed and incubated overnight at 4 °C. Following overnight incubation, plates were brought to room temperature before commencement of the assay. Wells were aspirated and washed 3 times with 300 μl per well wash buffer. After the last wash, plates were inverted and blotted onto absorbent paper to remove any residual buffer.

2.4.4. Plate blocking

Coated microtitre plates were blocked with 300 $\mu l.well^{-1}$ of assay diluents (PBS/Tween/FCS) and incubated at room temperature for 1 h. Plates were aspirated and washed 3 times with 300 μl per well wash buffer. After the last wash, plates were inverted and blotted onto absorbent paper to remove any residual buffer.

2.4.5. Addition of standards and samples

From a standard cytokine solution of (10,000 pg ml^{-1}), a 1000 pg ml^{-1} standard solution of cytokine was prepared by diluting the stock in RPMI 1640 (1:10). A further 1 in 2 dilution of this solution was made in RPMI 1640 medium to yield a cytokine standard solution of 500 $pg.ml^{-1}$. Successive serial dilutions of the standard were prepared to correspond to six tubes labelled as 250 pg ml^{-1}, 125 pg ml^{-1}, 62.5 pg ml^{-1}, 31.3 pg ml^{-1}, 15.6 pg ml^{-1} and 7.8 pg ml^{-1} respectively. At each stage the content of the tubes were thoroughly mixed with a vortex mixer. The assay buffer or RPMI 1640 medium served as zero standard (0 $pg.ml^{-1}$). 100 µl of each standard, sample standard or control were added into appropriate wells and plates were sealed and incubated at room temperature for the indicated period of time. After 2 h incubation with standards and samples, wells were aspirated and washed 5 times.

2.4.6. Detection Step.

100 µl of working detector reagent was added to each well plates were sealed and incubated for 1 h at room temperature. Wells were then aspirated and washed a total of 7 times. In the final wash step, the plates were soaked in wash buffer for between 30 seconds and 1 min for each wash. 100 µl of substrate solution (tetra methyl benzidine ;TMB) was added to each well and incubated (without a plate sealer) for 30 min at room temperature in the dark. 50 µl of stop solution (1 M H_2SO_4) was added to each well. The absorbance was read at 450 nm within 30 minutes of stopping reaction in a labsystems micro titre plate reader.

2.4.7. TNF-α measurement

TNF-α release from THP-1 cells was measured by Enzyme linked immunosorbent assay (ELISA) as described above. The capture anti-human TNF-α monoclonal

antibody (Cat No.18631D) was paired with biotinylated anti-human TNF-α monoclonal detection antibody (Cat. No. 186420; Pharmingen, B.D., Oxford, UK

2.4.8. IL-2 measurement

IL-2 release from Jurkat E6.1 cell line or PBMC was measured by Enzyme linked immunosorbent assay (ELISA) as described above. The capture anti-human IL-2 monoclonal antibody (Cat. No. 555051) was paired with biotinylated anti-human IL-2 monoclonal detection antibody (Cat No. 555040; Pharmingen, B.D. Oxford, UK). Using a one step detection procedure (1.0 μg ml^{-1} biotin/streptavidin) reagent was added and incubated at 37°C for 1 h

2.4.9. IL-8 measurement

IL-8 release from HT-29 cell line was measured by Enzyme linked immunosorbent assay (ELISA) as described above. The capture anti-human IL-8 monoclonal antibody(Cat. No. 554718 Pharmingen, B.D Oxford, UK) was paired with biotinylated anti-human IL-8 monoclonal detection antibody (Cat No.554716)

2.5. Western Blotting

2.5.1. Preparation of reagents

Resolving Gel (10 ml): The resolving gel was prepared with the salts listed below and dissolved accordingly.

Ultra Pure water	4.64 ml
30% acrylamide/bis acrylamide	2.66 ml
1.5 M Tris-HCl	2.50 ml
10% SDS	0.10 ml
10% ammonium persulphate	0.10 ml
TEMED (BDH, UK)	6.00 μl

Stacking Gel (4 ml): The resolving gel was prepared with the salts listed below and dissolved accordingly.

Ultra Pure Water	2.44 ml
30% acrylamide/bis acrylamide	0.52 ml
0.5 M Tris-HCl	1.00 ml
10% SDS	0.04 ml
10% ammonium persulphate	0.02 ml
TEMED	4.00 µl

Transfer buffer (1000 ml): The transfer buffer was prepared with the salts listed below and the volume adjusted to (1000 ml).

39 mM glycine	2.93 g
48 mM Tris-Base	5.82 g
0.0375% SDS	0.0375 g
20% methanol (BDH, UK)	200.00 ml

Wash Buffer (1000 ml): The wash buffer was prepared with the salts listed below and the volume adjusted to 1000 ml

10 mM Tris-Base	1.21 g
100 mM NaCl	5.84 g
0.1% Tween 20	1.00 ml

2.5.2. Determination of the protein content of HT-29 cell lysates

HT-29 cells were cultured in McCoys 5A medium to confluence in a 75-cm^2 standard culture flask, Falcon, (B.D Oxford, UK) as described above in section 2.3.5. The medium was removed and washed twice with ice-cold phosphate buffered saline

(PBS). 1 ml of boiling lysis buffer (100°C) was added to the flask and cells were removed with a cell scraper. The lysis buffer contained 10% glycerol, 2% SDS and 76.5 mM Tris. The cell lysate was transferred to an Eppendorf tube and immediately heated to 95°C for 5 min. The cell lysate was sonicated for 15 seconds to reduce the viscosity of the sample, before being centrifuged for 5 min at 5000 rpm in a Hettich EBA12 centrifuge (Hettich Zentrifugen, Germany). To every 100 µl of cell lysate, 2 µl of 2 % bromophenol blue, and 5 µl of β-mercaptoethanol was added in a fume cupboard. Each sample (2 µl) was added, in triplicate, to the inner wells of a 96-well plate, followed by 48 µl of ultra pure water (BDH, UK) to keep the volume in the wells constant. To the outer wells, 10 µl of a bovine serum albumin (Sigma-Aldrich Co, Dorset, Poole, UK) standard (1 to 40 µg/well) was added, again in triplicate. A blank was prepared by adding 10 µl of ultra pure water (BDH, U.K). Two micro litres of lysis buffer and 38 µl of ultra pure water were added to ensure all wells contained 50 µl. Finally, 200 µl of bicinchoninic acid (BCA) reagent (Pierce, UK) was added to each well, and incubated for 1 h at room temperature. The absorbency of the resulting product was measured at 652 nm in a microtitre plate reader and the total protein content of each sample calculated using a standard curve derived from known concentration of BSA solution treated in the same manner.

2.5.3. SDS-Page electrophoresis of protein samples

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the protein content of the sample lysates using a Mini-Protein 11 gel apparatus. The resolving gel was prepared and poured between the glass plates of the gel apparatus and a thin film of absolute ethanol (BDH, UK) was applied over the top of the gel to prevent the inhibition of polymerisation by air. The gel was left to polymerise at room temperature for 60 min. The ethanol was removed and stacking

gel poured on top of the resolving gel. Inserting a 0.75 mm comb into the top of the stacking gel created loading wells. The stacking gel was allowed to polymerise at room temperature for 60 min.

The sample lysates and molecular marker were heated to 95 °C for 5 min. The molecular marker (26 kDa to 180 kDa) was loaded into the first lane, 40 µg of HT-29 cells lysate in the second, third and fourth lanes. A blank lane was left, followed by lysate samples in duplicate. The gel apparatus was filled with tank buffer (0.25 M Tris, 0.192 M glycine, 1% SDS), and subjected to elecrophoresis at 200 volts until the dye reached the bottom of the resolving gel (~50 min).

2.5.4. Immunoblotting of protein

The gel apparatus was dismantled, and the stacking gel separated from the resolving gel. The gel was incubated for 10 min in transfer buffer. An immune-Blot polyvinylidene diflouride (PVDF) membrane (Amersham, UK) was cut to match the size of the gel, dipped briefly in absolute methanol, washed with Ultra pure water (BDH, UK) and placed in transfer buffer.

The base of Trans-Blot Semi-Dry electrophoretic cell was dampened with water, and a filter paper was placed in the middle of the cell, followed by PVDF membrane, then the gel and finally the second filter paper. Protein was transferred from gel to PVDF membrane by electrophoresis at 0.8 mA/cm^2 for 2 h. The membrane was removed from the transfer cell and immediately placed in blocking buffer (wash buffer supplemented with 5% dried milk) and left overnight at room temperature to block any non-specific sites.

After blocking, the membrane was washed three times for 5 min in wash buffer with agitation. The membrane was incubated with either the anti-cannabinoid CB_2 receptor antibody alone, or the anti-cannabinoid CB_2 receptor antibody pre-incubated with fusion protein (2 $\mu g\ ml^{-1}$). The cannabinoid CB_2 receptor antibody and fusion protein were generous gifts from Dr Ken Mackie (University of Washington, Seattle, WA, USA). The antibodies were diluted 1:1000 in blocking buffer and incubated with the membrane for 60 min at room temperature on a rotating plate. This was followed by 5 min washes of the membrane with the wash buffer, which was performed six times in total. The membrane was then incubated with secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) diluted 1:10 000 in blocking buffer, for 60 min at room temperature on a rotating plate. Finally, the membrane was washed for 5 min six times with wash buffer.

2.5.5. Detection of Chemiluminesence and film development

The membrane was placed onto cling-film, while equal volumes of ECL detection solutions A and B (Amersham, UK) were mixed and added to the protein side of the membrane. Following incubation for 1 min, excess detection solution was removed and membrane wrapped in cling-film, ensuring no air-bubbles were present. The membrane was exposed to autoradiography film (Hyperfilm-ECL from Amersham, UK) for 1 to 5 min in the dark, followed by incubation in DEKTOL developer (Kodak, UK) and finally UNIFIX fixer (Kodak, U.K) before being washed with distilled water. All materials were obtained from Sigma, UK, unless otherwise stated.

2.6. Isolation of genomic DNA

2.6.1. Preparation of reagents

Lysis buffer: The lysis buffer was prepared with the reagents listed below and the volume and pH adjusted accordingly

TRIS. HCl	20 mM pH 7
EDTA	10 mM
Triton X-100)	0.2% (v/v)

TE Buffer: The Tris/EDTA buffer was prepared with the reagents listed below and the volume and pH adjusted accordingly

Tris. HCl	10 mM (pH 8.0)
EDTA	1 mM (pH 8.0)

Genomic DNA from neutrophils, Jurkat cells or HT-29 cells were isolated using a modification of an original method (Blin and Stafford, 1976). Following drug treatment of cells and incubation for the indicated period of time, neutrophils (1 x 10^6 cells ml^{-1}) and Jurkat cells (1x 10^6 cells ml^{-1}) or trypsinised HT-29 cells were washed in cold PBS by centrifugation at 500 x g for 5 min in Hettich EBA 12 centrifuge (Hettich Zentrigen, Germany). Supernatants were discarded and cellular content was resuspended in 0.5 ml of lysis buffer in an eppendorf tube and left on ice for 10 min. Lysates were centrifuged for 15 min at 12,000 x g to separate fragmented DNA (supernatant) from chromatin (pellet). Supernatants were incubated with proteinase K (100 $\mu g.ml^{-1}$) at 37°C overnight to prevent DNA degradation by proteolytic enzyme activity. Supernatants were extracted with a 1:1 dilution of phenol/chloroform (v/v) reagent at 4 °C in a Sigma 2K15 centrifuge for 15 min at 5000 x g. The DNA was precipitated at–20 °C for 30 min with 1/5 volume of 5 M ammonium acetate and 1 volume of isopropanol. Supernatants were discarded and DNA pellets washed with 70% ethanol at 4 °C in Sigma 2K15 centrifuge for 15 min at 5000 x g. Supernatants

were discarded and pellets were resuspended with 50 µl TE buffer and residual RNA was digested with 50 µg ml^{-1} of RNAse A for 1 h at 37 °C.

2.6.2. Estimation of genomic DNA content

The purity of DNA sample was estimated spectrophotometrically by measuring the absorbance at UV 260 nm /280 nm respectively and ratios greater than 1.75.was considered sufficiently pure. Samples with a lower ratio suggested the presence of significant amounts of protein and therefore was subjected to a further protein extraction by repeating phenol/chloroform procedure as described in section 2.6.1 above. The DNA concentration was calculated from the absorbance using the formula of Blin and Stafford (1976). From this formula, a solution with OD_{260} of 1.0 contains approximately 50 µg.ml^{-1} of DNA.ml^{-1}.

2.6.3. Agarose gel electrophoresis of genomic DNA

DNA samples were loaded into 1 % agarose gel containing 1 µg. ml^{-1} ethidium bromide and subjected to electrophoresis in TEA buffer at 3 V/cm. The DNA was visualised by UV transillumination for photography. A 1 kb plus ladder (Gibco BRL, Paisely, UK) was used for sizing linear double stranded DNA fragments from 100 bp to 12 k b p.

2.7. Apoptosis Assays

2.7.1. May and Grunwald-Giemsa Staining

Apoptotic neutrophils were identified morphologically following May and Grunwald-Giemsa staining of cytocentrifuge preparations according to the modification of an original method (Dacie and Lewis, 1991). Briefly, cytoprep slides of human neutrophils were fixed in methanol for 5 min. Slides were then stained with May and Grunwald stain 1:2 dilutions (v/v) in phosphate buffered pH 6.8 for 5 min. Slides were stained with Giemsa stain (1:9 dilution (v/v) in phosphate buffer (pH 6.8)

without washing for 15 min. Slides were thoroughly washed with phosphate buffer (pH 6.8). For complete differentiation to be achieved, slides were flooded with phosphate buffer (pH 6.8) for a further 5 min. Slides were air dried and examined under light microscope with oil immersion objective under a x 100 objective. At least 500 cells per slide were counted from different parts of the slide and the data used to assess the percentage of apoptotic cells. Criteria for apoptosis included condensed nuclei and cytoplasmic vacuolation (Savill *et al.*, 1989).

2.7.2. DAPI staining

Apoptosis of human neutrophils, Jurkat cells and HT-29 cells were assessed by a modification of a nuclear staining method with DAPI (Kroning and Lichtenstein, 1998). Briefly, cytocentrifuge preparations of neutrophils or Jurkat cells were fixed in 3.7% formaldehyde in PBS at room temperature for 10 min. Following drug treatment and incubation for different times, a cytocentrifuge preparation of these cells was made onto polylysine coated glass slides. For the adherent cell line HT-29, a culture of these cells was made on Lab-Tek chamber slides. Slides were fixed in 3.7% formaldehyde in PBS at room temperature for 10 min. Slides were thoroughly washed in PBS, and then stained with DAPI (1 $\mu g\ .ml^{-1}$) in PBS at room temperature for 15 min in the dark. Stained cells were then washed three times with PBS and resuspended in 10:1 dilution of glycerol/PBS (v/v). Cells were covered with coverslip. The slide was examined under 400 x magnification using a fluorescent microscope with a 340/380 nm excitation filter and an LP 430 nm barrier filter. At least 500 cells per slide were and used to assess the percentage of apoptotic cells. Slides were photographed with a Leitz fluorescent microscope using UV excitation for DAPI staining (Fig. 2.7 summarises the procedure for apoptotic assays).

2.8. Cell Viability assays

2.8.1. MTT Assay

MTT tablets were dissolved in PBS to produce a concentration of 5 mg ml^{-1} and filtered to remove any insoluble residue. Cells were cultured with drugs. At the end of the incubation period, media were removed and 100 $\mu l.ml^{-1}$ of MTT reagent was added to all wells and incubated at 37 °C for 2 h. Cells were transferred onto 96 well plates and 100 $\mu l.well^{-1}$ of DMSO was added to each well and thoroughly mixed to dissolve the dark crystals. Absorbance was measured at 570 nm wavelength and results were expressed as % of the control values.

2.8.2. Trypan Blue dye exclusion method

One volume of trypan blue dye (0.4% in PBS) was added to 5 volumes of cells in suspension and incubated at room temperature for 5 min. The cell suspension was then counted in an improved Neubauer counting chamber. All counts were performed in duplicate. Cellular viability was expressed as percentage of cells that excluded the dye from the total number of cells counted.

2.9. Intracellular free calcium measurement

2.9.1. Preparation of reagents

HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] buffered saline (Prepared as 10 x stock solution): The HEPES buffered saline was prepared by dissolving the agents listed below

NaCl	4.2 g
KCl	0.19 g
$MgSO_4.6\ H_20$	0.10 g
HEPES (free acid)	1.19 g
Distilled water	50 ml

This solution was mixed with a magnetic stirrer until the contents were dissolved and the solution was stored at 4 °C until required)

Working solution

D Glucose	180 mg
stock HEPES buffered saline	10 ml

50 ml of distilled water was added and pH adjusted to 7.4 with a pH meter (Contents were transferred to 100 ml volumetric flask and made up to 100 ml with distilled water). Final concentrations were NaCl, 145 mM, KCl, 5 mM, $MgSO_4.6\ H_2O$, 1 mM, HEPES, 10 mM, D-Glucose, 10 mM.

2.9.2. Fura-2/AM loading of HT-29 cells

HT-29 cells were cultured as described in section (2.3.5). Adherent HT-29 cells were washed with PBS and detached (0.05% trypsin/0.02% EDTA) from the tissue culture flask. The action of trypsin was stopped by addition of McCoy's 5A medium supplemented with 10% FCS. Before experimentation, cells were incubated in

McCoy's 5A medium for at least 2 h in a humidified atmosphere of 95% air and 5% CO_2. Prior to loading, cells were resuspended in HEPES buffer pH 7.4 containing (10 mM Glucose/1 mM $CaCl_2$) and incubated for 10 - 15 min at 37 °C. Fura-2/AM was added to the cell suspension from a 1 mM stock solution to give a final concentration of 4 μM. Cells were incubated at 37 °C for 45 min in the dark. After loading, cells were washed twice in HEPES containing 10 mM Glucose/1 mM $CaCl_2$ by centrifugation and resuspended in this buffer at a final density of 2×10^7 cells ml^{-1}. Cells were stored at room temperature and protected from sunlight. To minimise problems associated with Fura 2 leakage from cells, all experiments were performed within 2 h of Fura-2/AM loading.

2.9.3. Intracellular Ca^{2+} measurement

Intracellular calcium $[Ca^{2+}]_i$ flux was measured in response to cannabinoids, TNF-α and ACh using a Perkin-Elmer LS5 spectrofluorimeter controlled by a desktop computer. Excitation wavelengths alternated between 340 nm and 380 nm every 4 seconds and fluorescence was monitored at 509 nm. Graphical plots were prepared using commercial software (GraphPad Prism Inc.). Calibration of each individual experiment was performed as described by Thomas and Delaville (1991), where the maximum fluorescence was measured following cell lysis with digitonin (10 μM) and minimum fluorescence by quenching with EGTA (20 mM). The intracellular calcium concentration $[Ca^{2+}]_i$ (nM) was calculated as described by the equation of Grynkiewicz *et al.*, (1985) where $[Ca^{2+}]_i$ (nM) $=K_d.\{R\text{-}R\ min)/(R\ max\text{-}R)\}.\{(F\ max,$ 340 nM)/F min, 380 nm)}, where is 225, and the remaining parameters are defined as in (Fig. 6.4.5 a and b) in chapter 6 respectively.

2.9.4.Calibration of ionised Ca^{2+} measurement with Fura-2/AM preloaded HT-29 cells.

The calibration of ionised intracellular Ca^{2+} measurement was carried out to obtain the maximum and minimum response of the intracellular dye. This maximum Ca^{2+} response was achieved by adding digitonin (10 μM) in the presence of 1 mM extracellular calcium to saturate the dye. The minimum response was achieved by adding EGTA (20 mM) (Fig. 6.4.5 a and b, chapter 6). Data obtained from these experiments were stored as ASCII files post run and were latter retrieved to calculate the $[Ca^{2+}]_i$ (nM). The response produced on the addition of the agonists employed in this study was calculated with reference to data stored in the file.

2.10 Determination of intracellular cyclic adenosine monophosphate (cAMP) in HT-29 cells

HT-29 cells were cultured as described in section 2.3.5 of this chapter. The determination of $[cAMP]_i$ was performed as described by the manufacturer (Amersham International PLC, Amersham UK).

The kit is made of the following components

Microtitre plate

This plate contains 12 x 8 well strips coated with donkey anti-rabbit IgG, ready to use.

Assay buffer

Assay buffer concentrate 1 bottle. On dilution of this bottle, the solution contains 0.05 M sodium acetate buffer, pH 5.8 containing 0.02 % bovine serum albumin and 0.01% preservative.

cAMP standards (for non-acetylation assay)

cAMP standard for non-acetylation assay in the range 12.5-3200 $fmol.well^{-1}$, lyophilised. On reconstitution, this bottle contains 32 p mol $cAMP.ml^{-1}$.

Antibody

Rabbit anti-cAMP was supplied in lyophilised form.

Peroxidase conjugate

cAMP-horse radish peroxidase also lyophilised.

Wash buffer concentrate

Wash buffer concentrate (1 bottle). On dilution, this reagent contains 0.01 M phosphate buffer, pH 7.5 plus 0.05% Tween 20.

TMB substrate

TMB is the enzyme substrate containing 3,3',5,5'-tetramethyl benzidine (TMB)/ hydrogen peroxide in 20% (v/v) dimethylformamide, ready for use.

Acetic anhydride (supplied as ready for use).

Triethylamine (supplied as ready for use).

Lysis reagent 1. containing dodecyltrimethylammonium bromide, 2 g .

Lysis reagent 2. Description of this reagent was not given.

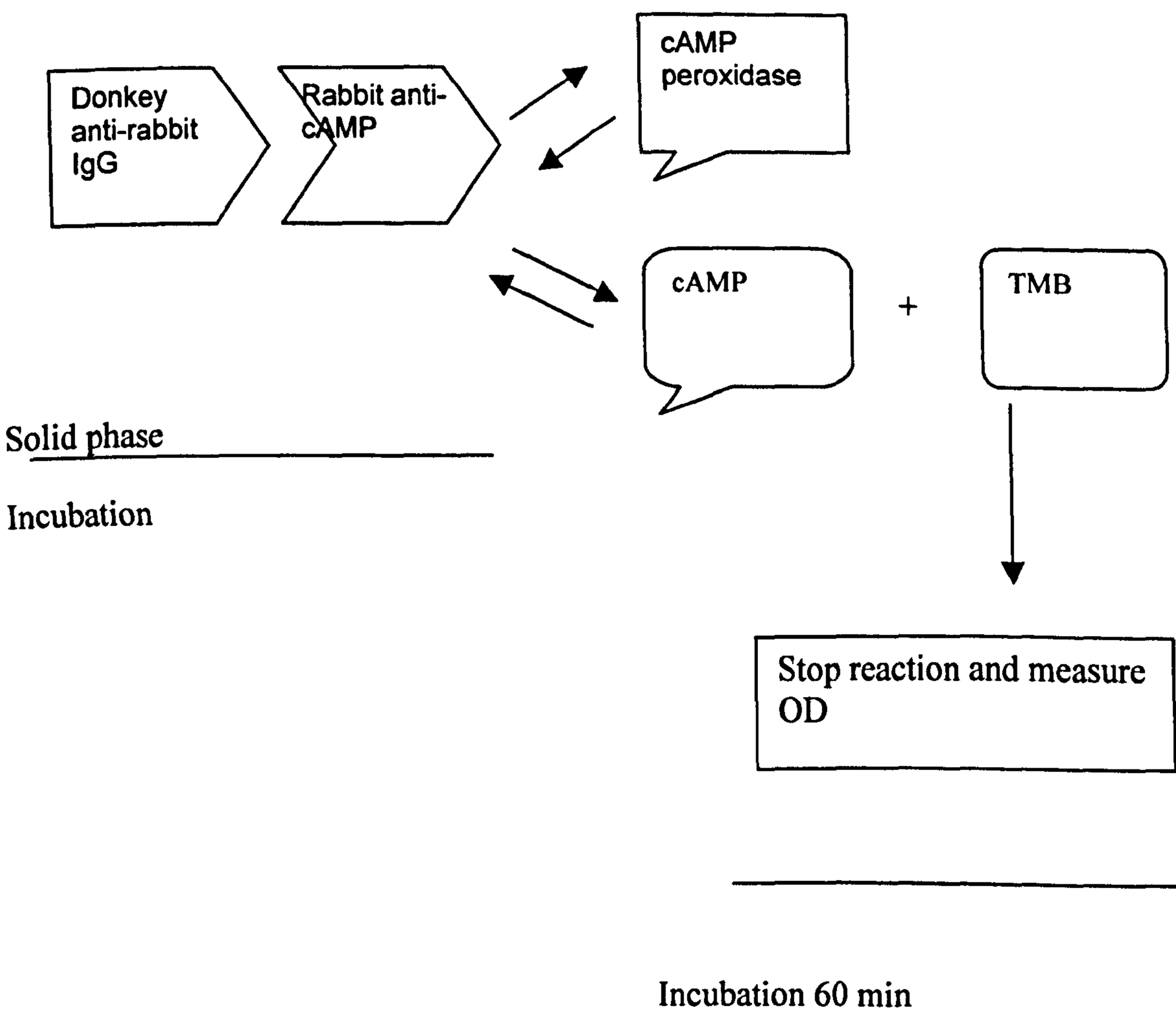
2.10.1 Preparation of working standards.

Working cAMP standards was prepared by labelling 8 propylene tubes (12 x 75 mm) 12.5, 25, 50, 100, 200, 400, 800, and 1600 f mol. 500 µl of lysis reagent 1B was added into all tubes. Into the 1600 f mol tube, 500 µl of stock non-acetylation (32 p mol.ml^{-1}) was added and mixed thoroughly. 500 µl was transferred from 1600 fmol tube to 800 f mol tube and mixed thoroughly by vortexing. This doubling dilution was repeated successively with the remaining tubes and mixed by vortexing after each dilution.

2.10.2 Principle of assay

The lysis reagent 1 hydrolyses cell membranes to release intracellular cAMP. Lysis reagent 2 sequesters the key component in lysis reagent 1 and ensures cAMP is free

for subsequent analysis. The detergent/sequestrant complex does not interfere with antigen: antibody binding. Lysis reagent 1 is simply added to cultured cells, followed by 5-10 min incubation before assay (Figure 2.10) below. The antiserum is reconstituted with lysis reagent 2. The assay is based on competition between competition between unlabelled camp and a fixed quality of peroxidase-labelled cAMP, for a limited number of binding sites on a cAMP specific antibody (figure 2.10).



(Figure 2.10 Principles of enzyme immunoassay of cAMP)

2.10.3 EIA assay protocol for the measurement of cAMP in HT-29 cells

HT-29 cells were maintained in culture as described in section 2.3.5 of this chapter. For the assay of cAMP, trypsinised HT-29 cells were seeded in a standard 96 well microtitre plates (tissue culture grade) with cell density of 10^6 cells.ml^{-1}. The plate was incubated overnight at 37 °C (5% CO_2/95% humidity). 100 µl of the drugs was added and incubated for the indicated time period. Excess culture media was decanted and 200 µl per well of diluted lysis reagent 1B added into the cultures. Following the addition of lysis reagent 1B, shaking the plate on a micro titre plate shaker for 10 min facilitated cell lysis. In order to check whether complete lysis of HT-29 cells has taken place, microscopic evaluation using trypan blue dye assay was done as described in section 2.8.2. Once cells were lysed, the enzyme immunoassay protocol was processed as described by the manufacturers of the cAMP kit (Amersham International PLC, Amersham UK).

Briefly, 100 µl of samples or standards was added to the wells of the pre-coated EIA plates supplied in the Biotrak kit. To each well, 50 µl of cAMP-peroxidase conjugate was added and incubated at 5 °C for 2 h. The Plate was washed thoroughly with PBS/Tween and 150 µl of enzyme substrate (Trimethylbenzidine) was added to all the wells and incubated at room temperature for 1 h. The reaction was stopped by the addition of 100 µl of 1 M H_2SO_4. Absorbance was measured at 450nm on a Labsystems micro titre plate reader.

Chapter 3; The effect of cannabinoids on the secretion of tumour necrosis factor-α (TNF-α) and interleukin 2 (IL-2) from immune cell lines (THP-1 and Jurkat E6.1 cells)

3.1 Introduction

There is evidence suggesting that cannabinoids may modulate immune cell functions (for reviews, see Specter, *et al,* 1990). A number of studies have implicated a variety of immune cell functions in cannabinoid-induced immunomodulation. For example, the proliferative responses of T and B cells to specific mitogens, natural killer (NK) cell killing ability and expression of TNF-α by macrophages were all suppressed by cannabinoids (Klein *et al.*, 1998). Other immunological responses affected by cannabinoids include interleukin 2 (IL-2), production by T cells (Condie *et al.*, 1996). In our laboratory, CP55,940 inhibited mitogen-induced release of reactive oxygen species generated in rat peritoneal mast cells (Brook's *et al.*, 1999), suggesting that cannabinoids may also alter mast cell function.

The pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) has been shown to be an important component of cellular immune responses (Beutler, 1995). Consequently animals treated with anti-TNF-α were found to have an unusual susceptibility to infection by *listeria monocytogens* (Havell, 1989). Furthermore, TNF-α was demonstrated to exert a general anti-viral effect on infected cells (Mestan *et al.*, 1986; Beutler *et al.*, 1995). TNF-α, primarily a product of activated macrophages and monocytes can also be synthesised by other cells of the immune system including epithelial cells and endothelial cells (Beutler *et al.*, 1984; Beutler, 1995). Many *in vitro* studies have shown that cannabinoids modulate pro-inflammatory cytokine release from monocyte cell lines. In particular, Δ^9-THC inhibited LPS-induced release of IL-1β or TNF-α from the monocyte cell line, THP-1 (Shivers *et al.*, 1994, Halfpenny *et al.*, 1998) respectively. However, inhibition was

observed at relatively high concentrations (>1 μM) and no attempts were made at characterising the cannabinoid receptors mediating these effects.

Another pro-inflammatory cytokine interleukin-2 (IL-2) is responsible for T lymphocyte signalling during proliferation. Expression of functional IL-2 receptors is another important variable that determines how long the clonal proliferation of T cells occurs following antigen stimulation (Smith, 1988). IL-2 receptors are not detectable on the majority of freshly isolated T-cells but they appear following polyclonal e g (PHA-induced) activation of T cell receptors (TCR). In general, IL-2 can regulate both antigen-specific and non-specific proliferation of T-cells. Given the importance of IL-2 in T cell signalling, and the role of TNF-α in reactions directed at removing intracellular pathogens, the modulation of their release from immune cells would present an attractive pharmacological target for treatment of various immune conditions.

3.2 Aims of study

The aims of the experiments described in this chapter are:

To investigate the effect of synthetic, classical and endogenous cannabinoids on the secretion of IL-2 from the lymphocytic cell line, Jurkat and TNF-α from the monocytic cell line THP-1. To assess whether THP-1 and Jurkat cells express functional cannabinoid receptors. To characterise the cannabinoid receptor responsible for any observed effects.

3.3 Experimental Protocols

Maintenance of THP-1 and Jurkat E6.1 cell lines were carried out as described in chapter 2 (sections 2.3.1 and 2.3.2) of this thesis. Cell viability was assessed as described in chapter 2 (sections 2.8.1 and 2.8.2).

3.3.1 Treatment of cells.

3.3.1.1 THP-1 cells

THP-1 cells were seeded into 24 well plates at a density of $3x10^5$ cell.ml^{-1} in 1 ml of fresh RPMI 1640 medium. For time course experiments, cells were stimulated with LPS (3 μg.ml^{-1}) and supernatants were harvested hourly, following centrifugation of cultures at 250 x g for 5 min. Cell free supernatants were harvested and assayed for TNF-α release by ELISA as described in chapter 2 (section 2.4).

For studies on the effect of ethanol (vehicle) on TNF-α release, cells were incubated with graded concentrations of ethanol (0-1%) for 2 h prior to stimulation with LPS (3 μg.ml^{-1}) and the supernatants harvested after incubation for a further 2 h. In experiments involving the effects of cannabinoid receptor agonists, cells were incubated with CP55,940 (10^{-6} M – 10^{-4} M), Δ-THC (10^{-6} M - 10^{-4} M) or anandamide (10^{-6} M-10^{-4} M) for 2 h prior to addition of LPS (3 μg.ml^{-1}) for a further 2 h and TNF-α release was measured by ELISA. For experiments involving the study of the effects of antagonists, the cells were first incubated with the appropriate drug 30 min prior to addition of CP55,940. Cells were incubated with CP55940 ± cannabinoid receptor antagonists for 2 h and stimulated with LPS for a further 2 h. The supernatant harvested from cell cultures was assayed for TNF-α by ELISA. In experiments involving the study of G-proteins, cells were first treated with pertussis toxin (PTX)

(100 ng.ml^{-1}) or cholera toxin (CTX) (10 ng.ml^{-1}) for 18 h. Cells were washed by centrifugation before being dosed with CP55,940 (10^{-6} M).

3.3.1.2 Jurkat E6.1 cells

Jurkat cells were seeded into 24 well plates at a density of 1 x 10^6 cells ml^{-1} in 1 ml of fresh RPMI 1640 medium. In experiments where the effect of cannabinoid agonists was measured, cells were dosed with CP55,940 (10^{-6} M-10^{-4} M), Δ^9-THC (10^{-6} M-10^{-4} M) or anandamide (10^{-6} M-10^{-4} M) and incubated for 2 h at 37 °C prior to addition of PHA (2.5 μg.ml^{-1}) and PMA (25 μg.ml^{-1}) and the incubation continued for a further 18 h. IL-2 release into the culture supernatant was measured by ELISA. Where the effect of cannabinoid receptor antagonists were measured cells were first treated with antagonist 30 min prior to treatment with CP55,940. Cells were incubated with cannabinoid receptor antagonist and CP55,940 for 2 h prior to the addition of PHA (2.5 μg.ml^{-1})/PMA (25 μg.ml^{-1}) and then incubated for a further 18 h. The cell free supernatants were harvested and assayed for IL-2 by ELISA.

3.4 Data Analysis

Concentration-response curves were analysed using GraphPAD prism (GraphPAD Software Inc., CA, USA). Other results were represented as bar graphs. In experiments where a single concentation of stimulant was used, inhibitory effects of cannabinoids on the release of TNF-α or IL-2 was normalised and expressed as % inhibition from the control (TNF-α or IL-2) treated cells alone. Calculation of EC_{50} values was made with GraphPAD Prism statistical software. All values were expressed as geometric mean and variability as 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 followed by the appropriate Post hoc test. Significance was assumed if a *P* value of <0.05 or less was obtained.

3.5 Results

3.5.1 Time course of TNF-α release from THP-1 cells.

Non-stimulated THP-1 cells (3 x 10^5 cell.ml^{-1}) secreted small amounts of TNF-α (21.46 ± 38.46 pg.ml^{-1}) after 4 h incubation in RPMI medium at 37°C. Following stimulation with LPS (3 μg.ml^{-1}) there was a small rise in TNF-α secretion in the first hour followed by a rapid increase in TNF-α by 2 h incubation (Figure 3.5.1). TNF-α levels in culture supernatants then remained stable for up to 5 h after stimulation. Cumulative release of TNF-α from THP-1 cells following stimulation with LPS for 5 h was (9686.2 ± 537.1 pg.ml^{-1}, n=6) (Figure 3.5.1).

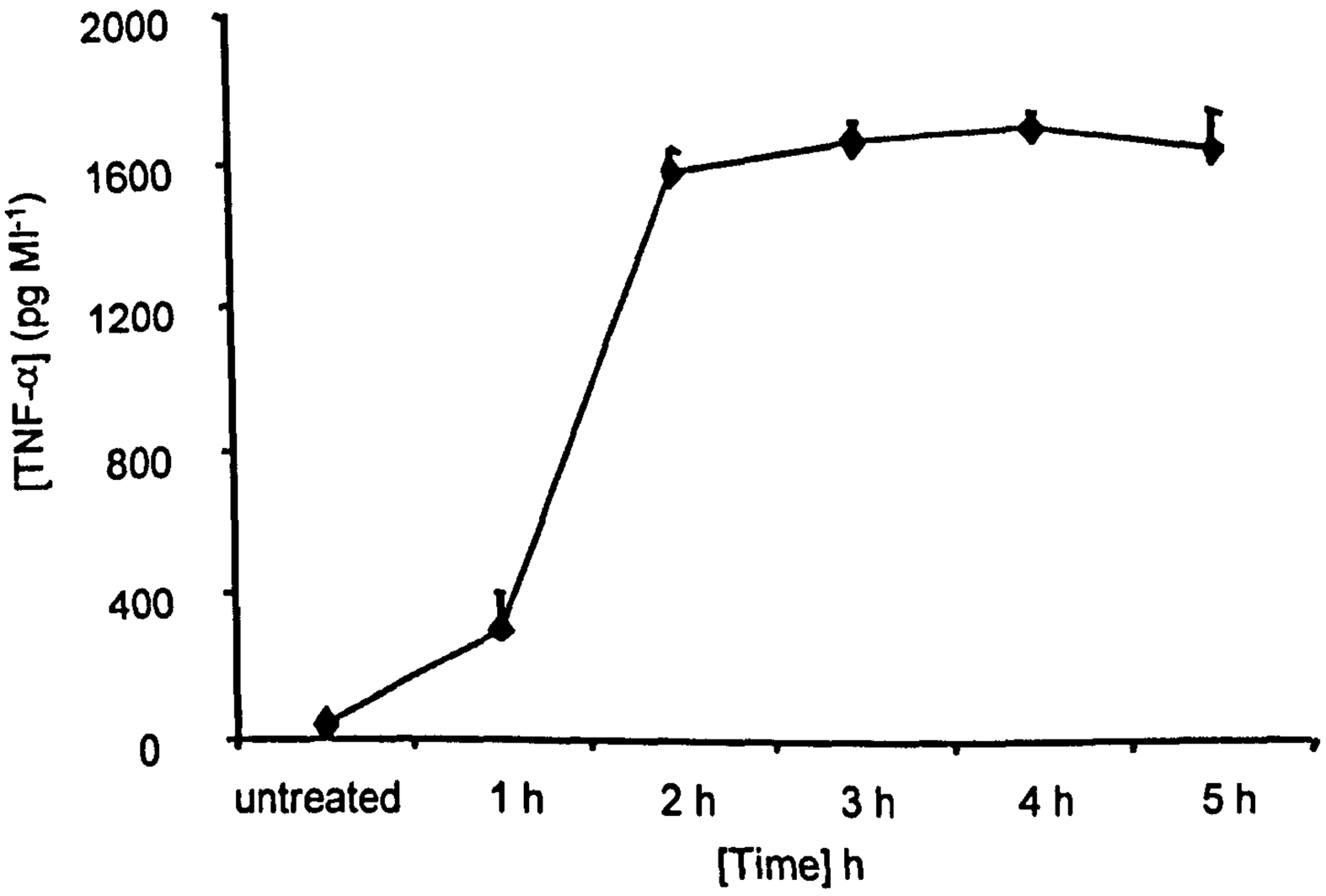


Figure 3.5.1. Time course of TNF-α release from THP-1 cells.
THP-1 cells were stimulated with LPS (3 μg ml^{-1}) for 1 h, 2 h, 3 h, 4, 5 h. Cell free supernatants were harvested for TNF-α assay by ELISA as described in chapter 2, section 2.4. Data are mean ± SE mean of six separate experiments.

3.5.2 The effect of vehicle on LPS-induced release of TNF-α from THP-1 cells.

The effect of increasing concentrations of ethanol (0-2%) on LPS-induced release of TNF-α from THP-1 cells was investigated because this was the vehicle for the cannabinoid receptor agonists CP55,940 and Δ^9-THC respectively. Higher concentrations of ethanol (0.5-2.0%) inhibited LPS-induced TNF-α release from THP-1 cells whereas lower concentrations of ethanol (0-0.1%) had no significant ($P<0.05$) inhibitory effect on the release of TNF-α (Figure 3.5.2).

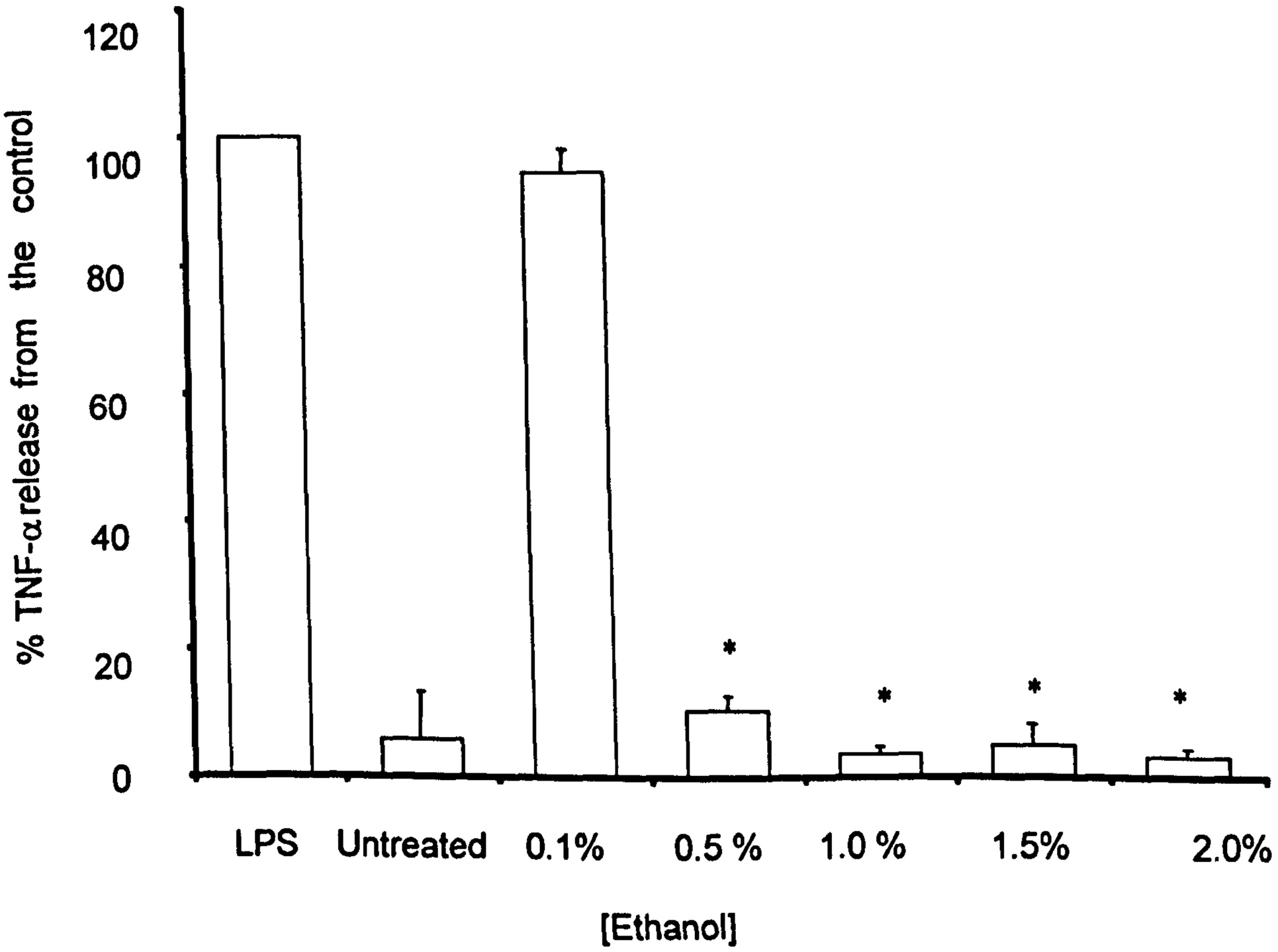


Figure 3.5.2 Effect of Ethanol on LPS induced release of TNF-α from THP-1 cells.

THP-1 cells (3×10^5 cells.ml^{-1}) were incubated with or without ethanol for 2 h prior to stimulation with LPS (3 μg ml^{-1}) for 2 h. Cell free supernatants were harvested for TNF-α assay by ELISA as described in chapter 2, section 2.4. Data are mean±SE mean of six separate experiments. * Denotes significant difference ($P<0.05$) from the control LPS treated cells (Student's *t*-test)

3.5.3 The effect of CP55,940, Δ^9-THC and anandamide on LPS-induced release of TNF-α from THP-1 cells.

We examined the effect of the non-selective cannabinoid receptor agonists CP55,940 (10^{-6} M-10^{-4} M), Δ^9-THC (10^{-6} M-10^{-4} M) and anandamide (10^{-6} M-10^{-4} M) on LPS-induced secretion of TNF-α from THP-1 cells. All three cannabinoid receptor agonists produced a concentration-related inhibition of TNF-α secretion (Figure 3.5.3) and the following EC_{50} values were calculated; CP55,940 (4.8×10^{-5} M, 95% confidence Limits (C.L.)=2.6×10^{-5} M - 8.8×10^{-5} M, n=6), Δ^9-THC (3.1×10^{-5} M, 95 % C.L. = 2.8×10^{-5} M - 3.5×10^{-5} M, n = 6) and anandamide (1.86×10^{-5} M, 95% C.L.=1.6×10^{-5} M - 2.1×10^{-5} M, n = 6). All the cannabinoid agonists employed in this study produced approximately the same maximum inhibition of LPS-induced release of TNF-α (~100%). Within the concentration-ranges tested, CP55,940 (10^{-6} M - 10^{-4} M), Δ^9-THC (10^{-6} M - 10^{-4} M) and anandamide (10^{-6} M - 10^{-4} M) significantly ($P<0.05$) inhibited LPS-induced TNF-α release from THP-1 cells (one way ANOVA followed by Dunnet's post hoc test, n=6) (Figure 3.5.3).. The rank order of agonist potency obtained from our study was as follows; anandamide > Δ^9-THC > CP55,940.

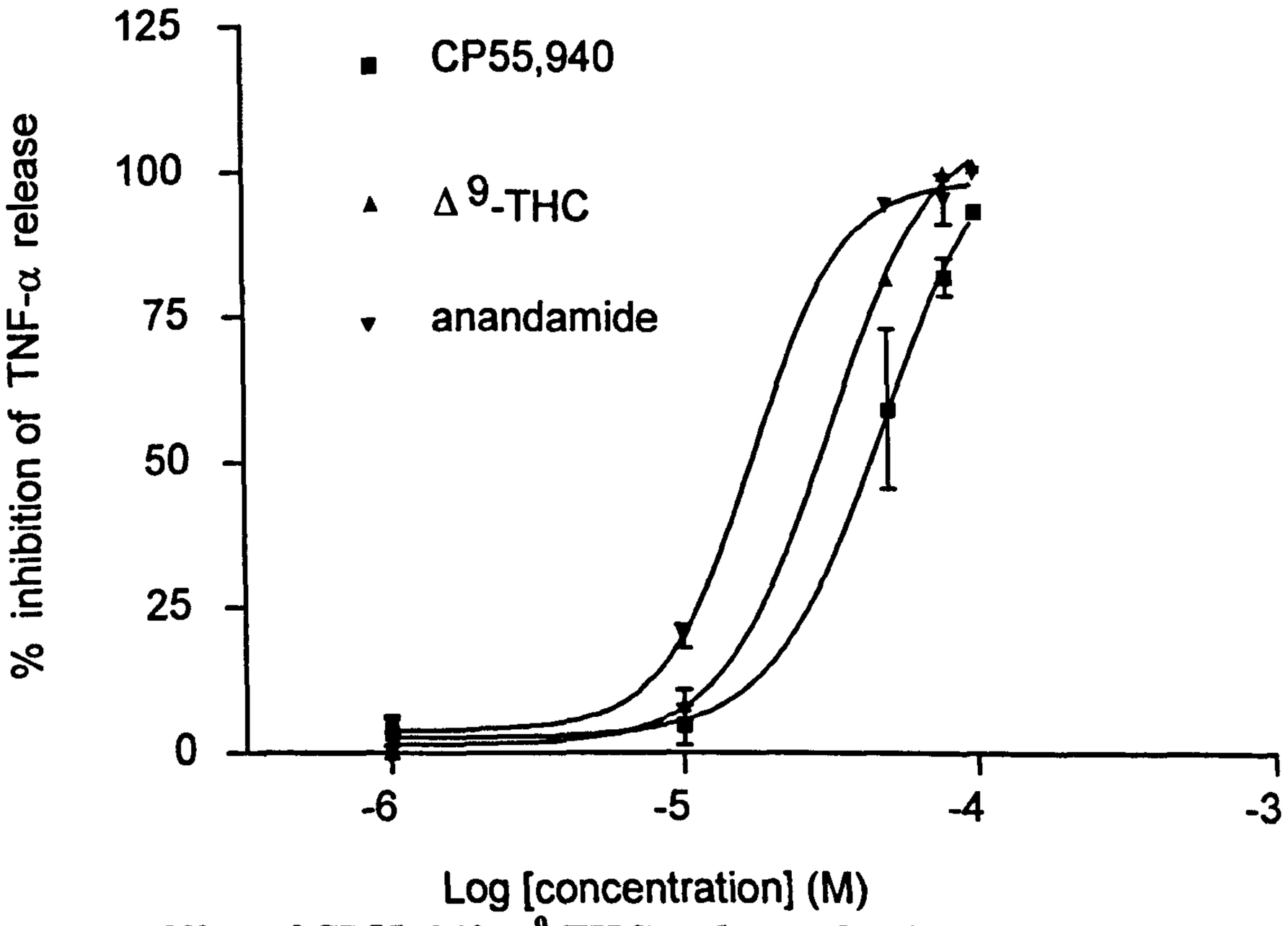


Fig. 3.5.3 Effect of CP55, 940, Δ^9-THC and anandamide on LPS-induced release of TNF-α from THP-1 cells.

THP-1 cells (3×10^5 cell.ml^{-1}) were treated with CP55,940 (10^{-6} M - 10^{-4} M), Δ^9-THC (10^{-6} M - 10^{-4} M) and anandamide (10^{-6} M - 10^{-4} M) for 2 h before stimulation with LPS (3 μg ml^{-1}). Incubation was continued for a further 2 h. Cell free supernatants were assayed for TNF-α by ELISA as described in chapter 2, section 2.4. Data are presented as % inhibition of TNF- α release from control (LPS treated cells alone). Error bars represent mean ± S E mean of six separate experiments.

3.5.4 The effect of cannabinoid receptor antagonists.

The cannabinoid CB_2 receptor antagonist SR144528 (10^{-6} M), significantly ($P<0.05$, 2 way ANOVA followed by Bonferroni's post hoc test, n=6) antagonised the inhibitory effects of CP55,940 ($pA_2 = 6.1 \pm 0.1$, n = 6) on LPS-induced TNF-α release. In contrast, the cannabinoid CB_1 receptor antagonist SR141716A (10^{-6} M) did not antagonise the effect of CP55,940 (Figure 3.5.4).

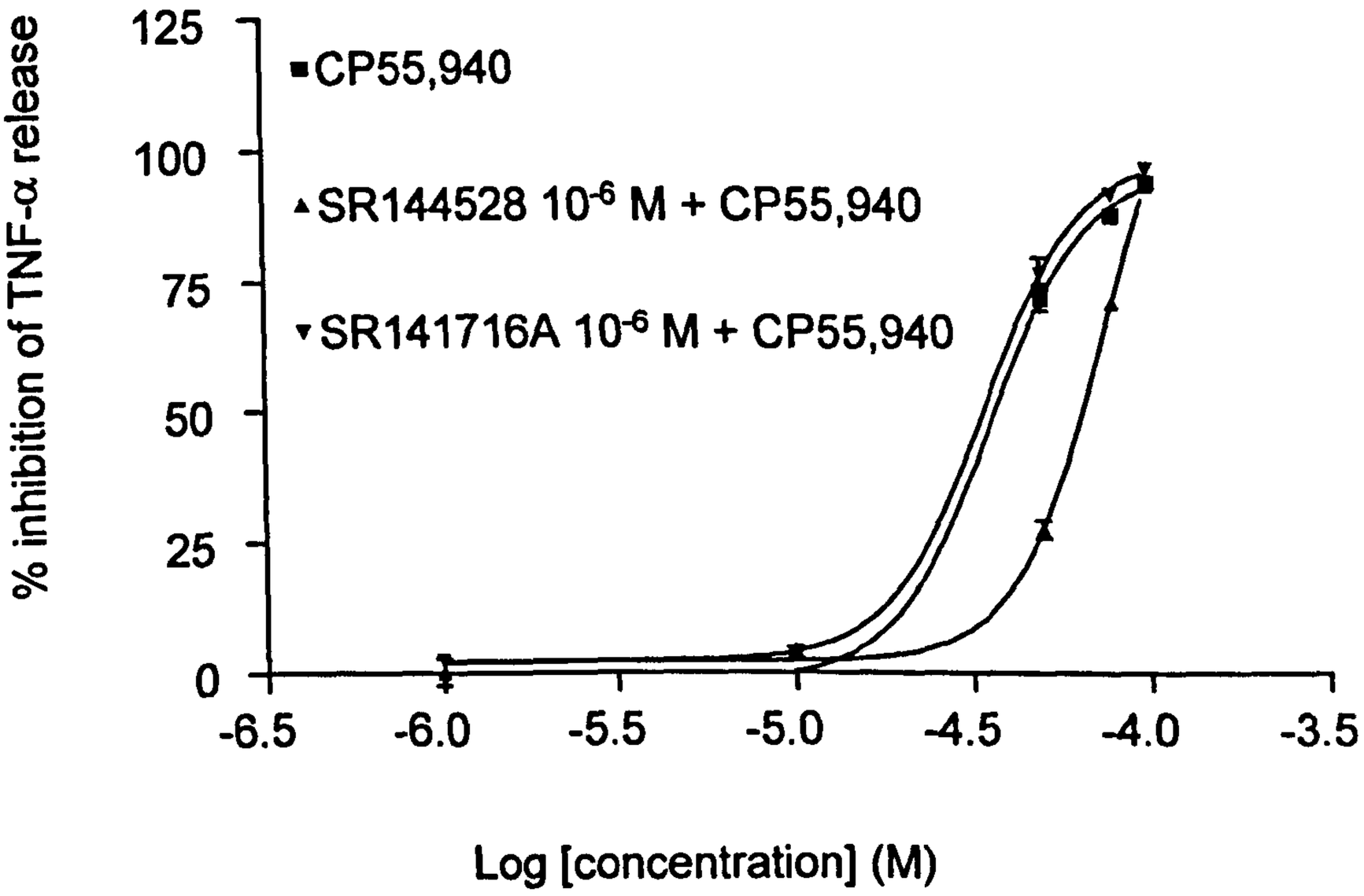


Figure 3.5.4 Effect of SR144528 and SR141716A on CP55,940 induced inhibition of TNF-α release from THP-1 cells.

THP-1 cells (3 x 10^5 cell.ml^{-1}) were incubated with SR141716A (10^{-6} M) or SR144528 (10^{-6} M) for 30 min before treatment with CP55,940 (10^{-6} M – 10^{-4} M) for 2 h. Cells were stimulated for a further 2 h with LPS (3 μg.ml^{-1}). Supernatants were assayed for TNF-α release by ELISA as described in the chapter 2, section 2.4. Bars represent mean ± S.E. mean of six separate experiments.

3.5.5 The effect of serum on CP55,940 induced inhibition of LPS-induced TNF-α release from THP-1 cells.

In order to determine the effect of serum on cannabinoid-induced inhibition of TNF-α release from THP-1 cells, cells were treated with CP55,940 (10^{-6} M - 10^{-4} M) in the presence or absence of 10 % FCS for 2 h before stimulation with LPS (3 μg.ml^{-1}) for a further 2 h. Under the experimental conditions described in this chapter, 10% FCS evoked a small but non-significant (P>0.05) shift of CP55,940 concentration-effect curves to the left (Figure. 3.5.5).

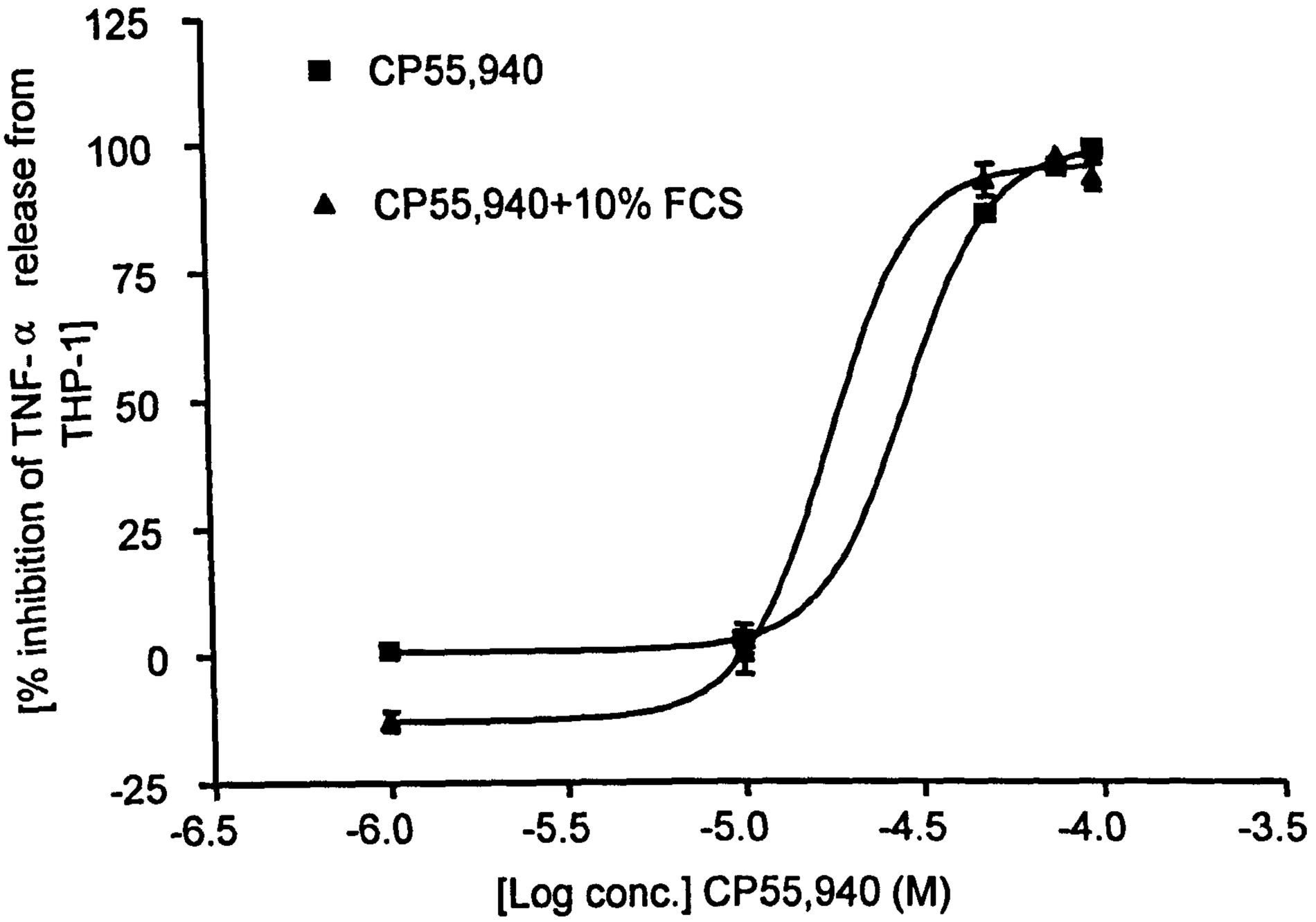


Figure 3.5.5 Effect of 10% FCS on CP55,940 concentration-effect curves.
THP-1 cells (3 x 10^5 cells.ml^{-1}) were treated with CP55,940 for two hours in RPMI medium in the presence or absence of 10% FCS. The cells were stimulated with LPS (3 μg.ml $^{-1}$) for 2 h and TNF-α secretion assayed by ELISA as described in chapter 2, section 2.4. Data are mean ± SE mean of six separate experiments.

3.5.6 The effect of PTX and CTX on LPS- induced secretion of TNF-α release from THP-1 cells.

To study the involvement of G-proteins in cannabinoid-induced inhibition of TNF-α release, THP-1 cells were incubated with PTX (100 ng.ml^{-1}) or CTX (10 ng.ml^{-1}), followed by treatment with or without CP55,940 (10^{-5} M) for 2 h and stimulation with LPS (3 μg.ml^{-1}) for a further 2 h. Treatment of THP-1 cells with PTX (100 ng.ml^{-1}) abolished the inhibitory effect of CP55,940 on LPS-induced release of TNF-α. Treatment with CTX (10 ng.ml^{-1}) or a combination of the two toxins attenuated the inhibitory responses of CP55,940 (Figure 3.5.6). PTX (100 ng. ml^{-1}) had no effect on the release of TNF-α on its own (data not shown).

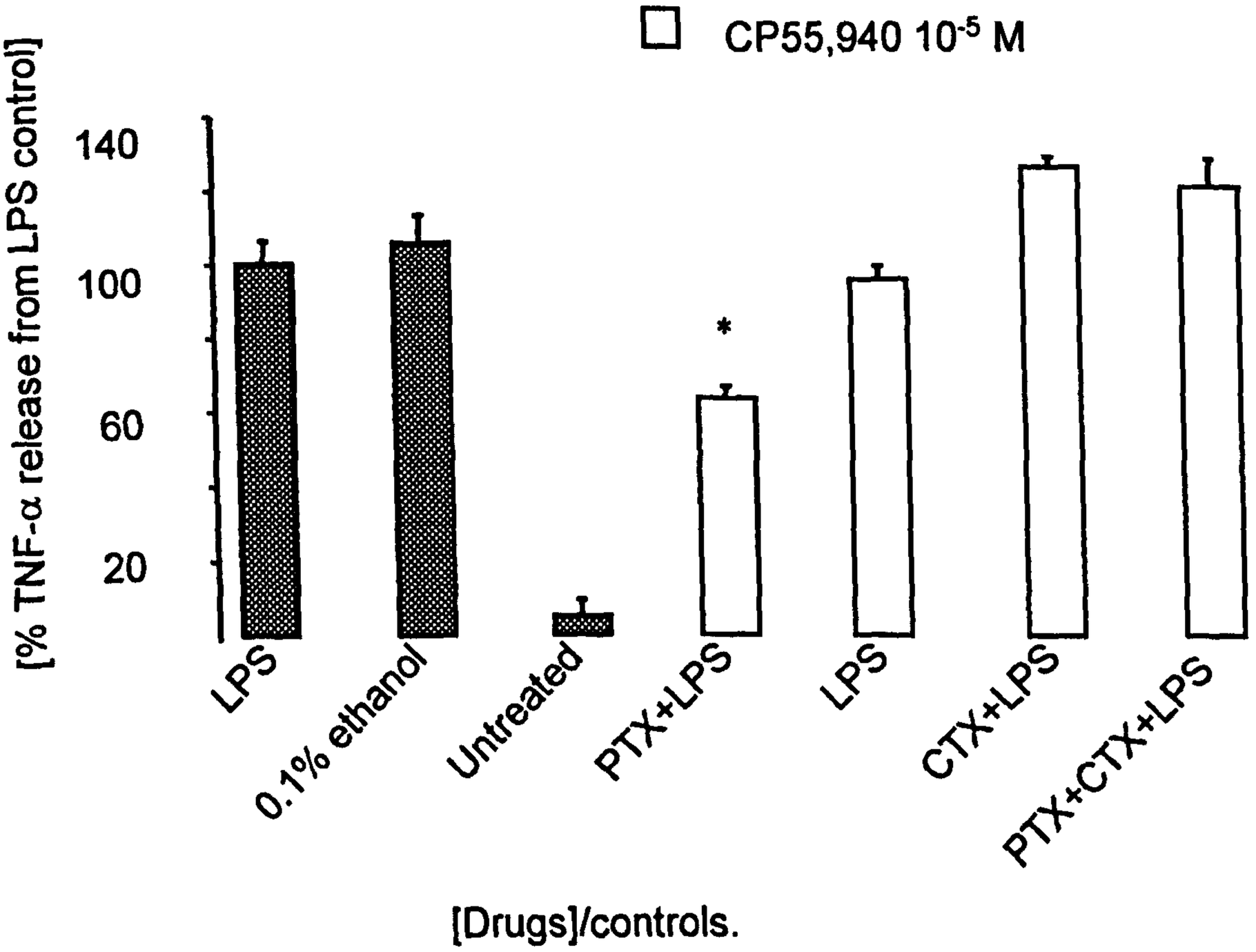


Figure 3.5.6 Effect of PTX and CTX on LPS induced secretion of TNF-α fromTHP-1 cells.

THP-1 cells were treated with PTX (100 ng ml^{-1}) and or CTX (10 ng ml^{-1}) for 18 h. Cells were washed and incubated in the presence or absence of CP55,940 (10^{-5} M) before stimulation with LPS. TNF-α release was measured as described in chapter 2, section 2.4. Data are presented as mean ± SEM of 6 independent experiments. * Denotes significant difference ($P<0.05$) from the control LPS treated cells (Student's *t*-test).

3.5.7 The effect of CP55,940, Δ^9-THC and dexamethasone on the release of TNF-α from THP-1 cells.

To compare the inhibitory effects of cannabinoids and dexamethasone on LPS-induced release of TNF-α, THP-1 cells were incubated with CP55,940 (10^{-7} M - 10^{-5} M), Δ^9-THC (10^{-7} M - 10^{-5} M) and dexamethasone (10^{-7} M - 10^{-5} M) or vehicle for 2 h before stimulation with LPS (3 $\mu g.ml^{-1}$) for further 2 h. CP55,940, Δ^9-THC and dexamethasone produced concentration-related decreases in TNF-α release from THP-1 cells. Dexamethasone evoked significant ($P<0.05$) concentration-related

inhibition of TNF-α release when compared with the LPS treated control at all the concentrations tested whereas Δ^9-THC and CP55,940 only produced significant ($P<0.05$) inhibition at 10^{-5} M, the highest concentration of the drugs used, when compared with control (Figure 3.5.7).

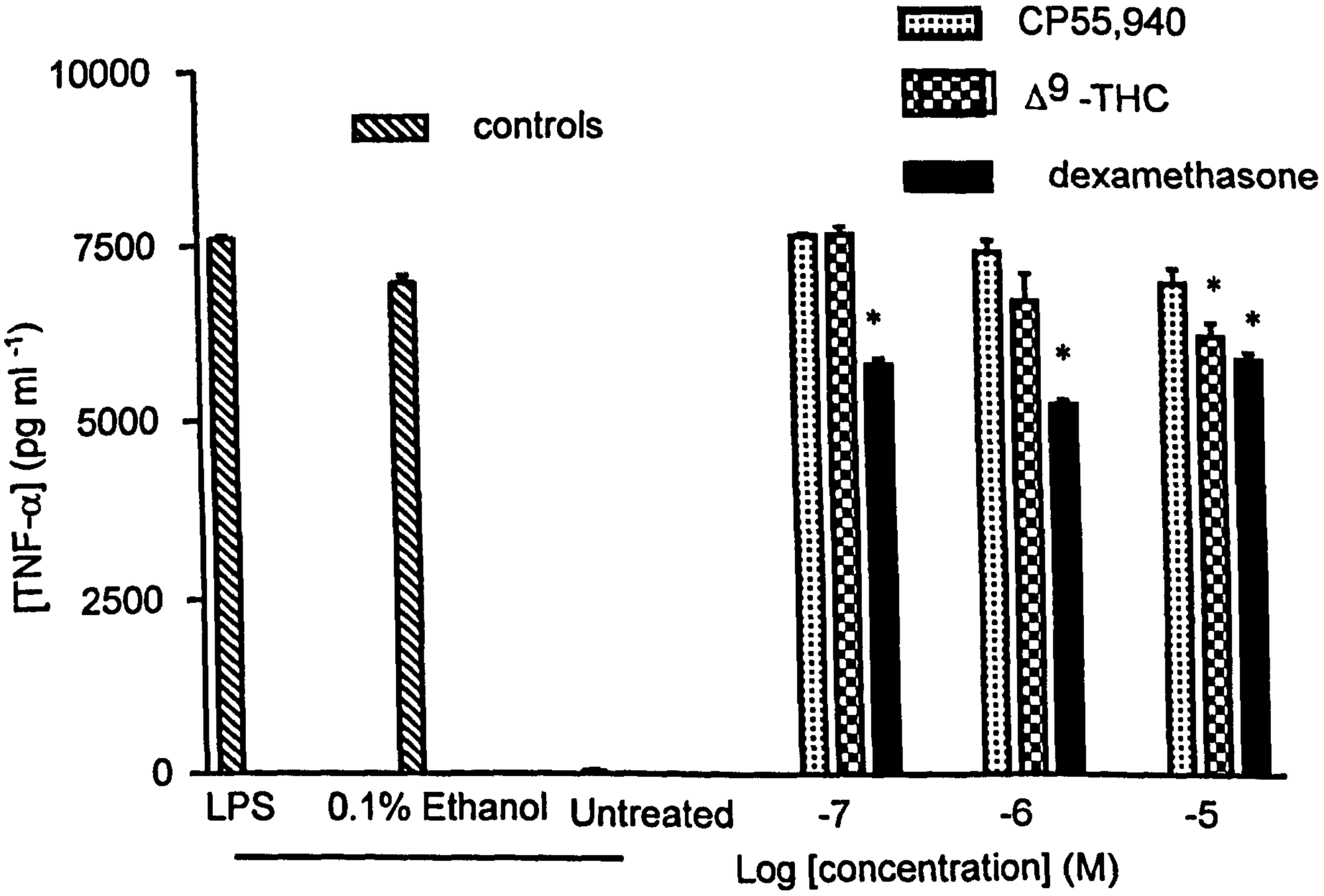


Figure 3.5.7 . Effect of CP55,940, Δ^9-THC and Dexamethasone on the release of TNF-α from THP-1 cells.

CP55,940 (10^{-7} M-10^{-5} M), Δ^9-THC(10^{-7} M-10^{-5} M) and dexamethasone (10^{-7} M - 10^{-5} M) were incubated with THP-1 cells for 2 h prior to stimulation with LPS (3 μg ml^{-1}). Cells were incubated for further 2 h and TNF-α secretion was assayed by ELISA as described in chapter 2, section 2.4. Error bars represent SE of the mean for six independent determinations. * Denotes significant difference ($P<0.05$) from the control LPS treated cells Students *t*-test

3.5.8 Determination of the viability of THP-1 cells with CP55,940 using MTT assay.

The viability of THP-1 cells, as determined by the ability of cells to reduce MTT to formazan was between 77% and 100% following incubation with CP55,940 for 2 h and stimulation with LPS for a further 2 h (see Table.3.1 below

Table 3.1 MTT assay on THP-1 cells

[Drugs] (M)/Control	**Cell viability % of control**
LPS (3 $\mu g.ml^{-1}$)	100±7.1, SEM, n=6
Untreated	91.6±1.5, SEM, n=6
CP55,940 10^{-6} M	89.2±12.5, SEM, n=6
CP55,940 10^{-5} M	86.1±4.6, SEM, n=6
CP55,940 $5x10^{-5}$ M	92.7±4.6, SEM, n=6
CP55,940 $7.5x10^{-5}$ M	77.5±2.4, SEM, n=6
CP55,940 10^{-4} M	94.8±5.0, SEM, n=6

Cell viability of THP-1 cells was determined by MTT assay as described in chapter 2, section 2.8.2 of this thesis. The data are mean ± SEM of 6 different experiments. * Significant difference (* $P<0.05$) from control (untreated cells)

3.5.9 Time course for IL-2 release from Jurkat cells.

Non-stimulated Jurkat cells constitutively released minimal amounts of IL-2 (2.7 ±0.1 $pg.ml^{-1}$) following 24 h incubation at 37°C. Following stimulation with PHA (2.5 μg ml^{-1}) and PMA (25 $\mu g.ml^{-1}$) a small increase in IL-2 release was observed during the first 6 h. This was followed by a rapid increase in IL-2 secretion between 6 and 12 h. This was followed by a further, small increase in IL-2 release, which peaked at 18 h. There was a small decline in IL-2 release by 24 h. Thus, the maximum release of IL-2 occurred at 18 h (220 ± 11.14 $pg.ml^{-1}$) (Figure 3.5.8).

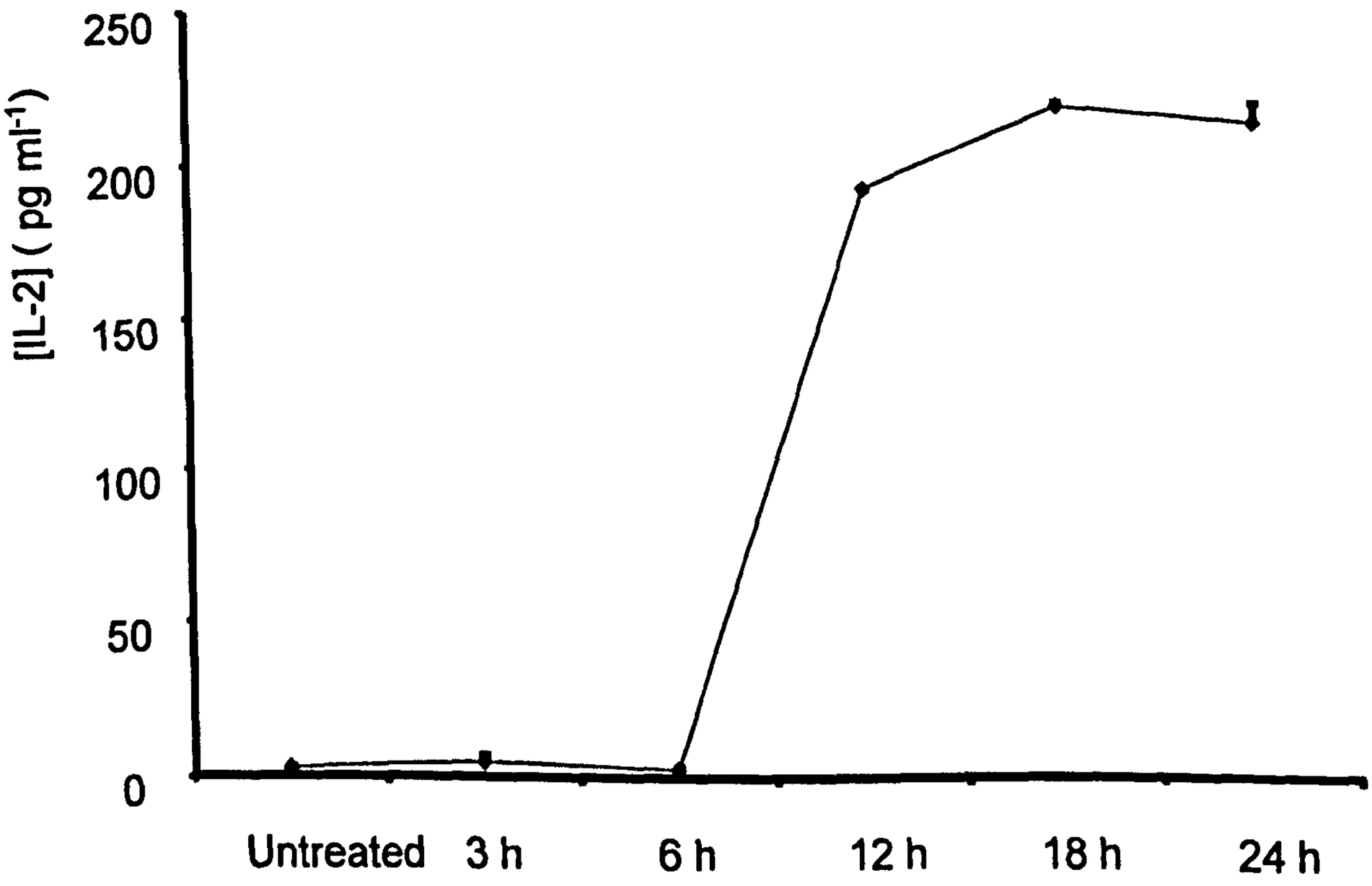


Figure 3.5.8 Time course of IL-2 release from Jurkat cells.
Jurkat cells (1 x 10^6 $cell.ml^{-1}$) were stimulated with PHA (2.5 μg ml^{-1}) and PMA (25 μg ml^{-1}) for 0 h, 3 h, 6 h, 12 h, 18 h and 24 h. Cell free supernatants were harvested for IL-2 assay by ELISA as described in chapter 2, section 2.4. Data are the mean and SE mean of six separate experiments.

3.5.10 The effect of CP55,940, Δ^9-THC and anandamide on PHA-induced release of IL-2 from Jurkat cells.

The effect of the non-selective cannabinoid agonists CP55,940 (10^{-6} M - 10^{-4} M), Δ^9-THC (10^{-6} M - 10^{-4} M) and anandamide (10^{-6} M - 10^{-4} M) on PHA/PMA-induced secretion of IL-2 from Jurkat cells was examined. All three agonists produced a concentration-related inhibition of PHA/PMA-induced IL-2 release and the following EC_{50} values were calculated; CP55,940 (2.3×10^{-5} M, 95% confidence limits (C.L.)=1.5×10^{-5} M - 3.5×10^{-5} M, n=6), Δ^9-THC (3.2×10^{-5} M, 95 % C.L. = 2.1×10^{-5} M - 4.8×10^{-5} M, n=6) and anandamide (7.1×10^{-6} M, 95% C.L.=6.1×10^{-5} M - 8.3×10^{-5} M, n=6). All the cannabinoid agonists employed in this study produced approximately the same maximum inhibition of PHA and PMA-induced IL-2 release (~100%). Within the concentration ranges tested, CP55,940 (10^{-6} M - 10^{-4} M), Δ^9-THC (10^{-6} M - 10^{-4} M) and anandamide (10^{-6} M - 10^{-4} M) significantly ($P<0.05$) inhibited PHA/PMA-induced IL-2 release from Jurkat cells (one way ANOVA followed by Dunnet's post hoc test, n=6). The rank order of agonist potency obtained from our study was as follows; anandamide> Δ^9-THC>CP55,940 (Figure 3.5.9).

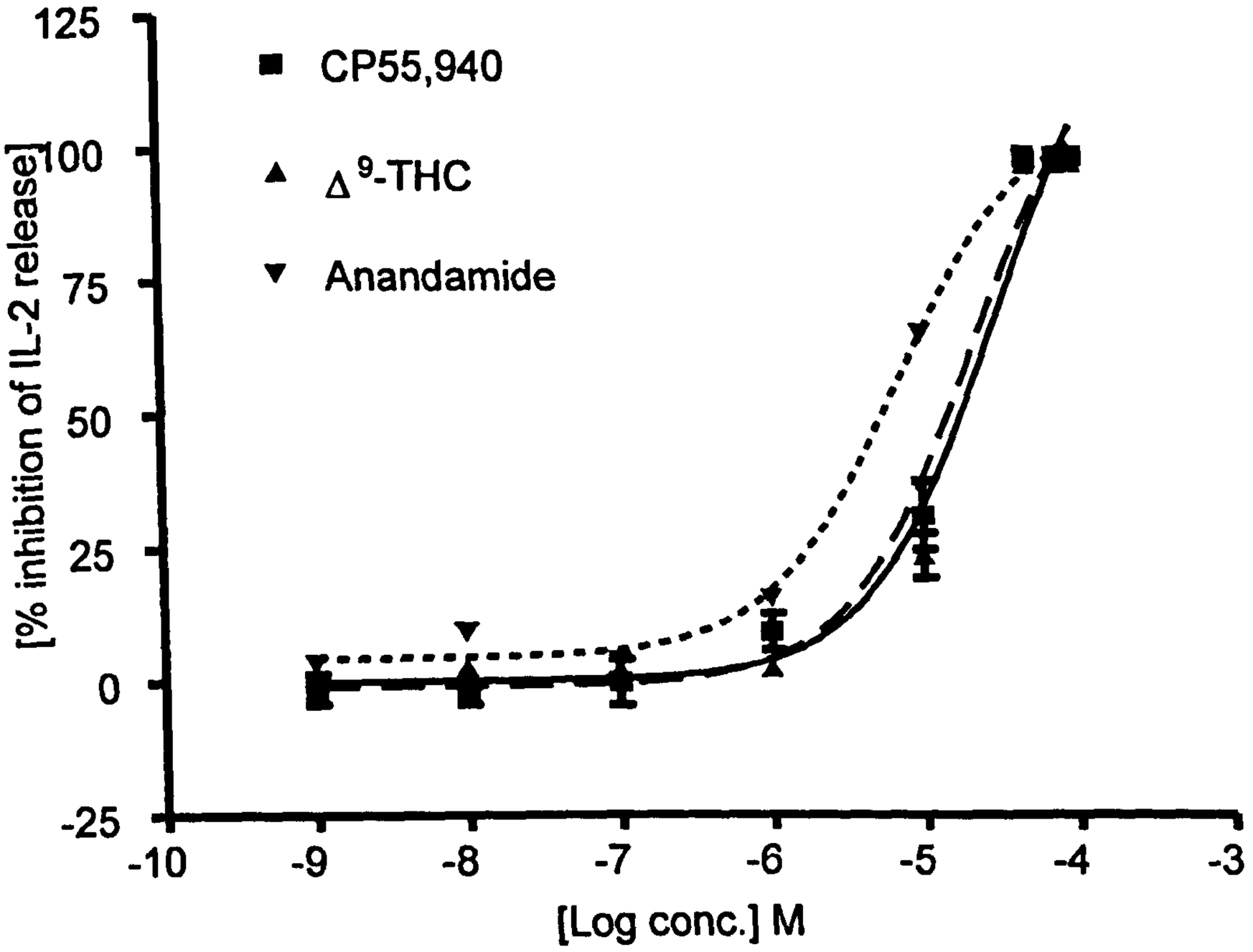


Figure 3.5.9 Effect of CP55,940, Δ9-THC and Anandamide on the release of IL-2 from Jurkat cell line.

Jurkat cells (1 x 10^6 cell.ml^{-1}) were treated with CP55,940 (10^{-6} M - 10^{-4} M), Δ^9-THC (10^{-6} M - 10^{-4} M) and anandamide (10^{-6} M - 10^{-4} M) for 2 h before stimulation with PHA (2.5 μg ml^{-1})/PMA (25 μg ml^{-1}) for a further 18 h. Cell free supernatants were assayed for IL-2 by ELISA as described in the chapter 2, section 2.4. Data are presented as % inhibition of IL-2 release from control PHA (2.5 μg ml^{-1})/PMA (25 μg ml^{-1}). Error bars represent the mean ± S E mean of six separate experiments.

3.5.11 The effect of cannabinoid receptor antagonists SR141716A and SR144528 on CP55, 940 induced inhibition of PHA and PMA-induced release of IL-2 from Jurkat cells.

Neither SR141716A (10^{-6} M) nor SR144528 (10^{-6} M) antagonised the effect of CP55,940 (Figure 3.5.10 a). However SR141716A (10^{-6} M) and SR144528 (10^{-6} M) evoked significant (P<0.05) inhibition of PHA/PMA induced IL-2 release from Jurkat cells (Figure 3.5.10 b).

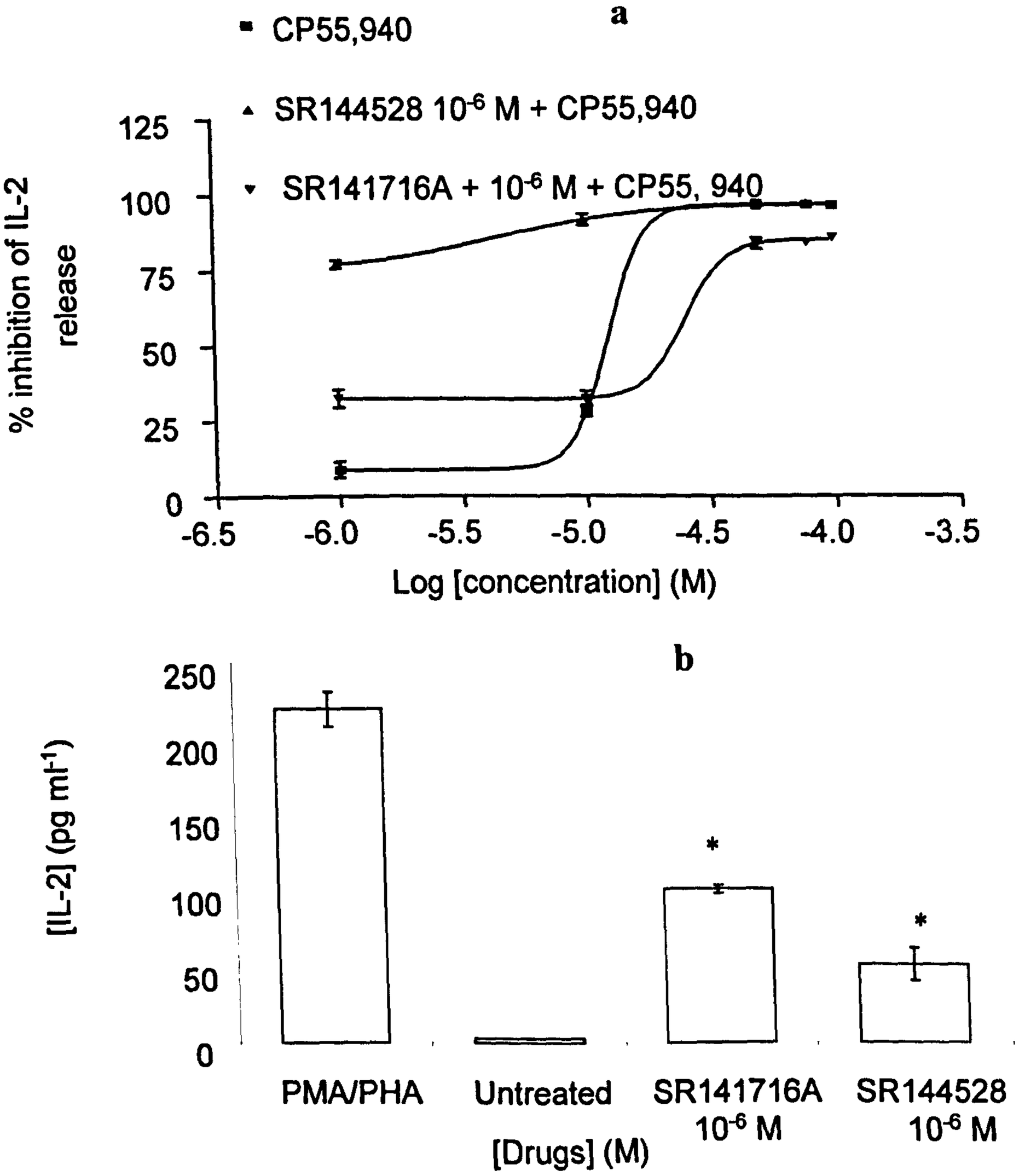


Figure 3.5.10 Effect of SR141716A and SR144528 on CP55,940 induced inhibition of IL-2 release from Jurkat cell lines.

Jurkat cells (1 x 10^6 cell.ml^{-1}) were incubated with SR141716A (10^{-6} M) or SR144528 (10^{-6} M) for 30 min before treatment with CP55,940 (10^{-6} M – 10^{-4} M) for 2 h. Cells were stimulated for a further 18 h with PHA (2.5 μg ml^{-1})/PMA (25 μg ml^{-1}) . Supernatants were assayed for IL-2 release by ELISA as described in chapter 2, section 2.4. Bars represent mean ± mean of six separate experiments. Figure 3.5.10 a shows the effect of SR141716A and SR144528 on CP55,940-induced release of IL-2 from Jurkat cells. Figure 3.5.10 b shows a concentration-related inhibition of IL-2 release by SR141716A and SR144528 from Jurkat cells. Error bars indicate S.E mean of 6 independent experiments. * Denotes significant difference $P< 0.05$, from the control PHA/PMA stimulated cells (Student's *t*-test).

3.5.12 Determination of the viability of Jurkat cells with CP55,940 using MTT assay

Table 3.2 shows the viability of Jurkat cells following treatment with CP55,940 for 2 h and stimulation with PHA/PMA for a further 18 h. There was a concentration-related reduction in the mitochondria oxidative metabolism of Jurkat cells treated with increasing concentration of CP55,940 (10^{-6} M – 10^{-4} M).

Table 3.2. MTT assay on Jurkat cells

[Drugs] M/Control	**Cell viability % of control**
Control (PHA/PMA) stimulated cells alone	100.25±.4, SEM, n=6
CP55,940 10^{-6} M What	94.9±0.8, SEM, n=6
CP55,940 10^{-5} M	95.05±2.3, SEM, n=6
CP55,940 $5x10^{-5}$ M	71.6±.5, SEM, n=6
CP55,940 $7.5x10^{-5}$ M	13.5±1.0, SEM, n=6
CP55,940 10^{-4} M	7±1.2, SEM, n=6

Cell viability of Jurkat cells was determined by MTT assay as described in chapter 2, section 2.8.2 of this thesis. The data are mean ± SEM of 6 different experiments. * Significant difference (* $P<0.05$) from control (untreated cells). Abbreviations: *SEM, standard error of the mean, PHA, Phytohemagglutinin, PMA, Phorbol-13- myristate-14- acetate.*

3.6 Discussion.

3.6.1. The effect of cannabinoids on the release of TNF-α from THP-1 cells

The present study demonstrates that *in vitro* treatment of THP-1 cells with cannabinoids decreased LPS-induced TNF-α secretion. These events occurred at high micromolar concentrations i.e. higher than those reported in binding studies (Rinaldi-Carmona *et al.*, 1998), and were attenuated by treatment with pertussis toxin but not cholera toxin. The cannabinoid CB_1 receptor antagonist, SR141716A (10^{-5} M) (Rinaldi-Carmona *et al.*, 1994) was ineffective in antagonising the CP55,940 responses in this cell line whereas the cannabinoid CB_2 receptor antagonist SR144528 (10^{-5} M) (Rinaldi-Carmona *et al.*, 1998) antagonised the inhibitory effects of CP55,940. The rank order of agonist potency for inhibition of LPS-induced TNF-α release by cannabinoids was anandamide>Δ^9-THC>CP55,940. These observations suggest that the immune suppression of TNF-α release from the monocytes by cannabinoids is mediated via cannabinoid CB_2-like receptors.

THP-1 cells have been shown to express cannabinoid CB_2 receptors (Shivers *et al.*, 1994; Halfpenny *et al.*, 1998). To our knowledge, there is no evidence for the presence of cannabinoid CB_1 receptor protein or the mRNA for cannabinoid CB_1 receptors in these cells (Halfpenny *et al.*, 1998). The concentration-dependent inhibition of LPS-induced TNF-α release observed in the present study suggests that immune responses produced by THP-1 cells can be modulated by classical, non-classical and endogenous cannabinoids through an action on cannabinoid CB_2 receptors.

Both cannabinoid CB_1 and CB_2 receptors are negatively coupled to adenylate cyclase through pertussis toxin-sensitive G_i/G_o-protein (Felder *et al.*, 1995) and to investigate whether the inhibitory actions of cannabinoids observed in the present study were G-protein mediated, THP-1 cells were incubated with PTX and CTX respectively for 18 h before treatment with CP55,940 (10^{-6} M) followed by stimulation with LPS. In the presence of PTX, CP55,940-evoked inhibition of LPS-induced TNF-α release was significantly attenuated suggesting the involvement of G_i/G_o protein coupled receptors. In the presence of CTX or a combination of CTX and PTX, CP55,940 (10^{-6} M) evoked inhibition of LPS-induced TNF-α release by CP55,940 was still attenuated suggesting that Gs protein may not be involved in cannabinoid-induced inhibition of TNF-α release.

The mechanism of LPS-induced TNF-α release in monocytes is complex however the findings reported in this chapter is in agreement with those of Altavilla *et al.*, (1986), who demonstrated that LPS-induced TNF-α release in macrophages is a G-protein mediated event and with those of Halfpenny *et al.*, (1998), who showed that cannabinoid receptor agonists could inhibit LPS-induced TNF-α release from THP-1 cells albeit at higher concentrations (1 μM). However, the scenario is more complex and indeed it is difficult to assign a particular receptor subtype to the effects reported here. For example, cannabinoids are highly lipid soluble substances (Dewey, 1986) and the effects observed in the present study could be interpreted as membrane effects although their blockade by a cannabinoid CB_2 receptor antagonist but not by a cannabinoid CB_1 receptor antagonist may be suggestive of a cannabinoid CB_2 receptor-mediated event. Alternatively, whether the reported presence of the cannabinoid CB_2 receptors is found only in differentiated THP-1 cells or is also seen

in undifferentiated cells is not known (Halfpenny *et al.*, 1998). If the former is true, a situation may arise where the expressed receptors are insufficient to evoke a functional response and this may account at least in part to the effects observed in the present study. However to asses these possibilities, it may be worth investigating the effect of the inactive enatiometer of CP55,940 on LPS induced release of TNF-α from THP-1 cells. Regrettably, the inactive enantiomer to CP55,940 was not available at the time of these experiments.

Derocq *et al* (1995) showed that cannabinoid receptor mediated effects on B cell responses were enhanced in medium containing low concentrations of serum. Thus, the effect of cannabinoid receptor agonists on the release of TNF-α in THP-1 cells in the presence and absence of 10% FCS was studied. An enhanced inhibitory effect of cannabinoids in serum free medium was shown. It has been shown that cannabinoid receptor ligands bind to serum protein (Dewey, 1986), and this may in part account for higher drug concentrations needed to evoke functional responses in the present study and other similar *in-vitro* studies in comparison to receptor binding studies employing cannabinoid receptor ligands. (Watzl *et al.*, 1991; Halfpenny *et al.*,1998; Shivers *et al.*, 1994)

From the data described above and the finding of others, it can be concluded that cannabinoids suppress LPS-induced TNF-α release from activated THP-1 cells. These effects occur at high cannabinoid concentration and are attenuated by a cannabinoid CB_2 receptor antagonist and they are also G_i/G_o protein sensitive, suggesting an effect mediated through cannabinoid CB_2 receptors. Given the importance of TNF-α in immune responses, the role of macrophage/monocyte in

inflammation and modulation of these effects by cannabinoids as demonstrated herein, cannabinoids could be considered a potential target for anti-inflammatory drug therapy.

3.6.2 The effects of cannabinoids on the release of IL-2 from Jurkat E6.1 cells

These experiments investigated the possible immunomodulatory effects of cannabinoids on Jurkat cells. These cells are a T-helper type-1 human pro-lymphocyte cell line capable of secreting IL-2 following stimulation with appropriate mitogen (Werge *et al.*, 1994). In the present study, it has been shown that CP55,940, Δ^9-THC and anandamide inhibited PHA/PMA-induced secretion of IL-2 release from Jurkat cell line. These effects occur in a concentration-dependent fashion and are observed at micromolar cannabinoid concentrations. Additionally, higher cannabinoid concentrations inhibited the mitochondria oxidative metabolism in these cells as assessed by the ability of cells to reduce MTT to formazan.

Receptor mRNA specific for cannabinoid CB_2 receptors but not cannabinoid CB_1 receptors has previously been identified in the Jurkat E6-1 cell line (Schatz, *et al.*, 1997). Whether the mRNA isolated from this cell line is transcribed to form functional cannabinoid CB_2 receptor protein has remained a controversial subject. This controversy largely stems from the aberrant nature of the cannabinoid CB_2 receptor mRNA isolated from Jurkat cells and partly due to the inactivity of cannabinoid receptor ligands to evoke inhibition of forskolin-stimulated cAMP in these cells (Schatz *et al.*, 1997). However, in the present study, CP55,940, Δ^9-THC and anandamide inhibited PHA/PMA-induced IL-2 release from Jurkat cells albeit at higher concentrations than those reported in some other studies (Condie *et al.*, 1996). These observations would appear to agree with a previous study, which implicated Δ^9-

THC and cannabinol in suppressing IL-2 secretion and inducing a steady state IL-2 mRNA expression in primary mouse splenocytes and EL4 T-cell line (Condie *et al.*, 1996) but at odds with the study reported by Schatz *et al.* (1997). However, these groups did not employ cannabinoid receptor antagonists to identify the receptor mediating cannabinoid-induced inhibition of IL-2 release from these cells. In the present system SR141716A and SR144528 exhibited marked partial agonist activity in that these cannnabinoid receptor antagonists evoked responses on their own. Under these circumstances, it would be difficult to demonstrate clearly the antagonist properties of these compounds on CP55,940-induced action on Jurkat cells since they evoke responses on Jurkat cells on their own. The partial and inverse agonist effects of these compounds are well documented (Portier *et al.*1999). It would be worth employing selective agonists such as ACEA, JWH 015 (Hillard *et al.*, 1999) devoid of any inverse or partial agonist properties to characterise the receptor mediating the effect of cannabinoids in this system, which were not available at the time when these experiments were performed.

Taken together, it is concluded that the pharmacological profile exhibited by cannabinoid receptor ligands as inhibitors of PHA and PMA-induced IL-2 release from Jurkat cells is inconsistent with the presence of typical functional cannabinoid receptors as reported elsewhere (Kaminski *et al.*, 1992). Firstly, the agonist rank order of agonist potency; (Anandamide>Δ^9-THC> CP55,940> SR144528> SR141716A) is not in agreement with other published work (Pertwee *et al* , 1999). Secondly, the cannabinoid CB_1 and CB_2 receptor antagonists were unable to attenuate the effect of CP55,940 in this system. Whether this profile represents the presence of a unique subtype of a variant of a previously described receptor, such as vanilloid

receptors, would require further studies. Furthermore, Jurkat cells are apoptosis-sensitive cells (Neuzil *et al*, 1999). The unusual susceptibility of these cells to metabolic oxidative damage in response to increasing concentration of CP55,940 may suggest an up regulation of apoptotic signals in this system, a topic studied in a greater detail in chapter 7 of this thesis. Taken together, these observations point to the existence of an alternative immune inhibitory pathway in T cell responses. However, further studies such as the effect of cannabinoids on Jurkat cell apoptosis as reported in chapter 7 of this thesis is required to confirm our hypothesis.

In conclusion, the findings described in this chapter show that cannabinoid receptor agonists inhibit PHA and PMA-induced IL-2 release from Jurkat cells. These data could not conclusively show whether these actions are receptor mediated or simply a non-specific membrane effect, hence this study has been extended to peripheral blood mononuclear cells (PBMC) as described in chapter 4 of this thesis. Finally, it has been demonstrated that Jurkat cells are highly susceptible to mitochondria oxidative damage when treated with cannabinoids.

Chapter 4; The effect of cannabinoids on the release of interleukin 2 (IL-2) from peripheral blood mononuclear cells (PBMC)

4.1 Introduction

In the previous chapter, the effects of cannabinoids on the release of pro-inflammatory cytokines, TNF-α and IL-2 from a pro-monocytic cell line, THP-1 and from a pro-lymphocytic cell line, Jurkat cells were investigated. These cell lines are known to express cannabinoid CB_2 receptors (Halfpenny *et al.*, 1998; Schatz *et al.*, 1997). They are human derived immature white blood cells and therefore the effects observed in these systems may not necessarily be representative of the mature human blood mononuclear cells. In the present chapter, experiments are described where the effects of cannabinoids on the release of IL-2 from human peripheral blood mononuclear cells (primary cells) are studied. In a recent study, the effects of cannabinoids on the release of TNF-α from human peripheral blood mononuclear cells were described (Germain *et al.*, 2002), therefore, no attempt was made at replicating this study.

Interleukin-2 is an important cytokine responsible for T lymphocyte signalling during proliferation and macrophage/monocyte activation during inflammatory episodes (Herrman *et al.*, 1989). The expression of functional interleukin-2 receptors is another variable that determines how long the clonal proliferation of T cells occurs after antigen stimulation (Smith, 1988). In general, interleukin-2 regulates both antigen-specific and non-antigen specific proliferation of T-cells, natural killer (NK) cells and B cells.

The discovery and cloning of two cannabinoid receptors, CB_1 and CB_2, has begun to give new clues as to how these drugs affect the immune system (Matsuda *et al.*, 1990; Munro *et al*, 1993). Cannabinoid receptors are members of the G-protein coupled

receptor family (Bayewitch *et al.*, 1995). While cannabinoid CB_1 receptors are found in the brain with low levels of expression in the peripheral tissues, cannabinoid CB_2 receptors are expressed primarily in immune tissues (Bouaboula *et al.*, 1993; Galiegue *et al.*, 1995; Kaminski *et al.*, 1992), suggesting that the majority of the immunomodulatory properties of cannabinoids may be mediated via cannabinoid CB_2 receptors, although to date, very few studies have been reported to support this hypothesis.

The density of cannabinoid CB_2 receptors on immune cells is 10-100 times that of cannabinoid CB_1 receptors, as shown by semi-quantitative reverse transcriptase polymerase chain reaction and Northern blotting studies (Galigue *et al.*, 1995). The rank order of cannabinoid CB_2 receptor expression on human blood leukocytes is B cells> NK cells> monocytes> polymorphonuclear neutrophils> T8 cells> T4 cells (Parolaro, 1999). Furthermore, it has been shown that cannabinoid receptor expression in peripheral blood mononuclear cells is altered upon stimulation with phytohaemagglutinin (Daaka *et al.*, 1996), suggesting an active role for the cannabinoid system in immune responses.

Given the pro-inflammatory properties of interleukin-2, modulation of its release via cannabinoid receptors would present an attractive pharmacological target for the treatment of various inflammatory conditions.

4.2 Aims of study

The aims of this chapter are:

1 To investigate the effect of cannabinoid receptor agonists on the secretion of IL-2 from peripheral blood mononuclear cells (PBMC).

2 To characterise the cannabinoid receptor responsible for any observed effects using selective cannabinoid receptor ligands.

4.3 Experimental Protocol

Isolation of human peripheral blood mononuclear cells from buffy coat was as described in chapter 2 (section 2.3.3) of this thesis. Cell viability was assessed as described in chapter 2 (section 2.8.1 and 2.8.2.).

4.3.1 Treatment of cells

The isolated human peripheral blood mononuclear cells was adjusted to a density of 1 x 10^6 cells.ml^{-1} with RPMI 1640 medium and cultured in 24-well plates (Falcon, Becton Dickinson, Pont De Claire, France) in foetal calf serum-free RPMI-1640 medium, at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were pre-incubated with CP55940 (10^{-10} M - 10^{-5} M), WIN55212-2 (10^{-10} M - 10^{-5} M), Δ^9Tetrahydrocannabinol (10^{-10} M - 10^{-5} M), JWH 015 (10^{-10} M - 10^{-5} M) or dexamethasone (10^{-10} M - 10^{-6} M) for 2 h before stimulation with phytohaemagglutinin (10 μg ml^{-1}). Supernatants were harvested after 18 h incubation and stored at–70 °C until assayed for interleukin-2 by ELISA as described in chapter 2 (section 2.4.8). In experiments where the effects of antagonists were studied, cells were pre-incubated with SR141716A (10^{-6} M), SR144528 (10^{-6} M), CP55940 (10^{-6} M) or Δ^9-Tetrahydrocannabinol (10^{-6} M) for 30 min before the addition of the cannabinoid agonist or dexamethasone.

4.4 Data Analysis

Concentration-effect curves were analysed by Prism (GraphPAD Inc., San Diego, U.S.A.). Other results are shown as bar graphs. In some experiments, the results are expressed as percentage inhibition of IL-2 release from PHA treated cells. $IC_{1/2max}$ 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 ($IC_{1/2max}$ values) ± S.E.M (standard error of the mean) or 95 % confidence limit as appropriate. Statistical significance was determined using a one sample *t*-test or analysis of variance followed by an appropriate post hoc test. Statistical significance was assumed if *P* value was ≤ 0.05

4.5 Results

4.5.1 Purity and viability of human peripheral blood mononuclear cells.

Under the experimental conditions described in this thesis, the viability of human peripheral blood mononuclear cells isolated from buffy coat cells exceeded 95 % on all occasions, when determined by trypan blue dye exclusion and by the MTT assay. This viability was not significantly (P>0.05) altered by incubation of human peripheral blood mononuclear cells for 18 h with phytohaemagglutinin, dexamethasone or any of the cannabinoid receptor ligands studied in foetal calf serum free RPMI 1640 medium.

Human peripheral blood mononuclear cell preparations, prepared from buffy coat cells, comprised approximately 95% lymphocytes and 5% monocytes as measured by differential leukocyte counts.

4.5.2 The effect of phytohaemagglutinin on interleukin-2 secretion from human peripheral blood mononuclear cells.

Non-stimulated human peripheral blood mononuclear cells constitutively released minimal amounts of interleukin-2 (14 ± 10 pg ml^{-1}, n = 5) after 18 h incubation at 37 °C (Figure 4.5.8). Following stimulation with phytohaemagglutinin (10 μg ml^{-1}), a marked release of interleukin-2 was observed over 18 h (1869 ± 54 pg.ml^{-1}, n = 5, Figure 4.5.1). Stimulation of human peripheral blood mononuclear cells with phytohaemagglutinin (10 μg ml^{-1}) evoked a minimal release of interleukin-2 within the first 6 h and a rise between 12 and 18 h. The peak release of interleukin-2 was seen at 18 h (Figure 4.5.1). There was no significant change (P>0.05) in cell numbers between phytohaemagglutinin (10 μg ml^{-1}) stimulated and non-stimulated cells over 18 h following incubation at 37 °C in foetal calf serum-free medium (data not shown).

Vehicle controls (0.1 % ethanol and 0.1 % DMSO) had no significant ($P<0.05$) inhibitory effect on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells.

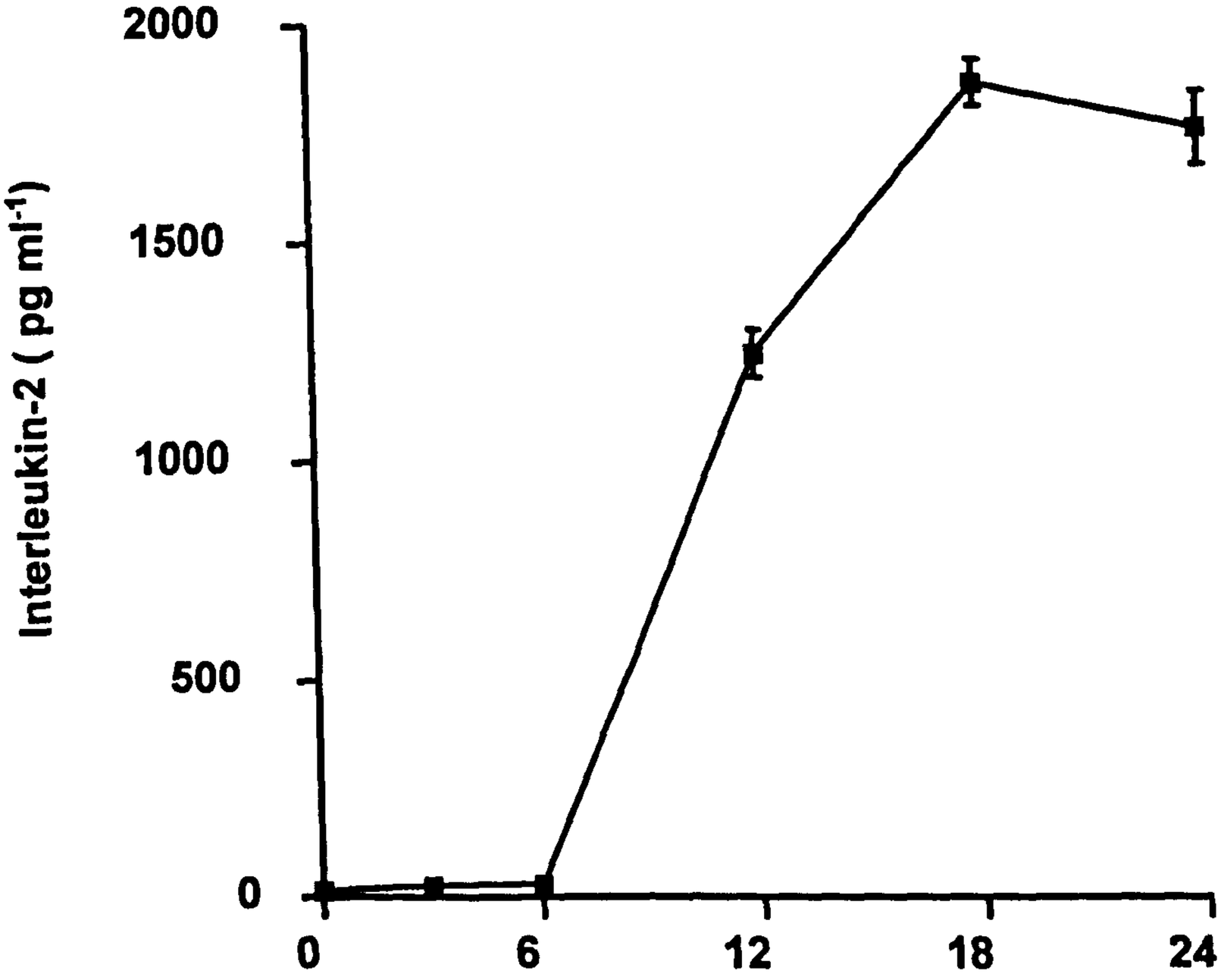


Figure 4.5.1 Time course of phytohaemagglutinin -induced interleukin-2 release from human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 $\mu g.ml^{-1}$) for 3, 6, 12, 18 and 24 h. Cell free supernatants were harvested for interleukin-2 assay by ELISA as described chapter 2, section 2.4.8. Data are means and S.E. of the means of 5 separate experiments.

4.5.3 The effect of cannabinoid receptor agonists on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells.

The non-selective cannabinoid receptor agonist WIN55212-2 (10^{-10} M – 10^{-5} M) and a selective cannabinoid CB_2 receptor agonist JWH 015 (10^{-10} M - 10^{-5} M) inhibited phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells (Figure 4.5.2). This inhibition was concentration-related and significant ($P<0.05$) over the concentration range 10^{-6} M - 10^{-5} M ($IC_{1/2max}$, WIN55212-2 = $8.8x10^{-7}$ M, 95 % C.L. = $2.2x10^{-7}$ M – $3.5x10^{-6}$ M, JWH 015 = $1.8x10^{-6}$ M, 95 % C.L. = $1.2x10^{-6}$ M – $2.9x10^{-6}$ M, n = 5). The non-selective cannabinoid receptor agonist CP55,940 (10^{-10} M – 10^{-6} M), produced a small, non-significant ($P>0.05$), inhibition of interleukin-2 release from human peripheral blood mononuclear cells (Figure 4.5.2). The non-selective cannabinoid receptor agonist Δ^9-Tetrahydrocannabinol (10^{-10} M – 10^{-6} M) and the selective cannabinoid CB_1 receptor agonist ACEA (10^{-10} M – 10^{-6} M) also had no significant ($P>0.05$) inhibitory effect on the release of interleukin-2 from human peripheral blood mononuclear cells. As a positive control, dexamethasone (10^{-10} M - 10^{-6} M) a glucocorticoid, significantly ($P<0.05$) inhibited phytohaemagglutinin-induced interleukin-2 release from human peripheral blood mononuclear cells ($IC_{1/2max}$ = 1.3 x 10^{-8} M, C.L. = 5.4 x 10^{-9} M – 3.2 x 10^{-8} M, n = 5, Figure 4.5.3). The maximum inhibition produced by JWH 015 was greater than that produced by WIN55212-2 (Figure 4.5.3).

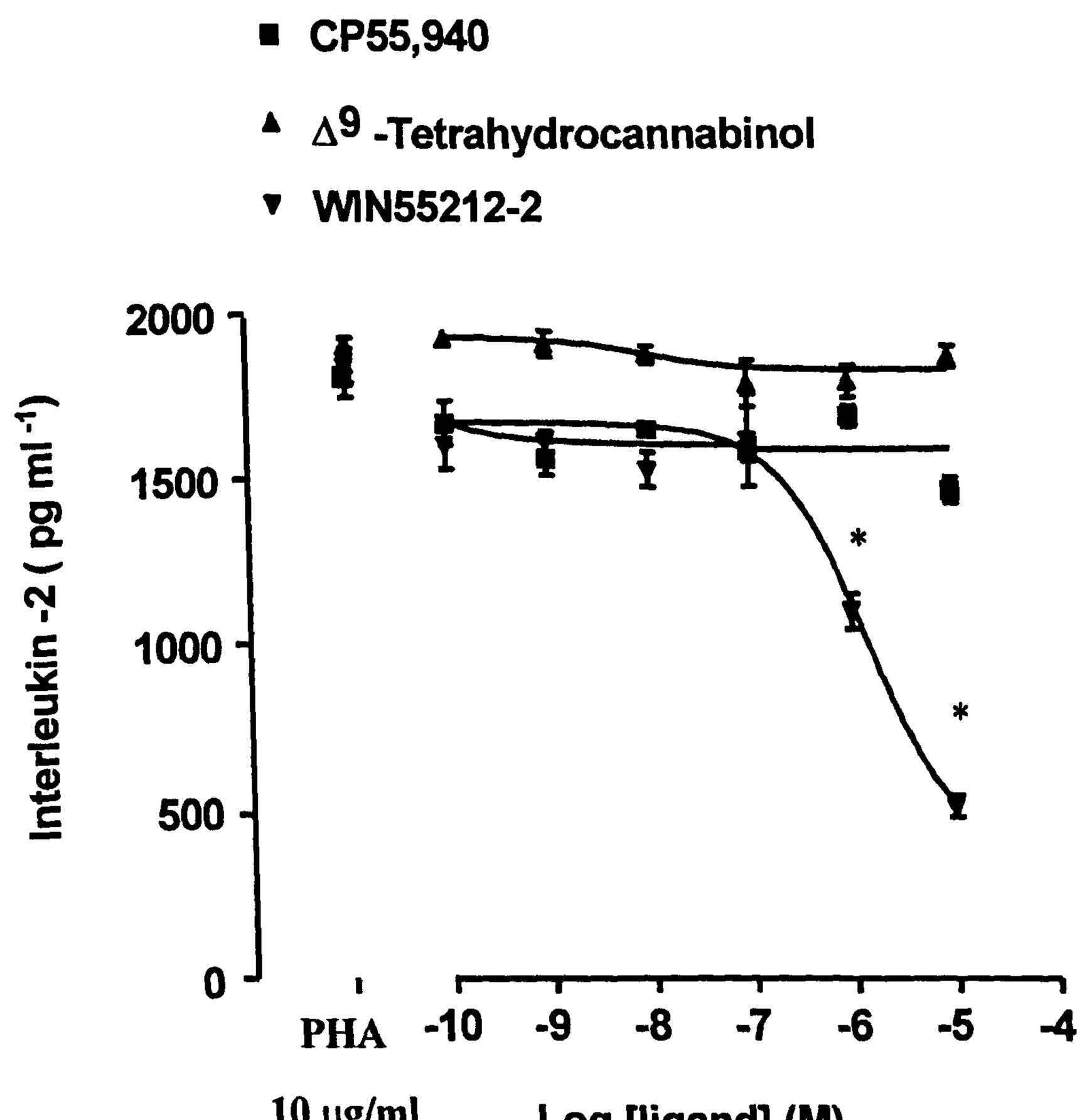


Figure 4.5.2 The effect of non-selective cannabinoid agonists on phytohaemagglutinin -induced release of interleukin -2 from human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells were treated with CP55,940 (10^{-10} M - 10^{-5} M), Δ^9-Tetrahydrocannabinol (10^{-10} M - 10^{-5} M) or WIN55212-2 (10^{-10} M - 10^{-5} M) for 2 h before stimulation with phytohaemagglutinin (10 μg ml^{-1}) for a further 18 h. Cell free supernatants were harvested and assayed for interleukin-2 by ELISA as described in the chapter 2, section 2.4.8. Data are means and S.E. of the means of 5 separate experiments. *Denotes significant difference ($P< 0.05$) from the control (phytohaemagglutinin treated cells) (Student's *t*-test).

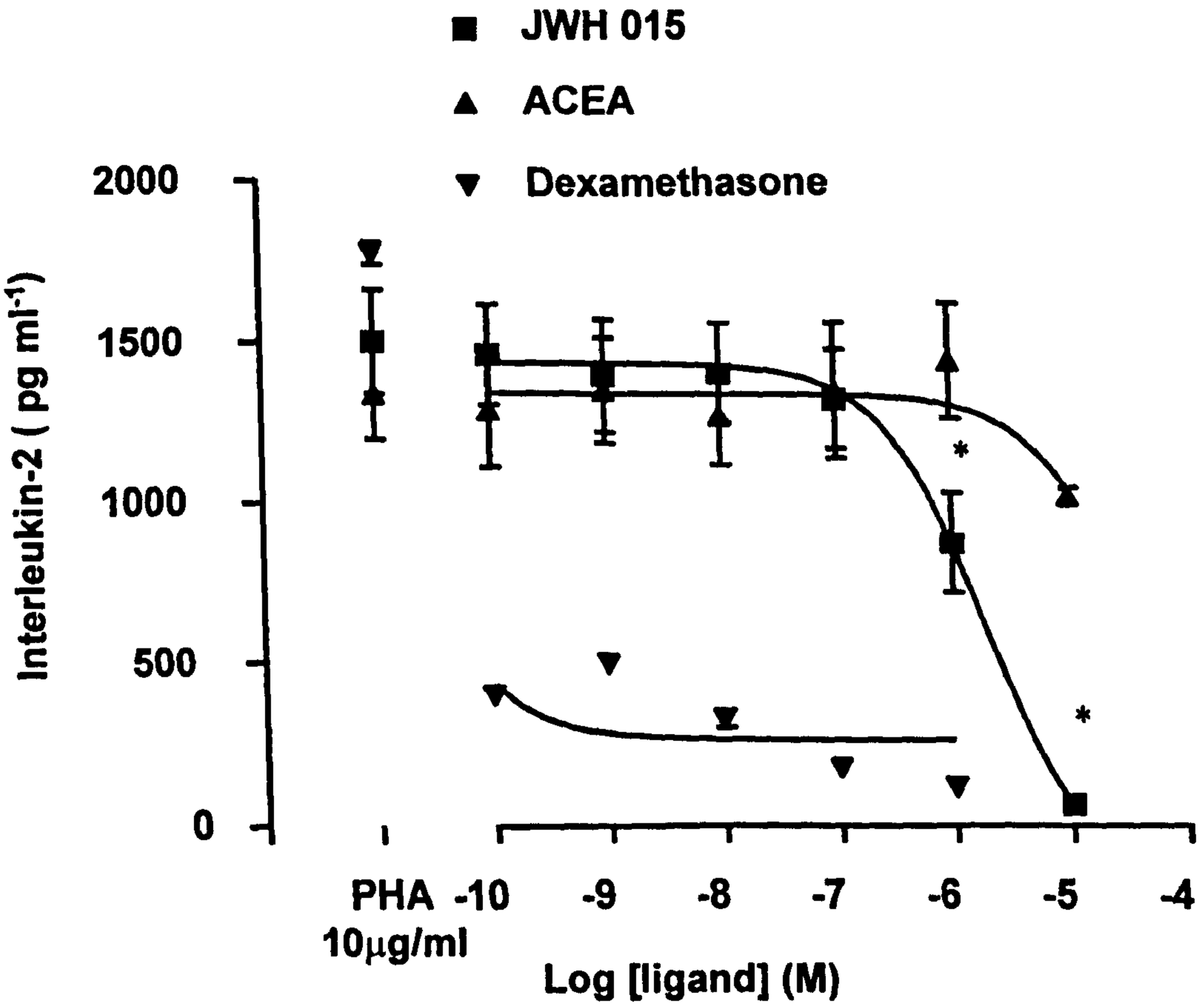


Figure 4.5.3 The effect of selective cannabinoid agonists and dexamethasone on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells were treated with ACEA (10^{-10} M - 10^{-5} M), JWH 015 (10^{-10} M - 10^{-5} M) or dexamethasone (10^{-10} M – 10^{-5} M) for 2 h before stimulation with phytohaemagglutinin (10 $\mu g.ml^{-1}$) for a further 18 h. Cell free supernatants were harvested and assayed for interleukin-2 by ELISA as described in the chapter 2, section 2.4.8. Data are means and S.E. of the means of 5 separate experiments. *Denotes significant difference ($P< 0.05$) from the control (phytohaemagglutinin treated cells) (Student's *t*- test).

4.5.4 The effect of SR141716A and SR144528 on WIN55212-2 and JWH 015-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells.

When incubated with human peripheral blood mononuclear cells for 18 h, neither SR141716A (10^{-6} M) nor SR144528 (10^{-6} M) had any significant effect on phytohaemagglutinin-induced interleukin-2 release (interleukin-2 release =1530.5 ± 80.8 pg.ml^{-1} (n=5) and 1653.4 ± 65.5 pg.ml^{-1} (n=5) respectively) when compared with phytohaemagglutinin treated controls (1655.7 ± 52.8 pg.ml^{-1} (n=9). SR141716A (10^{-6} M) had no significant (P>0.05) effect in attenuating the inhibitory action of WIN55212-2 on phytohaemagglutinin-induced release of interleukin-2 (Figure 4.5.4). In contrast, SR144528 (10^{-6} M) significantly (P<0.05, 2 way ANOVA followed by Bonferroni's post hoc test, n = 5) antagonised the inhibitory effects of WIN55212-2 on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells (pA_2 = 6.3 ± 0.1, n = 5) (Figure 4.5.4). Similarly, SR141716A (10^{-6} M) had no significant (P>0.05) effect in attenuating the inhibitory effect of JWH 015 on phytohaemagglutinin-induced release of interleukin-2. In contrast, SR144528 (10^{-6} M) significantly (P<0.05, 2 way ANOVA followed by Bonferroni's post hoc test, n = 5) antagonised the inhibitory effects of JWH 015 on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells (pA_2 = 6.5 ± 0.1, n=5) (data not shown).

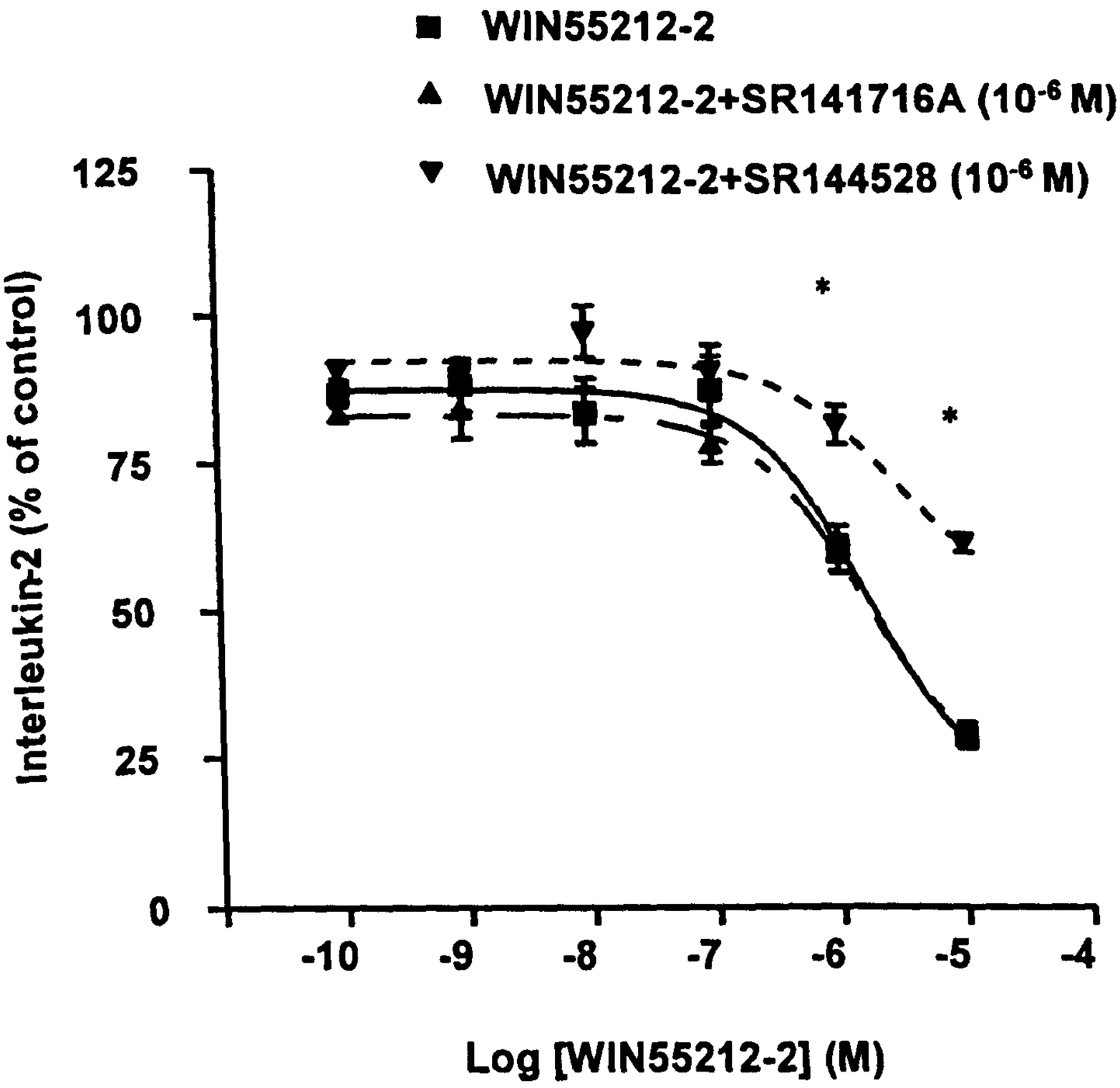


Figure 4.5.4 Effect of SR141716A or SR144528 on WIN55212-2-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells were pre-incubated with SR141716A (10^{-6} M) or SR144528 (10^{-6} M) for 30 min before addition of WIN55212-2 (10^{-10} M - 10^{-5} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 $\mu g.ml^{-1}$) for further 18 h. Cell free supernatants were harvested for interleukin-2 assay by ELISA as described in the chapter 2, section 2.4.8. Data are means and S.E. of the means of 5 separate experiments. * Denotes significant difference from WIN55212-2 treated cells ($P<0.05$, 2 way ANOVA followed by Bonferroni's post hoc test, n=5).

4.5.5 *The effect of CP55,940 and Δ^9-Tetrahydrocannabinol on WIN55212-2-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells.*

CP55,940 (10^{-6} M) and Δ^9-Tetrahydrocannabinol (10^{-6} M) significantly ($P<0.05$, 2 way ANOVA followed by Bonferroni's post hoc test, n = 5) antagonised the inhibitory effects of WIN55212-2 on phytohaemagglutinin-induced release of

interleukin-2 from human peripheral blood mononuclear cells (Figure 4.5.5 and Figure 4.5.6)

When pA_2 values were calculated from these data, a value of 6.1 ± 0.1, n = 5 was obtained for CP55940 and a value of 6.96 ± 0.16, n = 5 for Δ^9-Tetrahydrocannabinol.

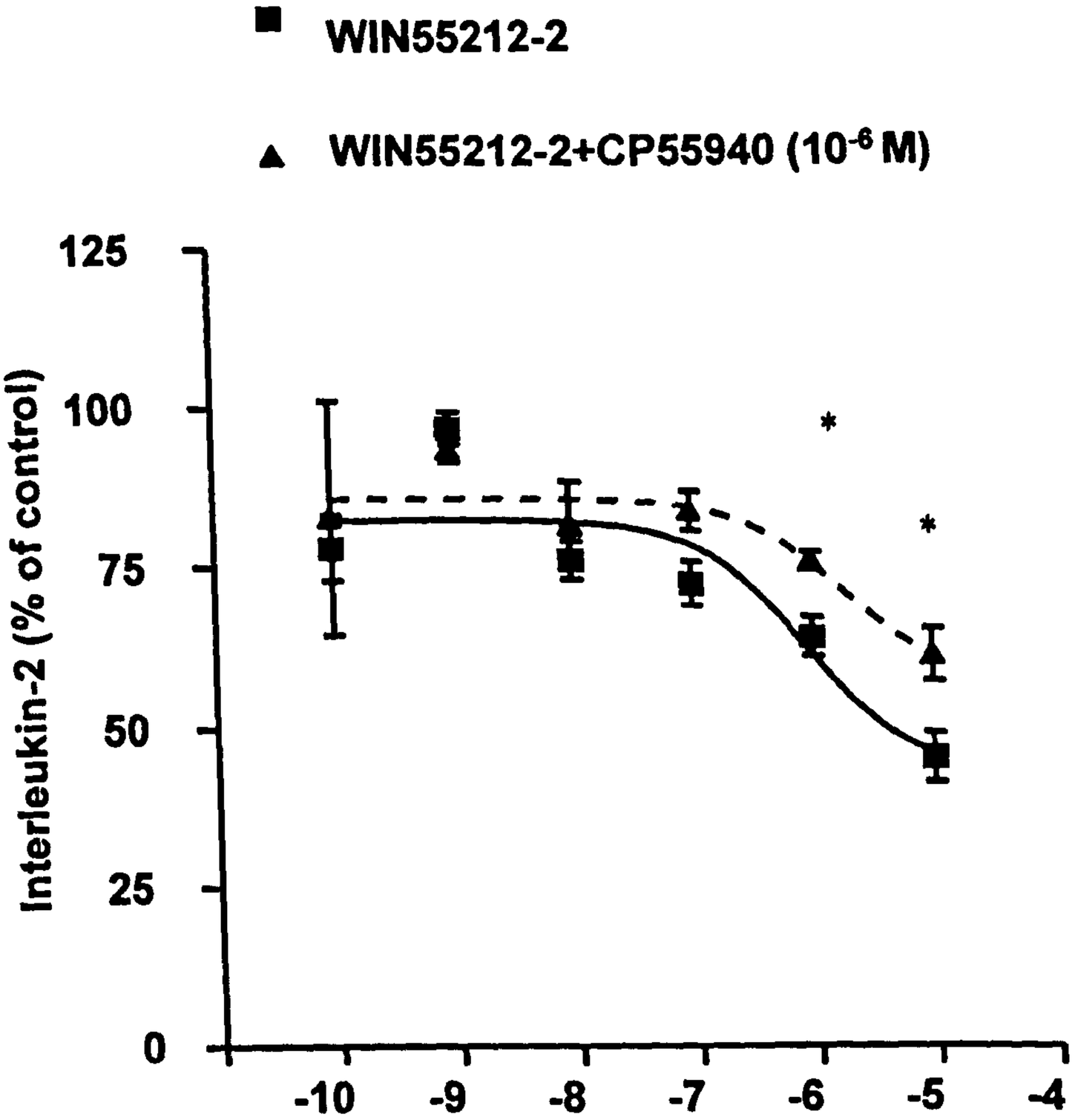


Figure 4.5.5 Effect of CP55940 on WIN55212-2 -induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells were pre-incubated with CP55, 940 (10^{-6} M) for 30 min before addition of WIN55212-2 (10^{-10} M - 10^{-5} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 $\mu g.ml^{-1}$) for a further 18 h. Cell free supernatants were harvested for interleukin-2 assay by ELISA as described in the chapter 2, section 2.4.8. Data are means and S.E of the means of 5 separate experiments. * Denotes significant difference from WIN55212-2 treated cells ($P<0.05$, 2 way ANOVA followed by Bonferroni's post hoc test, n=5)

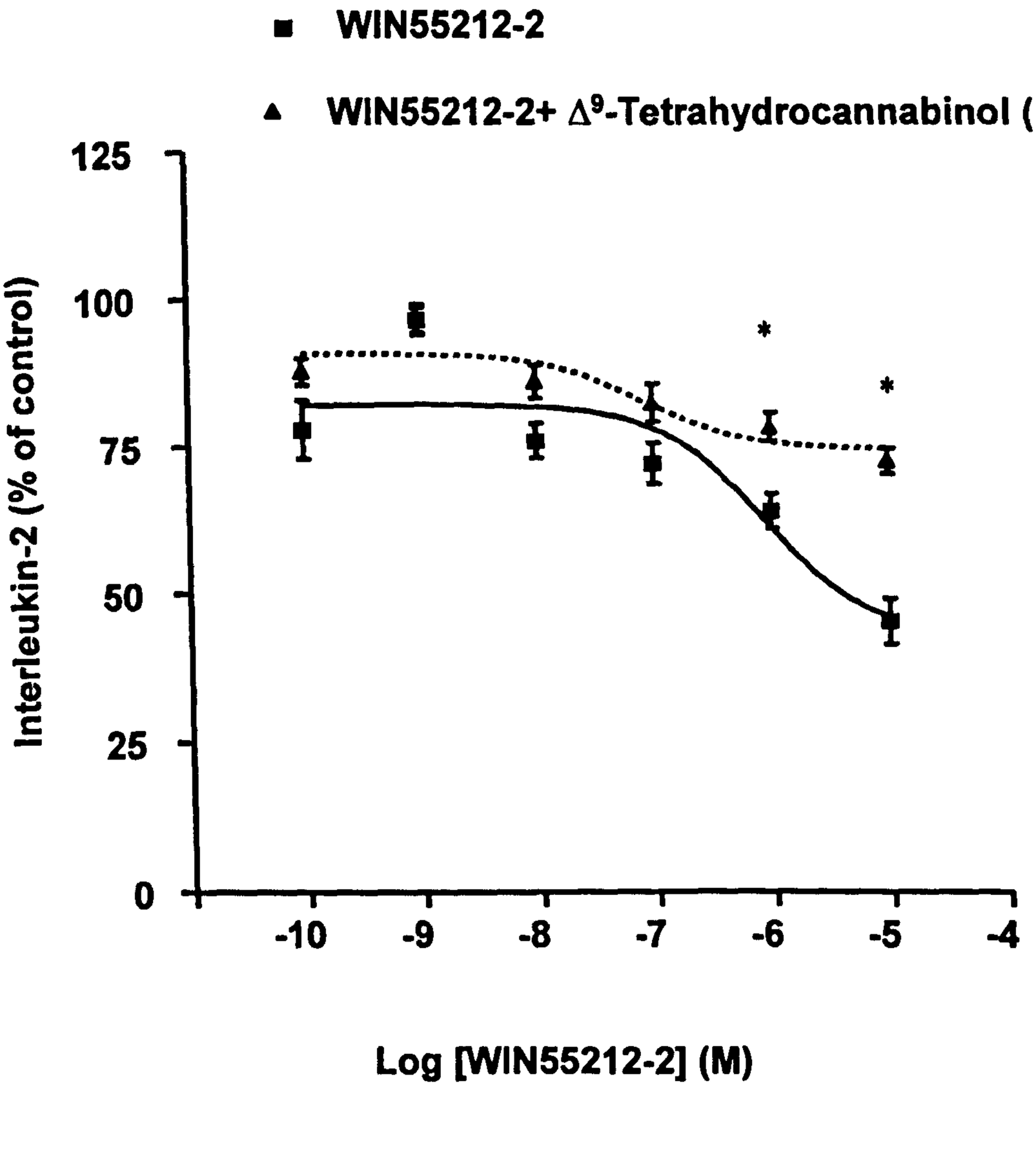


Figure 4.5.6 Effect of Δ^9-Tetrahydrocannabinol on WIN55212-2-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells were pre-incubated with Δ^9-Tetrahydrocannabinol (10^{-6} M) for 30 min before addition of WIN55212-2 (10^{-10} M - 10^{-5} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 $\mu g.ml^{-1}$) for a further 18 h. Cell free supernatants were harvested for interleukin-2 assay by ELISA as described in the chapter 2, section 2.4.8. Data are means and S.E of the means of 5 separate experiments. * Denotes significant difference from WIN55212-2 treated cell ($P<0.05$, 2 way ANOVA followed by Bonferroni's post hoc test).

4.5.6 The effect of CP55,940 on dexamethasone-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells.

CP55,940 (10^{-6} M) had no significant ($P>0.05$) effect in antagonising the inhibitory actions of dexamethasone on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells (Figure 4.5.7)

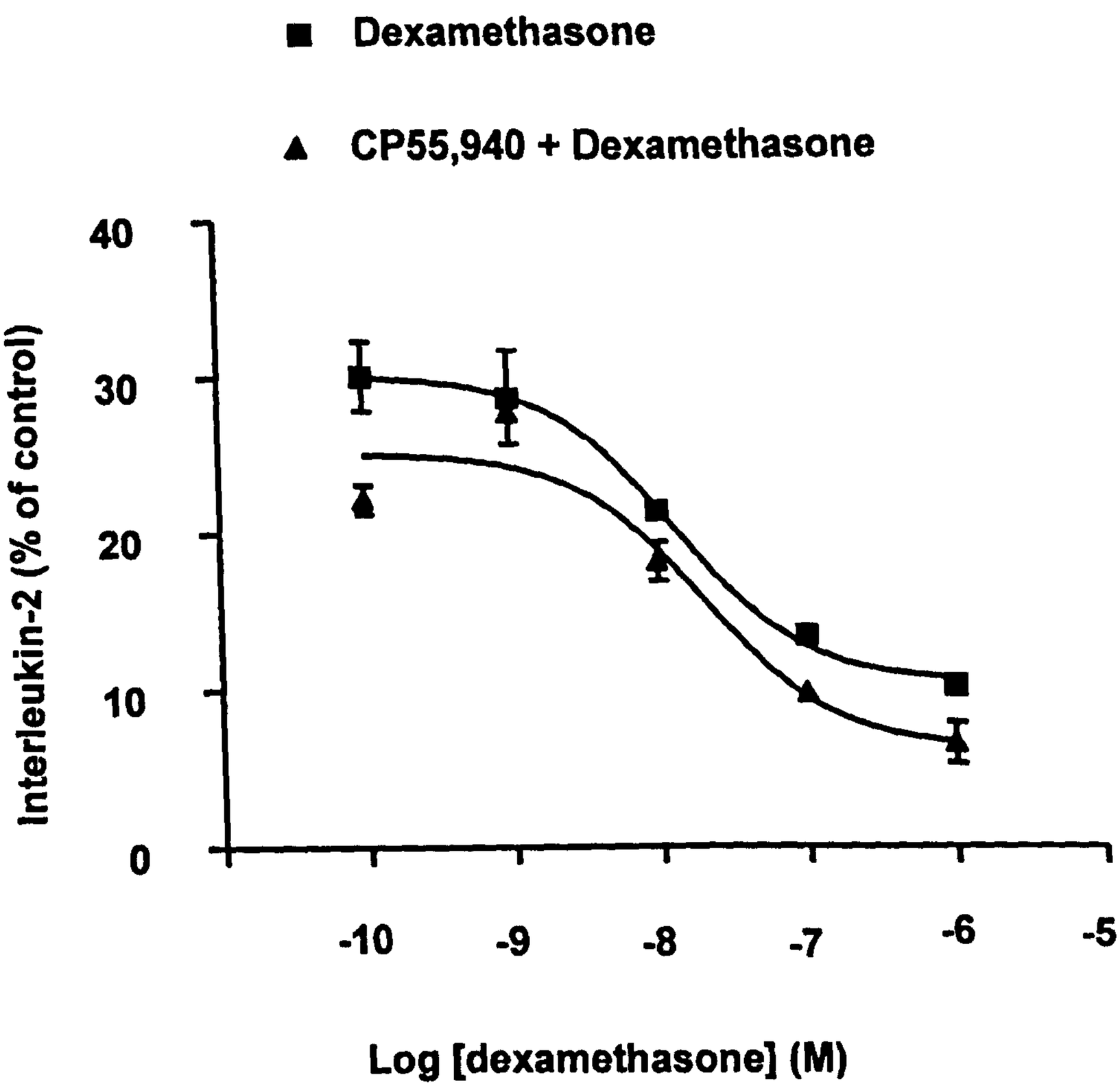


Figure 4.5.7 Effect of CP55,940 on dexamethasone-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells were pre-incubated with CP55,940 (10^{-6} M) for 30 min before addition of dexamathasone (10^{-10} M - 10^{-6} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg ml^{-1}) for a further 18 h. Cell free supernatants were harvested for interleukin-2 assay by ELISA as described in chapter 2, section 2.4.8. Data are means and S.E of the means of 5 separate experiments

4.5.7 The effect of CP55,940 on the release of interleukin-2 from non-stimulated human peripheral blood mononuclear cells.

Addition of CP55,940 (10^{-5} M) to non-stimulated human peripheral blood mononuclear cells followed by incubation at 37 °C for 18 h evoked a minimal release of interleukin-2 (21.8 ± 6.3 pg ml^{-1}, n = 5), which was not significantly (P>0.05) different from the basal release (Figure 4.5.8)

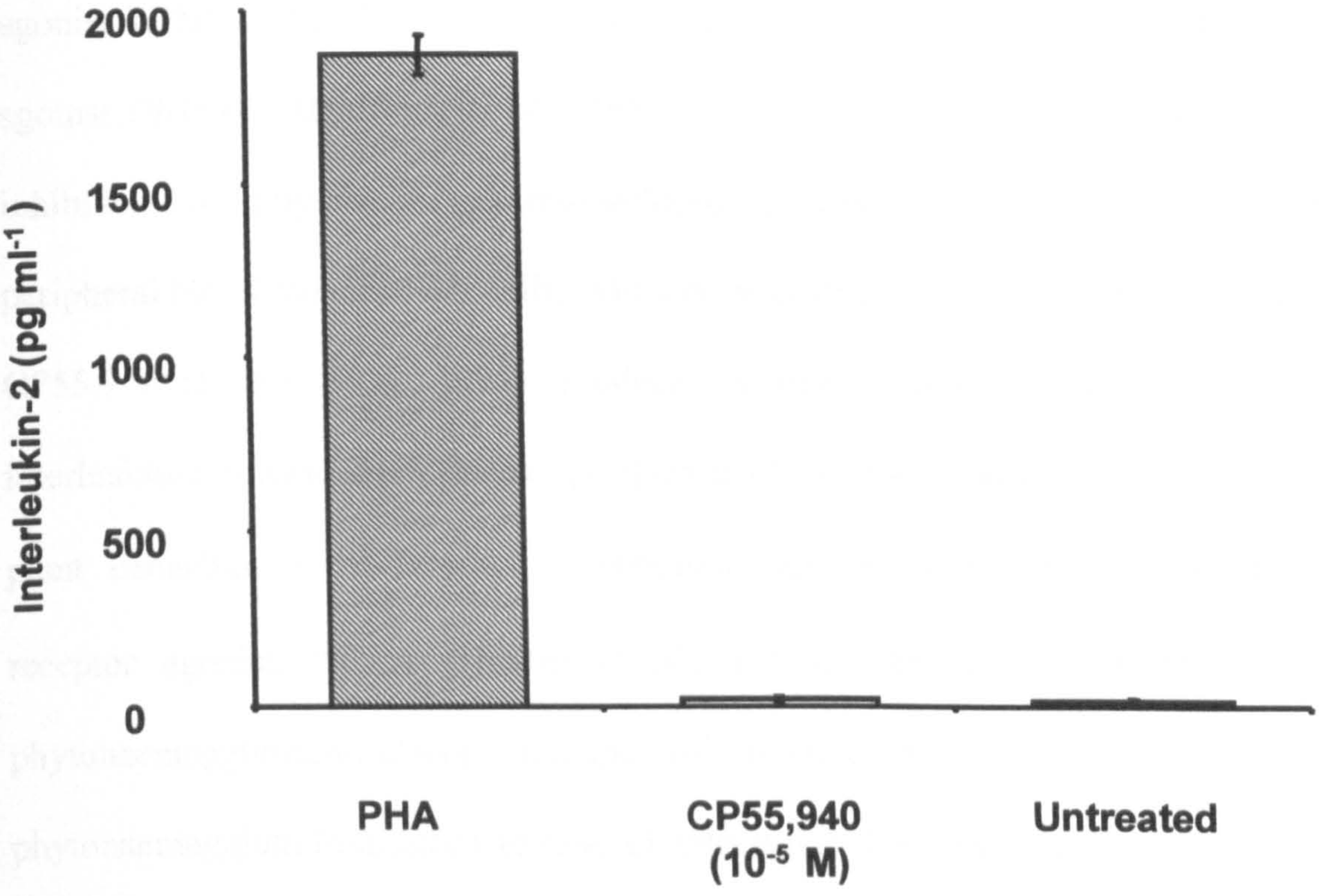


Figure 4.5.8 Effect of CP55,940 on the secretion of interleukin-2 from human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg ml^{-1}) or CP55,940 (10^{-5} M) for 18 h. Cell free supernatants were harvested for interleukin-2 assay by ELISA as described in chapter 2, section 2.4.8. Data are means and S.E. of means of 5 separate experiments.

4.6 Discussion

In the present study, it has been shown that a non-selective cannabinoid receptor agonist WIN55212-2 (Felder *et al.*, 1995) and a selective cannabinoid CB_2 receptor agonist JWH 015 (Huffman *et al.*, 1996) evoked a significant concentration-related inhibition of phytohaemagglutinin-induced interleukin-2 release from human peripheral blood mononuclear cells. The non-selective, synthetic cannabinoid agonist CP55,940 (Felder *et al*, 1995), produced a small, non-significant inhibition of interleukin-2 release from human peripheral blood mononuclear cells whereas the plant cannabinoid, Δ^9-Tetrahydrocannabinol and the selective cannabinoid CB_1 receptor agonist, ACEA (Hillard *et al.*, 1999), were ineffective in inhibiting phytohaemagglutinin-induced release of interleukin-2. The inhibition of phytohaemagglutinin-induced release of interleukin-2 evoked by WIN55212-2 was not antagonised by pre-treatment of the cells with SR141716A, a cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona *et al.*, 1994). However, SR144528, a cannabinoid CB_2 receptor antagonist (Rinaldi-Carmona *et al.*, 1998) significantly attenuated the inhibitory effects of WIN55212-2. Taken together, these data suggest that the observed effects were mediated by a cannabinoid CB_2-like receptor.

Cannabinoid receptor ligands have potential utility as anti-inflammatory drugs for the treatment of many disease conditions primarily because of their immunosuppressive actions, but their psychoactive effects limit their therapeutic benefits. Emerging evidence suggests that cannabinoids produce many of their immunosuppressive effects by inhibiting T-cell responses (see Klein *et al.*, 1998; Parolaro, 1999, for reviews). A significant proportion of these studies has been conducted on cell lines and transfected cells derived from rats or mice (Kaminski *et al.*, 1992; Condie *et al.*,

1996; Massi *et al.*, 2000). While these systems provide useful information for the understanding of the functional properties of cannabinoid receptors, extrapolating these data to man may be hindered by problems of species differences and the artificial nature of the cell lines and transfected cells in which receptors are over expressed (Kenakin, *et al.*, 1995). Consequently, the effects of a range of cannabinoid receptor ligands on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells, a human immune cell were investigated.

In the present study, the human periperal blood mononuclear cells suspended in foetal calf serum-free medium was cultured. While it is conventional to include foetal calf serum in cell culture medium (for example, Corrigan *et al.*, 1995), it was a choice not to include it because plasma proteins have been shown to bind cannabinoids and reduce their potency (Dewey, 1986) that is this process acts as an agonist uptake/removal process. Furthermore, if this binding were saturable, over the concentration range studied, then this could influence the data obtained particularly when attempting to characterise antagonist activity (Kenakin and Beek, 1981). Thus, it was elected to negate the influence of protein binding in our experiments by omitting foetal calf serum from the medium.

In the present study, inhibition of PHA-induced release of interleukin-2 by WIN55212-2 and JWH015 was observed at concentrations greater than those required to displace a radio-labelled cannabinoid receptor ligand in receptor binding studies (> 1 μM) (Felder *et al.*, 1995; Showalter *et al.*, 1996). However, the potency of WIN55212-2 in the present study is similar to that reported by others in studies on a murine macrophage cell line (RAW264.7) (Ross *et al*, 2000). It is noteworthy that the

K_d values reported from cannabinoid binding studies are usually higher in experiments where purified receptors or transfected cells have been used (Howlett *et al.*, 1995; Slipetz *et al.*, 1995). This difference has been ascribed to loss of activity of lipophilic cannabinoids due to non-specific interactions with cells and serum (Howlett *et al.*, 1995; Slipetz *et al.*, 1995). Furthermore, the pA_2 value for the cannabinoid CB_2 receptor antagonist SR144528 reported in this study is significantly lower than the pK_i value reported for this compound on Chinese hamster ovary cells transfected with CB_2 receptors (Iwamura *et al.*, 2001). It is lower than that previously obtained by us in studies on epithelial cells (Ihenetu *et al.*, 2003), although the potency of SR144528 in the present study is similar to that reported by others in experiments on a murine macrophage cell line (Ross *et al.*, 2000). One explanation for this difference may be due to the level of cannabinoid CB_2 receptor expression in mononuclear cells compared to that in other tissues, coupled with the lipophilic nature of these compounds reducing the actual concentration of antagonist available at the receptor. Clearly further experiments are required to determine why SR144528 is apparently less potent as a cannabinoid CB_2 receptor antagonist on monocytes compared with other tissues.

In line with the present study, it is noteworthy that few studies to date have reported functional effects of cannabinoids via cannabinoid CB_2 receptors at concentrations below 1 μM (Ross *et al.*, 2000). Furthermore, in transfected cell lines, the stoichiometry of key regulatory proteins may be altered resulting in responses distinct from those found in primary cells (Kenakin *et al.*, 1995). Thus, it seems possible that our finding that cannabinoid agonists were less potent in human peripheral blood

mononuclear cells when compared to data published by others may reflect a low level of cannabinoid receptor expression in these cells.

Other published work suggests that cannabinoids can stimulate cytokine release. In contrast to our findings, Derocq *et al.* (1995) were able to show that low concentrations of CP55,940 significantly ($P<0.05$), increased DNA synthesis in human tonsilar B-cells, a primary cell system that expresses high levels of cannabinoid CB_2 receptors (Galigue *et al.*, 1995). Other studies showing effects of cannabinoids at low concentrations include experiments in which the cannabinoid receptor agonists CP55,940 or WIN55212-2 caused increased expression of IL-8 in HL-60 cells transfected with cannabinoid CB_2 receptors (Jbilo *et al.*, 1999; Derocq *et al.*, 2000). However, these cannabinoid CB_2 receptor agonists still increased IL-8 expression when wild type HL-60 cells were used (Derocq *et al.*, 2000; Jbilo *et al.*, 1999). These findings suggest that HL-60 cells have a higher level of endogenous cannabinoid CB_2 receptor expression than human peripheral blood mononuclear cell since, in the present study, the cannabinoid receptor agonist CP55,940 did not induce the release of IL-2 from PBMC even after incubation for 18 h.

Other published work has also shown that cannabinoids may either increase or decrease IL-2 release from immune cells depending on the experimental conditions and the cells studied (Pross *et al.*, 1992; Watzl *et al.*, 1991). In the murine lymphocyte cell line, EL4.IL-2, Δ^9-Tetrahydrocannabinol and cannabidiol inhibited phorbol myristyl acetate/Ionophore-induced interleukin-2 mRNA expression and interleukin-2 release in a concentration-dependent manner (Condie *et al.*, 1996; Jan *et al.*, 2002). In contrast, in phytohaemagglutinin activated human peripheral blood

monouclear cells, Δ^9-Tetrahydrocannabinol and cannabidiol did not inhibit interleukin-2 release, although these cannabinoid receptor ligands did inhibit the release of other cytokines (Watzl *et al.*, 1991), findings that are consistent with those reported in the present study. Thus, it appears that the choice of cell and the stimulus used to provoke cytokine release may influence the inhibitory activity of cannabinoid receptor agonists. Such an effect is not unique to cannabinoid receptor agonists and has been noted in studies with other classes of agonists (e.g. Kenakin, 1982; Kenakin *et al.*, 1995). The exact reason for the differences between the findings of the present study and those described above is still unclear and additional experiments are necessary to resolve these discrepancies.

Previous studies in our laboratory and others have shown that a range of cannabinoid ligands including WIN55212-2, CP55,940 and Δ^9-Tetrahydrocannabinol act as agonists at the peripheral cannabinoid CB_2 receptor to cause inhibition of tumour necrosis factor-α-induced release of interleukin-8 in HT-29 cells (Ihenetu *et al.*, 2001) and to inhibit adenylate cyclase activity in Chinese hamster ovary cells transfected with cannabinoid CB_2 receptors (Bayewitch *et al.*, 1995) respectively. However in the present study CP55,940 only marginally and non-significantly inhibited phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells while Δ^9-Tetrahydrocannabinol had no effect in inhibiting this release. Receptor binding studies have demonstrated that these two agonists have affinity for cannabinoid CB_2 receptors on immune cells (Bouaboula *et al.*, 1993; Galiegue *et al.*, 1995; Kaminski *et al.*, 1992). Thus, one explanation for this lack of activity could be due to a low level of efficacy combined with a relatively low level of cannabinoid CB_2 receptor expression. Similar effects have been reported in

experiments with partial agonists in other receptor systems (Kenakin and Beek, 1982). This hypothesis is supported by the ability of CP55,940 and Δ^9-Tetrahydrocannabinol to inhibit the effects of WIN55212-2. In the present study both compounds shifted concentration-effect curves for WIN55212-2-induced inhibition of interleukin-2 release, to the right. In the case of CP55,940, the small inhibitory effect on interleukin-2 release adds further weight to the hypothesis that it is acting as a weak partial agonist at cannabinoid CB_2 receptors relative to the effect observed with WIN55212-2.

Given the apparent potency of CP55,940 at cannabinoid CB_2 receptors, reported by others (Showalter *et al.*, 1996), it is possible that the lack of inhibitory effect on phytohaemagglutinin-induced interleukin-2 release is because the inhibitory effect is negated by additional release of interleukin-2 induced by CP55,940. Such an effect has been reported by others (Jbilo *et al.*, 1999) and could also explain the apparent antagonism of the inhibitory action of WIN55212-2 by CP55,940. However, this is clearly not the case since when human peripheral blood mononuclear cells were incubated with CP55,940 for 18h, no release of interleukin-2 was seen adding support to the hypothesis that in our experiments CP55,940 acts at cannabinoid CB_2 receptors on human peripheral blood mononuclear cells to antagonise the effects of WIN55212-2.

To test the specificity of CP55,940 in antagonising the effect of WIN55212-2, the effect of CP55,940 in antagonising dexamethasone-evoked inhibition of phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells was studied. CP55,940 did not antagonise dexamethasone-evoked

inhibition of phytohaemagglutinin-induced release of interleukin-2 but marginally potentiated its effect. In order to investigate whether high concentration of CP55,940 evoked the release of interleukin-2 on its own, a point which could account for its poor activity in inhibiting phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells, the effect of CP55,940 (10^{-5} M) on the release of interleukin-2 from human peripheral blood mononuclear cells in the absence of phytohaemagglutinin. In these experiments, CP55,940 alone did not stimulate the release of interleukin-2 from phytohaemagglutinin. Taken together, these results show that CP55,940 appears to be specific in antagonising WIN55212-2-mediated inhibition of phytohaemagglutinin-induced interleukin-2 release from human peripheral blood mononuclear cells and does not, on its own, evoke the release of interleukin-2. Δ^9-Tetrahydrocannabinol exhibited similar profiles (data not shown). Previously, other laboratories have demonstrated that Δ^9-Tetrahydrocannabinol antagonised HU293a and HU210 (non-selective cannabinoid receptor agonists)–induced inhibition of forskolin stimulated adenylyl cyclase in Chinese hamster ovary cells transfected with CB_2 receptors (Bayewitch *et al.*, 1996). To our knowledge, the present study is the first report of CP55,940 acting as a partial agonist/antagonist at a cannabinoid CB_2 receptor-mediated event in a native system.

In summary, it has been demonstrated that WIN55212-2 and JWH 015 evoke inhibition of interleukin-2 release from human peripheral blood mononuclear cells. The selective cannabinoid CB_2 receptor antagonist SR144528 antagonised WIN55212-2 inhibition of phytohaemagglutinin -induced release of interleukin-2 from human peripheral blood mononuclear cells whereas the cannabinoid CB_1 receptor antagonist, SR141716A had no effect. Furthermore CP55,940 and Δ^9-

Tetrahydrocannabinol behaved as partial agonists/antagonists under our experimental conditions indicating that they possess affinity for, but low efficacy at, cannabinoid CB_2 receptors. Thus, this study adds to and extends the body of knowledge suggesting that cannabinoids modulate immune cell function and suggests that some ligands have partial agonist activity at cannabinoid CB_2 receptors. The structures of the cannabinoid receptor ligands utilised in the above study could therefore serve as models for the synthesis of novel and more selective cannabinoid compounds for therapeutic use.

Chapter 5; The effect of cannabinoids on tumour necrosis factor-α (TNF-α)-induced release of interleukin 8 (IL-8) from human colonic epithelial cell line HT-29

5.1 Introduction

In the previous chapters, the effect of cannabinoids on the release of pro-inflammatory cytokines from a variety of immune cells derived from haematopoietic cells i.e. human mononuclear cells were studied. To date, there is little information on the effect of cannabinoids from immune cells derived from non- haematopoietic tissues. In this chapter therefore, the effect of cannabinoids on the release of IL-8 from human colonic epithelial cell line HT-29 was investigated.

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 gastro-intestinal 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 for the development of novel drug treatments for diseases such as ulcerative colitis and chronic bronchitis.

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

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). It is well established that human colonic epithelial cell play major immunological functions such as secretion of cytokines/chemokines (Schuerer-Maly *et al.*, 1994), but to our knowledge, there are no reports of the presence of functional cannabinoid receptors reported in these cells to date. The focus of this study is therefore to characterise cannabinoid receptors modulating cytokine/chemokine release from human colonic epithelial cells HT-29.

5.2 Aims of study

The aims of experiments described in this chapter are:

1. To describe the pharmacological actions of a range of cannabinoid receptor ligands on TNF-α-induced interleukin-8 release from HT-29 cells *in vitro*.
2. To characterise the functional cannabinoid receptors in the human colonic epithelial cell line, HT-29.

5.3 Experimental Protocol

The culture and maintenance of HT-29 cells were carried out as described in chapter 2 (section 2.3.5). Enzyme linked immunosorbent assay (ELISA) for interleukin-8 (IL-8) was carried out as described in chapter 2 (section 2.4 and 2.4.9). Assessment of cell viability was also as described in chapter 2 (sections 2.8.1 and 2.8.2). Western immunoblotting for cannabinoid CB_2 receptors was carried out as described in chapter 2 (section 2.5)

5.3.1 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^{-1}) 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 g for 5 min. Cell free supernatants were stored at –70 °C until assayed for interleukin-8 release by ELISA. For time course studies, TNF-α (100 ng ml^{-1}) 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} M - 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^{-1}) 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 in chapter 2 of this thesis.

5.4 Data analysis

Concentration-response curves were analysed by Prism (GraphPad Inc., 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 ($EC_{½\ max}$ values) ± S.E.M (standard error of the mean) or 95% confidence limits as appropriates. 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.

5.5 Results

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

Figure 5.5.1 b shows the time course of interleukin-8 release from HT-29 cells after stimulation with TNF-α (100 ng ml^{-1}). 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^{-1}), 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 (4,578 ± 378 pg ml^{-1}, n = 6) after the 24 h incubation period. HT-29 cells constitutively released low levels of interleukin-8 (33.8 ± 3.8 pg ml^{-1}, n = 6) after 24 h incubation at 37 °C. Following stimulation with TNF-α (0.1- 100 ng ml^{-1}), there was a concentration-dependent increase in the release of interleukin-8 from HT-29 cells (Figure 5.5.1 a).

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

The effect of the non-selective cannabinoid receptor agonists CP55,940, Δ^{9}-Tetrahydrocannabinol, WIN55212-2 (10^{-10} M - 10^{-4} M) and a selective cannabinoid CB_2 receptor agonist, JWH 015, (10^{-10} M - 10^{-4} M) on TNF-α-induced secretion of interleukin-8 from HT-29 cells was examined. 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 x 10^{-7} M, 95 % confidence limits (C.L.) = 3.8 x 10^{-8} M - 3.6x10^{-7} M, n = 6), Δ^{9}-Tetrahydrocannabinol (5.3 x 10^{-8} M, 95 % C.L.= 9.7 x 10^{-9} M - 2.9 x 10^{-7} M, n = 6), WIN55212-2 (1.7 x 10^{-7} M, 95 %C.L.= 1.2 x 10^{-7} M - 2.5 x 10^{-7} M, n = 6) and JWH 015 (9.8 x 10^{-8} M, 95 % C.L. = 6.8 x 10^{-8} M - 1.3 x 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). (Figure 5.5.2).

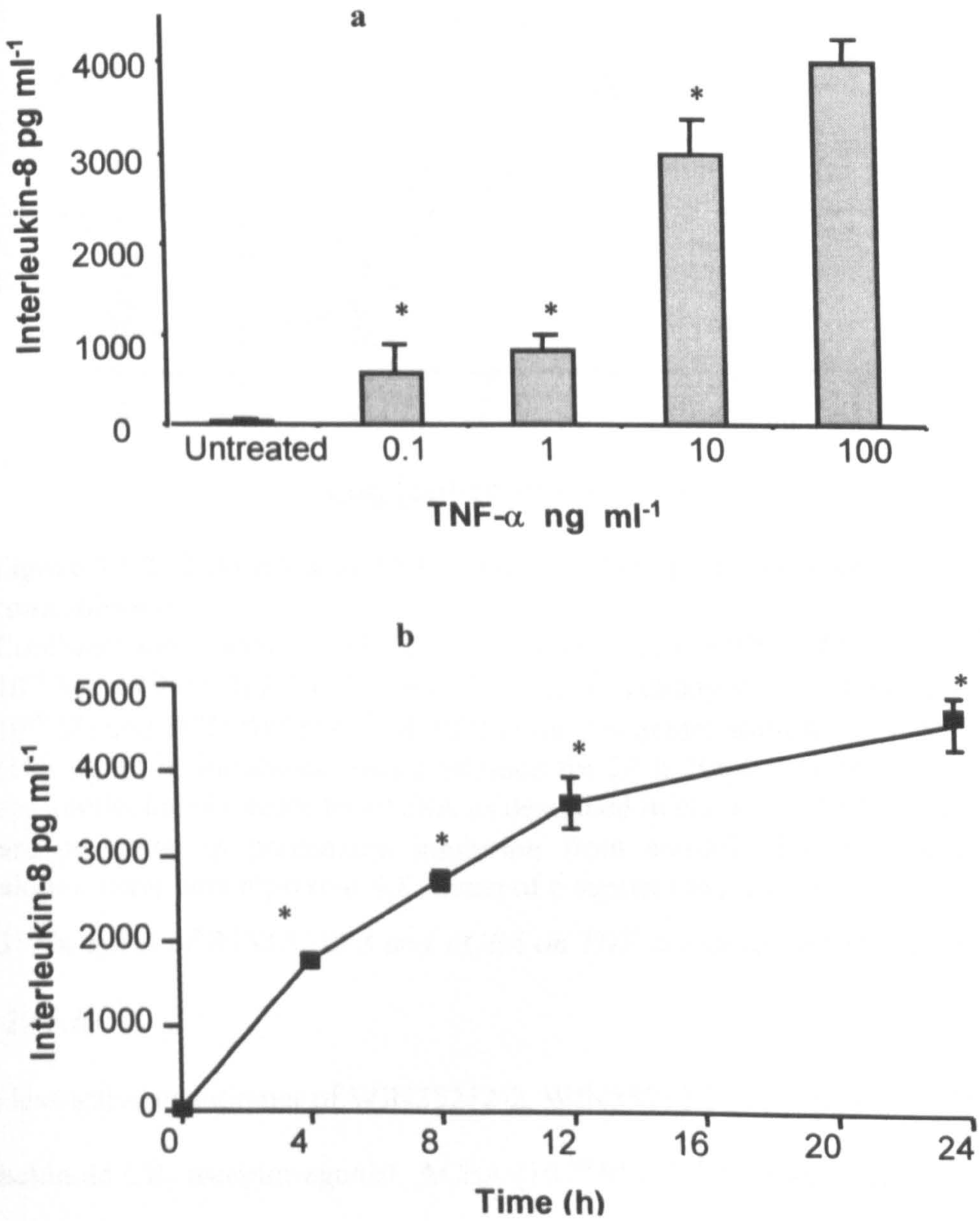


Figure 5.5.1 TNF-α-induced release of interleukin-8 from HT-29 cells *in vitro*
(a) Confluent monolayers of HT-29 cells were stimulated with TNF-α (0.1 - 100 ng ml^{-1}) in foetal calf serum free McCoy's 5A medium for 24 h. (b) Confluent monolayers of HT-29 cells were stimulated with TNF-α (100 ng ml^{-1}) 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 chapter 2 (section 2.4). Data are means and S.E. means of at least 6 experiments. * Significant difference from control P<0.05.

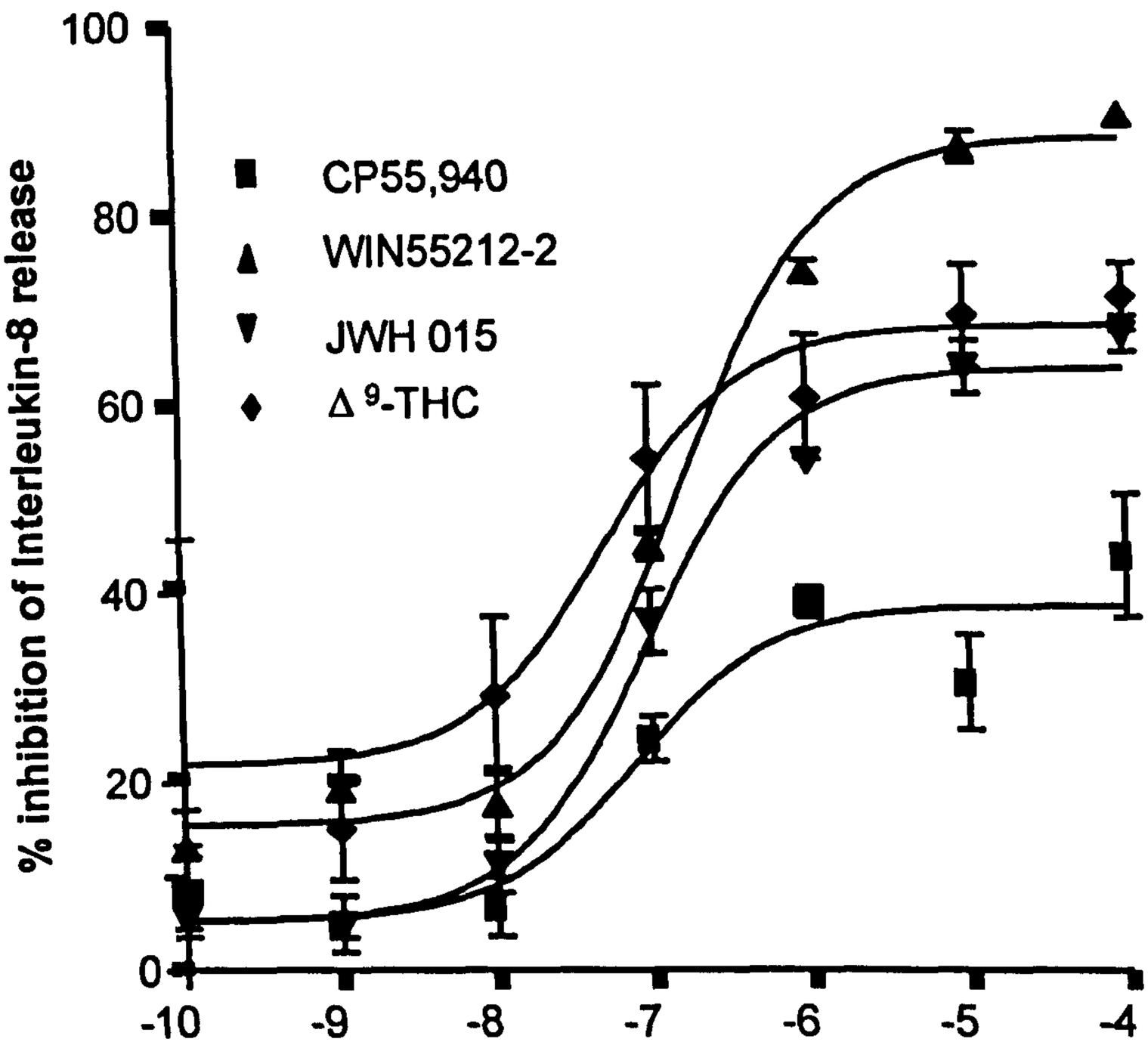


Figure 5.5.2. Inhibition of TNF-α-induced interleukin-8 release by cannabinoids.
Confluent monolayers of HT-29 cells were treated with CP55,940 (10^{-10} M- 10^{-4} M), WIN55,212-2 (10^{-10} M- 10^{-4} M), Δ^9-Tetrahydrocannabinol (10^{-10} M- 10^{-4} M) and JWH 015 (10^{-10} M-10^{-4}M) for 2 h before stimulation with TNF-α (100 ng ml^{-1}). Incubation was continued for 24 h. Supernatants were assayed for interleukin-8 release by ELISA as described in chapter 2 (section 2.4). Data are presented as percentage inhibition from control (TNF-α treated cells alone). Error bars represent S.E. mean of 6 separate experiments.

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

The less active enantiomer of WIN55212-2, WIN55212-3 (10^{-10} M - 10^{-4} M) and the cannabinoid CB_1 receptor agonist, ACEA (10^{-10} M - 10^{-4} M) had no significant ($P > 0.05$, n = 6), inhibitory effect on TNF-α (100 ng ml^{-1})-induced release of interleukin-8 from

HT-29 cells (refer to Figure 5.5.3). 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 x 10^{-5} M). Under these conditions, ACEA (10^{-10} M - 10^{-4} M) still did not significantly alter interleukin-8 secretion (data not shown).

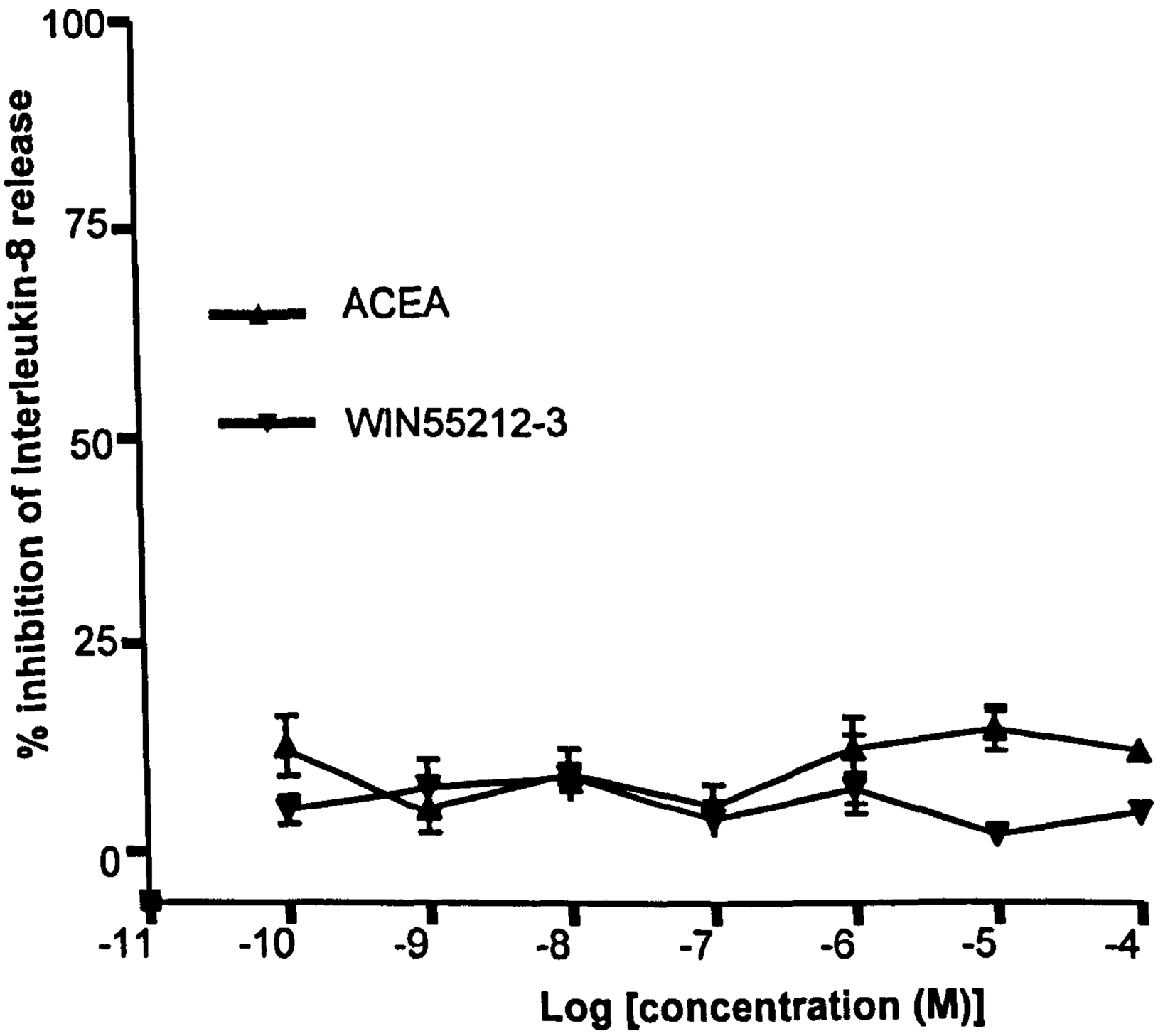


Figure 5.5.3 The effect of ACEA and WIN55212-3 on the release of interleukin-8 from HT-29 cells.

Confluent monolayers of HT-29 cells were treated with ACEA (10^{-10} M - 10^{-4} M) or WIN55212-3 (10^{-10} M - 10^{-4} M) for 2 h before stimulation with TNF-α (100 ng ml^{-1}). Incubation was continued for 24 h. Supernatants were assayed for interleukin-8 release by ELISA as described in chapter 2 (section 2.4). Data are presented as percentage inhibition from control (TNF-α treated cells alone). Error bars represent S.E. mean of 6 separate experiments.

5.5.4. 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 ($P < 0.05$, 2 way ANOVA followed by Bonferroni's post hoc test n=6) antagonised the inhibitory effects of CP55,940 ($pA_2 = 8.3 \pm 0.2$, n = 6), but did not antagonise the effects of WIN55212-2 ($pA_2 < 6$) or JWH 015 ($pA_2 < 6$) (Figure 5.5.4 a, 5.5.5a and 5.5.6 a). In contrast, the cannabinoid CB_2 receptor antagonist, SR144528 (10^{-6} M) significantly ($P < 0.05$, 2 way ANOVA followed by Bonferroni's post hoc test n = 6) 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 ($pA_2 = 7.6 \pm 0.4$, n = 6) respectively (Figure 5.5.4 b, 5.5.5 b and 5.5.6 b).

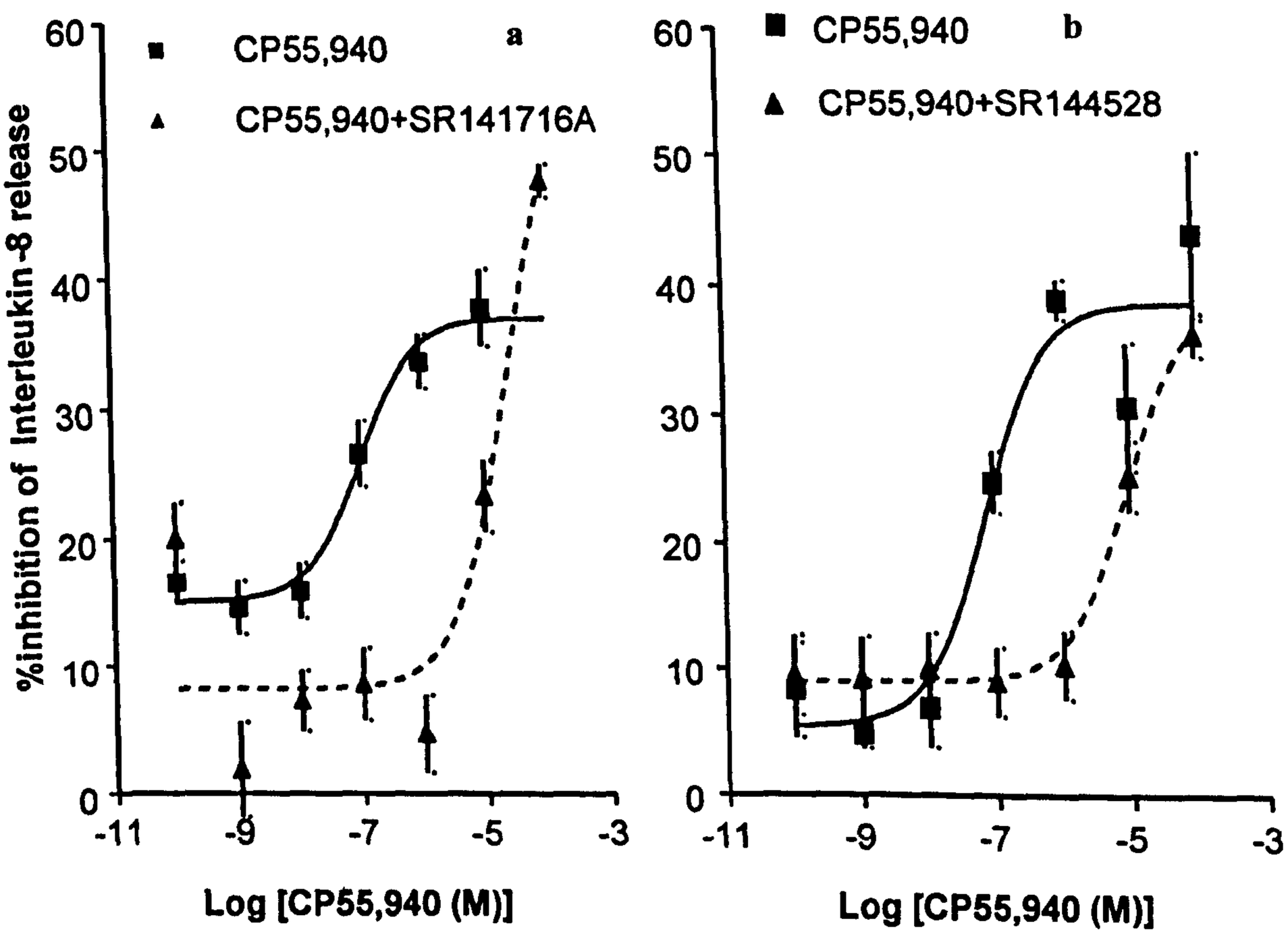


Figure 5.5.4 The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by CP55,940.
Confluent monolayers of HT-29 cells were incubated with SR141716A (10^{-6} M) (a) or SR144528 (10^{-6} M) (b) for 30 min before treatment with CP55,940 (10^{-10} M - 10^{-4} M) for 2 h. Cells were stimulated for further 24 h with TNF-α (100 ng ml^{-1}). Supernatants were assayed for interleukin-8 by ELISA as described in the chapter 2 (section 2.4). Bars represent S.E. mean of 6 separate experiments.

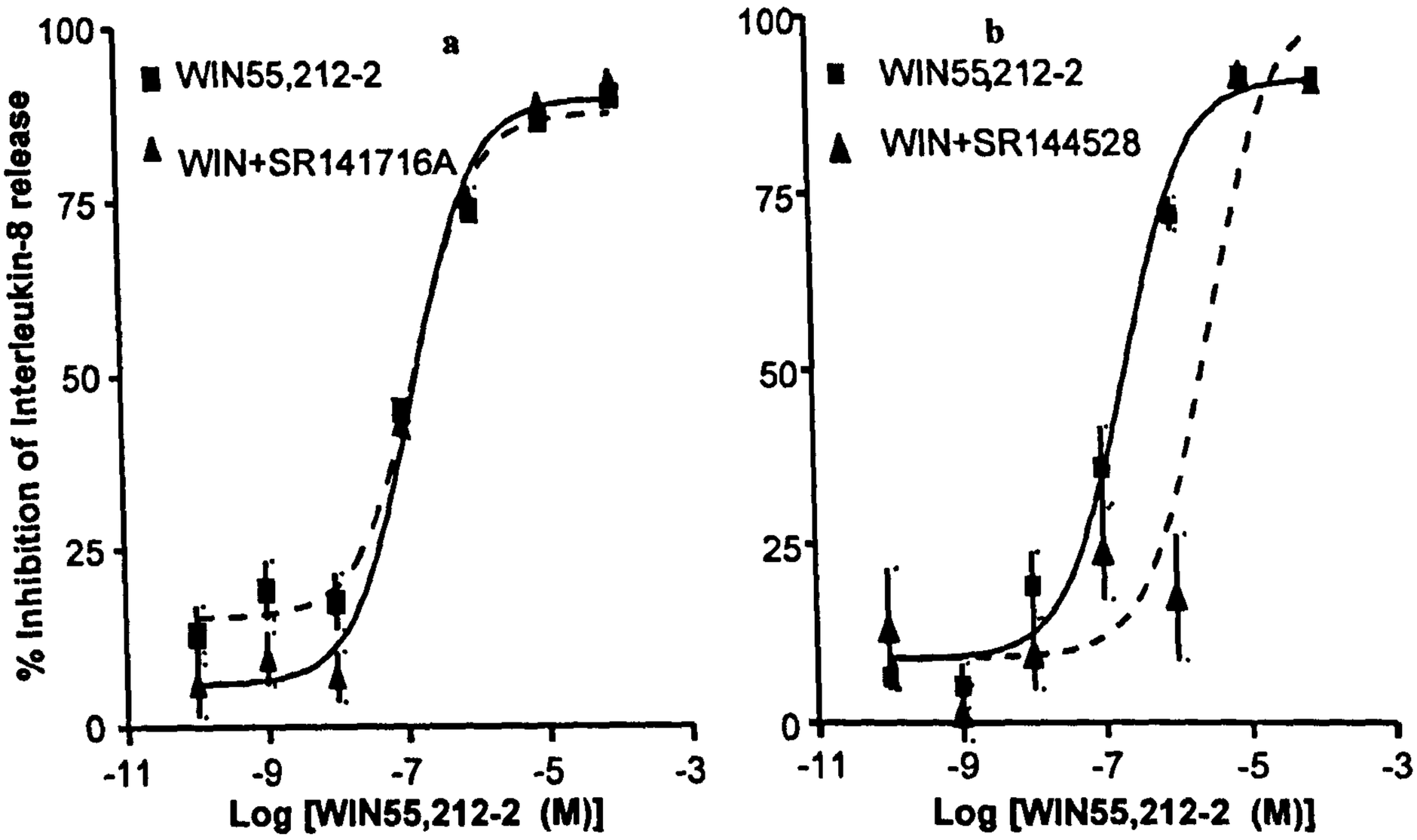


Figure 5.5.5 The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by WIN55212-2.

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} M - 10^{-4} M) for 2 h. Cells were stimulated for further 24 h with TNF-α (100 ng ml^{-1}). Supernatants were assayed for interleukin-8 release by ELISA as decribed in chapter 2, section 2.4. Vertical bars represent S.E mean of 6 separate experiments.

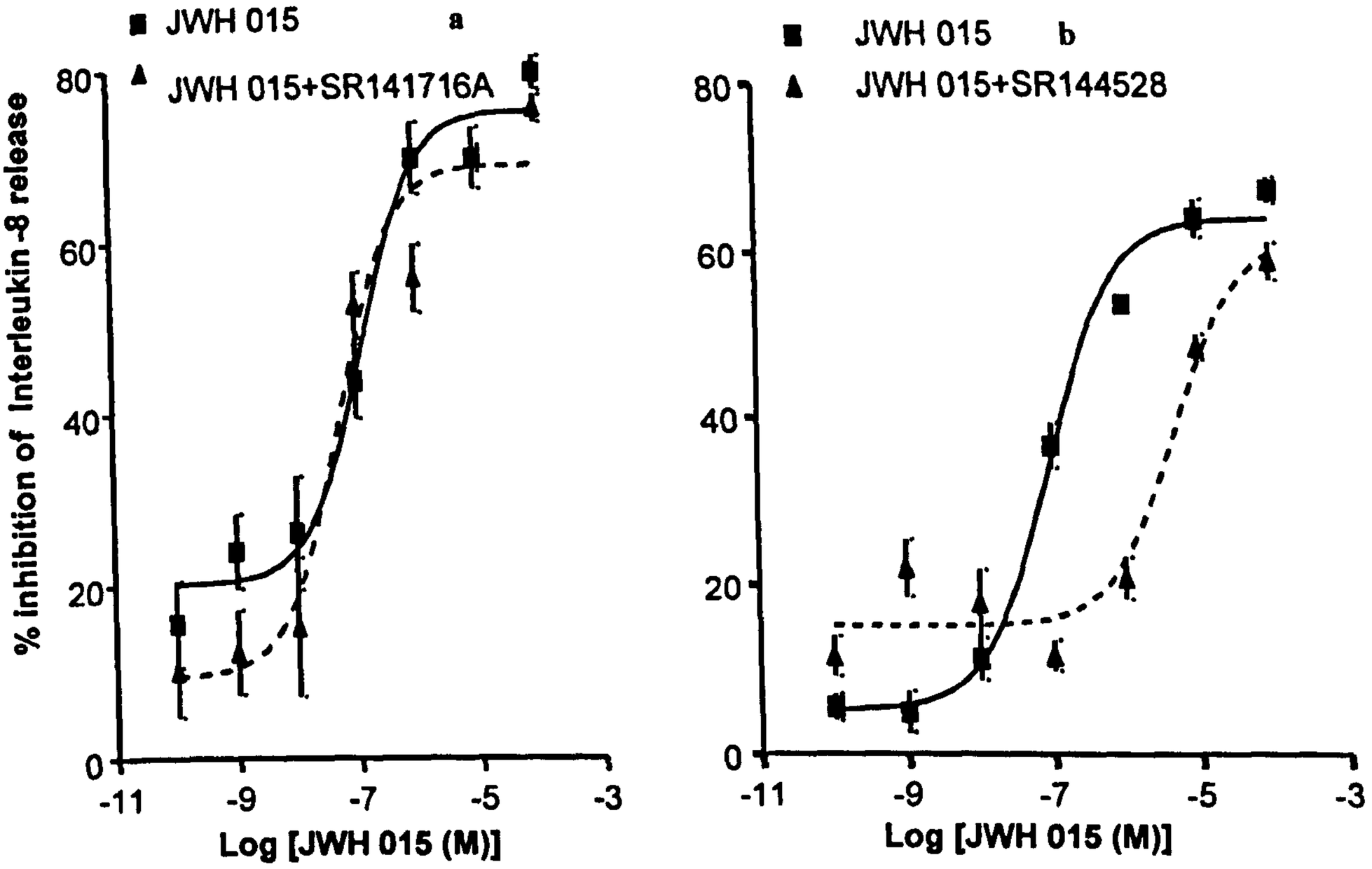


Figure 5.5.6 The effect of SR141716A (10-6 M) and SR144528 (10-6 M) on the inhibition of TNF-α-induced interleukin-8 release by JWH 015.

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} M - 10^{-4} M) for 2 h. Cells were stimulated for further 24 h with TNF-α (100 ng ml^{-1}). Supernatants were assayed for interleukin-8 release by ELISA as described in chapter 2, section 2.4. Bars represent S.E. mean of 6 separate experiments.

5.5.5. Immuno-localization 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 immuno-blots 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 immuno-reactivity with a molecular weight of 40 kDa, along with other minor bands in the HT-29 cells (lanes 1 - 3, Figure 5.6.7). In the lanes where this antibody was pre-incubated with fusion protein, these bands were completely absent

(lanes 4 - 6, Figure 5.5.7). Figure 5.5.7 is a representative blot of 6 separate experiments, all of which gave similar results.

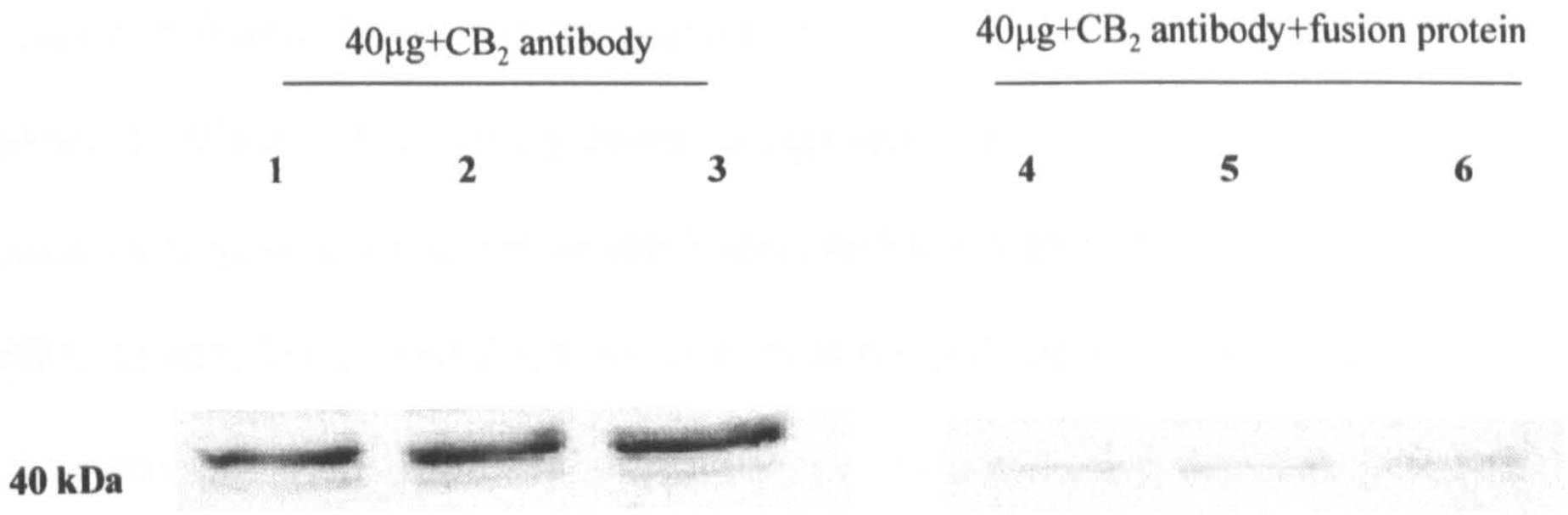


Figure 5.5.7. Western immunoblotting for cannabinoid CB_2 receptor protein in HT-29 cells.

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.

5.5.6 The effect of Drugs on cell viability.

The HT-29 cells were tested for viability by the MTT assay. Under the experimental conditions described in this thesis, 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 (Figure 5.5.2) a concentration where cell viability was > 95%.

5.6. Discussion.

In the experiments described above, the effects of cannabinoid receptor ligands on the secretion of interleukin-8 from the human colon epithelial cell line HT-29 were studied. Epithelial cells are increasingly being recognised to play a pivotal role in host defence against microganisms 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.*, 1998). 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 up regulated 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^{-1}) 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 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 2 to 7 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 *et al.*, 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 mRNA 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, interleukin-8 release after 24 h incubation with cannabinoid receptor agonists was not observed (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 *et al.*, 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 *et al.*, 1998). However, experiments in which second messenger concentrations are measured will be necessary to investigate the pathways mediating inhibition of cytokine release by cannabinoids. This fact is considered in chapter 6 of this thesis.

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 enantiometric 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; Rinaldi-Carmona *et al.*, 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. The reason for the unusual susceptibility of inhibition of CP55,940 to reversal by both classes of cannabinoid antagonists is not known 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, the 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, I 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 immuno-blotting. An intense band of immunoreactivity at the 40 kDa position was found, 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, the data described in this chapter 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 immuno-blotting revealed immuno-reactive protein at a region with a size consistent with that of cannabinoid CB_2 receptor protein. It was therefore concluded 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.

Chapter 6; The effect of cannabinoid receptor agonists on basal and agonist-evoked increases in intracellular cyclic adenosine monophosphate $[cAMP]_i$ and intracellular free calcium $[Ca^{2+}]_i$ in HT-29 cells

6.1 Introduction

Data presented in chapter 5 of this thesis demonstrates that cannabinoids inhibit TNF-α-induced IL-8 release via the activation of cannabinoid CB_2 receptors. However the intracellular mechanisms underlying this event are incompletely understood. The current knowledge of cannabinoid signal transduction pathways, suggests that activation of cannabinoid receptors (CB_1 and CB_2) inhibit adenylate cyclase via a pertussis toxin sensitive G-protein (Howlett, 1995) and inhibit an N,P/Q-type calcium channel (Mackie and Hille, 1992). Like cannabinoid CB_1 receptors, cannabinoid CB_2 receptors are members of the G protein coupled receptor (GPCR) family and activation leads to inhibition of adenylyl cyclase and activation of mitogen activated protein (MAP) kinases (Felder *et al.*, 1995). Until recently, inhibition of adenylate cyclase, with a resultant decrease in intracellular cyclic adenosine monophosphate (AMP), is thought to account for most, if not all, of the immunosuppressive effects of cannabinoids (Kaminski *et al.*, 1994).

However, several data suggest that not all effects elicited by cannabinoid receptor activation are cAMP-dependent. For example, in mouse splenocytes the endogenous ligand for cannabinoid receptors, 2-AG, reduced both NF-AT-binding to DNA and promoter activity in a concentration-dependent manner but did not influence cAMP response element (CRE) binding activity or that of AP-1 and its octamer to DNA (Ouyang *et al.*, 1998). In another study, repeated *in vivo* pre-treatment with CP55,940 caused 50% reduction in the number of [^{3}H]CP55940 binding sites in the cerebellum and behavioural tolerance to CP55940 without producing tolerance to the inhibitory effect of

this agonist on cAMP production in cerebellar membranes (Fan *et al.*, 1996). These studies suggest the existence of another signalling response elicited by cannabinoid receptor agonists. As another possible signalling pathway modulated by cannabinoids, Yebra *et al.* (1992), have demonstrated that Δ^9-THC, the plant cannabinoid could inhibit agonist-evoked increases in intracellular calcium $[Ca^{2+}]_i$, a point investigated in the experiments described in this chapter.

An increase in intracellular free calcium $[Ca^{2+}]_i$, is one of the earliest changes observed after ligand-receptor interaction, which may result from mobilisation of calcium from intracellular stores, capacitative calcium entry or depolarisation of the cell membrane (Gelfand, 1987). Increases in $[Ca^{2+}]_i$ play key roles in many cellular processes: In neurones, rises in $[Ca^{2+}]_i$ usually results in neurotransmitter release (Khachaturian, 1994), whereas in non-neuronal tissues (e.g. leucocytes), inflammatory mediators such as histamine, arachidonic acid metabolites or even cytokines are released (Beavan and Baumgartner, 1996). Thus in epithelial cells, TNF-α initiates interleukin-8 synthesis through the activation of the transcription factor nuclear factor kappa B (NF-κB) (Gerwitz *et al.*, 2000). In many studies, activation of NF-κB has been shown to be dependent on increases in intracellular calcium e.g. (Pahl *et al.*, 1996). (see figure 6.5.1 below).

In addition to the expression of cannabinoid CB_2 receptors in HT-29 cells as shown in chapter 5 of this thesis, these cells also express acetylcholine (ACh) muscarinic M_3 receptors (Poronik *et al.*, 1999). Activation of these receptors evokes the liberation of

inositol triphosphate (IP_3), which mobilises $[Ca^{2+}]_i$ from internal stores (Poronik *et al.*, 1999). Thus, this system offers an interesting model to investigate cannabinoid-induced changes in both cytosolic free calcium and agonist-evoked increases in intracellular cyclic AMP.

6.2 Aims of Study

The aims of the experiments described in this chapter are:

1. To investigate the effect of cannabinoid receptor agonists on basal and agonist-evoked increases in intracellular cyclic AMP.
2. To investigate the effect of cannabinoid receptor agonists on basal and agonist–evoked increases in intracellular Ca^{2+} in HT-29 cells.
3. To assess whether cannabinoid-evoked changes in $[cAMP]_i$ and $[Ca^{2+}]_i$ may be related to cannabinoid-evoked inhibition of TNF-α-induced-release of IL-8 from HT-29 cells.

6.3 Experimental Protocol

Maintenance of HT-29 cells was carried out as described in chapter 2 (section 2.3.5).

Assessment of cell viability was as described in chapter 2 (sections 2.8.1 and 2.8.2).

6.3.1 Treatment of cells and determination of $[cAMP]_i$

The measurement of $[cAMP]_i$ was performed as described in chapter 2 (section 2.10).

For intracellular cyclic AMP measurements, HT-29 cells were plated in a 96 well culture plate at a density of 1 x 10^6 cells ml^{-1} for 24 h at 37 °C (95% air/5% CO_2). In experiments where the effects of cannabinoids on basal $[cAMP]_i$ were studied, cells were treated with 100 µl of CP55,940 (10^{-10} M - 10^{-5} M) or WIN55212-2 (10^{-10} M - 10^{-5} M) for

30 min. Cell free supernatants were removed and replaced with 200 µl of lysis buffer (supplied by the Biotrak kit). In experiments where the effects of cannabinoids on forskolin-induced rises in intracellular $[cAMP]_i$ were studied, cells were pre-treated with CP55,940 (10^{-10} M - 10^{-5} M) or WIN55212-2 (10^{-10} M - 10^{-5} M) for 30 min followed by treatment with forskolin (5 x 10^{-6} M) for 15 min. Cell-free supernatants were removed and replaced with lysis buffer. In experiments where the effect of cannabinoids on $[cAMP]_i$ in TNF-α treated cells were studied, cells were first treated with CP55,940 (10^{-10} M - 10^{-5} M) or WIN55212-2 (10^{-10} M - 10^{-5} M) for 2 h before stimulation with TNF-α for 18 h. Cell-free supernatants were removed and replaced with 200 µl of lysis buffer before the determination of $[cAMP]_i$

6.3.2 Treatment of cells and determination of intracellular Ca^{2+}

The treatment of cells and measurement of intracellular calcium was as described in chapter 2 (section 2.9) of this thesis. In experiments where the effect of WIN55212-2 or ACh-induced rises in $[Ca^{2+}]_i$ were studied, cells were pre-incubated with WIN55212-2 for 2 h before loading with Fura-2/AM. Fura-2/AM loading procedure was as described in chapter 2 (section 2.9.2) of this thesis.

6.4 Data Analysis

In all experiments, the relative intracellular $[Ca^{2+}]_i$ is expressed as the ratio of Fura 2 fluorescence that is due to excitation at 340 nm relative to that due to excitation at 380 nm (F_{340}/F_{380}) and finally converted to absolute $[Ca^{2+}]_i$ in nM. This was done because of inherent variability in the measurement of intracellular $[Ca^{2+}]_i$ fluorometrically, even with the new generation of intracellular Ca^{2+} indicators e.g. Fura-2/AM (Grynkiewicz *et*

al., 1985). Unless otherwise stated, all experiments were performed with at least two different cell passages and at least 5 replicates were obtained. Results for $[Ca^{2+}]_i$ and $[cAMP]_i$ are presented as the mean ± S.E. mean of the number of observations indicated, where necessary, data were tested for significance using an unpaired Student's *t*-test, where a value of $P \leq 0.05$ was considered significant. Concentration-response curves were analysed by Prism (Graph Pad Inc. San Diego, USA). Other results are shown as bar graphs or representative traces from at least 5 replicate experiments.

6.5 Results

6.5.1 Effects of cannabinoids on intracellular cyclic AMP

6.5.1.1 Calibration curve for intracellular cAMP

A calibration curve was generated using a non-acetylated cAMP standard supplied with the Biotrak cAMP detection kit. Generation of the standard curve was carried out according to the manufacturer's guidelines as described in chapter 2 (section 2.10) of this thesis. A typical standard curve is as shown in figure 6.5.1.1 below.

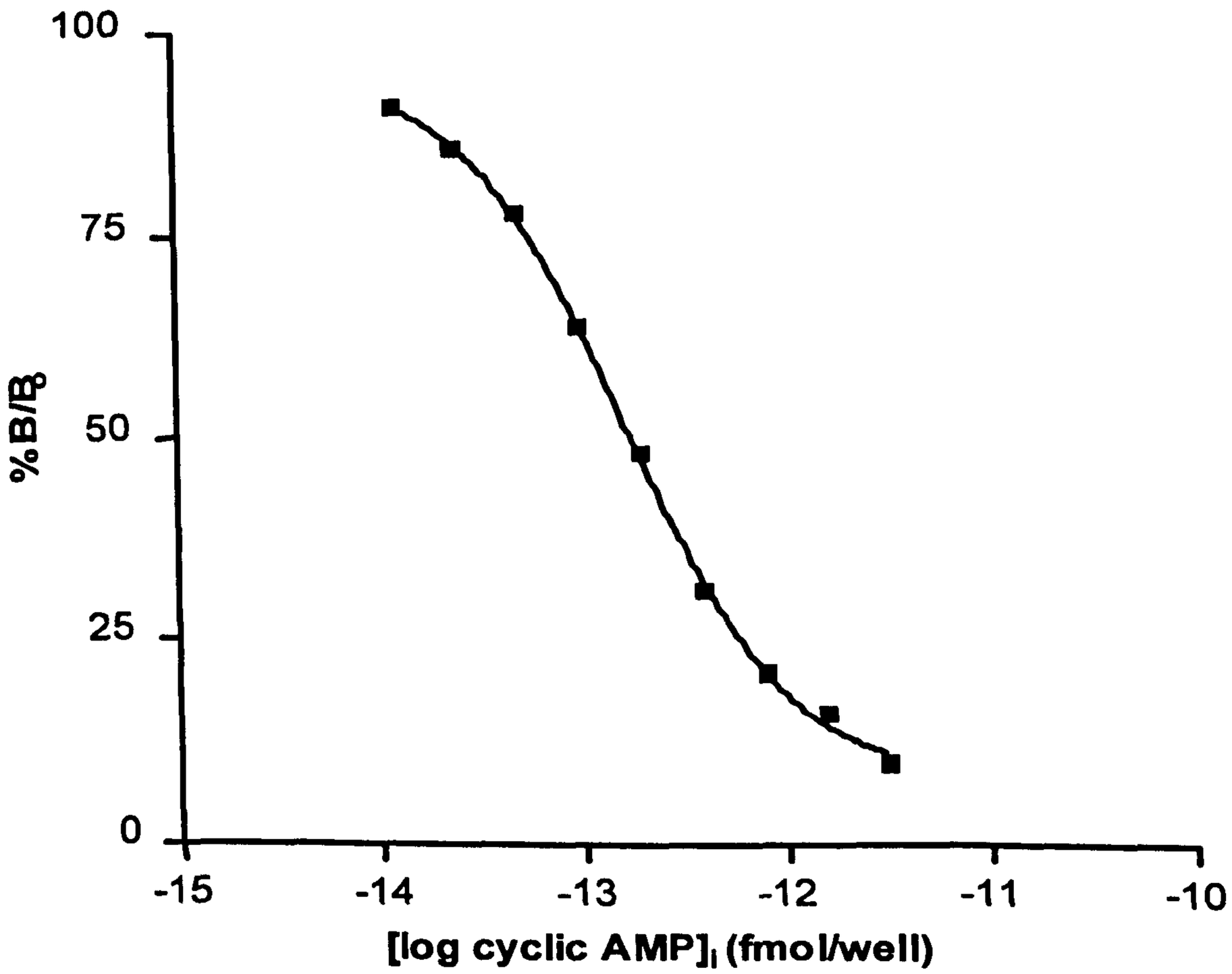


Figure 6.5.1.1. Typical standard curve for intracellular cyclic AMP measurement in HT-29 cells.

The standard curve was generated by plotting percent B/Bo as a function of log c AMP concentration, where $\%B/B_o$ = (Standard or sample OD-NSB x 100 / zero standard OD – (NSB OD). The $[cAMP]_i$ (fmol/well) value of sample was read directly from graph. Where OD = optical density, NSB = non-specific blank, NSB OD = non-specific blank optical density.

6.5.1.2. The effect of cannabinoids on $[cAMP]_i$ in HT-29 cells.

The basal $[cAMP]_i$ as measured by enzyme-immunoassay in HT-29 cells was (360 ± 46 fmol.well^{-1}, n = 8). Incubation of HT-29 cells with CP55,940 (10^{-7} M - 10^{-5} M) or WIN55212-2 (10^{-7} M - 10^{-5} M) for 30 min caused a concentration-related reduction in $[cAMP]_i$, from 360 ± 46 fmol.well^{-1}, (n = 8) to 152.5 ± 32.0; 135.1 ± 12.9; 117.5 ± 9.6 fmol.well^{-1}, (n = 4) and 132.5 ± 12.6; 145.0 ± 20.8; 130 ± 11.5 fmol.well^{-1}, (n = 4) respectively.. (Figure 6.5.1.2 a and b).

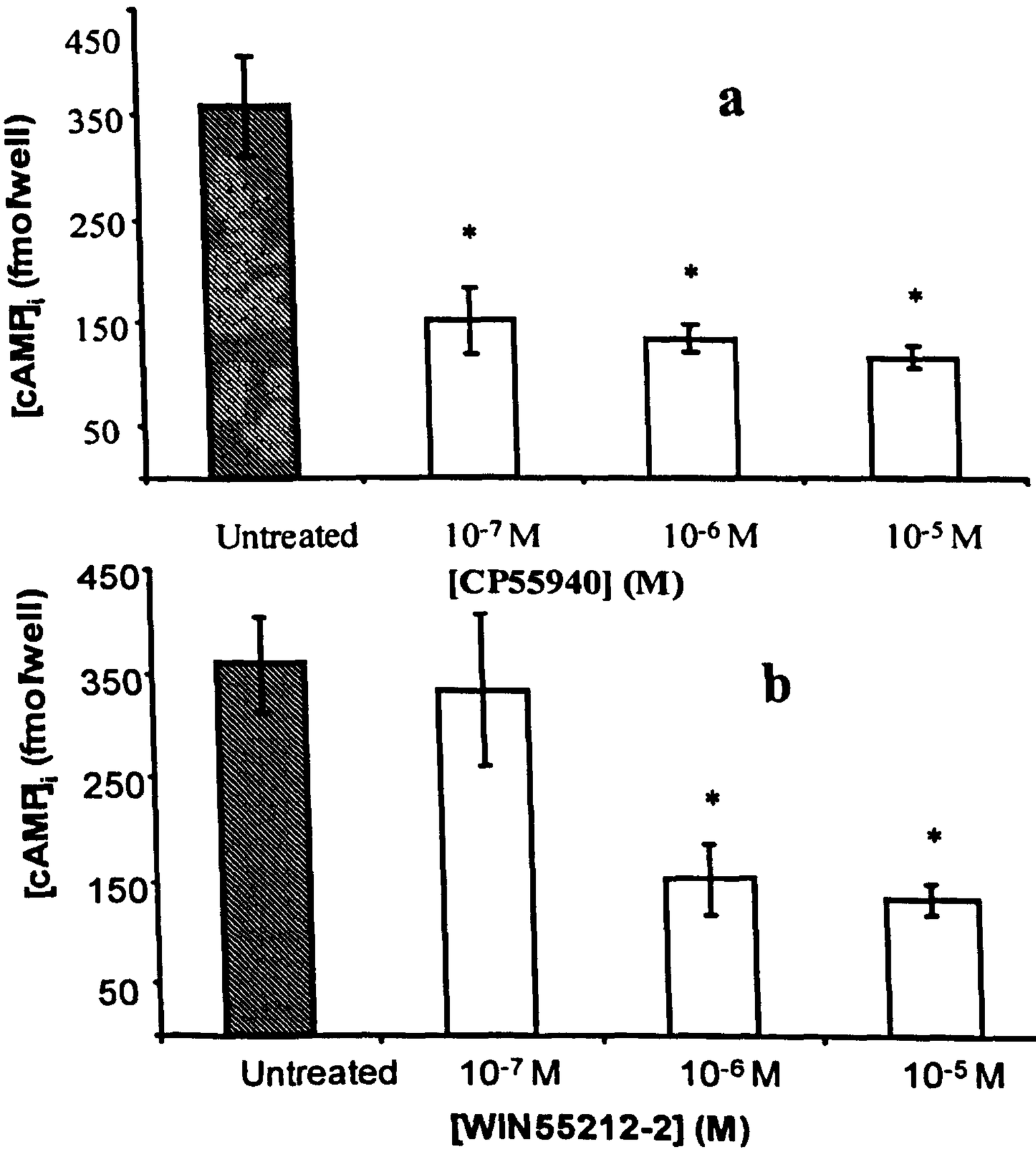


Figure 6.5.1.2. Effect of CP55940 and WIN55212-2 on resting $[cAMP]_i$ HT-29 cells

Cells were treated with CP55940 (10^{-10} M-10^{-5} M) (a) or WIN55212-2 (10^{-10} M-10^{-5} M) (b) for 30 min. Supernatants were removed and replaced with lysis buffer. Concentration of $[cAMP]_i$ was determined by ELISA. Each value represents mean ± SEM of 4 experiments. * denotes statistical significance ($P \leq 0.05$) by unpaired student's t-test as compared with control (untreated cells only).

6.5.1.3 The effect of WIN55212-2 on forskolin-stimulated increases in $[cAMP]_i$

Forskolin (5×10^{-6} M) increased the basal $[cAMP]_i$ from 360 ± 46 fmol.well^{-1}, (n=8) to 555 ± 51 fmol.well^{-1}, (n=4). Pre-treatment of the cells with WIN55212-2 (10^{-7} M-10^{-5} M) caused a concentration related decline in $[cAMP]_i$ at all concentrations tested 150.0 ± 14.1; 150.0 ± 8.2; 147.5 ± 5.0 fmol.well^{-1}, (n = 4) respectively (Figure 6.5.1.3).

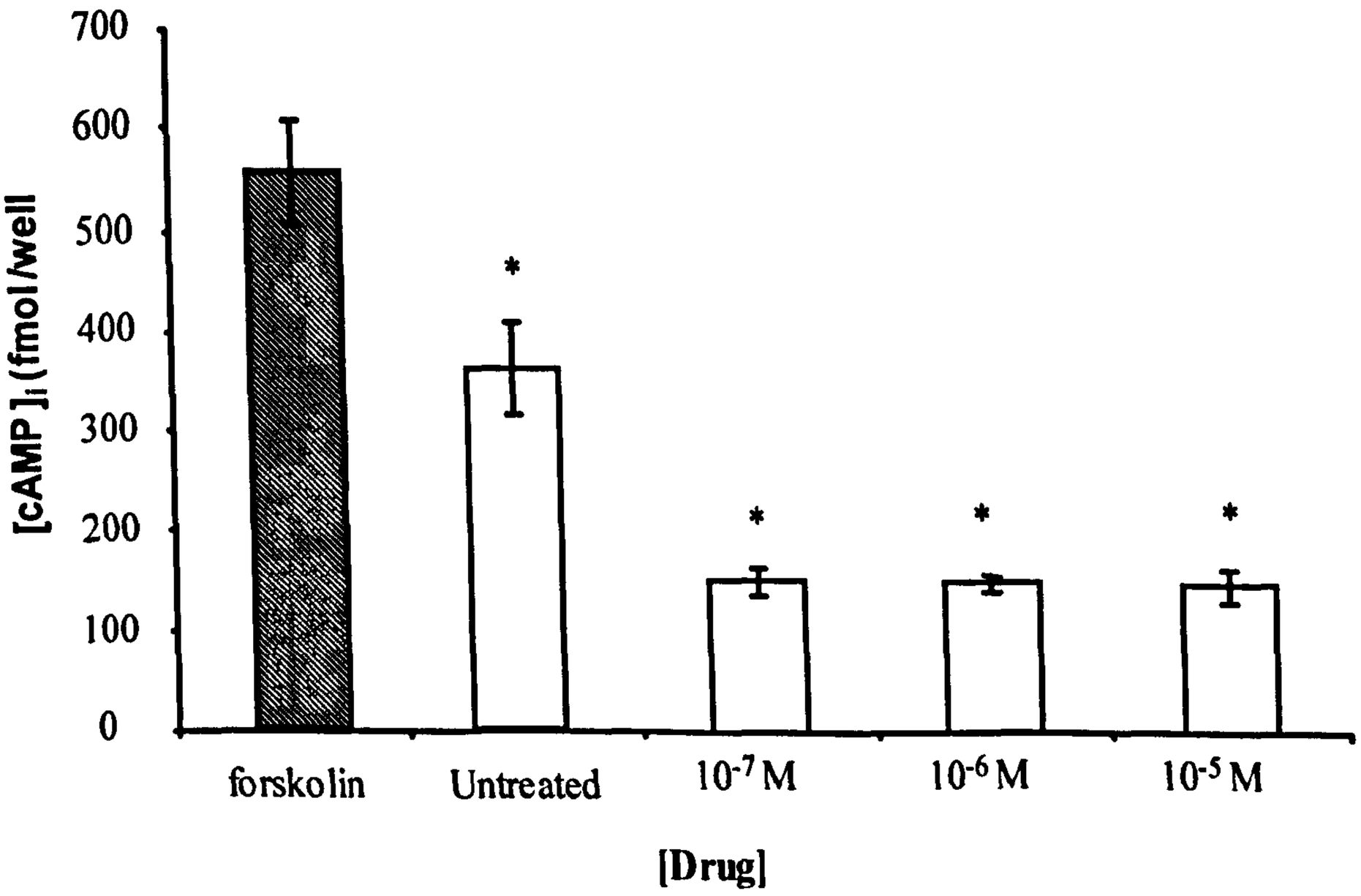


Figure 6.5.1.3. Effect of WIN55212-2 and forskolin on $[cAMP]_i$ in HT-29 cells

Cells were treated with or without WIN55212-2 (10^{-7} M-10^{-5} M) for 30 min before stimulation for a further 15 min. Supernatants were removed and replaced with lysis buffer. Concentration of intracellular cyclic AMP was determined by ELISA. Each value represents mean ± SEM of 4 experiments. * denotes statistical significance ($P \leq 0.05$) as determined by unpaired Student's *t*-test comparing data with forkolin treated cells only.

6.5.1.4 The effect of WIN55212-2 and TNF-α on [cAMP]$_i$ in HT-29 cells.

TNF-α significantly ($P \leq 0.05$) decreased basal [cAMP]$_i$ in TNF-α treated cells alone from 360.0 ± 46.2 fmol.well^{-1}, (n = 8) to 125.0 ± 10.0, fmol.well^{-1}, (n = 4). Similarly, WIN55212-2 (10^{-7} M-10^{-5} M) caused significant ($P \leq 0.05$) decrease in [cAMP]$_i$ in TNF-α treated cells to 132.5 ± 12.6; 145.0 ± 20.8 and 130.0 ± 11.5 fmol.well^{-1}, (n = 4) respectively (Figure 6.5.1.4).

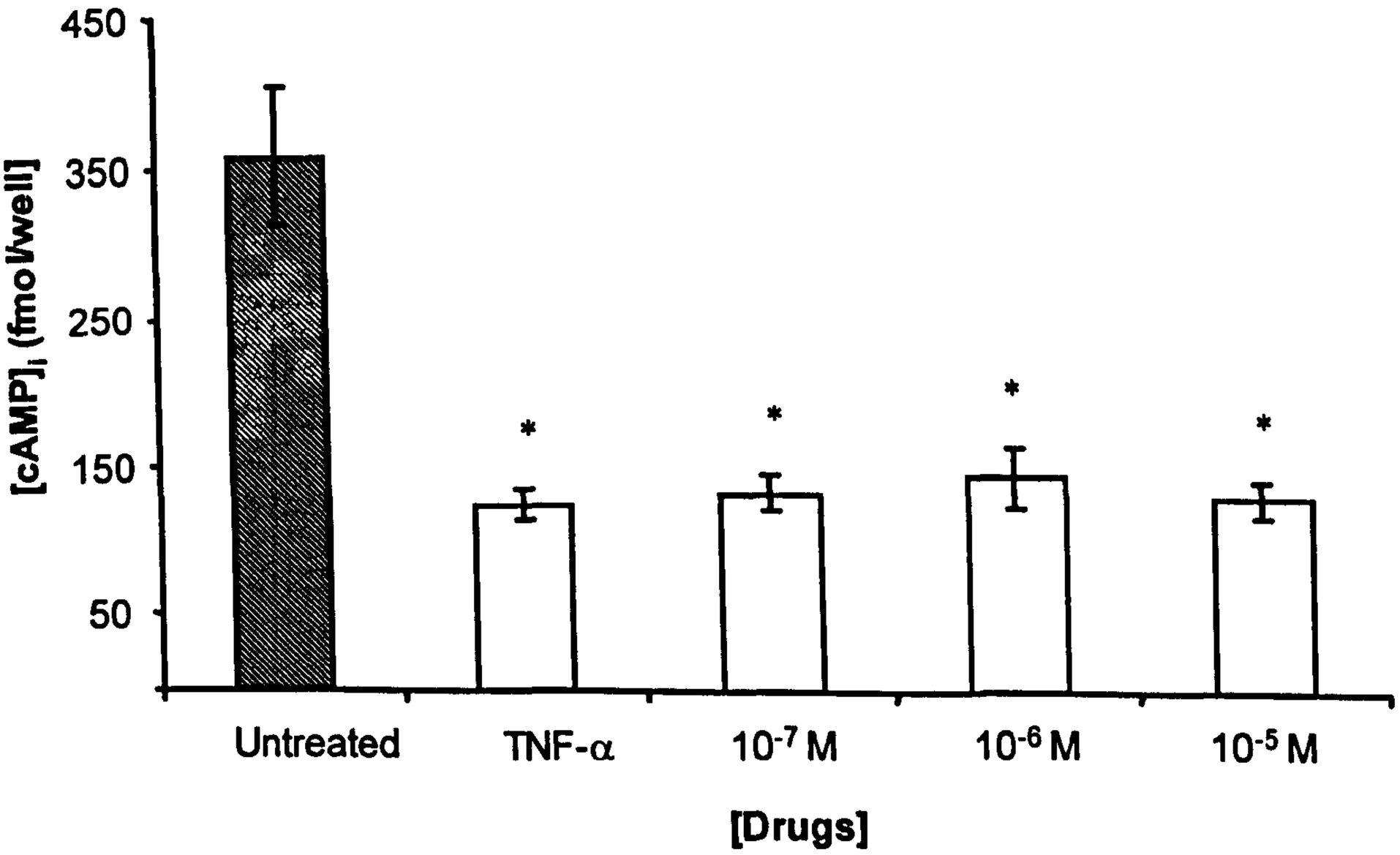


Figure 6.5.1.4 . Effect of WIN55212-2 and TNF-α on intracellular cyclic AMP in HT-29 cells.

Cells were treated with or without WIN55212-2 (10^{-7} M-10^{-5} M) for 2 h before stimulation with TNF-α (100 ng.ml^{-1}) for a further 18 h. Supernatants were removed and replaced with lysis buffer. Concentration of intracellular cyclic AMP was determined by ELISA. Each data represents mean ± SEM of 4 experiments. * denotes statistical significance ($P \leq 0.05$) as determined by unpaired student's *t*-test comparing data with the control (untreated cells).

6.5.2 Determination of intracellular calcium

6.5.2.1 Calibration of ionized free Ca^{2+} in HT-29 cells

Figure 6.5.2.1 is a representative graph showing the calibration of ionized Ca^{2+} in HT-29 cells.

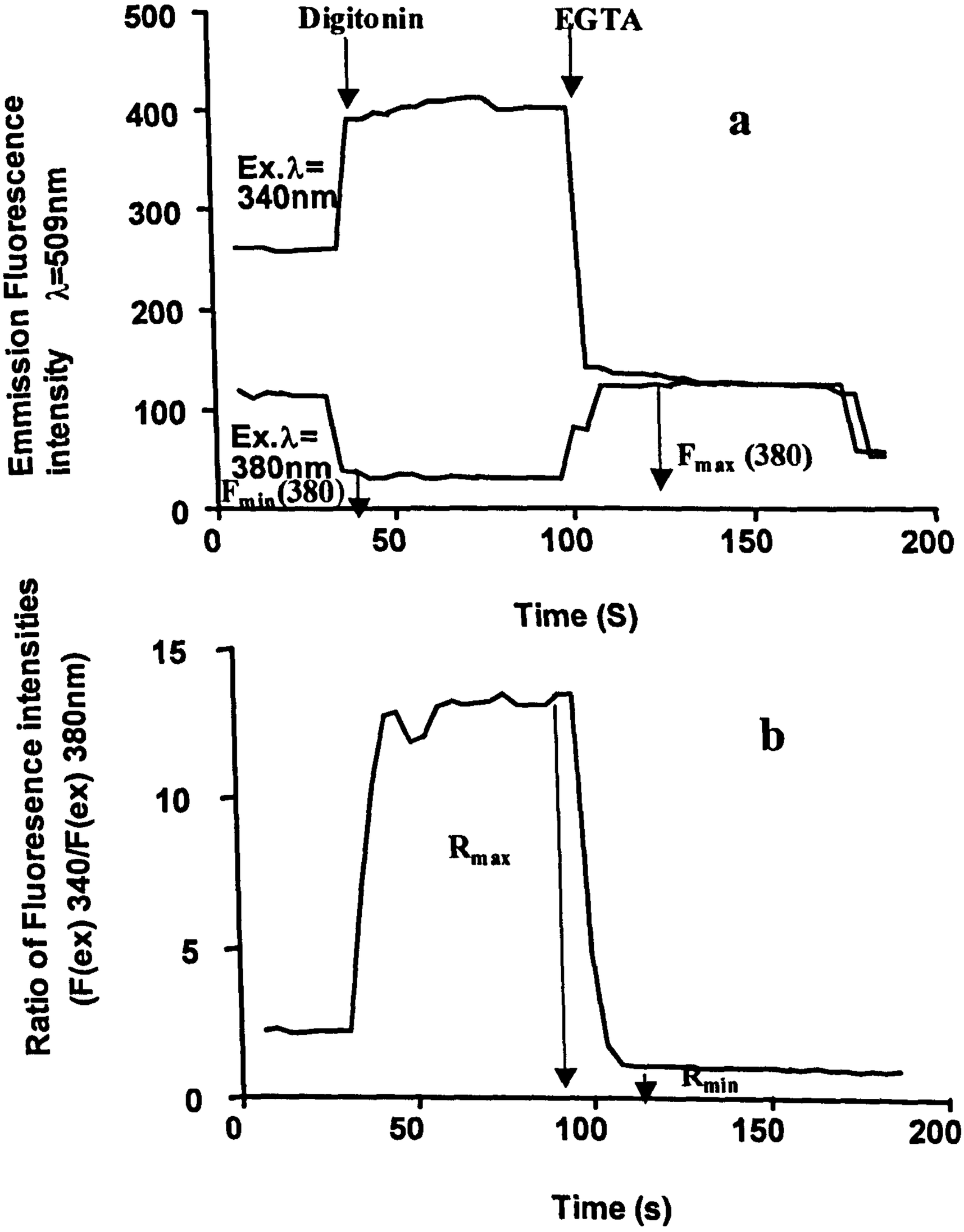


Fig. 6.5.2.1 Calibration of free Ca^{2+} measurement with Fura2 loaded HT29 cells. Fluoresence at saturating concentrations of Ca^{2+} was determined after cell lysis with digitonin and under conditions where the chelator was essentially Ca^{2+}-free by addition of EGTA/Tris pH 8.5. Definitions of parameters required to calculate $[Ca^{2+}]_i$ (nM) by the equation of Grynkiewitz *et al.* (1985) are indicated. Further details are given in Chapter 2 (section 2.9.3).

6.5.2.2 The effect of WIN55212-2 on basal $[Ca^{2+}]_i$ in HT-29 cells

In the presence of an extracellular calcium concentration of 1 mM, the basal $[Ca^{2+}]_i$ in Fura-2/AM loaded HT-29 cells was (141.4 ± 19.1 nM, n=9). WIN55212-2 (10^{-5} M and 10^{-6} M) resulted in a small, concentration-related decrease in $[Ca^{2+}]_i$ (11.6 ± 1.1 nM and 18.9 ± 3.9 nm respectively, n=5), 120 sec after addition of WIN55212-2. These values amounted to a significant ($P < 0.05$) reduction in basal $[Ca^{2+}]_i$ of 8.2 % and 13 % respectively, (Figure 6.5.2.2). Figure 6.5.2.2 a and b are representative traces showing the effects of WIN55212-2 (10^{-6} M and 10^{-5} M) on basal intracellular $[Ca^{2+}]_i$

To assess the effect of time on WIN55212-2-induced inhibition of basal $[Ca^{2+}]_i$, HT-29 cells were pre-treated for 2 h with WIN55212-2 (10^{-5} M) before loading cells with Fura-2/AM for 2 h. In these experiments, WIN55212-2 (10^{-5} M) caused an even greater decrease in $[Ca^{2+}]_i$ (56.1 ± 11.6 mM, n=5), than in experiments where WIN55212-2 was added following Fura-2/AM loading and $[Ca^{2+}]_i$ measured for 120 sec. This value amounts to 45% reduction of basal $[Ca^{2+}]_i$, Figure 6.5.2.2 a.

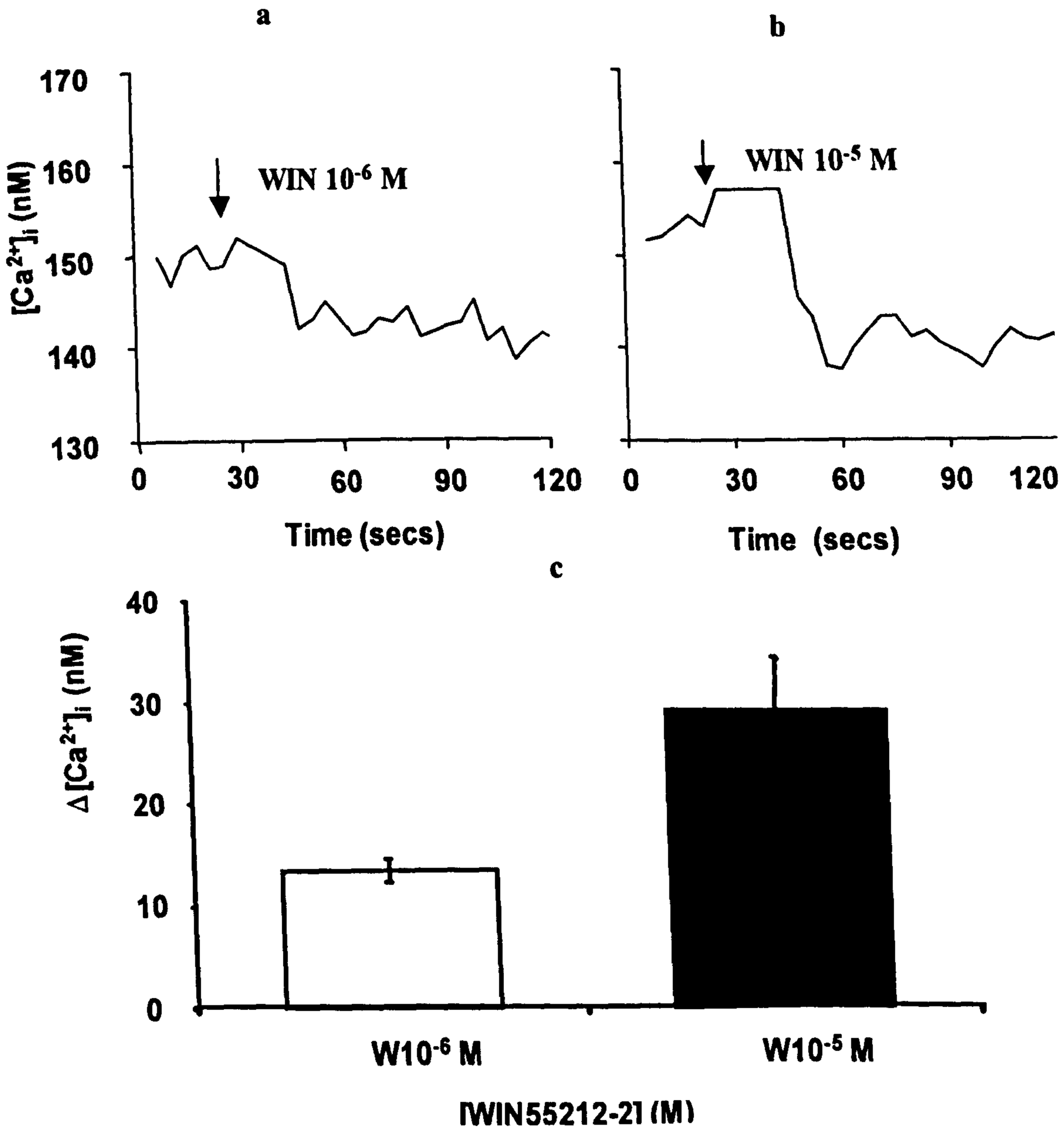


Figure 6.5.2.2. Effect of WIN55212-2 on basal $[Ca^{2+}]_i$ in HT-29 cells

HT-29 cells in HEPES/$CaCl_2$ (1mM) buffer were pre-loaded with Fura 2 and $[Ca^{2+}]_i$ monitored over 120 sec with discrete measurement taken every 4 sec interval upon addition of WIN55212-2 as described in Chapter 2 (section 2.9.3). Fig. (6.5.2.2a) WIN55212-2 (10^{-6} M) and (6.5.2.2b) WIN55212-2 (10^{-5} M) are representative traces. Fig.(6.5.2.2c) Data presented as bar graph and represents mean ± SEM of at least 6 independent experiments.

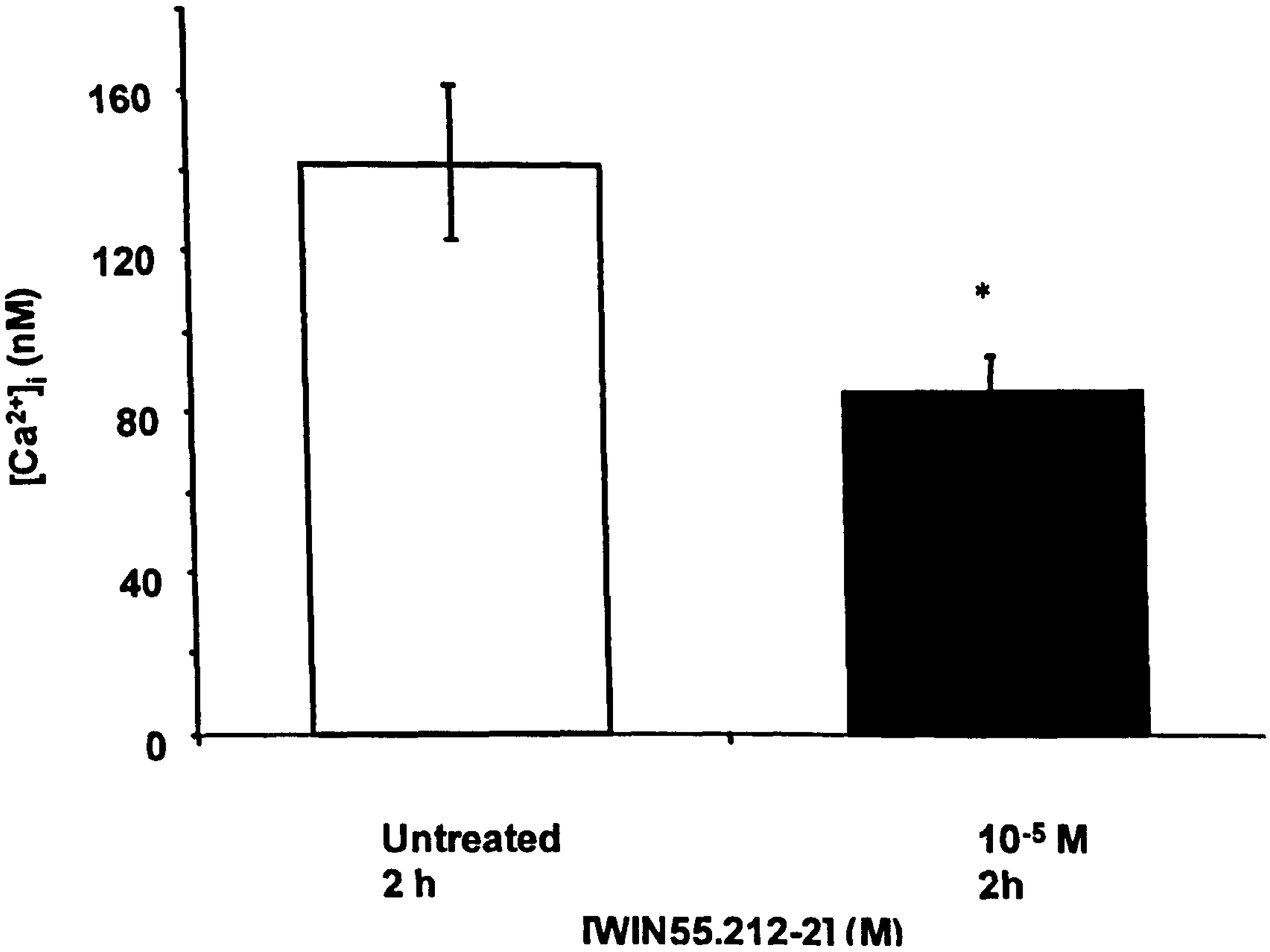


Figure 6.5.2.2 d. Effect of WIN55212-2 on basal $[Ca^{2+}]_i$ in HT-29 cells after 2 h incubation.

Suspension of HT-29 cells was incubated with WIN55212-2 for 2 h. Following a wash in HEPES/$CaCl_2$ (1 mM) buffer, HT-29 cells were pre-loaded with Fura-2/AM and $[Ca^{2+}]_i$ monitored over 120 sec with discrete measurements taken every 4 sec interval as described in Chapter 2 (section 2.9.3). Results are mean ± s.e mean of 5 separate experiments. * denotes significant difference from untreated cells as determined by Student's unpaired *t*-test.

6.5.2.3 Effect of CP55940 on basal $[Ca^{2+}]_i$ in HT-29 cells

Figure 6.5.2.3. a and 6.5.2.3 b are representative traces showing the effect of CP55940, on basal intracellular $[Ca^{2+}]_i$ in Fura-2/AM preloaded HT-29 cells.

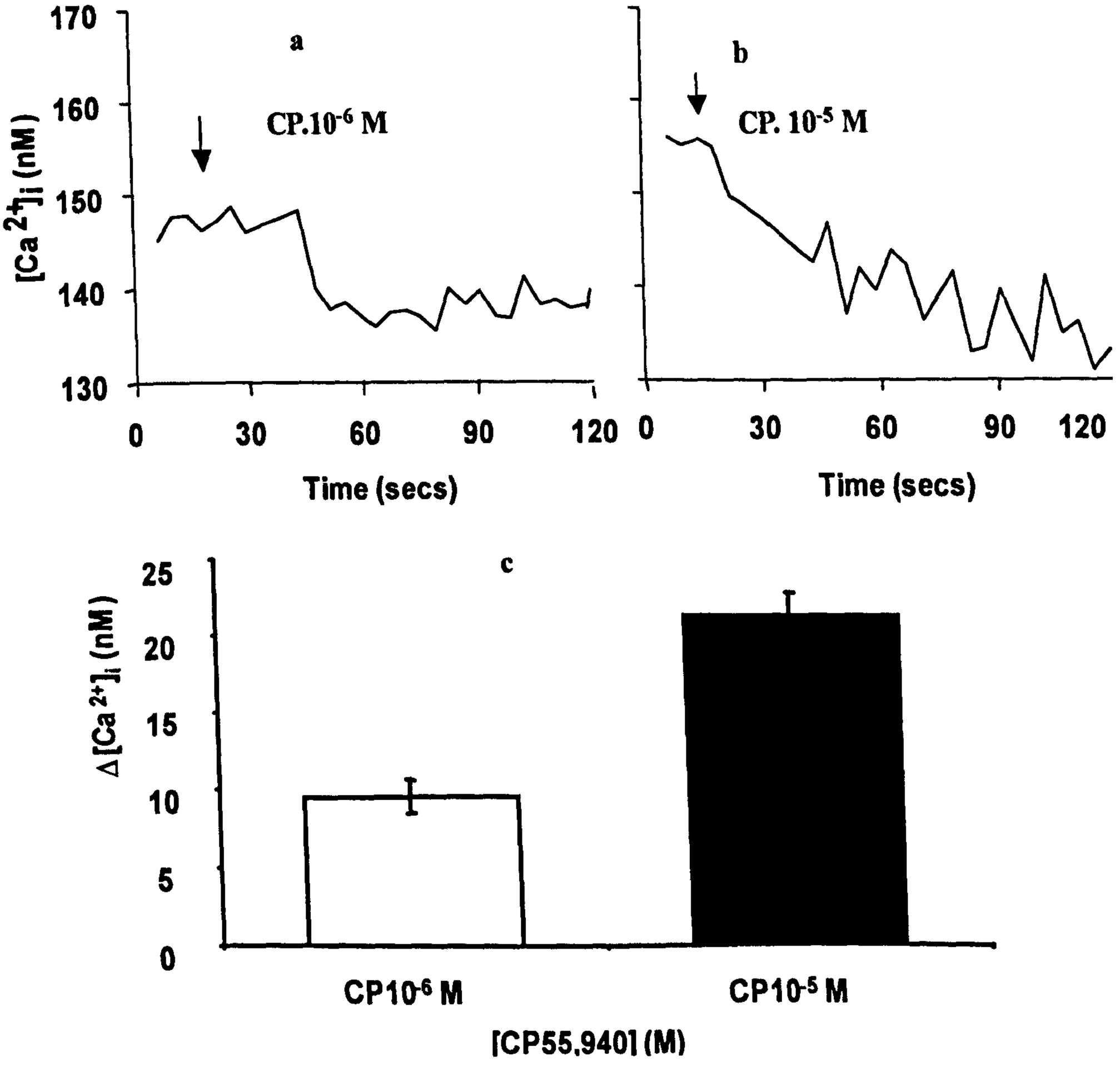


Figure 6.5.2.3 Effect of CP55,940 on basal $[Ca^{2+}]_i$ in HT-29 cells

HT-29 cells in HEPES/$CaCl_2$ (1 mM) buffer were loaded with Fura-2/AM and $[Ca^{2+}]_i$ monitored over 120 sec with discrete measurements taken every 4 sec interval following addition of CP55,940. Fig (6.5.2.3 a) CP55940 (10^{-6} M) and Fig.(6.5.2.3 b) CP55940 (10^{-5} M) are representative traces. Fig. 6.5.2.3 d data presented as bar graph and represent mean ± SEM of at least 5 independent experiments.

Addition of CP55940 (10^{-6} M and 10^{-5} M) to Fura-2/AM preloaded HT-29 cells caused a small, concentration-dependent inhibition in basal $[Ca^{2+}]_i$ (9.5 ± 2.1 nM and 21.3 ± 2.9 nM, n=5) respectively, when monitored for 120 sec. These values amounted to 6.7% and 15.1 % reductions in basal $[Ca^{2+}]_i$ respectively (figure 6.5.2.3 c).

6.5.2.4 The effect of ACh on $[Ca^{2+}]_i$ in HT-29 cells

In the presence of an extracellular calcium concentration of 1 mM, ACh (10^{-7} M- 10^{-4} M) induced a rapid, concentration-related increase in $[Ca^{2+}]_i$ when monitored over 120 sec (Figure 6.5.2.4a). The $EC_{1/2\,max}$ for ACh-induced increases in $[Ca^{2+}]_i$ was (1.6×10^{-5} M, 95 % confidence limits (C.L.) = 1.1×10^{-5} M- 2.5×10^{-5} M, n = 5). At a maximum effective concentration (10^{-4} M), ACh induced an increase in $[Ca^{2+}]_i$ of 221.0 ± 8.2 nM, n = 5).

6.5.2.5 The effect of WIN55212-2 on ACh-induced increases in $[Ca^{2+}]_i$

In the presence of an extracellular calcium concentration of 1 mM, pre-incubation of HT-29 cells with WIN55212-2 (10^{-5} M) for 10 min before the addition of ACh (10^{-7} M-10^{-4} M), resulted in a significant ($P<0.05$) shift of the ACh concentration-effect curve. The inhibitory effect of WIN55212-2 on ACh-induced increases in $[Ca^{2+}]_i$ appeared to result from a decrease in the maximum response (and reduction of baseline), rather than a shift to the right of the concentration–effect curve for ACh (Fig 6.5.2.4b). Thus WIN55212-2 (10^{-5} M) reduced the response to a maximum effective concentration of ACh (10^{-4} M) from (221.0 ± 8.2 nM, n=5) to (77.4 ± 5.6 nM, n=5)

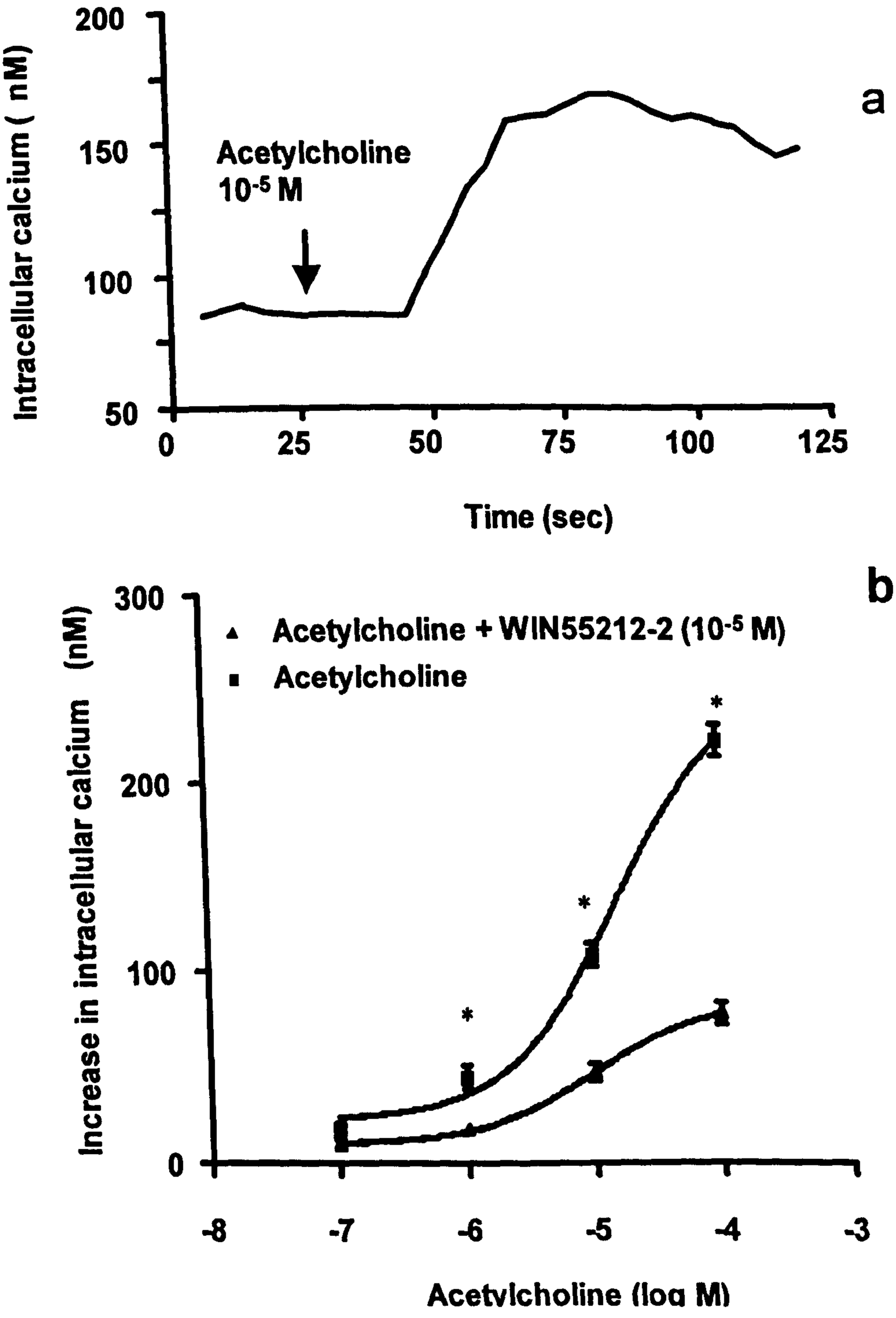


Figure 6.5.2.4. Concentration-dependent increases in $[Ca^{2+}]_i$ induced by ACh. HT-29 cells, suspended in HEPES/$CaCl_2$ (1 mM), were pre-loaded with Fura 2 and $[Ca^{2+}]_i$ monitored over 120 sec with discrete measurements taken every 4 sec interval following addition of ACh. (a) A representative trace for increases in $[Ca^{2+}]_i$ induced by ACh. Arrow indicates point of addition of ACh. (b) Effect of WIN55212-2 (10^{-5} M) on ACh-induced increases in $[Ca^{2+}]_i$. Results are mean ± SEM of 5 separate experiments. * Denotes significant difference from ACh treated cells ($P< 0.05$, 2 way ANOVA followed by Bonferroni's post hoc test).

6.5.2.6 The effect of TNF-α on $[Ca^{2+}]_i$ in Fura 2 preloaded HT-29 cell.

In the presence of an extracellular calcium concentration of 1 mM, TNF-α (10 – 1000 ng ml^{-1}) induced slow, significant ($P < 0.05$), concentration-related increases in $[Ca^{2+}]_i$ in HT-29 cells (figure 6.5.2.5a). The $EC_{1/2\ max}$ for TNF-α-induced increases in $[Ca^{2+}]_i$ was (522.8 ng ml^{-1}, 95 % C.L. = 297.1 – 920.1 ng ml^{-1}) and the maximum concentration of TNF-α (1000 ng ml^{-1}) studied, induced an increase in $[Ca^{2+}]_i$ of (259.6 ± 11.6 nM, n= 5) (Figure 6.5.2.6).

6.5.2.7 The effect of WIN55212-2 on TNF-α-induced increases in $[Ca^{2+}]_i$

In the presence of an extracellular calcium concentration of 1 mM, pre-incubation of HT-29 cells with WIN55212-2 (10^{-5} M) for 10 min before the addition of TNF-α (100 ng ml^{-1}), resulted in a significant ($P<0.05$) reduction in TNF-α-induced increases in $[Ca^{2+}]_i$. WIN55212-2 (10^{-5} M) reduced the increase in $[Ca^{2+}]_i$ induced by TNF-α from 80.9 ± 15.5 nM to 29.9 ± 3.8 nM (n = 5) (Figure 6.5.2.5 b).

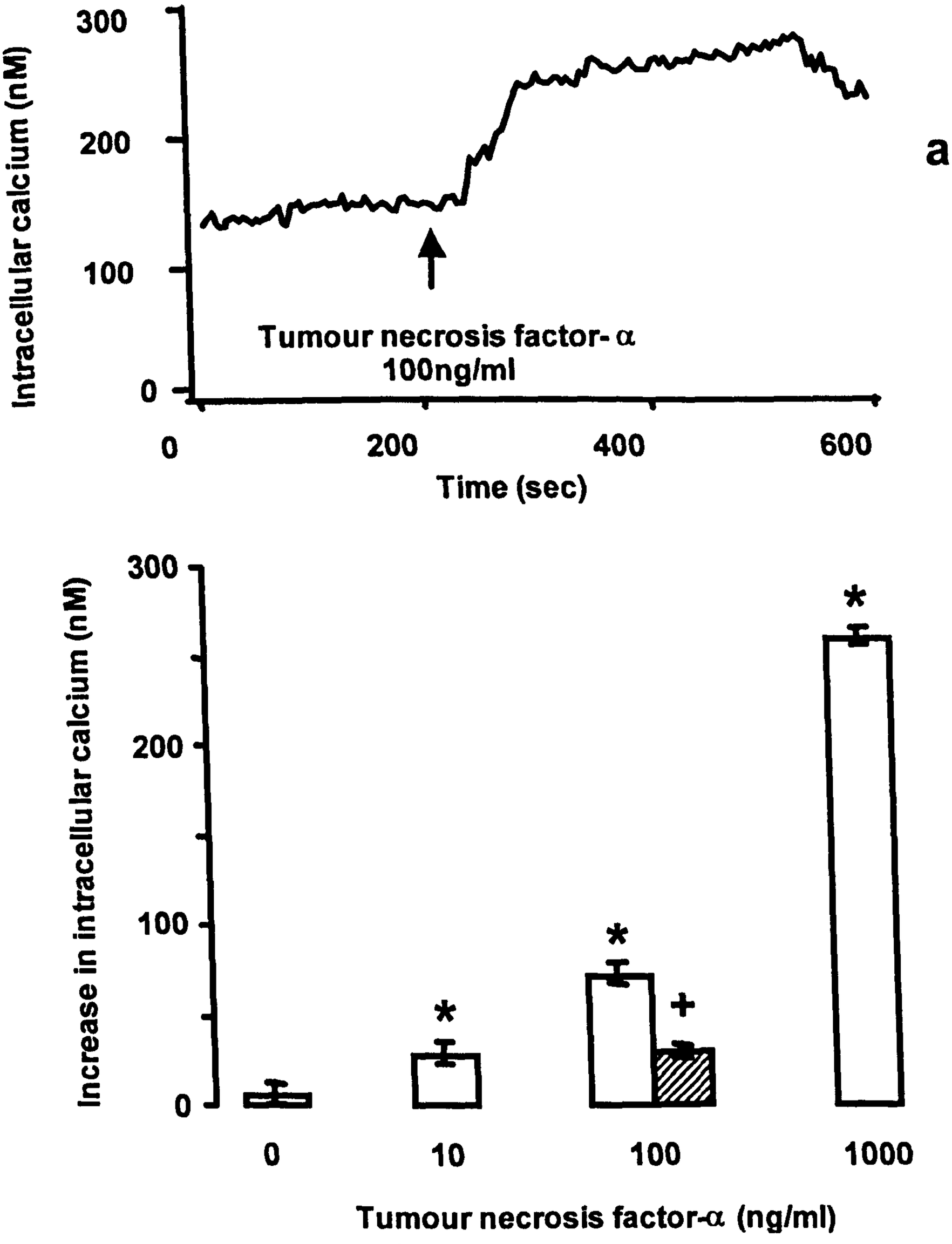


Figure.6.5.2.5. Increases in $[Ca^{2+}]_i$ in HT-29 cells induced by TNF-α
HT-29 cells in suspension were pre-loaded with Fura 2 and $[Ca^{2+}]_i$ was monitored. Figure 4 a. representative trace of increase in $[Ca^{2+}]_i$ induced by TNF-α 100 ng ml^{-1}. Arrow indicates point of addition of TNF-α. Fig. 4 b Increases in $[Ca^{2+}]_i$ induced by TNF-α (10 – 100 ng ml^{-1}) (open bars). Results are mean ± SEM of 5 separate experiments. * denotes significant difference from ACh treated cells ($P < 0.05$, 2 way ANOVA followed by Bonferroni's post hoc test). Hatched bar shows the increase in $[Ca^{2+}]_i$ induced by TNF-α (100 ng ml^{-1}) in cells pretreated with WIN55212-2 (10^{-5} M). Results are mean ± SEM of 5 separate experiments. + denotes a significant difference from TNF-α (100 ng ml^{-1}) treated cells.

6.6 Discussion

6.6.1 The effect of cannabinoids on basal and agonist evoked increases in $[cAMP]_i$

In the experiments described in the present chapter, CP55,940 and WIN55212-2 have been shown to inhibit basal and forskolin stimulated increases in $[cAMP]_i$ over the same concentration ranges that inhibited TNF-α-induced release of IL-8 by cannabinoids in HT-29 cells (Chapter 5). Furthermore, incubation of HT-29 cells with WIN55212-2 for 2 h followed by stimulation with TNF-α have also been shown to lead to inhibition of $[cAMP]_i$ suggesting that a decrease in $[cAMP]_i$ may be related to cannabinoid-evoked inhibition of TNF-α-induced release of IL-8 in this cell line as reported in chapter 5 of this thesis.

Adenylate cyclase is ubiquitously distributed in the mammalian tissue where it synthesizes cAMP from adenosine triphosphate (ATP). The function of this cyclic nucleotide is to act as an intracellular second messenger through activation of protein kinases. Elevation of intracellular cAMP by addition of cell permeable stable analogues eg dibutyryl-cAMP or drugs which increase intracellular cAMP e.g. rolipram have been generally associated with inhibition of immune cell function (Haraguchi *et al.*, 1995). However emerging evidence suggest that a decrease in intracellular cAMP as seen following activation of cannabinoid receptors may also lead to inhibition of immune cell function. These observations suggest that the role of the cAMP-signalling cascade in immune cell function may have been oversimplified (Kaminski *et al.*, 1998). Thus an increase in intracellular cAMP has been shown to bind to and activate protein kinase A (PKA), an enzyme that in turn phosphorylates transcription factors, which bind to cAMP

response elements (CREBs) in the DNA thereby resulting in the activation or suppression of cytokine gene expression (Sassone-Corsi, 1995).

A direct activation or inhibition of cAMP responsive element in the promoter region of the IL-8 gene is probably not involved because, to our knowledge, no such site has been identified. However, multiple classes of transcription factors have been implicated in the regulation of IL-8 gene expression. The promoter region of IL-8 contains potential binding sites for nuclear factors such as AP-1, AP-2, interferon regulatory factor-1, hepatocyte nuclear factor-1, glucocorticoid receptor, NF-κB and NF-IL6 (Mukaida *et al.*, 1990, Kunsch *et al.*, 1995). Since the binding activity of transcription factors such as NF-κB, c Fos and Jun B are influenced by cAMP via activation of CREB, they might well be targets of cannabinoid receptor activation. In addition, the possibility that the effect of cannabinoid receptor activation on cAMP may be secondary to a regulatory effect on the expression of other intermediate proteins, e.g. cytokines such as IL-10, which have been shown to inhibit IL-8 release in immune cells cannot be excluded (Siegmund *et al.*, 1997).

Opinions are divided on the role of $[cAMP]_i$ as the main signal producing cannabinoid-induced actions in immune cells (See Berdyshev, 2000). Other intracellular signalling events may also be involved. For example, Δ^9-THC has been shown to suppress concanavalin A-induced increases in cytosolic free calcium in murine thymocytes (Yebra *et al.* (1992). Given this fact, the alterations in $[Ca^{2+}]_i$ and the inhibition of IL-8 release from HT-29 cell line could result from a combined effect of cannabinoid on $[cAMP]_i$ and

$[Ca^{2+}]_i$ as shown below including a multitude of the resulting changes in the signalling cascade downstream.

In summary, CP55,940 and WIN55212-2 have been shown to inhibit basal and forskolin-induced increases in $[cAMP]_i$ in HT-29 cells. It was also shown that incubation of WIN55212-2 for 2 h followed by stimulation with TNF-α could lead to a decrease in $[cAMP]_i$. Taken together, these studies demonstrate that cannabinoid-evoked inhibition of adenylate cyclase/cAMP signalling pathway may be related to inhibition of TNF-α-induced release of IL-8 from this cell line via inhibition of transcription factor binding at IL-8 promoter regions necessary for IL-8 transcriptional regulation. The possible site of action may be AP-1, AP-2, interferon regulatory factor-1, hepatocyte nuclear factor-1, glucocorticoid receptor, NF-κB or NF-IL6 via CREB/fos or CREB/Jun heterodimers however, further studies are needed to confirm this hypothesis. Thus these data adds to a body of knowledge supporting the negative regulatory effect of the cannabinoids in the immune system.

6.6.2 The effect of cannabinoids on basal and agonist evoked increases in $[Ca^{2+}]_i$

The aim of the experiments described in this chapter was to determine whether the cannabinoid agonists WIN55212-2 and CP55,940 inhibited tumour necrosis factor-α-induced increases in intracellular calcium in HT-29 cells.

In the present study, spectrofluorimetry and the intracellular calcium indicator molecule Fura2/AM were used to measure intracellular calcium in HT-29 cells. Tumour necrosis

factor-α induced a slow concentration-dependent increase in intracellular calcium, when monitored for 10 min, similar to that reported by others in microglia (McLarnon *et al.*, 2001). This slow increase in intracellular calcium, induced by tumour necrosis factor-α, appears to result from the release of calcium from intracellular stores since, in studies in microglia, this response was not inhibited when experiments were conducted in calcium free media nor was the fluorescence quenched by the presence of manganese in the extracellular medium (McLarnon *et al.*, 2001). In contrast, Gewirtz *et al.* (2000) found that tumour necrosis factor-α induced activation of NF-κB and synthesis of interleukin-8 in the epithelial cell line T84 was not calcium dependent. However, the experiments described by McLarnon *et al.* (2001), those previously published by us (Chapter 5; Ihenetu *et al.*, 2003) and those described above were all conducted with a concentration of tumour necrosis factor-α of 100 $ng.ml^{-1}$ whereas those described by Gewirtz *et al.* (2000) used a lower concentration (10 $ng.ml^{-1}$). Interestingly in the present study, 10 $ng.ml^{-1}$ of tumour necrosis factor-α did not cause a marked increase in intracellular calcium whereas in parallel experiments where interleukin-8 release was measured this concentration of tumour necrosis factor-α caused a marked increase in interleukin-8 release (Chapter 5). Thus the concentration-effect curve for tumour necrosis factor-α-induced increases in intracellular calcium appears to be to the right of that for interleukin-8 release, suggesting that tumour necrosis factor-α-induced increases in intracellular calcium and interleukin-8 release may not be causally related in HT-29 cells. However, it was not the aim of the present study to investigate the mechanism of tumour necrosis factor–α-induced cell activation and further experiments are required to determine the calcium dependency of responses induced by tumour necrosis factor-α.

In contrast to tumour necrosis factor-α, in the present study, ACh induced a rapid, concentration–dependent increase in intracellular calcium. These findings are consistent with other published data that also show that this rapid increase in intracellular calcium results from the release of calcium from intracellular stores (Gerwitz *et al.*, 2000). Epithelial cells have been shown to contain muscarinic M3 receptors (Poronnik *et al.*, 1999) and published data show that increases in intracellular calcium induced by the muscarinic receptor agonist carbachol also activates NF-κB and induces interleukin-8 expression in epithelial cells (Gerwitz *et al.*, 2000). Thus, it appears that in epithelial cells, an increase in intracellular calcium results in an increase in NF-κB activation and interleukin-8 synthesis.

In the present study, incubation of WIN55212-2 and CP55,940 for 2 min caused a concentration-related reduction of basal intracellular calcium in HT-29 cells. Incubation of HT-29 cells with WIN55212-2 for 2 h resulted in an even greater reduction in basal intracellular calcium suggesting that this decrease was a slow event. Furthermore, when HT-29 cells were incubated with WIN55212-2 prior to the addition of either tumour necrosis factor-α or acetylcholine, WIN55212-2 inhibited the increase in intracellular calcium induced by these agents. WIN55212-2 reduced basal intracellular calcium and shifted concentration-effect curves for acetylcholine-induced increases in intracellular calcium in a non-parallel fashion with a marked reduction in the response produced by the maximum concentration of acetylcholine tested. Similarly, WIN55,212-2 significantly antagonised increases in intracellular calcium in HT-29 cells induced by

tumour necrosis factor-α (100 ng.ml^{-1}), the lowest concentration of tumour necrosis factor-α that significantly increased intracellular calcium in the present study and that used previously to study the cannabinoid receptors modulating tumour necrosis factor-α-induced interleukin-8 release (Ihenetu *et al.*, 2003). Published evidence suggests that tumour necrosis factor-α releases calcium from intracellular stores (reviewed above) as acetylcholine (Gerwitz *et al.*, 2000). The finding that cannabinoid receptor agonists, such as WIN55212-2, reduce intracellular calcium and reduce increases in intracellular calcium induced by tumour necrosis factor-α and acetylcholine suggest that cannabinoid receptor agonists reduce the availability of calcium within the cell.

With respect to previous studies, our data with HT-29 cells agree in part with those of Yebra *et al.* (1992), who reported that Δ^9-tetrahydrocannabinol suppressed concanavalin A-induced increases in cytosolic free calcium in murine thymocytes but are at odds with those of Felder *et al.*, (1995) who demonstrated that activation of cannabinoid CB_2 receptors in CHO cells did not induce changes in intracellular calcium although the latter experiments were conducted in transfected cells where the appropriate intracellular signalling mechanism may not be present.

In summary, it has been shown that WIN55212-2 and CP55,940 reduce basal intracellular calcium in HT-29 cells. Furthermore, WIN 55212-2 inhibited increases in intracellular calcium induced by acetylcholine and tumour necrosis factor-α. These data suggest that the immunosuppressive effects of cannabinoids in HT-29 cells may be related to a reduction in resting and agonist evoked increases in intracellular calcium.

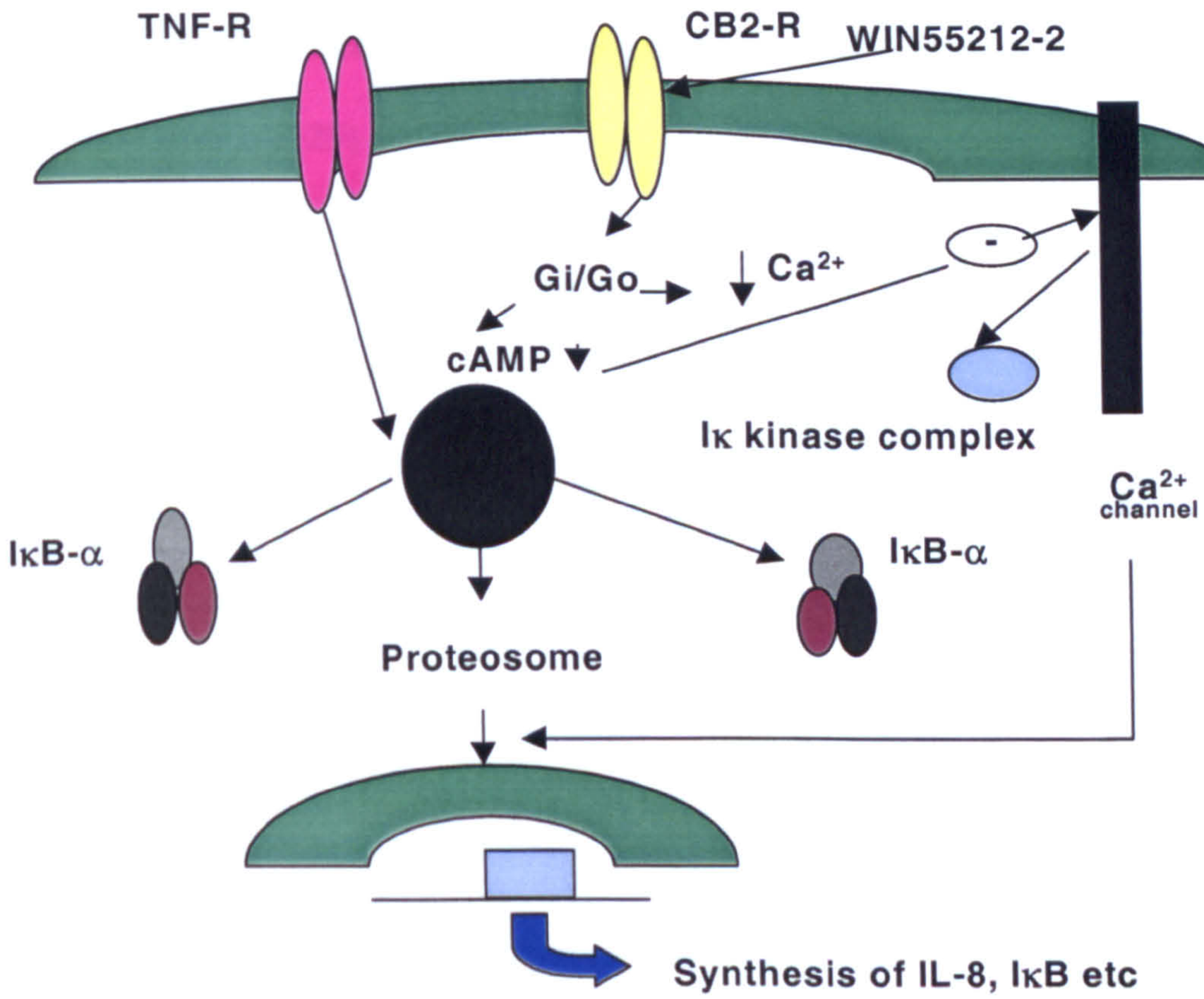


Figure 6.5.1. Possible mechanism of action of WIN55212-2 on HT-29 cells following stimulation with TNF-α

Following signal (TNF-α), IκB complex is activated by NF-κB inducible kinases. There is association of p50/p65-IκB α complex with IκB kinase-complex, IκB α is phosphorylated, followed by ubiquitination, degradation and release of p50/p65 to the nucleus. Once in the nucleus, p50/p65 induces transcription of many genes including IL-8. WIN55212-2 acting on cannabinoid CB_2 receptors via inhibition of adenylate cyclase and $[cAMP]_i$ also inhibits $[Ca^{2+}]_i$. These events eventually could lead to inhibition of IL-8 synthesis and release.

Chapter 7; The effect of cannabinoids on induction of apoptosis in immune cell lines (Jurkat and HT-29)

7.1 Introduction

In the previous chapters, data were presented showing that cannabinoids inhibit cytokine/chemokine release from a variety of immune cells and cell lines. However, these effects were often observed at high cannabinoid concentrations (>1 μM) i.e. greater than those required in cannabinoid binding studies (Rinaldi-Carmona *et al.*, 1998). Hence this work was undertaken to further characterise the immunosuppressive actions of cannabinoids and to investigate whether cannabinoids induce apoptosis in Jurkat cells and HT-29 cells at concentrations that inhibit cytokine release.

Cannabinoids have been shown to induce apoptosis in mononuclear cells (macrophages and lymphocytes) (Zhu *et al.*, 1998) and glial cells (Sanchez *et al.*, 1998) and to activate cell growth in haematopoietic cell lines (Derocq *et al.*, 1998). The endogenous cannabinoid, anandamide has also been shown to possess anti-proliferative actions in human breast carcinoma cells (De Petrocellis *et al.*, 1998) and to induce apoptosis in mononuclear cells (Schwartz *et al.*, 1994). Many other studies suggest that anandamide might have a pro-apoptotic activity both *in vitro* e.g. (Sarker *et al.*, 2000) and *in vivo* e.g. (Galve-Roperh *et al.*, 2000). However, the mechanism of cannabinoid–induced apoptosis is still unclear.

Cannabinoid CB_2 receptors are highly expressed in the immune system mainly in the cells of lymphoid origin, where they have been studied extensively (Berdyshev, 2000; Munro *et al.*, 1993). Whether such levels of expression are present in the cells of non-lymphoid origin such as epithelial cells is not yet clear. Activation of cannabinoid CB_2 receptors present in these cells can lead to the inhibition of

adenylate cyclase, mitogen activated protein (MAP) kinases and the induction of an immediate-early gene *krox 24* (Felder *et al.*, 1995). Although the mechanism of cannabinoid-induced apoptosis in immune cells is unclear, recent evidence suggests that it may involve both cannabinoid receptor-dependent (Mckallip *et al.*, 2002; De Petrocellis *et al.*, 1998) and cannabinoid receptor-independent pathways (Ruiz *et al.*, 1999; Galve-Roperh *et al.*, 2000). Published evidence, also suggests that cannabinoids may induce apoptosis via stress related signals e.g. nerve growth factor (NGF) or via the generation of ceramide (Kolesnick and Kronke, 1998; Galve-Roperh *et al.*, 1997). These signals have been demonstrated to be pro-apoptotic mediators (Kolesnick and Kronke, 1998; Galve-Roperh *et al.*, 1997). Furthermore, ligation of the vanilloid receptors by cannabinoid receptor agonists e.g. anandamide has also been shown to induce apoptosis (Maccarone *et al.*, 2000). The interactions of these pathways to the characterised cannabinoid receptors are not yet clear (refer to figure 1.10, chapter 1).

Apoptosis or programmed cell death is a normal physiological process that is essential for the maintenance of normal tissue homeostasis (for a review, see Cohen, 1992). Hence, the therapeutic induction of apoptosis has become a subject of an increasing interest. However, some recent studies suggest that there are unique differences in the control of apoptosis of various immune and inflammatory cells. For example, 50-70% of neutrophils in culture constitutively become apoptotic over 20 h (Meagher *et al.*, 1996; Ward *et al.*, 1999). In contrast, it can take up to 2 days for eosinophils to achieve an equivalent degree of apoptosis suggesting that distinct regulatory mechanisms control apoptosis in these cells. Thus, the advantage of a cannabinoid receptor-mediated apoptosis may depend on the fact that the exploitation of this action

of cannabinoids may lead to an anti-inflammatory drug treatment devoid of any psycho-activity particularly if this occurs via cannabinoid CB_2 receptors.

7.2 Aims

The aim of the experiments described in this chapter is:

- To investigate whether anandamide and other cannabinoid receptor agonists can induce apoptosis in Jurkat and HT-29 cells.

In this study, a nuclear fluorochrome, 4' 6-diamidino-2-phenyl indole (DAPI) was employed to identify apoptotic cells (Ruiz *et al.*, 1999). Furthermore, constitutive induction of apoptosis in neutrophils and Jurkat cells by aging and exposure to room temperature respectively (Meagher *et al.*, 1996; Shimura *et al.*, 1998) was used to validate this assay.

7.3 Experimental protocol

Isolation of human neutrophils from buffy coats and its maintenance in culture has been described in chapter 2, section 2.3.5. The culture and maintenance of Jurkat and HT-29 cells was also described in chapter 2, section 2.3.2 and 2.3.3 respectively.

7.3.1 Treatment of cells

Neutrophils (1×10^6 cells.ml^{-1}) or Jurkat cells (1×10^6 cells.ml^{-1}) were cultured in 75 cm^2 standard tissue culture flasks, Falcon (Beckton-Dickinson, Oxford, UK), supplemented with 10% foetal calf serum, 2 mM L-glutamate, 50 U.ml^{-1} penicillin, 50 μg.ml^{-1} streptomycin at 37 °C in a humidified 5% CO_2 atmosphere as described above. At 0, 24, 48 and 72 h, aliquots of neutrophils (250 μl) were harvested from culture and placed onto a polylysine coated glass slides by cytocentrifugation at 250 g for 5 min. Jurkat cells were treated with various concentrations of cannabinoids in a humidified atmosphere (95% air/5% CO_2) or incubated at room temperature for the

indicated period of time. Cytocentrifuge preparations of duplicate cell samples in each experiment were prepared on polylysine coated glass slides in a Heraeus Labofuge 400 (Heraeus Instrument Ltd, Brentwood, Essex, UK) at 250 g for 5 min. For assessment of apoptosis in neutrophils, slides were fixed in methanol for 5 min and stained in May and Grunwald-Giemsa or DAPI stains' whereas in the case of Jurkat cells only DAPI staining was performed.

HT-29 cells were cultured in Lab-Tek chamber slides. Following treatment with cannabinoids for different time points, slides were fixed with 3.7% formaldehyde for 10 min at room temperature before staining with DAPI for 15 min. Isolation of genomic DNA and apoptosis assays was described in chapter 2, section 2.6.

7.4 Data analysis

All data are expressed as means (± SE mean) of at least four independent experiments. Comparisons between groups were calculated using one-way ANOVA followed by Dunnett's post hoc test. Significance was assumed if $P \leq 0.05$.

7.5 Results

7.5.1 Neutrophil apoptosis

7.5.1.1 Viability of human neutrophils using MTT and trypan blue dye exclusion method

Table 7.1 **The Effect of aging on human neutrophil viability as assessed by trypan blue dye exclusion method and MTT assay respectively).**

Duration (h) following isolation of neutrophils	**Trypan blue dye exclusion assay.** Cell viability % of control	**MTT assay** Cell viability % of control
0	>98	>98
24	95.2±5.0	55.6±0.5 *
48	93.9±7.1	35.8±8.1 *
72	71.5±10.1 *	15.5±.12.1 *

Trypan blue dye exclusion technique and MTT assay were used to determine the viability of neutrophils as described in chapter 2, section 2.8.1 and 2.8.2 of this thesis. The data are mean ± SEM of 6 different experiments. * Denotes significant difference (* $P<0.05$) from the control (freshly isolated neutrophils).

In the first set of experiments, human neutrophils were assessed for viability after isolation by Histopaque-R gradient centrifugation and following incubation in complete RPMI medium for 24 h, 48 h or 72 h respectively. The viability of freshly isolated human neutrophils as assessed by the trypan blue dye exclusion technique and MTT assay exceeded 98 %. However, following incubation for 24 h, there was a small but non-significant ($P> 0.05$) reduction in viability (95.2 ± 5.0 %, n=6) as measured by trypan blue dye exclusion method when compared with the freshly isolated cells. In contrast, when the viability was assessed by MTT assay, there was a significant ($P<0.05$) reduction in cell viability (55.6 ± 0.5%, n = 6) after 24 h

incubation when compared to freshly isolated cells (> 98%). Incubation of human neutrophils for 48 and 72 h showed a small but time-dependent reduction in cell viability (93.9 ± 7.1 and 71. 5 ± 10.1 %, n = 6) as measured by trypan blue dye exclusion assay. In contrast, when viability was assessed by MTT assay, there was an increased reduction in the viability of neutrophils following incubation for 24, 48 and 72 h respectively when compared to freshly isolated cells (35.8 ± 8.1; 15.5%, n = 6) (data are summarised in table 7.1 above).

7.5.1.2 May and Grunwald-Giemsa staining of human neutrophils

Based on our results on cell viability, I investigated whether May and Grunwald-Giemsa staining of neutrophils could be used to measure apoptosis. Figure 7.4.2 a shows the morphological features of freshly isolated human neutrophils with multiple lobes of interconnecting nuclei staining bright red. In contrast, neutrophils cultured for 24 h and 72 h respectively (figure 7.4.2 b and c) show the characteristic apoptotic features of rounded deep blue staining nuclei with intact cytoplasmic membranes.

7.5.1.3 DAPI staining of human neutrophils

As control cells, human neutrophils were assessed for apoptosis by staining with the nuclear fluorescent dye DAPI. Figure 7.4.3 shows the features of apoptotic neutrophils with condensed chromatin bodies (apoptotic bodies) with brighter fluorescent intensity than the non-apoptotic cells. The quantitative analysis of apoptotic cells (x 1000 magnification) showed a time-dependent increase in the % of neutrophil apoptosis (0 h = 0 %; 24 h = 15.1 ± 4.6 %; 48 h = 50.2 ± 16.0 %; 72 h =71.0 ± 20.5%) (Figure 7.4.1).

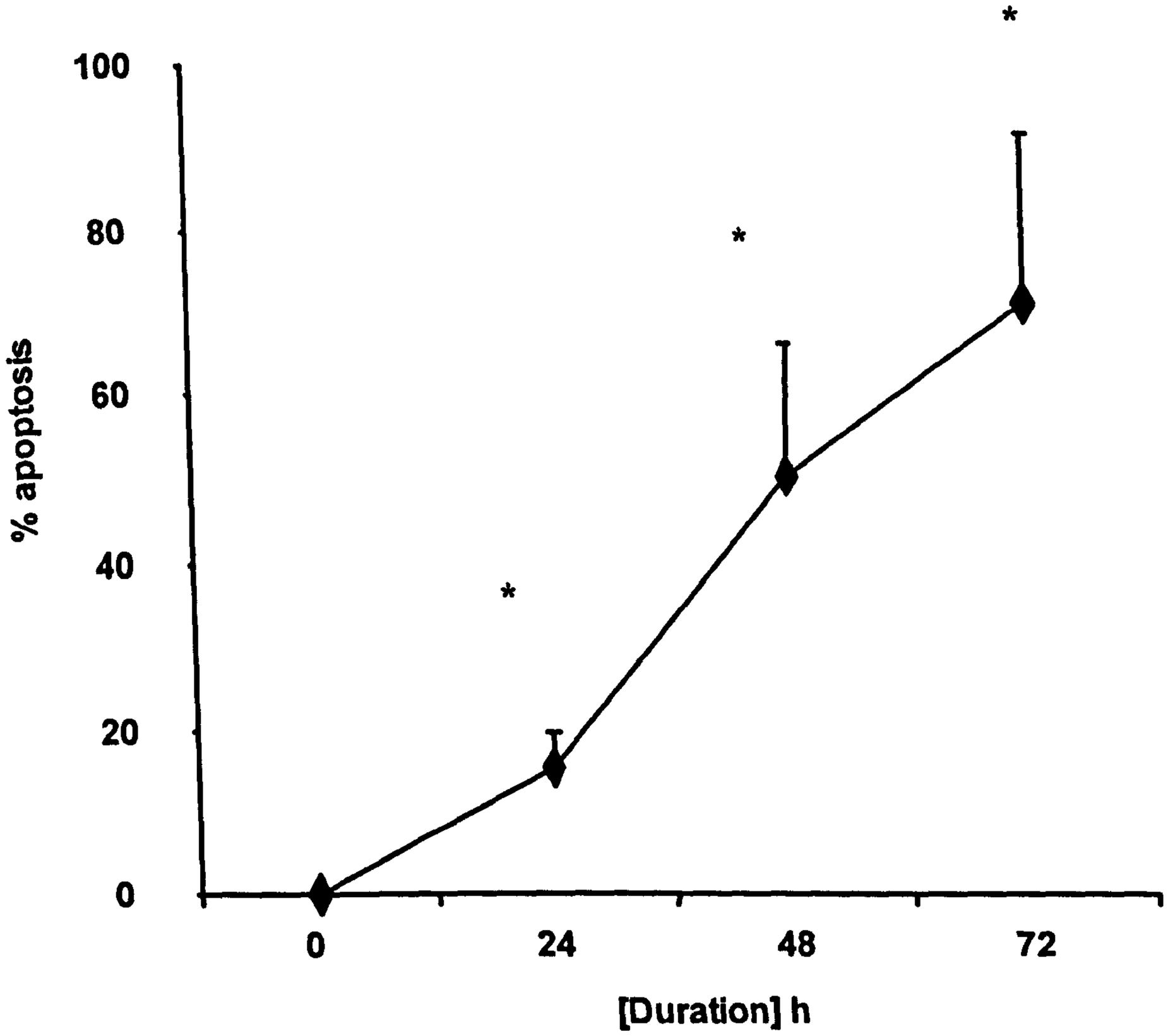
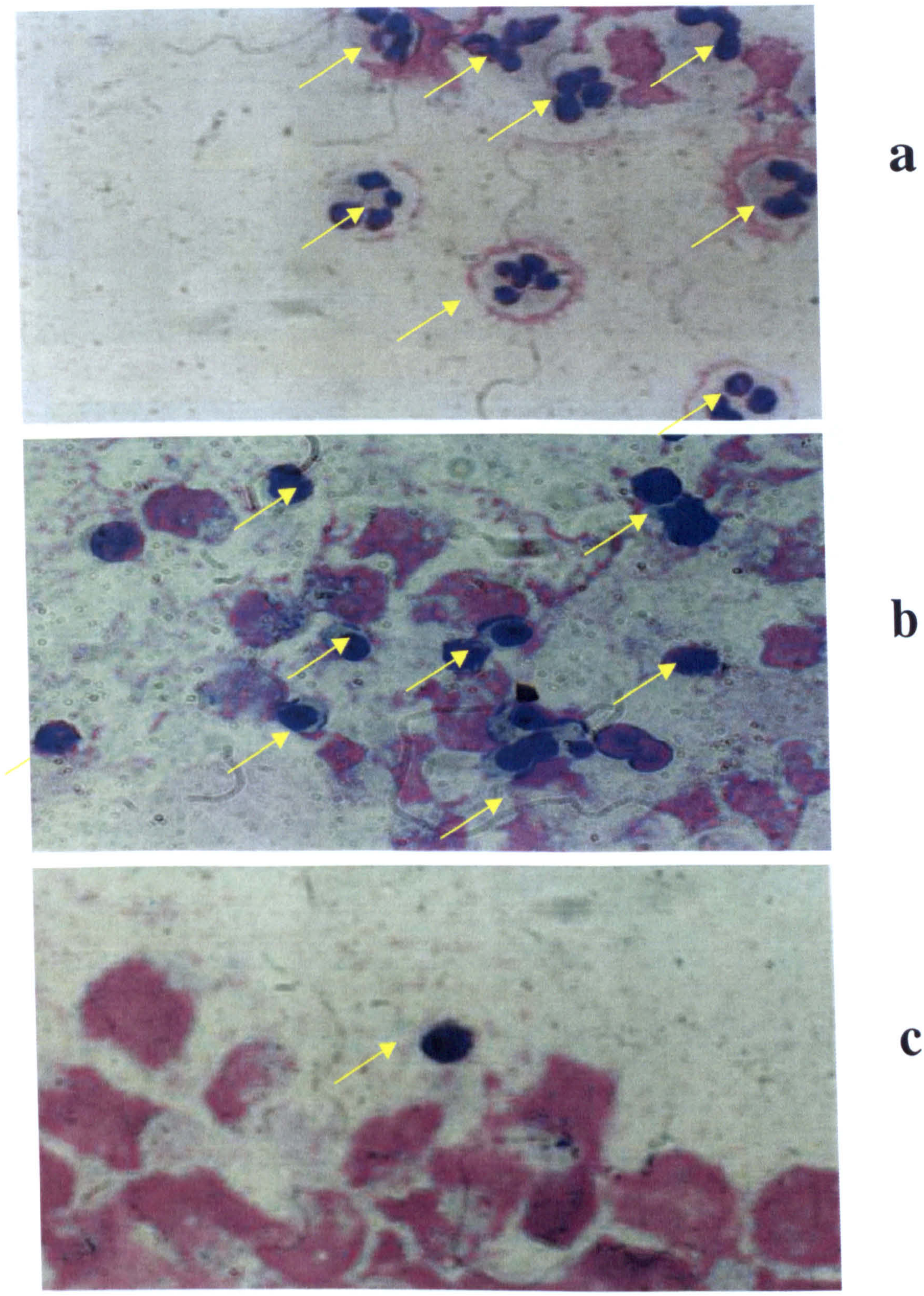


Figure 7.4.1. Effect of aging on human polymorphonuclear neutrophil apoptosis.

Neutrophils (1 x 10^6 cells.ml^{-1}) were cultured in complete RPMI 1640 medium as described in the materials and methods. Results represent quantitative analysis of apoptotic cells (x 1000 magnification) of 6 independent experiments ± SEM. * Significant difference (P<0.05) versus control (freshly isolated and stained cells)



a

b

c

Figure 7.4.2. Morphological features of age-induced apoptosis in human neutrophils (May and Grunwald-Giemsa staining, x 1000 magnification.

Untreated peripheral blood neutrophils incubated at 37 °C for (a) 0 h (b) 24 h and (c) 72 h. Fig. (7.4.2 a) shows normal neutrophil with the characteristic lobes and interconnecting filaments. Fig. (7.4.2 b and c) shows apoptotic neutrophils with condensed chromatin bodies. Result shown is representative of four independent experiments with similar results. Magnification x 1000.

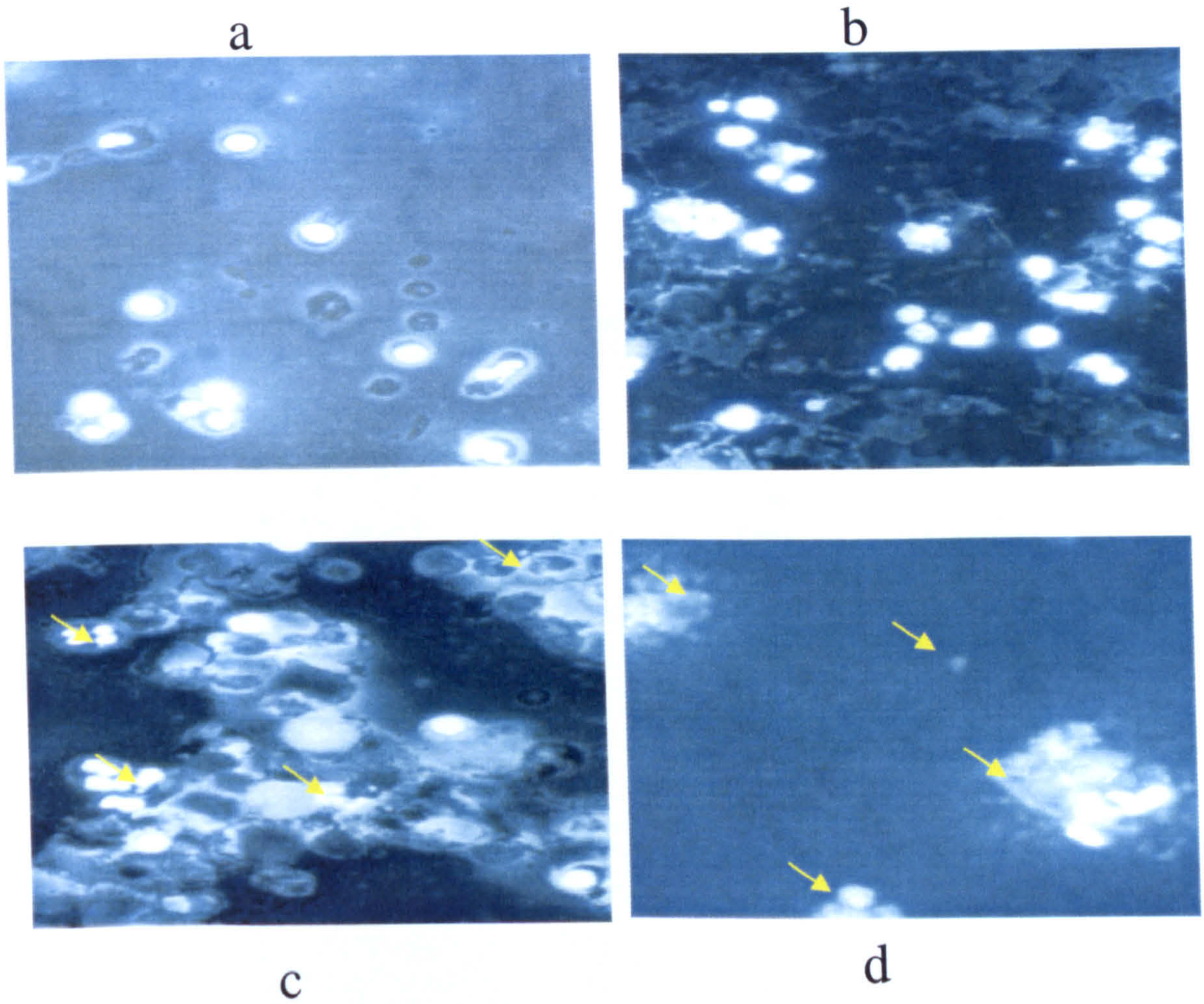


Figure 7.4.3. Morphological features of age-induced apoptosis in human neutrophils (DAPI staining, x 600 magnification).

Untreated peripheral blood neutrophils incubated at 37 °C for 0 h, 24 h and 72 h. Fig. (7.4.3 a) shows DAPI staining of normal neutrophils with no apoptotic features. Fig. (7.4.3 b and C) show DAPI staining of apoptotic neutrophils with condensed chromatin bodies (arrow) with increased fluorescent intensity, suggestive of apoptosis (x 600 magnification). Result shown is a representative of four independent experiments with similar results. Original magnification x 600

7.5.1.4 DNA fragmentation assay of human neutrophils

In order to confirm that neutrophils had undergone apoptotic cell death, genomic DNA was isolated and subjected to 2.0 % agarose gel electrophoresis. Figure 7.4.4

shows the characteristic "DNA ladders" of human genomic DNA from neutrophils after 24 h and 48 h incubation in a humidified atmosphere of (5 % CO_2/ 95 % O_2)

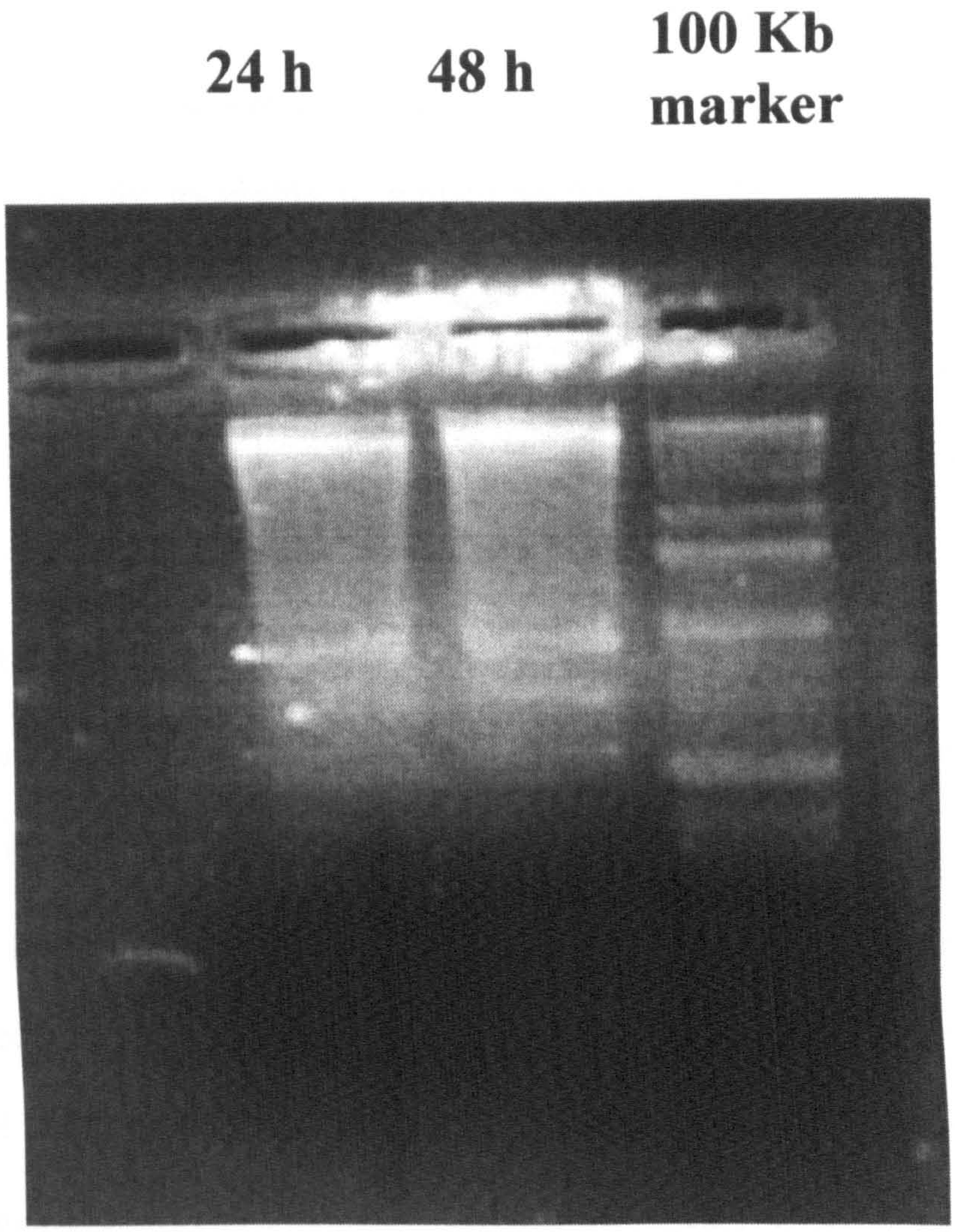


Figure 7.4.4. DNA fragmentation of aged human neutrophils in culture.
Isolated human neutrophils (1 x 10^6 cells.ml^{-1}) were cultured in complete RPMI 1640 medium. DNA were isolated after 24 and 48 h respectively and analysed as described in the chapter 2, section 2.6. Lane (a) shows molecular weight marker (12 kb –100 bp) (1 kb Plus DNA ladder, GIBco BRL Life Technologies, Cergy Pontoise, France). Lanes (b) and (c) show DNA from neutrophils incubated for 24 and 48 h respectively. DNA fragmentation was visualised as oligonucleosome-size fragments stained with ethidium bromide in 2 % agarose gel and transilluminated with UV light for photography. Result shown is a representative of four independent experiments with similar results.

7.5.2 Jurkat cell apoptosis

7.5.2.1. DAPI staining of Jurkat cells

Following the validation of DAPI staining technique with apoptotic neutrophils induced by aging, this method was employed to evaluate apoptosis in Jurkat cells. Anandamide was tested for its ability to induce apoptosis in Jurkat cells following treatment of these cells (1×10^6 cells.ml^{-1}) with anandamide (10^{-7} M - 10^{-4} M) for 24 h. Figure 7.4.6 shows a concentration-dependent increase in Jurkat cell apoptosis as measured by visual evaluation of DAPI stained preparations (Figure 7.4.5). A significant ($P<0.05$) degree of apoptosis was observed following treatment of cells with anandamide at 10^{-5} M (21.5% ± 5.3%, n = 6) for 24 h and 10^{-4} M (100.0 ± 0.0 %, n = 6) for 24 h. Jurkat cells (1×10^6 cells.ml^{-1}) exposed to room temperature for 24 h were included as positive control. Under these conditions 23.9 ± 10.6 % (n = 6) cells showed apoptotic features by visual evaluation of DAPI stained cytopreparations of anandamide treated Jurkat cells. The time course for anandamide-induced apoptosis in Jurkat cells and following exposure of these cells to room temperature also showed time-dependent increase in the apoptotic features by visual evaluation of DAPI stained preparations (Figure 7.4.7). The early features of apoptosis e.g. in Jurkat cells were seen in anandamide 10^{-5} M treated cells for up to 6 h following treatment (3.2 ± 2.8 %, n = 6). In contrast early signs of apoptosis were visible in Jurkat cells after 2 h exposures to room temperature (3.1 ± 1.3%, n = 6). By 24 h, anandamide 10^{-5} M induced more apoptotic features in Jurkats (17.5 ± 4.1%, n = 6) than was seen following incubation at room temperature (11.3 ± 1.6%) respectively (figure 7.4.7).

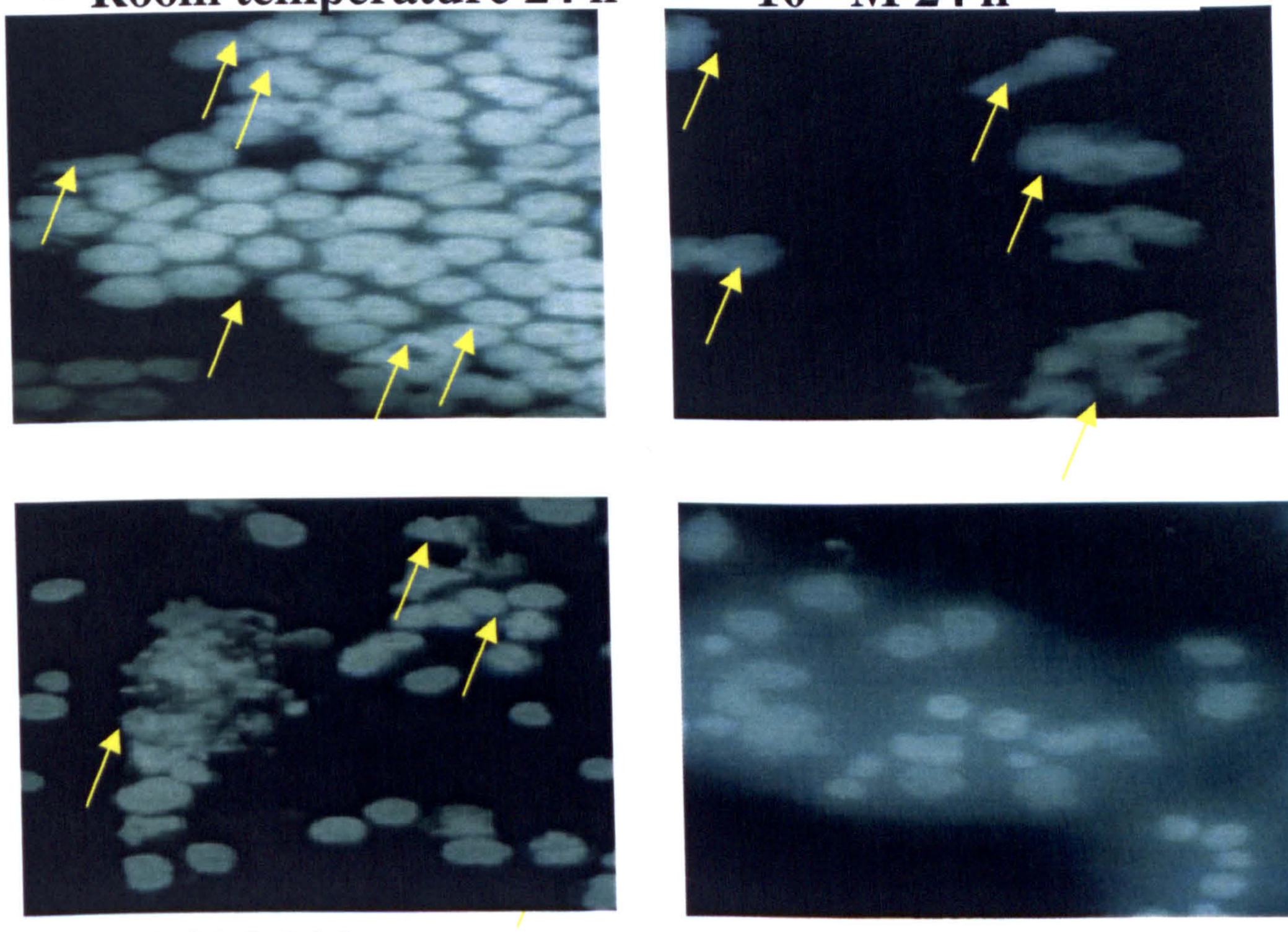


Figure 7.4.5. Effect of anandamide on Jurkat apoptosis

Morphological features of apoptosis induced in Jurkat cells (a) by exposure to room temperature (b) incubation with anandamide 10^{-5} M at 37 °C for 24 h (c) incubation with anandamide 10^{-4} M at 37 °C for 24 h (d) normal control incubated with vehicle (0.1 % ethanol) at 37 °C for 24 h. Jurkat cells were fixed in 3.7% formaldehyde and stained with DAPI as described in chapter 2 of this thesis. The characteristic features include condensed chromatin bodies (arrow) with increased fluorescent intensity, suggestive of apoptosis. Result shown is a representative of 4 independent experiments. Original magnification x 600.

To analyse the effect of synthetic cannabinoids, Jurkat cells were treated with CP55,940 (10^{-7} M - 10^{-4} M) for 24 h and the apoptotic features evaluated by DAPI staining. In these experiments, anandamide 10^{-5} M was included as a positive control based on its ability to induce apoptosis in this cell line. Apoptotic features were observed only in anandamide treated cells whereas CP55,940 did not appear to cause

any significant degree of apoptosis at any of the concentrations tested when compared to the control (data not shown)

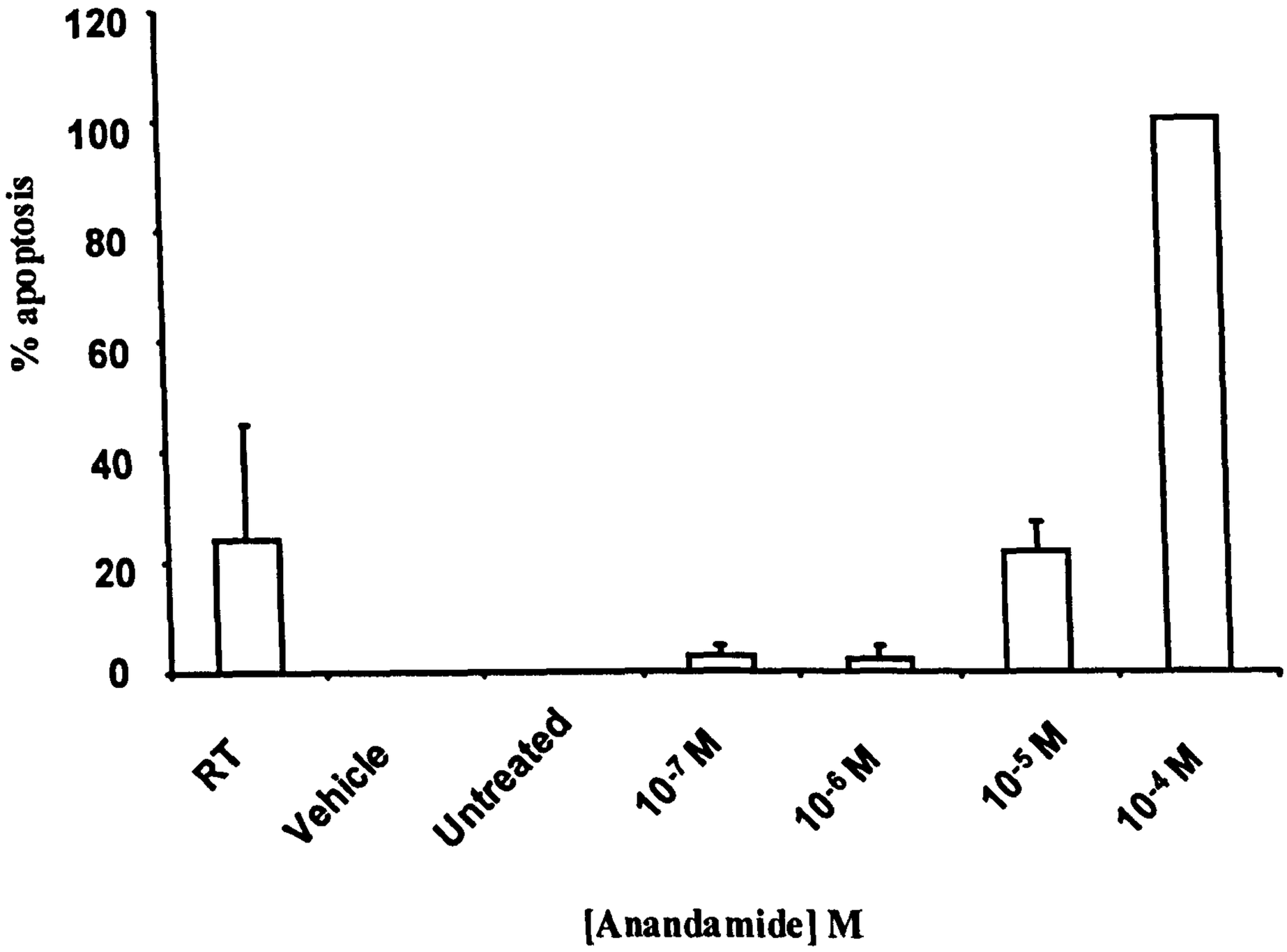


Figure 7.4.6. Effect of anandamide on induction of apoptosis in Jurkat cells after 24 h treatment.
Jurkat cells (1 x 10^6 cells.ml^{-1}) cultured in complete RPMI medium at 37 °C) in a humidified atmosphere (95% air/ 5% CO_2) and treated with increasing concentration of anandamide. Cytocentrifuge preparation of cells were made and stained with DAPI. Results represent quantitative analysis of apoptotic cells (x 1000) magnification) of six independent experiments. RT represents (room temperature induced apoptosis in Jurkat cells included as positive control). Values represent six independent experiments ± SEM.

7.5.2.2 The Viability of Jurkat cells as assessed by the MTT assay and trypan blue dye exclusion method

Based on the observation that a high concentration of anandamide (>1 μM) was required to induce a significant degree of apoptosis in Jurkat cells, it was tested whether another cannabinoid receptor ligand (CP55,940) was able to cause apoptosis

in Jurkat cells. Jurkat cells (1 x 10^6.ml^{-1}) were treated with anandamide, CP55,940 or exposed to room temperature for 24 h and viability was assessed by the MTT assay. Incubation of Jurkat cells with CP55,940 (10^{-6} M – 10^{-4} M) and anandamide (10^{-6} M - 10^{-4} M) showed a decrease in viability when compared with untreated cells. There was an even greater inhibition of cell viability in cells exposed to room temperature for 24 h (data are summarised in table 7.2 below). To test whether this reduction in viability was due to necrotic cell death or apoptotic cell death, cells were evaluated for their ability to exclude trypan blue dye. In all experiments, there was no significant (P> 0.05) reduction in the ability of Jurkat cells to exclude trypan blue (data summarised in table 7.2).

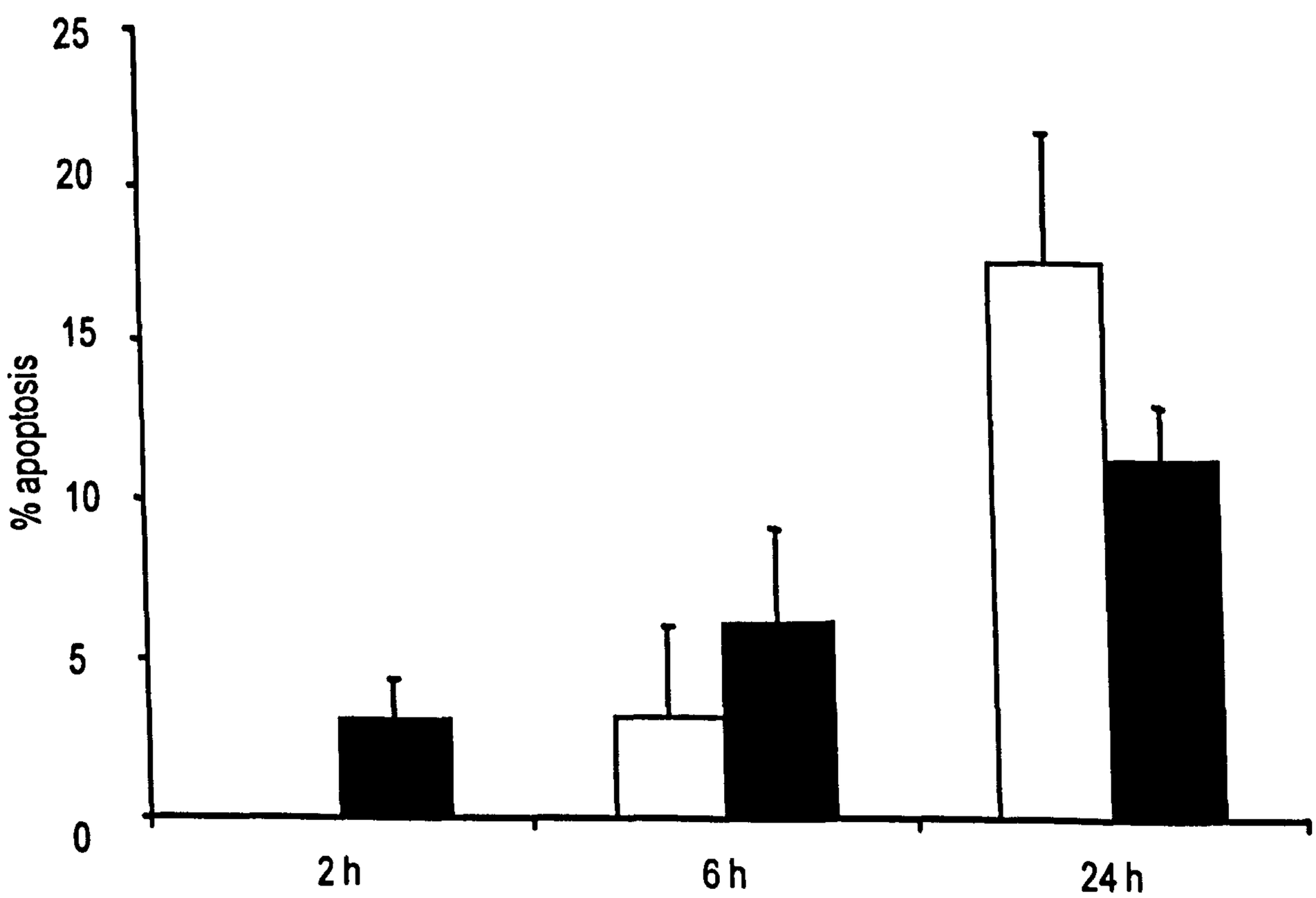


Figure 7.4.7. Time course of anandamide 10^{-5} M (open bars) and room temperature (closed bars)-induced apoptosis in Jurkat cells.
Jurkat cells (1 x 10^6 cell.ml^{-1}) cultured in complete RPMI medium. Following exposure to room temperature or incubation with anandamide (10^{-5} M) at 37 °C in a humidified atmosphere of 95 % air/ 5 % CO_2 . Cytopreparation of cells were made and stained with DAPI. Results represent quantitative analysis of apoptotic cells (x 1000 magnification) of six independent experiments. * Significantly different (P< 0.05).

Table 7.2. The effect of anandamide, CP55,940 or exposure to room temperature on Jurkat cell viability as assessed by trypan blue dye exclusion method and the MTT assay respectively).

Drugs/ Culture conditions	**Trypan blue dye exclusion assay** (Cell viability % of control)	**MTT assay** (Cell viability % of control)
37°C 24 h (95% air/5% CO_2)	100±0	97.1±2.9
Room temperature 24 h	93.9±0.3	18.3.0.3 *
Room temperature 48 h	82.7±10.7 *	8.3±5.0 *
Anandamide (10^{-6} M) 37°C for 24 h	95.1±5.3	74.6±6.0 *
Anandamide (10^{-5} M) 37°C for 24 h	89.8±2.2	53.8±2.7 *
CP55,940 (10^{-6} M) 37°C for 24 h	97.1±1.1	74.1±1.7 *
CP55,940 (10^{-5} M) 37°C for 24 h	95.2±3.4	60.5±7.5 *

Trypan blue dye exclusion technique and MTT assay were used to determine the viability of Jurkat cells as described in chapter 2, section 2.8.1 and 2.8.2 of this thesis. The data are mean ± SEM of 6 different experiments. * Denotes significant difference (* $P<0.05$) from the control.

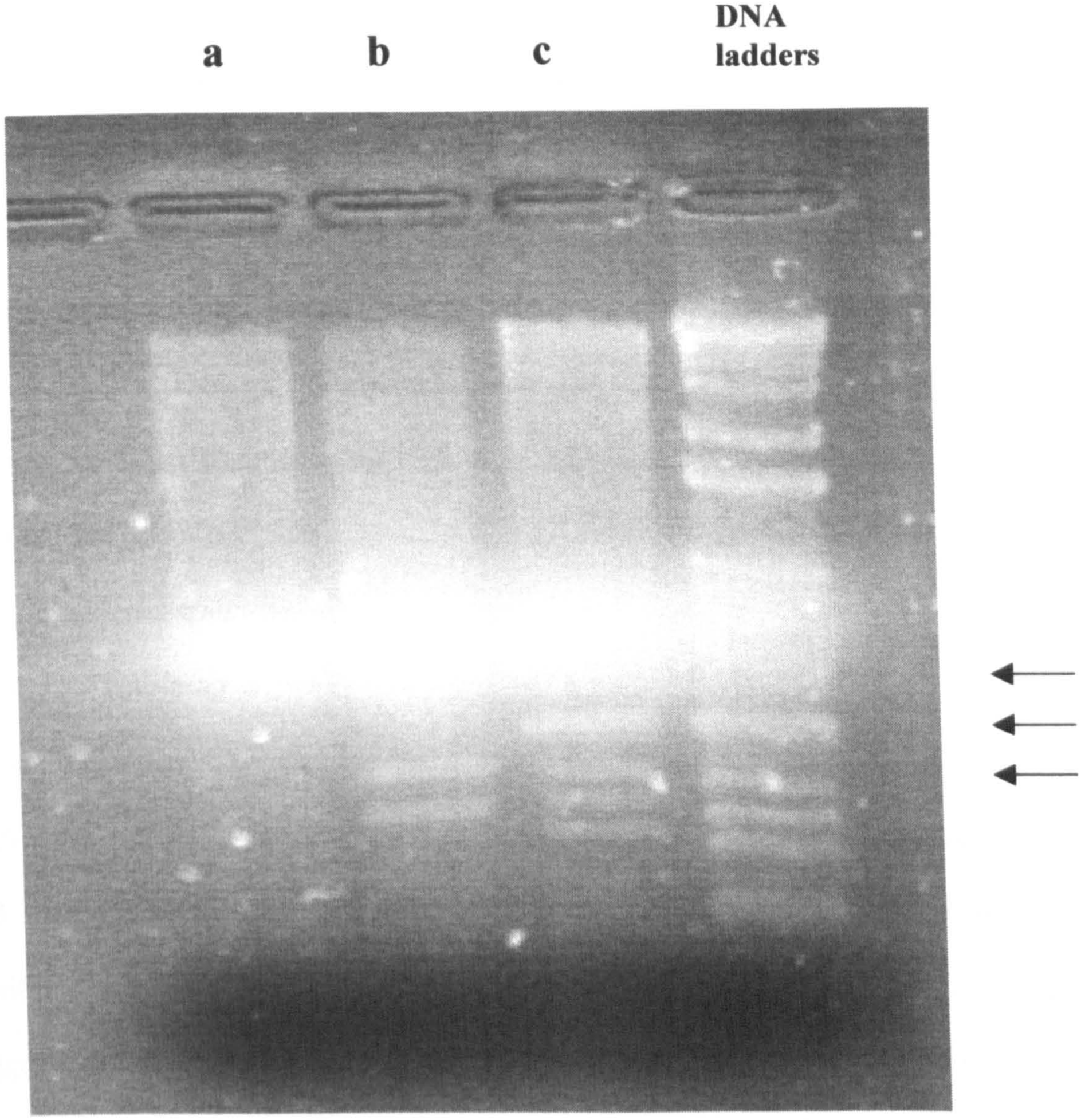


Figure 7.4.8 Characteristic apoptotic DNA laddering as shown by Jurkat cells on 2% agarose gel

Jurkat cells (1 x 10^6cells.ml^{-1}) were cultured in complete RPMI 1640 medium. Apoptosis was induced by exposure to room temperature or by treatment with anandamide for the indicated period of time. DNA were isolated and analysed as described in chapter 2 section 2.6. Lane (a) shows DNA from cultured and untreated Jurkat cells at 37 ^{o}C for 24 h. Lane (b) shows DNA from Jurkat cells exposed to anandamide (10^{-5} M) at 37^{o}C for 24 h. Lane (c) shows DNA from Jurkat cells exposed to room temperature for 24 h. Lane (d) shows molecular weight marker (12 kb –100 bp) (1 kb Plus DNA ladder, GIBco BRL Life Technologies, Cergy Pontoise, France). DNA fragmentation was visualised as oligonucleosome-size fragments stained with ethidium bromide in 2 % agarose gel and transilluminated with UV light for photography. Result shown is a representative of four independent experiments with similar results.

7.5.2.3 DNA fragmentation assay for Jurkat cell

To confirm whether anandamide treatment or exposure to room temperature induce internucleosomal DNA cleavage, a 2.0 % agarose gel electrophoresis was performed on the genomic DNA isolated from Jurkat cells following exposure to room temperature or treatment with anandamide for 24 h. Figure 7.4.8 shows the characteristic DNA fragmentation typical of 180 base pair multiples giving rise to "DNA ladders".

7.5.3 HT-29 cells apoptosis

7.5.3.1 DAPI staining

In order to test whether anandamide induced apoptosis in HT-29 cells, a culture of HT-29 cells on Lab-Tek slides starved with serum for 24 h to growth arrest cells were fixed in formaldehyde and stained for apoptosis with DAPI following treatment with or without anandamide. Anandamide induced significant ($P<0.05$) morphological changes consistent with apoptosis that became evident 24 h after treatment. Thus following anandamide (10^{-6} M) treatment a small but significant ($P < 0.05$) increase in apoptosis of 4.2 ± 0.5% (10^{-5} M=4.3 ± 0.6%, n=6) was seen. Anandamide-treated HT-29 cells showed irregular, condensed nuclei with increased fluorescent intensity (figure 7.4.11). Nuclear fragmentation, which is characteristically associated with apoptosis and clearly identifiable apoptotic bodies were also present. The cytoplasm appeared demarcated by an intact plasma membrane. Longer incubation periods induced a marked significant ($P < 0.05$) morphological time-dependent increase in characteristic apoptotic changes; 48 h (anandamide 10^{-6} M= 18.4 ± 1.8%; 10^{-5} M= 18.3 ± 2.4%, n = 6) and at 72 h (anandamide 10^{-6} M = 59.5 ± 2.1%; 10^{-5} M = 72.3 ± 5.0%, n = 6), respectively (figure 7.4.12). As a positive control, cells were also

treated with paclitaxel (100 nM) a drug known to induce apoptosis in HT-29 cells (Goncalves *et al.*, 2000). Pacliataxel induced an equivalent degree of apoptosis in HT-29 cells. Like anandamide, paclitaxel treatment showed a significant ($P < 0.05$), time-dependent increase in apoptosis (figure 7.4.12). To test whether the synthetic cannabinoid CP55,940 induced apoptosis in HT-29 cells, cells were treated with CP55,940 for 24 h and apoptotic changes evaluated using DAPI staining. Paclitaxel (100 nM) was included as a positive control. CP55,940 10^{-6} M - 10^{-5} M did not induce any significant ($P<0.05$) degree of apoptotic features in HT-29 cells as assessed by visual evaluation using DAPI stain after 24 h incubation (Figure 7.4.13).

Table 7.3. Cellular viability of HT-29 cells using MTT assay and trypan blue dye exclusion method

Drugs	**Trypan blue dye exclusion assay** (Cell viability % of control)	**MTT assay** (Cell viability % of control)
Untreated	>98%	>98%
Paclitaxel (10^{-7} M)	90.1 ± 5.0	75.5±2.5 *
Anandamide (10^{-5} M)	95.0±2.0	80.9±4.2 *
Anandamide (10^{-6} M)	93.4±5.0	84.4±2.1 *
CP55,940 (10^{-5} M)	90.0±1.0	74.3±5.1 *
CP55,940 (10^{-6} M)	88.2±1.0 *	84.1±3.1 *

Cell viability of HT-29 cells was determined by trypan blue dye exclusion technique and MTT assay as described in chapter 2, section 2.8.1 and 2.8.2 of this thesis. The data are mean ± SEM of 6 different experiments. * Significant difference (* $P<0.05$) from control (untreated cells)

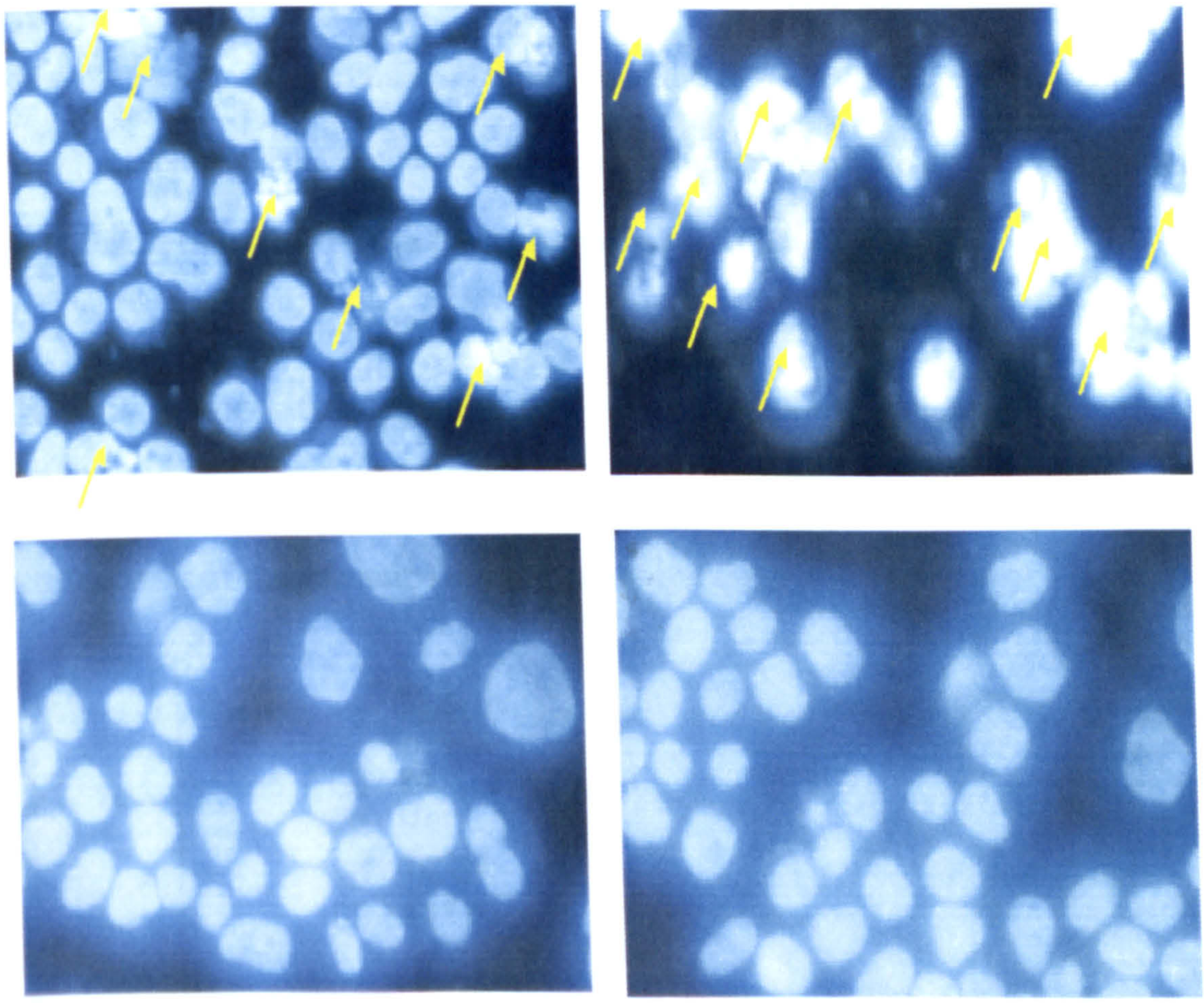


Figure 7.4.11 Effect of anandamide on induction of apoptosis in HT-29 cells stained with DAPI and visualised by fluorescent microscopy (x 1000 magnification).

HT-29 cells (1 x 10^6 cells.ml^{-1}) were cultured in polylysine-coated slides for 24 h in McCoy's medium. Cells were fixed in 3.7 % formaldehyde and stained with DAPI as described in the chapter 2, section 2.7.2 of this thesis. Characteristic apoptotic features (arrows) (a) cells treated with anandamide 10^{-4} M for 24 h (b) cells treated with anandamide 10^{-4} M for 24 h (c) and (d) untreated and 0.1% ethanol treated cells respectively with no evidence of apoptosis. Original magnification x 600.

That these changes were indeed induced by apoptosis and not necrosis was confirmed by the MTT assay and trypan blue dye exclusion methods. Treatment of HT-29 cells with anandamide (10^{-6} M – 10^{-5} M) or CP55,940 (10^{-6} M - 10^{-5} M) for 24 h showed a concentration-dependent decrease in cellular viability compared to untreated control. To test whether this reduction in viability was due to necrosis or apoptotic cell death, cells were evaluated based on their ability to exclude trypan blue dye. In all occasions, >94% of cells excluded the dye (data are summarised in table 7.3).

7.5.3.2 DNA fragmentation assay for HT-29 cells

HT-29 cells treated with anandamide (10^{-5} M - 10^{-6} M) for 24h showed no evidence of the DNA ladder pattern characteristic of apoptosis following a 2.0 % agarose gel electrophoresis of the genomic DNA (data not shown).

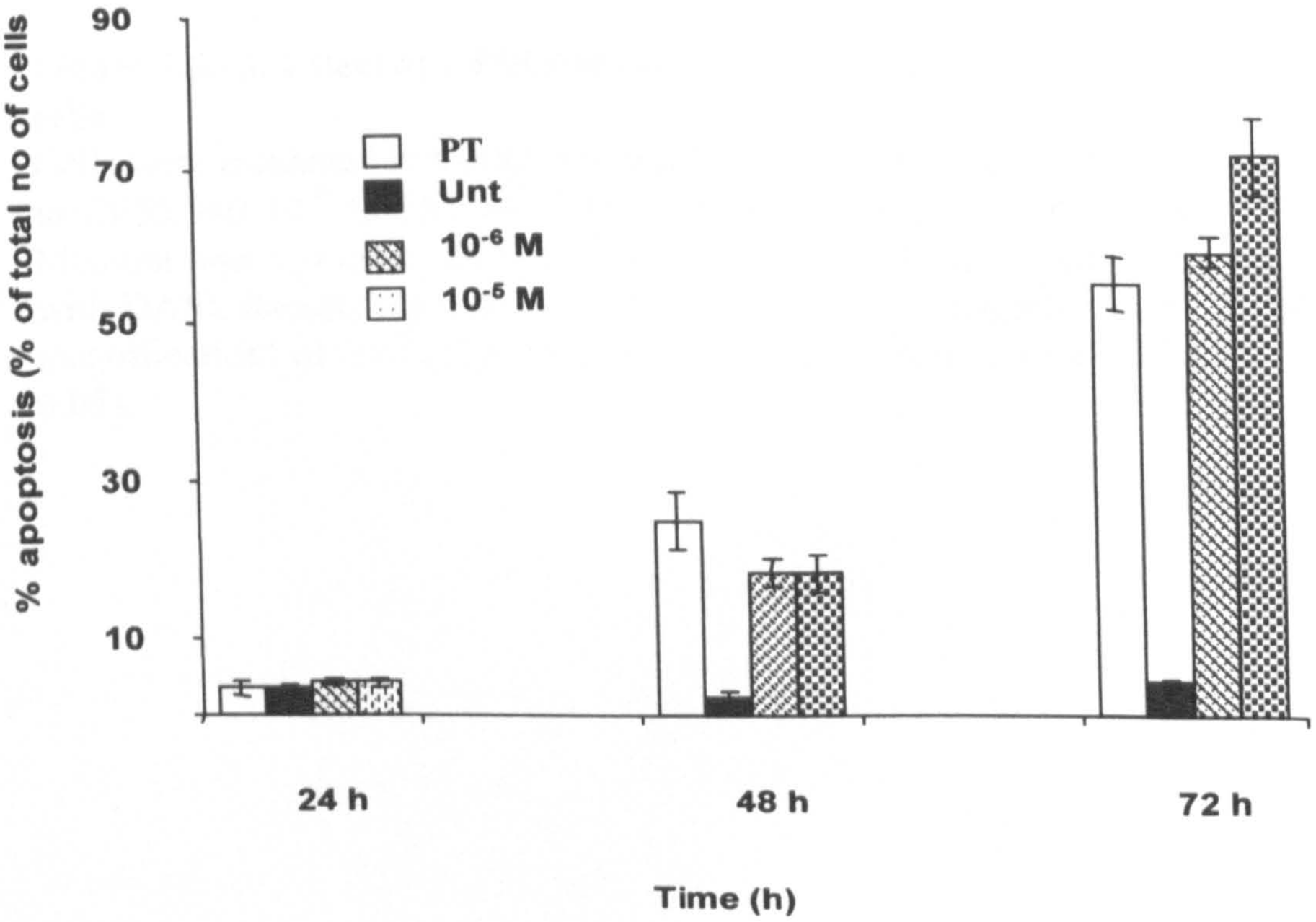


Figure 7.4.12. Effect of anandamide on induction of apoptosis in HT-29 cells.

Cells were incubated in a drug-free medium on a labtek glass slide or exposed to anandamide 10^{-6} M-10^{-5} M or PT control (Paclitaxel 10^{-7} M) for the indicated period of time. Medium was removed, fixed in 3.7% formaldehyde and cells were stained with DAPI. Results represent quantitative analysis of apoptotic cells (x 1000 magnification) of six independent experiments. * Significantly different ($P< 0.05$).

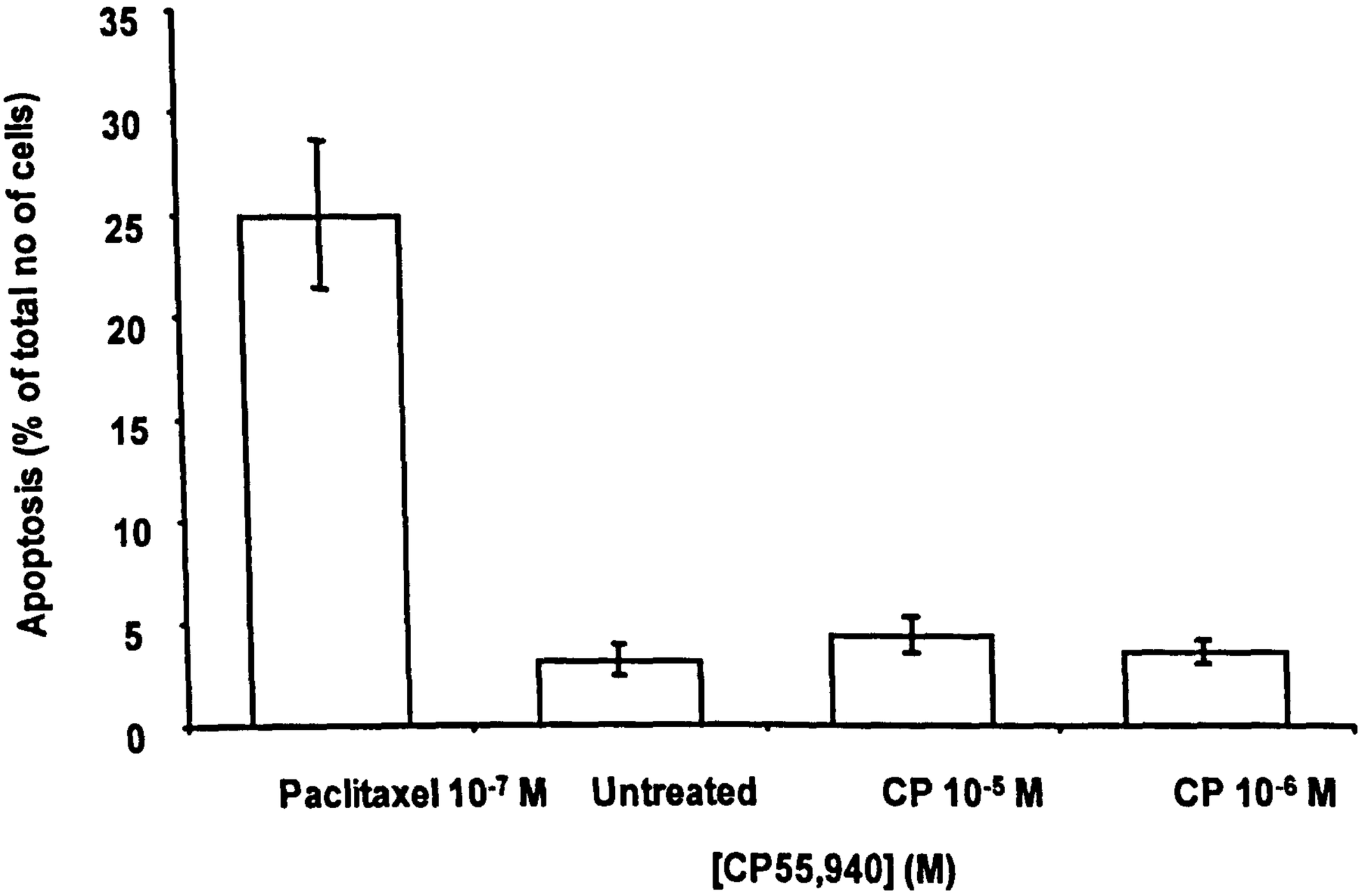


Figure 7.4.13. Effect of CP55,940 on induction of apoptosis in HT-29 cells.

Cells were incubated in a drug-free medium on a labtek glass slide or exposed to CP55,940 10^{-6} M-10^{-5} M or PT control (Paclitaxel 10^{-7} M) for 48 h. Medium was removed, fixed in 3.7% formaldehyde and cells were stained with DAPI. Results represent quantitative analysis of apoptotic cells (x 1000 magnification) of six independent experiments. * Significantly different ($P<$ 0.05).

7.6 Discussion

In the experiments described above, the endogenous cannabinoid, anandamide has been demonstrated to induces apoptosis and inhibit mitochondrial function in Jurkat and HT-29 cells albeit at higher concentrations (> 1 μM) than in untreated cells. The synthetic cannabinoid CP55,940 at higher concentrations also inhibited mitochondria oxidative metabolism as shown by MTT assay but in contrast to anandamide did not appear to cause apoptosis of either Jurkat or HT-29 cells. Neutrophils and Jurkat cells, unlike many other cells, undergo constitutive apoptosis in vitro (Haslett *et al.*, 1994; Shimura *et al*, 1998). Thus, the exploitation of the unique properties of these two cell types has been used to validate a sensitive assay for cannabinoid-induced apoptosis in immune cell lines using a nuclear fluorescent dye, DAPI.

The anti-inflammatory properties of cannabinoid receptors are well documented (Pertwee, 1997). However, the effect of cannabinoid receptor ligands and the endogenous cannabinoids in modulating immune cell function are unclear in spite of extensive research. Previously, an unusual susceptibility of Jurkat cells to mitochondria oxidative damage when exposed to cannabinoid receptor ligands by MTT assay has been reported (chapter 2 of this thesis). It was also shown that cannabinoids inhibit the release of a pro-inflammatory cytokine IL-8 from HT-29 cells, reported in chapter 4. Since the pro-lymphocytic cell line Jurkat cells and the colon epithelial cell line HT-29 express cannabinoid CB_2 receptors (Schatz *et al.*, 1997; Ihenetu *et al.*, 2001), the findings in the present study together with studies from other laboratories showing that cannabinoids may induce apoptosis *in vitro* in immune cells (Zhu *et al.*, 1998; Schwartz *et al.*, 1994; Mckallip *et al.*, 2002) suggests

the possible use of cannabinoid receptor agonists as an anti-inflammatory drug treatment.

In the current study, the effects of aging upon the induction of apoptosis of human neutrophils during *in vitro* culture was established, because the development of apoptosis is easy to observe in these cells (Meagher *et al.*, 1996). It was shown that neutrophils maintained in culture constitutively undergo apoptosis in a time-dependent manner as assessed by visual evaluation of their cell morphology when stained with May and Grunwald-Giemsa and DAPI stained preparations. Apoptosis was confirmed in this cell by "DNA laddering" following the isolation and electrophoresis of genomic DNA suggesting an activation of the endogenous endonucleases, a hallmark of late events in apoptosis (Fulthorpe *et al.*, 1997). Morphological criteria used to assess apoptosis in this study included the following; (a) cytoplasmic and nuclear shrinkage (b) chromatin condensation and deep blue stained nuclei (c) cytoplasmic blebbing with maintenance of integrity of cell membrane (zeiosis) (Cohen, 1992). Comparatively, non-apoptotic neutrophils maintained their characteristic interlobular structure with normal azurophilic staining. The DAPI staining of apoptotic neutrophils visualised under a fluorescence microscopy revealed dense granular nuclear fragments (apoptotic bodies) with a more intense fluorescence staining in neutrophils undergoing apoptosis as opposed to non-apoptotic neutrophils.

After the confirmation of the classical features of apoptosis in neutrophils using well-established techniques, the effect of cannabinoids on Jurkat and HT-29 cells was then studied. It was shown that higher concentration of anandamide induced apoptosis in

these two cell lines in a dose and time dependent manner as indicated by the characteristic morphological features of DAPI stained preparations. In time course studies, more Jurkat cells underwent apoptosis upon treatment with anandamide for 24 h than HT-29 cells. Furthermore, treatment of Jurkat cells with anandamide or exposure to room temperature induced intranucleosomal DNA cleavage when subjected to 2 % agarose gel electrophoresis as demonstrated by the characteristic"DNA ladders". In contrast, no such changes were detected on agarose gel electrophoresis of genomic DNA isolated from HT-29 cells after treatment with the same concentration of anandamide and incubated at an equivalent duration of time. The reason for this discrepancy was not known but taken together these observations suggest that staining cells with DAPI may be a more sensitive method than the DNA fragmentation assay for the detection of apoptosis in these cells. Furthermore, in line with previous studies, our data suggest that the key morphological changes as reported in the present experiments precede internucleosomal DNA cleavage, a common feature of late apoptotic events (Fulthorpe *et al.*, 1997). Additionally, cannabinoids are well known for their effects on cytokine network in lymphocytes (Klein *et al.*, 2000a). For example, cannabinoids have been shown to inhibit IL-2 release from lymphocytic cell lines via activation of cannabinoid CB_2 receptors (Schatz *et al.*, 1997; Ihenetu *et al.*, 2003). IL-2 in turn has been shown to play essential roles in the induction of lymphocyte apoptosis (Leonardo, 1991). Therefore, the inhibition of PHA/PMA-induced IL-2 release from the pro-lymphocytic cell line, Jurkat cells as shown in chapter 3 of this thesis may partly account for the increased apoptosis of Jurkat cells than the HT-29 cells. Taken together, these observations suggest that cannabinoids may inhibit proliferation in the pro-lymphocytic cell line Jurkat and induce apoptosis.

That these cells were undergoing apoptosis rather than necrosis is supported by additional findings.

1 There was a progressive loss of viability in Jurkat and HT-29 cells in the first 24 h following treatment with anandamide (10^{-6} M and 10^{-5} M) as indicated by the MTT assay, yet in both occasions less than 10 % of the cells excluded trypan blue dye.

2 At both concentrations (10^{-6} M and 10^{-5} M), characteristic morphological features of apoptosis were always present.

Thus, these observations agree in part with previous studies, which demonstrated that apoptotic cells possess an ability to exclude vital dyes whereas necrotic cells do not (Cohen, 1992; Zhu *et al.,* 1998; Walker and Quirke, 2001).

To examine whether the synthetic cannabinoid, CP55,940, induced apoptosis in these cells, Jurkat or HT-29 were treated with CP55,940 and the morphological features of apoptosis evaluated by DAPI staining. Cell viability was also investigated using MTT and the trypan blue dye exclusion test. In both cases, CP55,940 (10^{-6} M – 10^{-4} M) did not induce morphological features consistent with apoptosis, however the MTT and trypan blue dye exclusion assays revealed substantial loss of viability suggesting that high concentration of CP55,940 may predispose Jurkat and HT-29 cells to necrosis rather than apoptosis after 24 h of treatment. The reason behind the difference between the effects of anandamide and CP55,940 in the present study is not known. But these may reflect the differences between the bindings of these compounds to cannabinoid receptors in immune cells. Clearly additional studies may be necessary to assess the significance of these findings.

Whether or not anandamide-induced apoptosis in Jurkat and HT-29 cells is mediated via known cannabinoid receptors was not addressed in the present study. Interestingly previous studies demonstrating cannabinoid-induced apoptosis in immune cells have implicated both cannabinoid receptor dependent-mechanism (Zhu *et al.*, 1998; Schwartz *et al.* 1994) and non-cannabinoid receptor dependent mechanisms. It is possible that these actions are cannabinoid receptor dependent but clearly, additional studies are needed to elucidate the receptors mediating cannabinoid-induced apoptotis in immune cells.

In summary, anandamide but not CP55,940 have demonstrated to induce cell death in Jurkat and HT-29 cells by apoptosis. Higher concentrations of these compounds reduced cell viability. Collectively, whatever mechanisms underlie anandamide-induced apoptosis in these cell lines, it is important to note that the endogenous cannabinoid ligand anandamide may regulate important cellular functions such as proliferation and cell death. Hence, the apoptotic effects of endogenous cannabinoid as demonstrated in this study may provide the basis for the development of an anti-inflammatory drug for the future.

Chapter 8; General discussion

8.1 General discussion

In this section, an overview of the preceeding chapters is presented together with speculations on the potential clinical utilities of cannabinoid receptor ligands. However, it is prudent to state that among the reported cannabinoid receptors, the cannabinoid CB_1 is more convergent in that the nucleotide sequences in man, rats and mice are highly conserved than that in the cannabinoid CB_2 receptors (Chakrabarti *et al.*, 1995; Gerard *et al.*, 1991; Shire *et al.*, 1996). This may highlight the importance of the degree of interspecies differences existing within the cannabinoid CB_2 receptors in contrast to cannabinoid CB_1 receptors and their resultant effects on their binding sites, which has not been fully characterised to date (Berglund *et al.*, 1998). The aim of this thesis was to characterise the cannabinoid receptors mediating the inhibition of cytokine/chemokine release from a variety of immune cell lines and primary immune cells. In order to avoid the potential complication of differences between studies resulting from interspecies differences, all cells and cell lines studied in this thesis were derived from human sources.

8.2 General summary

The experimental work described in this thesis initially examined the effects of cannabinoid receptor agonists on LPS-induced release of TNF-α from THP-1 cells, a human promonocytic cell line. The effect of cannabinoids on PHA/PMA-induced release of IL-2 from a human pro-lymphocytic cell line, Jurkat, was also investigated.

In these studies, CP55,940, Δ^9-THC and anandamide inhibited LPS-induced TNF-α secretion from THP-1 cells in a concentration-dependent manner (see chapter 3 of this thesis). This inhibition was antagonised by SR144528, a cannabinoid CB_2 receptor antagonist but not by SR141517A, a cannabinoid CB_1 receptor antagonist

suggesting that these effects were mediated, at least partially, via cannabinoid CB_2 receptors. However, these inhibitory effects of cannabinoids were observed at concentrations (>1 μM), greater than those used in cannabinoid binding studies (Rinaldi–Carmona *et al.*, 1998), suggesting that THP-1 cells may not express sufficient cannabinoid receptors or that the effects observed may be non-cannabinoid receptor dependent. CP55,940, Δ^9-THC and anandamide also inhibited PHA/PMA-induced IL-2 release from Jurkat cells. In contrast to findings in THP-1 cells, neither cannabinoid CB_1 nor CB_2 receptor antagonists (SR144528 and SR141716A) antagonised the inhibitory effects of cannabinoids in Jurkat cells (chapter 3). However, when cell viability was measured using the MTT assay in Jurkat cells, a concentration-related loss of cell viability was seen suggesting that cannabinoid-evoked inhibition of IL-2 release may be due to a cytotoxic action and independent of cannabinoid receptors.

Having investigated the effects of cannabinoids on monocyte/macrophage and T-lymphocyte cell lines (THP-1 and Jurkat cell lines respectively), the focus of our studies was shifted to demonstrating these effects on primary cells. However previous studies have shown that WIN55212-2, but not CP55,940, inhibited LPS-induced release of TNF-α from PBMC, an effect that was antagonised by SR144528 but not by SR14617A, suggesting a cannabinoid CB_2 receptor-mediated effect (Germain *et al.*, 2002). Therefore, the experiments described on PBMC (chapter 4 of this thesis) were focussed on the effect of cannabinoids on the secretion of another cytokine, IL-2 known to play a role in inflammatory responses (Smith *et al.*, 1988).

In these experiments, WIN55212-2, a non-selective cannabinoid receptor agonist and JWH 015, a selective cannabinoid CB_2 receptor agonist inhibited PHA-induced release of IL-2 from PBMC in a concentration-dependent manner, an effect antagonised by SR144528, but not by SR141716A, suggesting that the inhibition was mediated via cannabinoid CB_2 receptors. CP55,940, a non-selective cannabinoid receptor agonist marginally inhibited PHA-induced IL-2 release from PBMC whereas Δ^9-THC had no effect in inhibiting this release. Furthermore, WIN55212-2 evoked inhibition of IL-2 was antagonised by CP55,940 and Δ^9-THC. Considering the fact that previous studies in our laboratory and others have shown that CP55,940 and Δ^9-THC are agonists at cannabinoid receptors (Bayewitch *et al.*, 1996; Ihenetu *et al.*, 2003; Chapters 4 of this thesis), the antagonist effect of these compounds as seen in the present study suggest that these compounds have an affinity for the cannabinoid CB_2 receptors in PBMC. However, the data described in Chapter 4 also suggests that these compounds have a low efficacy at these receptors in that they acted as cannabinoid receptor antagonists on PBMC. To our knowledge, this is the first report suggesting that CP55,940 may act as a partial agonist at cannabinoid CB_2 receptors.

The effects of cannabinoids were then studied on the epithelial cell line HT-29. HT-29 cells are a human colonic epithelial cell capable of secreting the chemokine, IL-8, in response to inflammatory cytokines in the same way as a native epithelium (Schuerer-Maly, *et al.*, 1994). In these experiments, described in chapter 5, it was shown that the cannabinoid receptor agonists CP55,940, Δ^9-THC, WIN55212-2 and JWH 015 significantly ($P<0.05$) inhibited TNF-α-induced release of IL-8 from HT-29 cells in a concentration-dependent manner. The endogenous cannabinoid agonist, anandamide and the cannabinoid CB_1 receptor agonist, arachidonyl-2-

chloroethylamide (ACEA) had no significant inhibitory effects on TNF-α-induced release of IL-8. The CB_1 receptor antagonist SR141716A (1 μM) antagonised the inhibitory effects of CP55,940 but did not antagonise the effects of the more selective cannabinoid CB_2 receptor agonists, WIN55,212-2 and JWH 015 (Felder *et al.*, 1995; Hillard *et al.*, 1999). The CB_2 receptor antagonist SR144528 (Rinaldi-Carmona *et al.*, 1998), antagonised the inhibitory effects of CP55,940, WIN55212-2 and JWH 015. Taken together, these results suggest that cannabinoids exert inhibitory effects on TNF-α-induced release of IL-8 from HT-29 cells. In support of this hypothesis, Western immunoblotting revealed immuno-reactive proteins at a region consistent with the size of cannabinoid CB_2 receptor proteins.

In an attempt to identify the intracellular events responsible for cannabinoid-evoked inhibition of IL-8 release from the human colonic epithelial cell line HT-29, the effect of cannabinoids was studied on basal and agonist evoked increases in two important cellular messengers, namely cyclic AMP and cytosolic free calcium. In this series of experiments, it was demonstrated that WIN55212-2 and CP55,940 inhibited basal $[Ca^{2+}]_i$ from HT-29 cells. In contrast, a published work showing that ACh and TNF-α induce increases in $[Ca^{2+}]_i$ from HT-29 cells (Poronnik *et al.*, 1999) was confirmed and it was also shown that WIN55212-2 inhibited these increases. Furthermore, WIN55212-2 and CP55,940 inhibited basal and forskolin-induced increases in cAMP. Given the fact that cannabinoid-evoked inhibition of basal and agonist-evoked increases in $[Ca^{2+}]_i$ and $[cAMP]_i$ occur at the same concentration ranges as cannabinoid-evoked inhibition of TNF-α-induced release of IL-8 from HT-29 cells, it could be argued that these events may be causally related. However, further studies

are required to identify the relative importance of these second messenger pathways to inhibition of IL-8 release (refer to Figure 6.5.1 of this thesis).

In experiments investigating the effect of cannabinoids on the induction of apoptosis, a nuclear sensitive fluorochrome DAPI was employed. In these studies, it was shown that anandamide, but not CP55,940, induced cell death in Jurkat and HT-29 cells by promoting apoptosis. However, higher concentrations of these compounds also reduced cell viability and caused necrosis in both of the cell lines studied suggesting that cannabinoid-induced inhibition of cytokine release in these cells may be due, in part, to induction of apoptosis.

8.3 Potential therapeutic utility of cannabinoid receptor ligands

Cannabinoids have a long history as medicinal preparations, mainly for indications such as induction of analgelsia, anti-emesis, ocular hypotension and anti-convulsion therapy (reviewed in Mechuolam *et al.*, 1998). Recent research *in vitro* and in animal models has led to increasing evidence that cannabinoids are also important modulators of immune system (Klein *et al.*, 1998). Thus, cannabinoid CB_2 receptor agonists could have a role in the treatment of chronic inflammatory diseases. The aim of the present study was to assess the potential anti-inflammatory properties of cannabinoid receptor agonists by investigating their effects on the release of pro-inflammatory cytokines from immune cells and cell lines. A detailed review of cytokine regulation of immune cell function will not be repeated in this section, but those aspects investigated in the present study and relevant to cannabinoid-evoked modulation of immune cell function and their prospective clinical utility will be discussed.

Monocytes/macrophages and neutrophils, the phagocytic cells of the immune system, are the most important cellular components of the host immune response. An important function of the monocytes and neutrophils is to migrate from the blood to the site of infection in response to inflammatory mediators such as interleukin-8 (IL-8) (refer to figure 1.8; chapter 1 of this thesis). Once at the site of inflammation, phagocytic cells eliminate many pathogens by phagocytosis. Lymphocytes of the T and B classes regulate subsequent steps in the immunological response by secreting cytokines and antibodies, which are crucial in all levels of cellular and humoral immune responses (Smith, 1988). In addition to monocytes/macrophages, neutrophils and lymphocytes, some studies have identified epithelial cells as the site of origin of IL-8 in inflammatory bowel disease lesions (Mazzucchelli *et al.*, 1994). Furthermore, IL-8 is up regulated in IBD and tissue expression correlates with the degree of inflammation (van Deventer, 1997; Mazzucchelli *et al.*, 1994). IL-8 is an 8 KDa member of CXC chemokine family which functions as a potent activator and chemoattractant for neutrophils, predominantly by binding to its surface receptors CXCR1 and CXCR2 (MacDermott *et al.*, 1998; Baggiollini *et al.*, 1997).

In the present study, general suppressive effects of cannabinoids on monocyte/macrophages, T cells and human colonic epithelial cell function have been described. Cannabinoid receptor agonists have been shown to impair T cell function by inhibiting IL-2 release and monocyte/macrophage function shown by suppressing the release of the pro-inflammatory cytokine TNF-α and the chemokine (IL-8) from an epithelial cell line. However, these findings differ according to the type of cell used, the experimental conditions, the concentration of cannabinoid required to produce an inhibitory effect and the type of cannabinoid receptor agonist studied.

Thus, given the potency of the aminoalkylindole cannabinoids (WIN55212-2 and JWH 015) in suppressing IL-8 release (chapter 5 of this thesis, table 8.1) and IL-2 release (chapter 4 of this thesis, table 8.1), it is plausible to suggest that these compounds may be useful anti-inflammatory and immunosuppressive drugs. They may therefore find a clinical utility in the treatment of inflammatory bowel disease (IBD) and chronic pulmonary obstructive disease (COPD), where the release of IL-8 is thought to play a crucial role in the pathogenesis of the disease (Mazzucchelli *et al.*, 1994). From a clinical standpoint, it is important that given species differences, the studies reported in this thesis were performed on human colonic epithelial cells (HT-29 cells). To our knowledge, these are the first observations to localise functional cannabinoid CB_2 receptors on a cell present in human colonic tissue.

That cannabinoid agonists inhibited TNF-α-induced IL-8 release in the present study (chapter 6 of this thesis), coupled with the fact that activation of cannabinoid receptors in the enteric neurons was able to suppress peristalsis in animal models via inhibition of acetylcholine-induced peristalsis (Tyler *et al.*, 2000; Heinmann *et al.*, 1999), holds out the promise that exploitation of the cannabinoid receptor system could be useful in the treatment of gastrointestinal motor disorders. Interestingly, cannabinoid receptor agonists have beneficial effects in the gut in inhibiting diarrhea in rodent models (Izzo *et al.*, 1999). Furthermore, cannabinoids have been shown to inhibit chloride ion secretion in studies using 'Ussing' chambers to measure transepithelial ion fluxes (Tyler *et al.*, 2000; Heinmann, *et al.*, 1999; Izzo *et al.*, 1999). Thus, exploitation of cannabinoid pharmacology may offer a promising new therapeutic target for the treatment of chronic inflammatory conditions where the secretion of pro-inflammatory chemokines such as IL-8, are known to play a major

role. Such conditions may include inflammatory bowel disease IBD (Crohn's disease and ulcerative colitis), chronic obstructive pulmonary diseases (COPD) e.t.c. (See Table 8.1 for the comparative efficacy of CP55,940 on inhibition of cytokine and chemokine on various cells employed in this thesis).

Table 8.1. A summary of the potencies of various cannabinoid receptor agonists on various cells employed in this study

Cell type	Effect measured	Agonist Potency	95% Confidence limit	No of observations
THP-1	**TNF-α release**	EC_{50} Anandamide=1.86×10^{-5} M	1.6×10^{-5} –8.8×10^{-5} M	6
		CP55,940=4.8×10^{-5} M	2.6 x 10-5 – 8.8×10^{-5} M	6
		Δ^9-THC=3.1×10^{-5} M	2.8 x 10-5 – 3.5×10^{-5} M	6
Jurkat	**IL-2 release**	EC_{50} Anandamide=7.1×10^{-6} M	6.1×10^{-5} - 8.3×10^{-5} M	6
		CP55,940=2.3×10^{-5} M	1.5×10^{-5} - 3.5×10^{-5} M	6
		Δ^9-THC=3.2×10^{-5} M	2.1×10^{-5} - 4.8×10^{-5} M	6
PBMC	**IL-2 release**	$IC_{1/2\ max}$ ACEA=ND	ND	5
		CP55,940=ND	ND	5
		Dexamethasone=1.3×10^{-8} M	5.4×10^{-9} - 3.2×10^{-8} M	5
		JWH 015=1.8×10^{-6} M	1.2×10^{-6} –2.9×10^{-6} M	5
		Δ^9-THC=ND	ND	5
		WIN55212-2=8.8×10^{-7} M	2.2×10^{-7} - 3.5×10^{-6} M	5
HT-29	**IL-8 release**	$EC_{1/2\ max}$ ACEA=ND	ND	6
		CP55,940=1.2×10^{-7} M	3.8×10^{-8} - 3.6×10^{-7} M	6
		Δ^9-THC=5.3×10^{-8} M	9.7×10^{-9} -2.9×10^{-7} M	6
		JWH 015=9.8×10^{-8} M	6.8×10^{-8} -1.3×10^{-7} M	6
		WIN55212-2=1.7×10^{-7} M	1.2×10^{-7} - 2.5×10^{-7} M	6
		WIN55212-3=ND	ND	6

Abbreviations: ND=not determined, CL= confidence limit, PBMC=peripheral blood mononuclear cells, EC_{50}=50% effective concentration, $EC_{1/2\ max}$=1/2 effective concentration, $IC_{1/2\ max}$=1/2 inhibitory concentration

8.4 Concluding remarks

An important message from this thesis is that the cannabinoid system is currently a promising pharmacological target not only for the treatment of CNS disorders but also for future development of immuno-modulating and anti-inflammatory drugs. The preferential expression of cannabinoid CB_2 receptors in immune cells and peripheral tissues holds out the promise for the use of this class of compounds to treat immune and inflammatory diseases with selective cannabinoids that are devoid of psychotropic effects. The development of "a so-called" non-steroidal steroid such as a cannabinoid CB_2 receptor agonist as an anti-inflammatory would become a medical milestone of the twenty second century taking into account the undesirable effects of the glucocorticoids and their congeners. Furthermore, the discovery of endogenous ligands to these receptors capable of mimicking the pharmacological actions of Δ^9-THC, including an ability to alter immune cell function (Lee *et al.*, 1995) has provided additional evidence for the immuno-regulatory roles of endogenous cannabinoids.

8.5 Future Work

8.5.1 An investigation into the effect of cannabinoids in the inhibition of IL-8 release from intact human colonic epithelium.

Data presented in chapter 6 of this thesis suggests that cannabinoids could inhibit IL-8 release from HT-29 cells. HT-29 cells employed in this study are human colonic epithelial cell line capable of secreting IL-8 (Schuerer- Maly *et al.*, 1994). While cell lines offer useful tools to explore pharmacological actions of candidate drugs, one should take into account the artificial nature of these cells and take care in extrapolating data obtained to native cells. Experience suggests that cell lines, such as HT-29 cells, differ in at least one respect from an intact human colonic epithelium.

For example, data presented in chapter 6 of this thesis suggest that HT-29 cells evokes minimal constitutive release of IL-8 whereas primary human colonic epithelial cells release large amounts of IL-8 in the absence of any external stimulus (Ihenetu and Baird, unpublished observation). These studies suggest that trauma or a different mechanism may also regulate the release of IL-8 from HT-29 cells and intact human colonic epithelial cells.

It would, therefore, be interesting to extend the observations described in this thesis (chapter 6), to investigate whether cannabinoid receptor agonists modulate cytokine-induced release of IL-8 from intact human primary epithelial cells. Interestingly, sections of intact human colon can be obtained from most gastro-intestinal (GI) surgery departments post-operatively, after obtaining the consent of an ethical committee and the patient. The entire underlying smooth muscle layer could be dissected off leaving the intact human epithelial cells (Mazzucchelli *et al.*, 1994). These tissues could in turn be sectioned into pieces and incubated in culture plates or custom designed manifolds. Following incubation for a chosen period of time, chemokine/cytokine release by ELISA could be assessed. Alternatively, tissues could be placed in "Ussing chambers" to study the effects of cannabinoids on intestinal secretion.

Another interesting possibility is to employ an *in vivo* model of rat colitis, such as that described by Sykes *et al.*, (1999) to assess the potential efficacy of cannabinoid receptor ligands in the treatment of inflammatory bowel disease. In this study, locally administered trinitrobenzenesulphonic acid (TNBS) was used to induce colitis in rats and to assess the anti-inflammatory actions of the matrix metalloproteinase inhibitor

marimastat by measuring cytokine release and assessing histological sections from treated and untreated groups. In this context, cannabinoids could be used instead of marimastat and its efficacy in alleviating the symptoms of IBD assessed.

8.5.2 Signalling pathways regulating cannabinoid evoked inhibition of chemokine release from the human colonic epithelial cells.

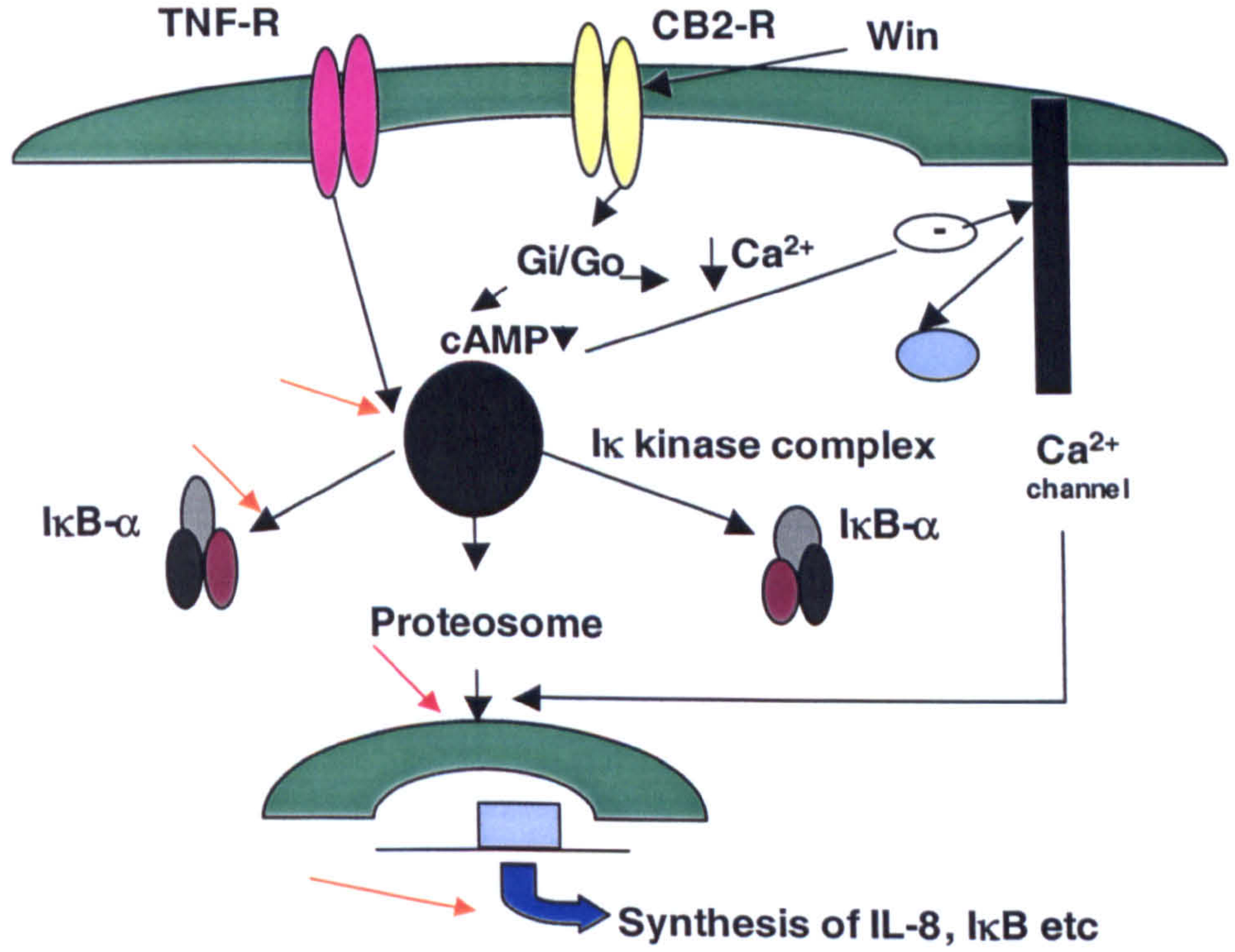


Figure 8.5.2. The schematic representation of events leading to IL-8 gene transcription and possible sites of action (red arrow) of cannabinoids.

Data presented in chapter 6 of this thesis suggests a role for intracellular free calcium and intracellular cyclic AMP in mediating cannabinoid – evoked inhibition of IL-8 release from TNF-α stimulated HT-29 cells. At present, the point at which all these intracellular signalling pathways converge to evoke the observed effects is not known. However, NF-κB (p65/p50) is known to play key roles in the expression of many genes including IL-8 as described elsewhere in this thesis (refer to figure 8.5.2). NF-κB exists in the cytoplasm as an inactive dimer bound to an inhibitory protein, IκB. A variety of extracellular signals including TNF-α have the ability to phosphorylate IκB at specific amino acid termini residues (DiDonato *et al.*, 1997; Mercurio *et al.*,

1997). The phosphorylated IκB is selectively ubiquitinated (Yaron *et al.*, 1998; Maniatis, 1999). In turn ubiquitinated IκB is degraded by a 26 s proteosome allowing NF-κB to translocate to the nucleus, where it binds to its target and initiates transcription of IL-8 (Yaron *et al.*, 1998; Maniatis, 1999).

Inhibition of IL-8 release from HT-29 cells by cannabinoids may result from an action at one or more of the multi-enzymic steps involved in this cascade as stated above (Figure 8.1). Techniques such as Western immunoblotting or gel shift assays (Gerwitz *et al.*, 2000) for any of these transcription factors could be used to localise the specific site of action of cannabinoids in this pathway.

8.5.3 Characterisation of cannabinoid receptors mediating cannabinoid-induced - apoptosis in Jurkat and epithelial cell line in vitro

Data presented in chapter 7 of this thesis suggests that endogenous cannabinoids have a unique ability to induce apoptosis in Jurkats and HT-29 cells. However, the cannabinoid receptors mediating these events were not studied. It would be worthwhile therefore to carry out these experiments in the presence of cannabinoid receptor antagonists and selective cannabinoid receptor ligands in order to characterise the receptors mediating these events. The identification of cannabinoid receptors mediating cannabinoid-induced apoptosis of human immune cells can then form the basis of rational drug design aimed at treating chronic inflammatory conditions and cancer.

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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.

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.

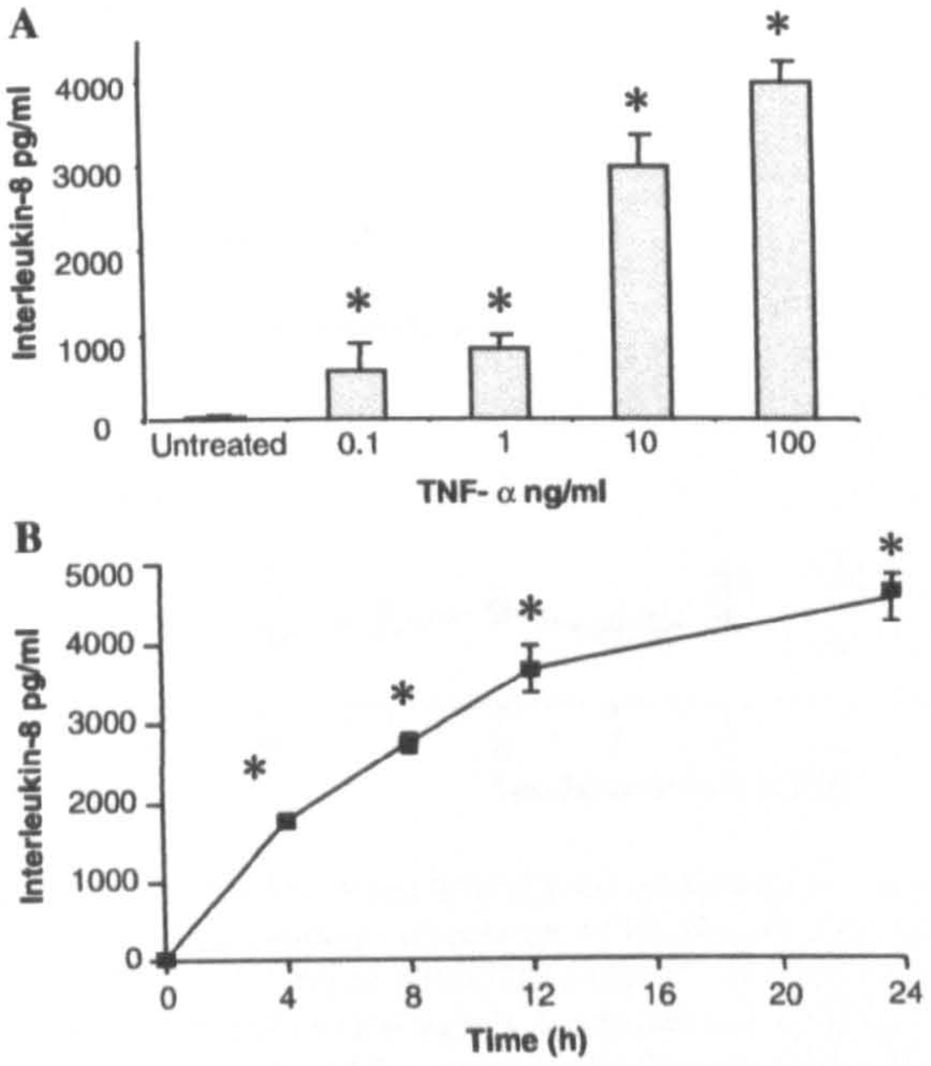

Fig. 1. TNF-α-induced release of interleukin-8 from HT-29 cells in vitro. (A) Confluent monolayers of HT-29 cells were stimulated with TNF-α (0.1–100 ng/ml) in foetal calf serum-free McCoy's 5A medium for 24 h. (B) Confluent monolayers of HT-29 cells were stimulated with TNF-α (100 ng/ml) in foetal calf 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.

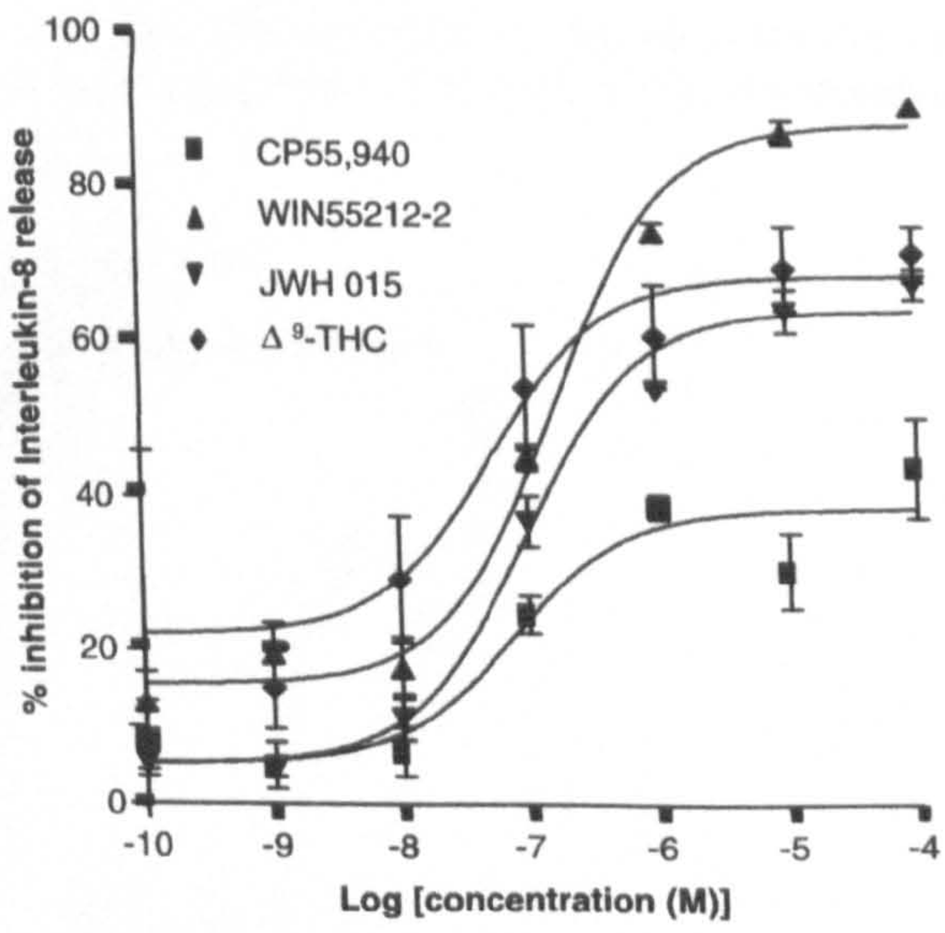

Fig. 2. Inhibition of TNF-α-induced interleukin-8 release by cannabinoids. Confluent monolayers of HT-29 cells were treated with CP55,940 (10^{-4}–10^{-10} M), WIN55,212-2 (10^{-10}–10^{-4} M), Δ^9-Tetrahydrocannabinol (10^{-10}–10^{-4} M) and JWH 015 (10^{-10}–10^{-4} M) for 2 h before stimulation with 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.

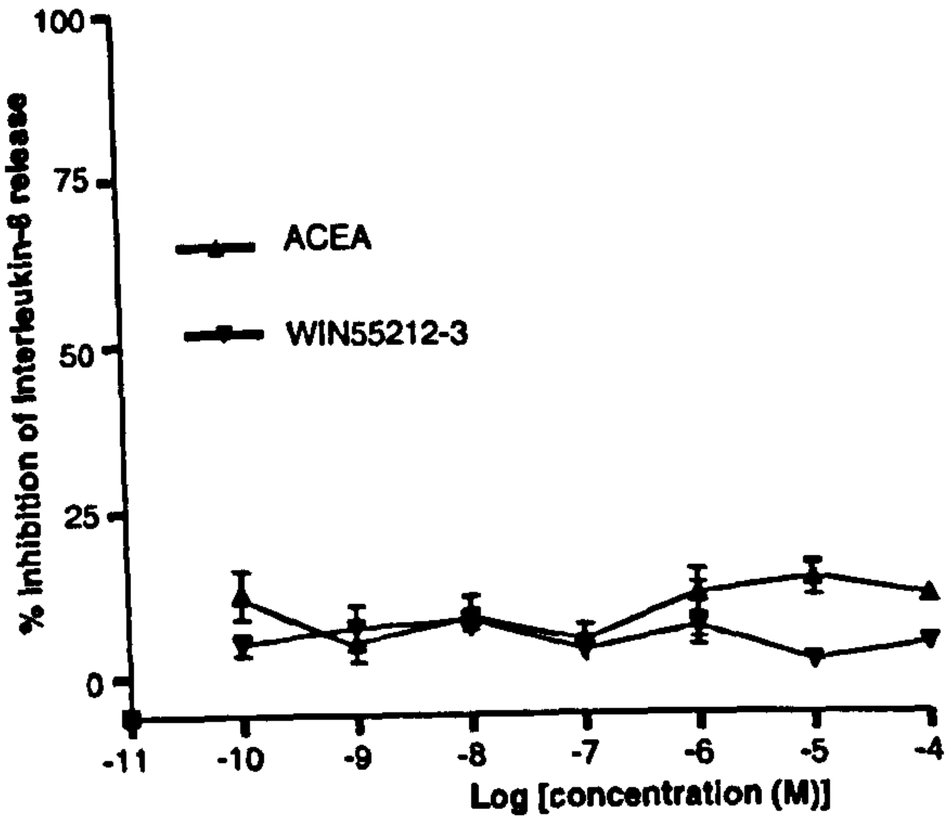

Fig. 3. The effect of ACEA and WIN55212-3 on the release of interleukin-8 from HT-29 cells. Confluent monolayers of HT-29 cells were treated with ACEA (10^{-10}–10^{-4} M) or WIN55212-3 (10^{-10}–10^{-4} M) for 2 h before stimulation with TNF-α (100 ng/ml). Incubation was continued for 24 h. Supernatants 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,

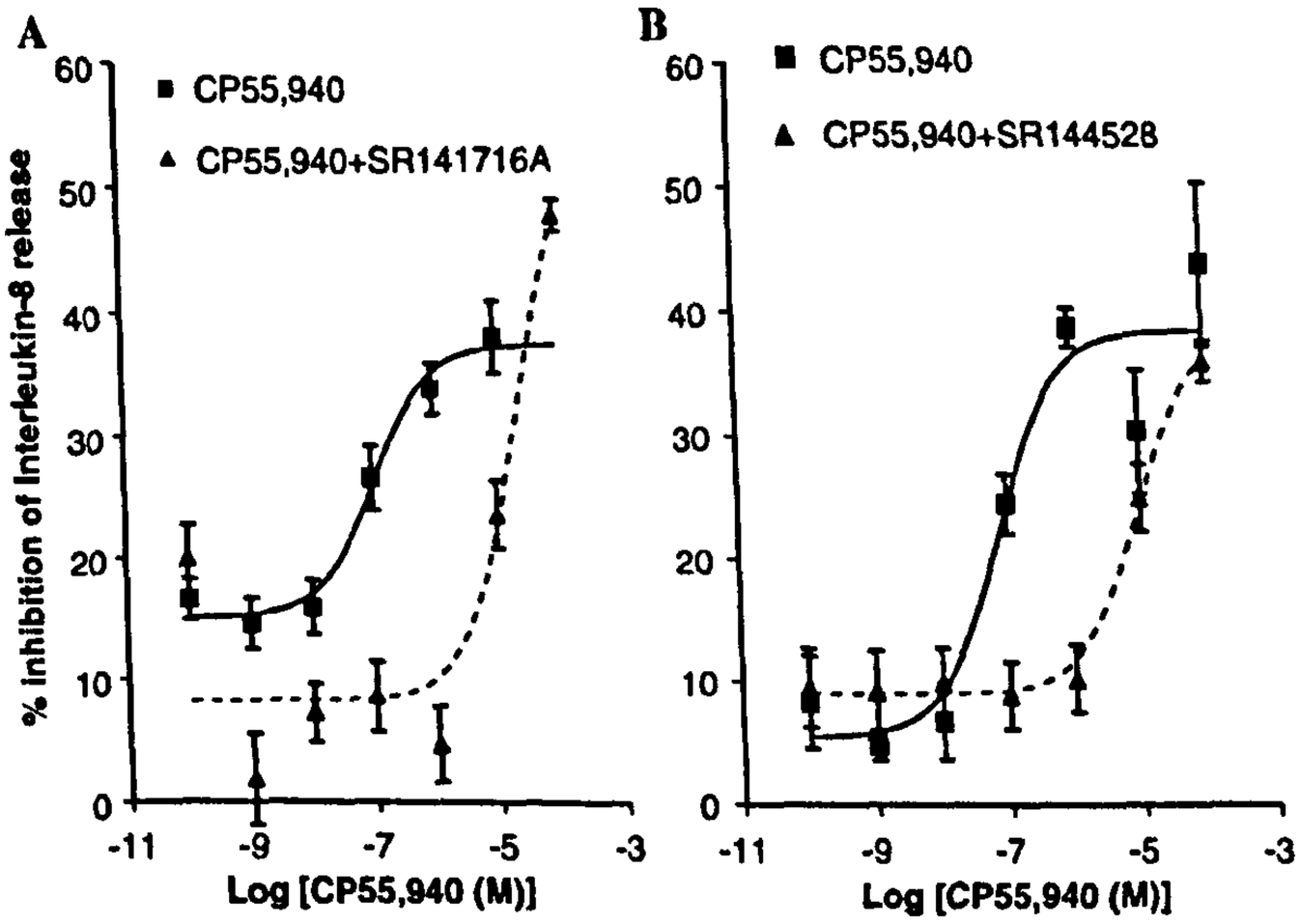

Fig. 4. The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by CP55,940. Confluent monolayers of HT-29 cells were incubated with SR141716A (10^{-6} M) (A) or SR144528 (10^{-6} M) (B) for 30 min before treatment with CP55,940 (10^{-10}–10^{-4} 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.

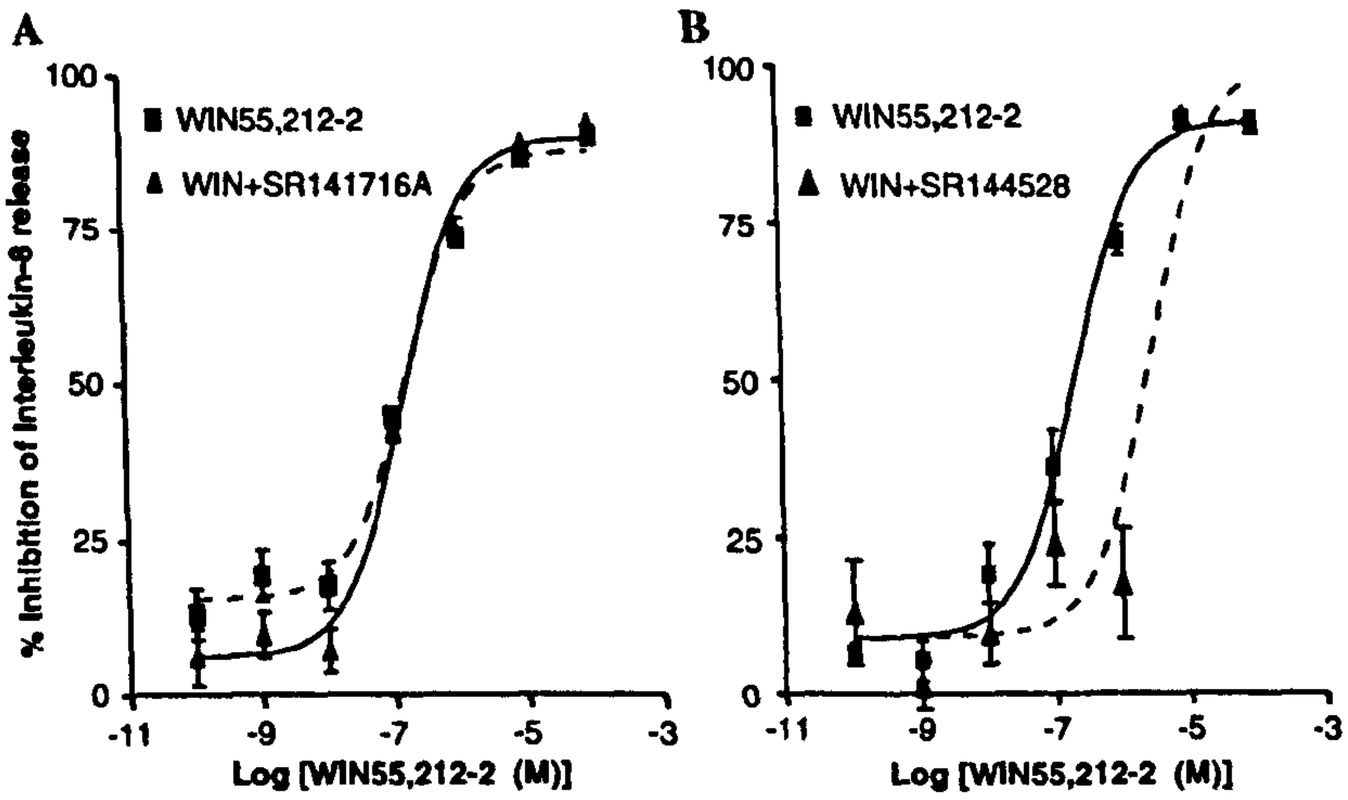

Fig. 5. The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by WIN55212-2. Confluent monolayers of HT-29 cells were incubated with SR141716A (10^{-6} M) (A) or SR144528 (10^{-6} M) (B) for 30 min before treatment with WIN55212-2 (10^{-10}–10^{-4} 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

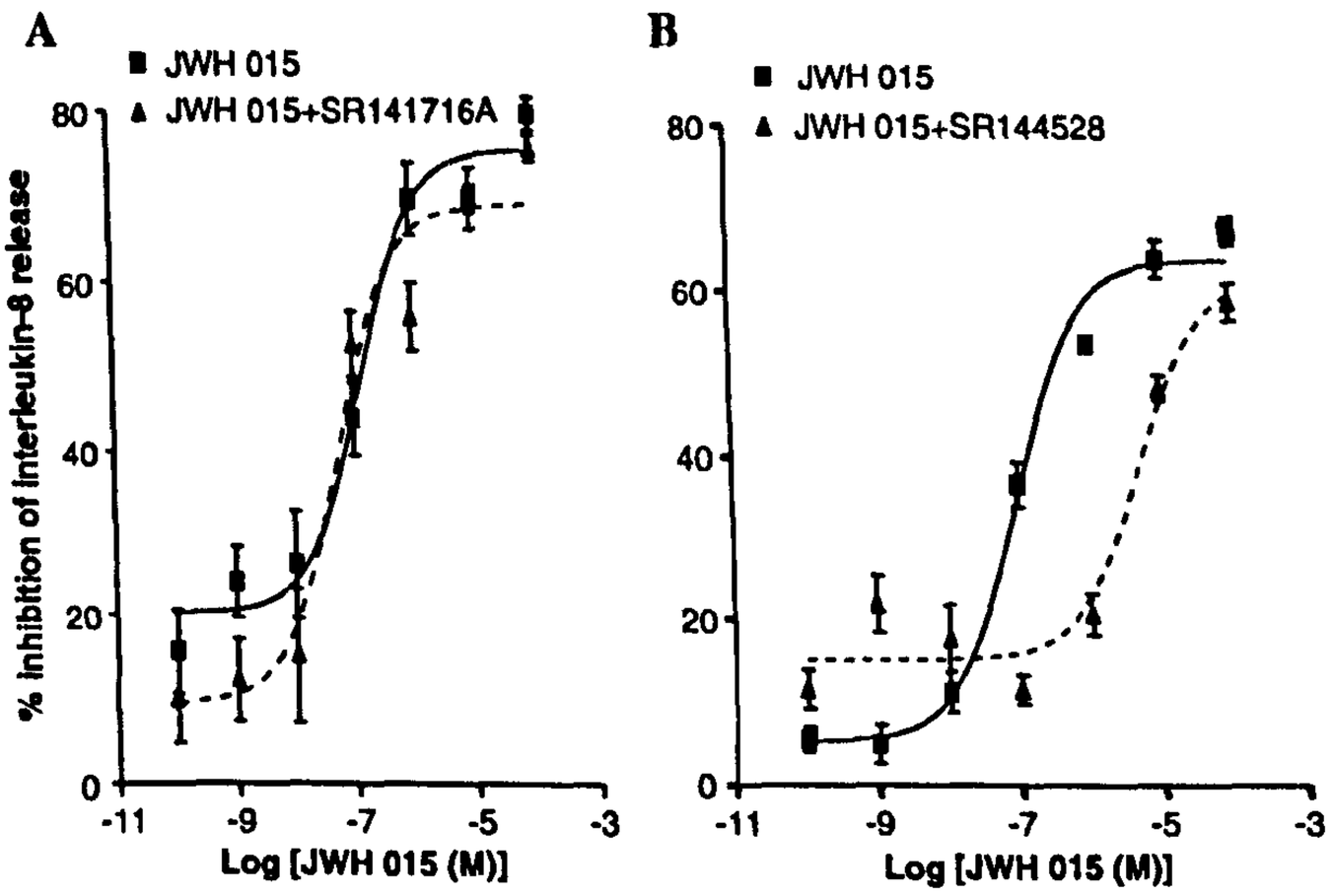

Fig. 6. The effect of SR141716A (10^{-6} M) and SR144528 (10^{-6} M) on the inhibition of TNF-α-induced interleukin-8 release by JWH 015. Confluent monolayers of HT-29 cells were incubated with SR141716A (10^{-6} M) or SR144529 (10^{-6} M) for 30 min before treatment with JWH 015 (10^{-10}–10^{-4} M) 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

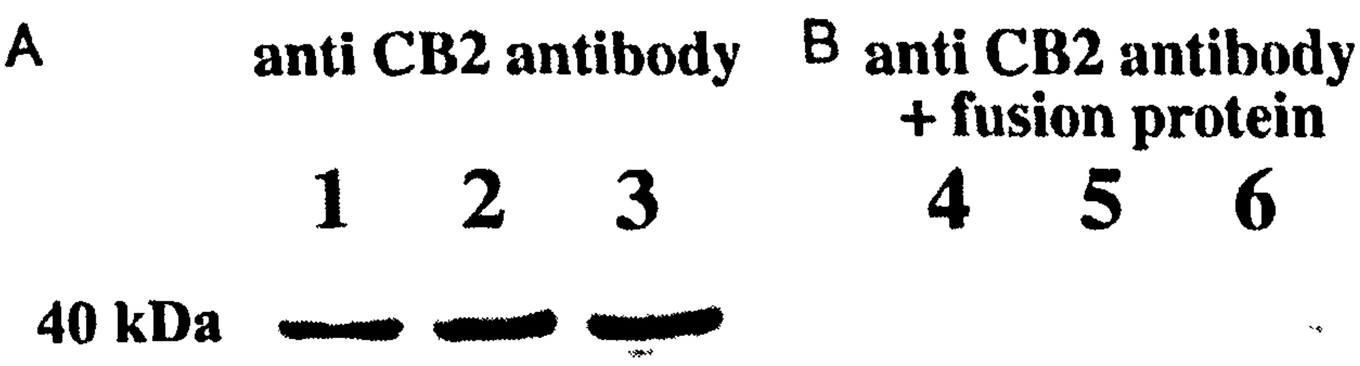

Fig. 7. Western immunoblotting for cannabinoid CB_2 receptor protein in HT-29 cells. Cell lysates (40 μg protein/lane) obtained from HT-29 cells were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and probed with polyclonal anti-cannabinoid CB_2 receptor antibody and anti-cannabinoid 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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Pharmacological characterisation of cannabinoid receptors inhibiting interleukin 2 release from human peripheral blood mononuclear cells

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Abstract

The effects of a range of cannabinoid receptor agonists and antagonists on phytohaemagglutinin-induced secretion of interleukin-2 from human peripheral blood mononuclear cells were investigated. The nonselective cannabinoid receptor agonist WIN55212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-[4-morpholinylmethyl]pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthyl) methanone mesylate) and the selective cannabinoid CB_2 receptor agonist JWH 015 ((2-methyl-1-propyl-1*H*-indol-3-yl)-1-napthalenylmethanone) inhibited phytohaemagglutinin (10 μg/ml)-induced release of interleukin-2 in a concentration-dependent manner ($IC_{1/2max}$, WIN55212-2 = 8.8×10^{-7} M, 95% confidence limits (C.L.) = 2.2×10^{-7}–3.5×10^{-6} M; JWH 015 = 1.8×10^{-6} M, 95% C.L. = 1.2×10^{-6}–2.9×10^{-6} M, $n = 5$). The nonselective cannabinoid receptor agonists CP55,940 ((−)-3-[2-hydroxy-4-(1,1-dimethyl-hepthyl)-phenyl]4-[3-hydroxypropyl]cyclo-hexan-1-ol), Δ^9-tetrahydrocannabinol and the selective cannabinoid CB_1 receptor agonist ACEA (arachidonoyl-2-chloroethylamide) had no significant ($P > 0.05$) inhibitory effect on phytohaemagglutinin-induced release of interleukin-2. Dexamethasone significantly ($P < 0.05$) inhibited phytohaemagglutinin-induced release of interleukin-2 in a concentration-dependent manner ($IC_{1/2max} = 1.3 \times 10^{-8}$ M, 95% C.L. = 1.4×10^{-9}–3.2×10^{-8} M). The cannabinoid CB_1 receptor antagonist SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride) (10^{-6} M) did not antagonise the inhibitory effect of WIN55212-2 whereas the cannabinoid CB_2 receptor antagonist SR144528 (*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) antagonised the inhibitory effect of WIN55212-2 ($pA_2 = 6.3 \pm 0.1$, $n = 5$). In addition, CP55,940 (10^{-6} M) and Δ^9-tetrahydrocannabinol (10^{-6} M) also antagonised the inhibitory effects of WIN55212-2 ($pA_2 = 6.1 \pm 0.1$, $n = 5$ and $pA_2 = 6.9 \pm 0.2$, $n = 5$). In summary, WIN55,212-2 and JWH 015 inhibited interleukin-2 release from human peripheral blood mononuclear cells via the cannabinoid CB_2 receptor. In contrast, CP55,940 and Δ^9-tetrahydrocannabinol behaved as partial agonists/antagonists in these cells.

Keywords: Cannabinoid; Interleukin-2; Phytohaemagglutinin; Peripheral blood mononuclear cell

1. Introduction

Cannabinoids have been shown to downregulate the immune system (for reviews, see Cabral and Dove Pettit, 1998; Berdyshev, 2000). This conclusion is partly based on an early in vivo study by Morahan et al. (1979) who demonstrated a decreased resistance of mice to *Listeria monocytogens* or *Herpes simplex* virus infections after treatment with Δ^9-tetrahydrocannabinol. Consistent with these findings are a number of in vitro studies in which cannabinoids have been reported to inhibit T cell mitogenesis and interleukin-2 production from T lymphocyte cell lines (for reviews, see Klein et al., 1998a,b).

Interleukin-2 is an important cytokine responsible for T lymphocyte signalling during proliferation and macrophage/monocyte activation during inflammatory episodes (Herrman et al., 1989). The expression of functional interleukin-2 receptors is another variable that determines how long the clonal proliferation of T cells occurs after antigen stimulation (Smith, 1988). In general, interleukin-2 regulates both antigen-specific and non-antigen-specific proliferation of T cells, natural killer cells and B cells.

The discovery and cloning of two cannabinoid receptors, CB_1 and CB_2, has begun to give new clues as to how these drugs affect the immune system (Matsuda et al., 1990; Munro et al., 1993). Cannabinoid receptors are members of the G-protein-coupled receptor family (Bayewitch et al., 1995).

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While cannabinoid CB_1 receptors are found in the brain with low levels of expression in the peripheral tissues, cannabinoid CB_2 receptors are expressed primarily in immune tissues (Bouaboula et al., 1993; Galiegue et al., 1995; Kaminski et al., 1992), suggesting that the majority of the immunomodulatory properties of cannabinoids may be mediated via cannabinoid CB_2 receptors, although to date, very few studies have been reported to support this hypothesis.

The density of cannabinoid CB_2 receptors on immune cells is 10–100 times that of cannabinoid CB_1 receptors, as shown by semi-quantitative reverse transcription polymerase chain reaction and Northern blotting studies (Galiegue et al., 1995). The rank order of cannabinoid CB_2 receptor expression on human blood leukocytes is B cells>NK cells>monocytes>polymorphonuclear neutrophils>T8 cells>T4 cells (Parolaro, 1999). Furthermore, it has been shown that cannabinoid receptor expression in peripheral blood mononuclear cells is altered upon stimulation with phytohaemagglutinin (Daaka et al., 1996), suggesting an active role for the cannabinoid system in immune responses.

Given the proinflammatory properties of interleukin-2, modulation of its release via cannabinoid receptors would present an attractive pharmacological target for the treatment of various inflammatory conditions. In the present study, the effects of cannabinoid receptor ligands on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells have been investigated. A preliminary account of part of this report has been presented in abstract form to The International Cannabinoid Research Society (Ihenetu et al., 2002).

2. Materials and methods

2.1. Drugs and reagents

CP55,940 ((−)-3-[2-hydroxy-4-(1,1-dimethyl-heptyl)-phenyl]4-[3-hydroxypropyl]cyclo-hexan-1-ol) was a generous gift from Pfizer. SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride) and SR144528 (*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) were gifts from Sanofi Recherche (Montpellier, France). WIN55212-2 mesylate ((*R*)-(+)-[2,3-dihydro-5-methyl-3-[4-morpholinylmethyl]pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthyl) methanone mesylate), ACEA (arachidonoyl-2-chloroethylamide) and JWH 015 ((2-methyl-1-propyl-1*H*-indol-3-yl)-1-napthalenylmethanone) 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). CP55,940, SR141716A, SR144528 and ACEA were dissolved in ethanol whereas WIN55,212-2 and JWH 015 were dissolved in dimethyl sulphoxide (DMSO) and stored at −20 °C at a concentration of 10 mM. Accordingly, these solvents were included in all assays at a final concentration of 0.1% as vehicle controls.

2.2. Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from buffy coat cells purchased from the National Blood Transfusion Service (NBTS) (Brentwood, Essex, UK). Separation of peripheral blood mononuclear cells was done by density gradient centrifugation using Histopaque R-1077 (Sigma-Aldrich), based on the modification of the original method described by Boyum (1968). In brief, buffy coat cells were diluted (1:2, v/v) with sterile phosphate-buffered saline and human peripheral blood mononuclear cells were isolated by density gradient centrifugation (2500 × *g* for 25 min) in an Accuspin tube (Sigma-Aldrich). Cells recovered from the interface between the plasma and Histopaque solution were washed twice in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (1700 × *g* for 10 min). Peripheral blood mononuclear cells were resuspended in RPMI-1640 medium supplemented with L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml), and 10% heat-inactivated foetal calf serum. Aliquots were removed and cells were counted and assayed for viability by the trypan blue dye exclusion method and the MTT assay. Slides of the cell suspension were made, stained with a Romanowsky stain (May Grunwald-Giemsa) and a differential cell count obtained by examination of the slide under a microscope (magnification 1000 ×).

2.3. Interleukin-2 secretion

Human peripheral blood mononuclear cells were adjusted to a density of 1×10^6 cells/ml with RPMI-1640 medium and cultured in 24-well plates (Falcon, Becton Dickinson, Pont De Claire, France) in foetal calf serum-free RPMI-1640 medium, at 37 °C in a humidified atmosphere with 5% CO_2. Cells were preincubated with CP55940 (10^{-10}–10^{-5} M), WIN55212-2 (10^{-10}–10^{-5} M), Δ^9-tetrahydrocannabinol (10^{-10}–10^{-5} M), JWH 015 (10^{-10}–10^{-5} M) or dexamethasone (10^{-10}–10^{-6} M) for 2 h before stimulation with phytohaemagglutinin (10 μg/ml). Supernatants were harvested after 18 h incubation and stored at −70 °C until assayed for interleukin-2 by ELISA. In experiments where the effects of antagonists were studied, cells were preincubated with SR141716A (10^{-6} M), SR144528 (10^{-6} M), CP55940 (10^{-6} M) or Δ^9-tetrahydrocannabinol (10^{-6} M) for 30 min before the addition of the cannabinoid agonist or dexamethasone.

2.4. Enzyme-linked immunosorbent assay

Interleukin-2 release was measured by enzyme-linked immunosorbent assay (ELISA) of the culture supernatants according to the manufacturer's guidelines. In brief, anti-human interleukin-2 monoclonal capture antibody (Pharmingen B.D., Oxford, UK; cat. no. 555051) was paired with

biotinylated anti-human interleukin-2 monoclonal detection antibody (cat. no. 555040). Ninety-six-well plates (Nunc-immunoplates maxisorp F96, Pharmingen B.D.), were coated with 1 μg/ml capture antibody at 4 °C for 24 h. Following washing, blocking and addition of standards (10–2000 pg/ml) and samples (undiluted), a one-step detection comprising the use of biotinylated antibody/streptavidin-linked peroxidase (both 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.

2.5. Statistical analysis

Concentration–effect curves were analysed by Prism (GraphPad, San Diego, CA, USA). Other results are shown as bar graphs. In some experiments, the results are expressed as percentage inhibition of interleukin-2 release from phytohaemagglutinin-treated cells. $IC_{1/2max}$ 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 ($IC_{1/2max}$ values) ± standard error of the mean (S.E.M.) or 95% confidence limit (C.L.) as appropriate. Statistical significance was determined using a one-sample *t*-test or analysis of variance followed by an appropriate post hoc test. Statistical significance was assumed if *P* value was ≤ 0.05.

3. Results

3.1. Purity and viability of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cell preparations, prepared from buffy coat cells, comprised approximately 95% lymphocytes and 5% monocytes as measured by differential leukocyte counts. Furthermore, after 18 h incubation in serum-free medium, 99.17% ± 4.99% ($n=4$) of the lymphocytes were recovered from the medium.

Under our experimental conditions, the viability of human peripheral blood mononuclear cells isolated from buffy coat cells exceeded 95% on all occasions, when determined by trypan blue dye exclusion and by the MTT assay. This viability was not significantly ($P>0.05$) altered by incubation of human peripheral blood mononuclear cells for 18 h with phytohaemagglutinin, dexamethasone or any of the cannabinoid receptor ligands studied in foetal calf serum-free RPMI-1640 medium.

3.2. The effect of phytohaemagglutinin on interleukin-2 secretion from human peripheral blood mononuclear cells

Nonstimulated human peripheral blood mononuclear cells constitutively released minimal amounts of interleukin-2 (14 ± 10 pg/ml, $n=5$) after 18 h incubation at 37 °C (Fig. 8). Following stimulation with phytohaemagglutinin (10 μg/ml), a marked release of interleukin-2 was observed over 18 h (1869 ± 54 pg/ml, $n=5$, Fig. 1). Stimulation of human peripheral blood mononuclear cells with phytohaemagglutinin (10 μg/ml) evoked a minimal release of interleukin-2 within the first 6 h and a rise between 12 and 18 h. The peak release of interleukin-2 was seen at 18 h (Fig. 1). There was no significant change ($P>0.05$) in cell numbers between phytohaemagglutinin (10 μg/ml)-stimulated and nonstimulated cells over 18 h following incubation at 37 °C in foetal calf serum-free medium (data not shown). Vehicle controls (0.1% ethanol and 0.1% DMSO) had no significant ($P<0.05$) inhibitory effect on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells.

3.3. The effect of cannabinoid receptor agonists on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells

The nonselective cannabinoid receptor agonist WIN5521 2-2 (10^{-10}–10^{-5} M) and a selective cannabinoid CB_2 receptor agonist JWH 015 (10^{-10}–10^{-5} M) inhibited phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells (Fig. 2). This inhibition was concentration-related and significant ($P<0.05$) over the concentration range 10^{-6}–10^{-5} M ($IC_{1/2max}$, WIN55212-2 = 8.8×10^{-7} M, 95% C.L. = 2.2×10^{-7}–3.5×10^{-6} M, JWH 015 = 1.8×10^{-6} M, 95% C.L. = 1.2×10^{-6}–2.9×10^{-6} M, $n=5$). The nonselective cannabinoid receptor agonist CP55,940 (10^{-10}–10^{-6} M) produced a small, nonsignificant ($P>0.05$) inhibition of interleukin-2 release from human peripheral blood

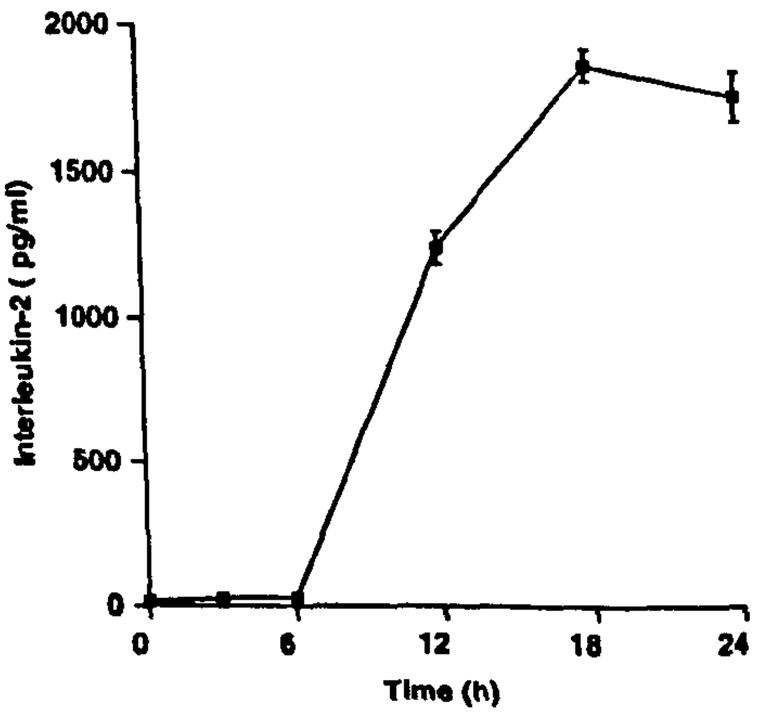

Fig. 1. Time course of phytohaemagglutinin-induced interleukin-2 release from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg/ml) for 3, 6, 12, 18 and 24 h. Cell-free supernatants were harvested for interleukin-2 assay by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments.

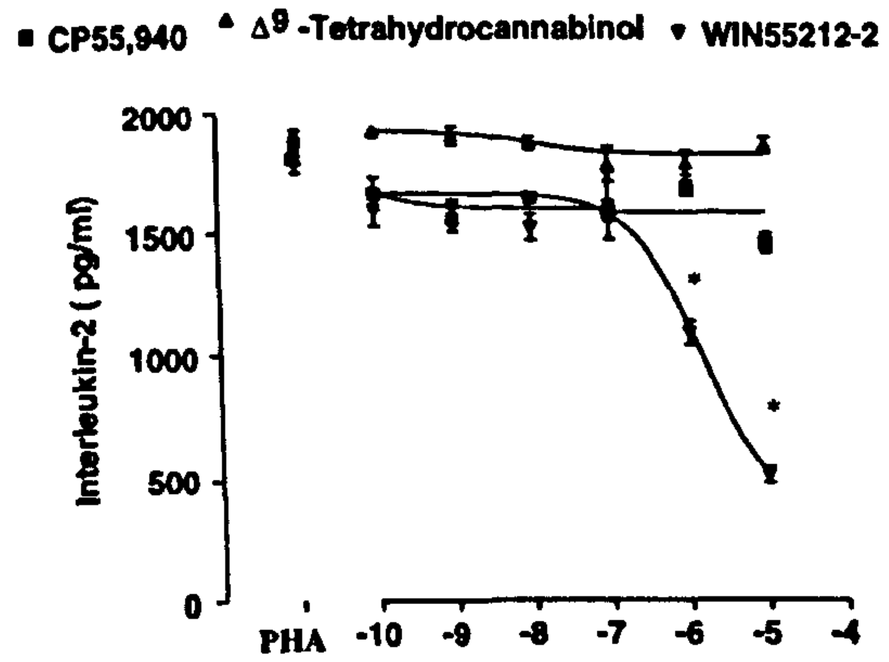

Fig. 2. Effect of nonselective cannabinoid agonists on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were treated with CP55,940 (10^{-10}–10^{-5} M), Δ^9-tetrahydrocannabinol (10^{-10}–10^{-5} M) or WIN55212-2 (10^{-10}–10^{-5} M) for 2 h before stimulation with phytohaemagglutinin (10 μg/ml) for a further 18 h. Cell-free supernatants were harvested and assayed for interleukin-2 by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments. *Denotes significant difference ($P<0.05$) from the control (phytohaemagglutinin-treated cells) (Student's *t*-test).

mononuclear cells (Fig. 2). The nonselective cannabinoid receptor agonist Δ^9-tetrahydrocannabinol (10^{-10}–10^{-6} M) and the selective cannabinoid CB_1 receptor agonist ACEA (10^{-10}–10^{-6} M) also had no significant ($P>0.05$) inhibitory effect on the release of interleukin-2 from human peripheral blood mononuclear cells. As a positive control, dexamethasone (10^{-10}–10^{-6} M), a glucocorticoid, significantly ($P<0.05$) inhibited phytohaemagglutinin-induced interleukin-2 release from human peripheral blood mononuclear cells ($IC_{1/2max} = 1.3 \times 10^{-8}$ M, C.L. = 5.4×10^{-9}–3.2×10^{-8} M, $n=5$, Fig. 3). The maximum inhibition produced by JWH 015 was greater than that produced by WIN55212-2 (Fig. 2).

3.4. The effect of SR141716A and SR144528 on WIN55212-2- and JWH 015-induced inhibition of interleukin-2 from human peripheral blood mononuclear cells

When incubated with human peripheral blood mononuclear cells for 18 h, neither SR141716A (10^{-6} M) nor SR144528 (10^{-6} M) had any significant effect on phytohaemagglutinin-induced interleukin-2 release (interleukin-2 release = 1530.5 ± 80.8 pg/ml ($n=5$) and 1653.4 ± 65.5 pg/ml ($n=5$), respectively) when compared with phytohaemagglutinin-treated controls (1655.7 ± 52.8 pg/ml ($n=9$)). SR141716A (10^{-6} M) had no significant ($P>0.05$) effect in attenuating the inhibitory action of WIN55212-2 on phytohaemagglutinin-induced release of interleukin-2 (Fig. 4). In contrast, SR144528 (10^{-6} M) significantly ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test, $n=5$) antagonised the inhibitory effects of WIN55212-2 on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells ($pA_2 = 6.3 \pm 0.1$, $n=5$) (Fig. 4).

Similarly, SR141716A (10^{-6} M) had no significant ($P>0.05$)effect in attenuating the inhibitory effect of JWH 015 on phytohaemagglutinin-induced release of interleukin-2. In contrast, SR144528 (10^{-6} M) significantly ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test, $n=5$) antagonised the inhibitory effects of JWH 015 on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells ($pA_2 = 6.5 \pm 0.1$, $n=5$) (data not shown).

3.5. The effect of CP55,940 and Δ^9-tetrahydrocannabinol on WIN55212-2-induced inhibition of interleukin-2 from human peripheral blood mononuclear cells

CP55,940 (10^{-6} M) and Δ^9-tetrahydrocannabinol (10^{-6} M) significantly ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test, $n=5$) antagonised the inhibitory effects of WIN55212-2 on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells (Figs. 5 and 6). When pA_2 values were calculated from these data, a value of 6.1 ± 0.1 ($n=5$) was obtained for CP55940 and a value of 6.96 ± 0.16 ($n=5$) for Δ^9-tetrahydrocannabinol.

3.6. Effect of CP55,940 on dexamethasone-induced inhibition of interleukin-2 from human peripheral blood mononuclear cells

CP55,940 (10^{-6} M) had no significant ($P>0.05$) effect in antagonising the inhibitory actions of dexame-

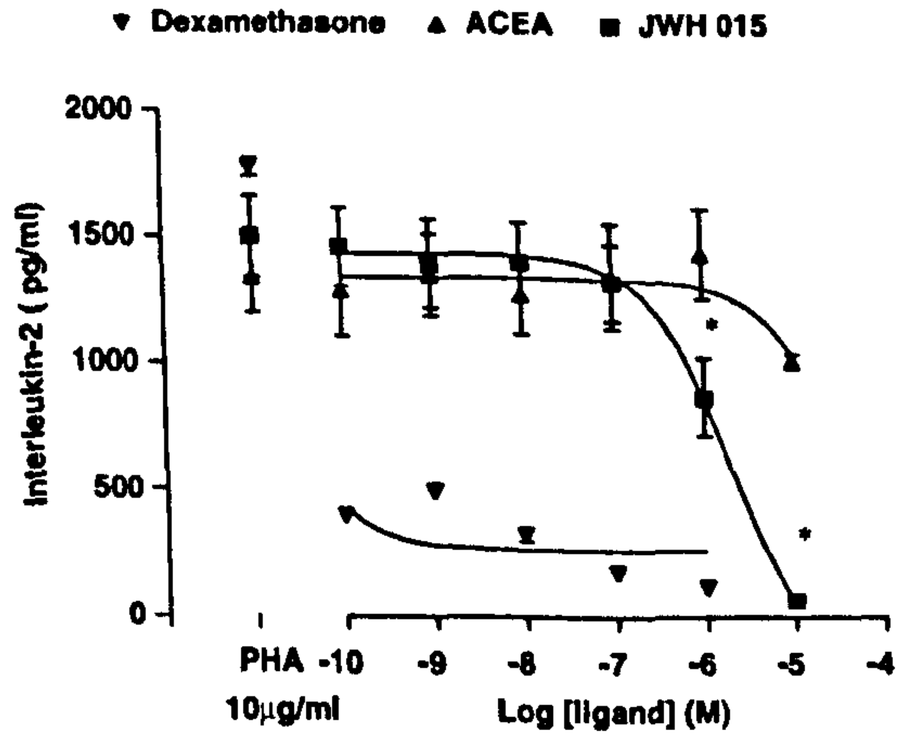

Fig. 3. Effect of selective cannabinoid agonists and dexamethasone on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were treated with ACEA (10^{-10}–10^{-5} M), JWH 015 (10^{-10}–10^{-5} M) or dexamethasone (10^{-10}–10^{-5} M) for 2 h before stimulation with phytohaemagglutinin (10 μg/ml) for a further 18 h. Cell-free supernatants were harvested and assayed for interleukin-2 by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments. *Denotes significant difference ($P<0.05$) from the control (phytohaemagglutinin-treated cells) (Student's *t*-test).

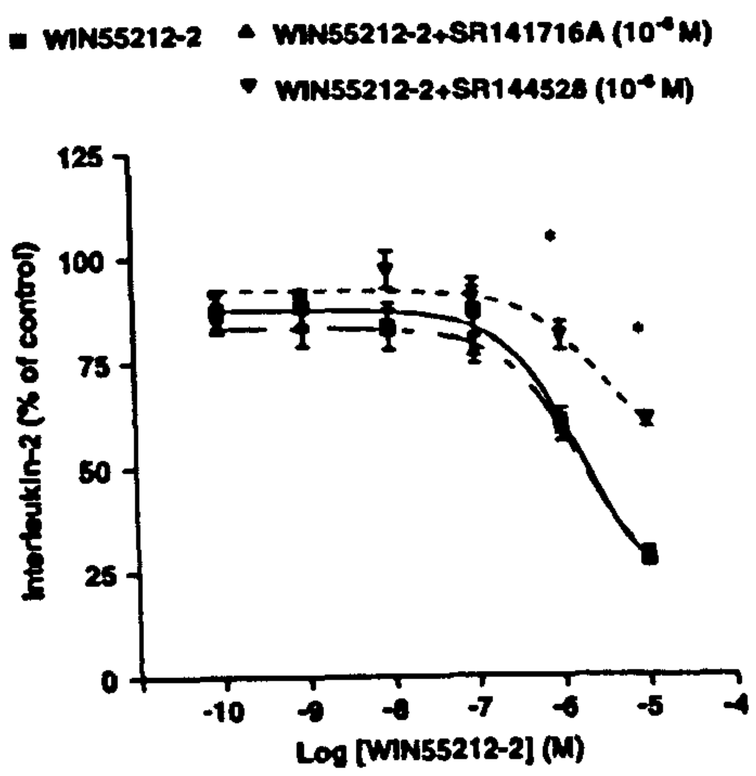

Fig. 4. Effect of SR141716A or SR144528 on WIN55212-2-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were preincubated with SR141716A (10^{-6} M) or SR144528 (10^{-6} M) for 30 min before addition of WIN55212-2 (10^{-10}–10^{-5} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg/ml) for further 18 h. Cell-free supernatants were harvested for interleukin-2 assay by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments. *Denotes significant difference from WIN55212-2-treated cells ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test, $n=5$).

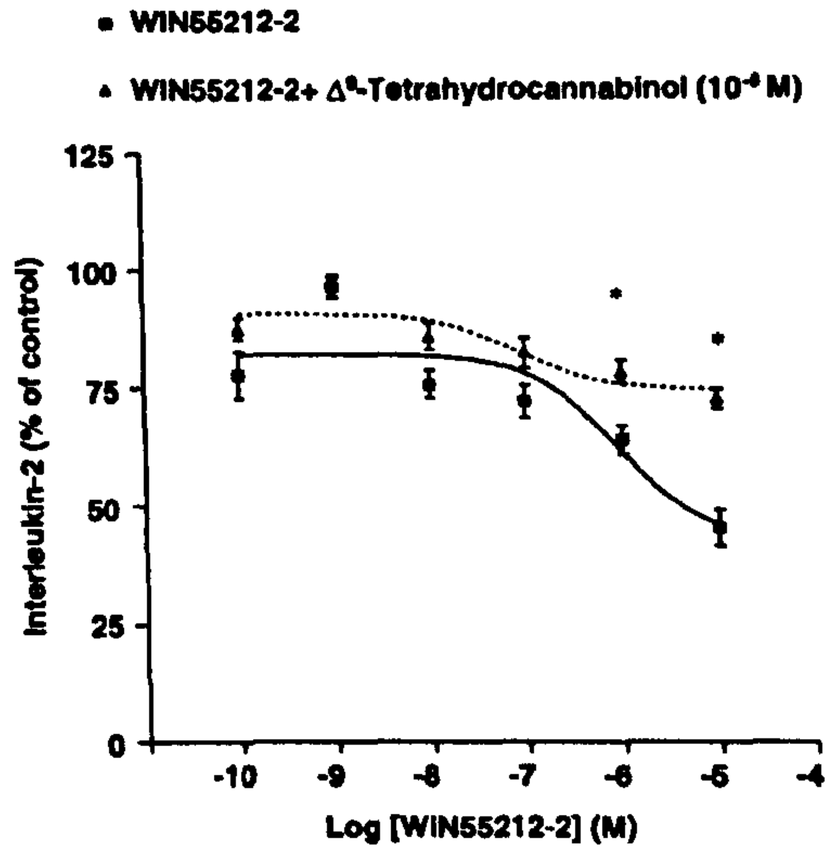

Fig. 6. Effect of Δ^9-tetrahydrocannabinol on WIN55212-2-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were preincubated with Δ^9-tetrahydrocannabinol (10^{-6} M) for 30 min before addition of WIN55212-2 (10^{-10}–10^{-5} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg/ml) for a further 18 h. Cell-free supernatants were harvested for interleukin-2 assay by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments. *Denotes significant difference from WIN55212-2-treated cell ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test).

thasone on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells (Fig. 7).

3.7. Effect of CP55,940 on the release of interleukin-2 from nonstimulated human peripheral blood mononuclear cells

Addition of CP55,940 (10^{-5} M) to nonstimulated human peripheral blood mononuclear cells followed by incubation at 37 °C for 18 h evoked a minimal release of interleukin-2

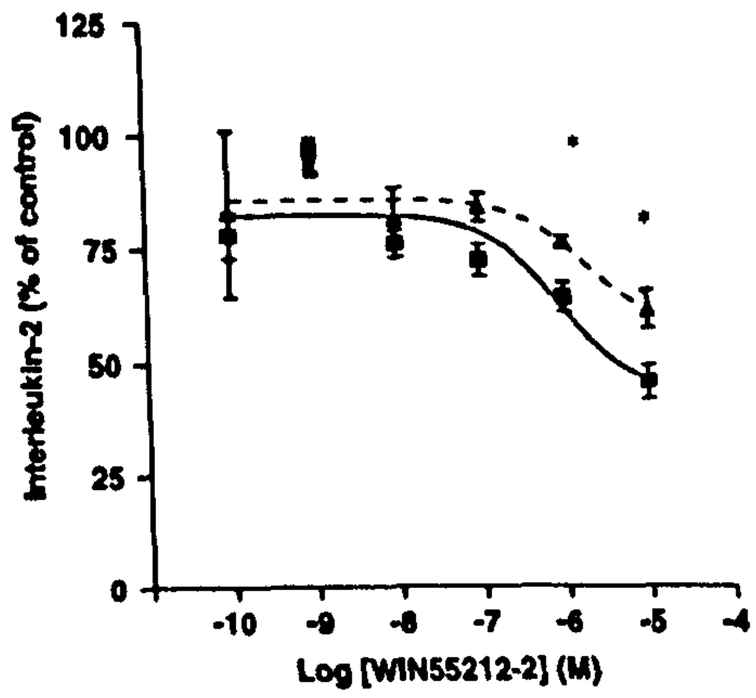

Fig. 5. Effect of CP55940 on WIN55212-2-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were preincubated with CP55, 940 (10^{-6} M) for 30 min before addition of WIN55212-2 (10^{-10}–10^{-5} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg/ml) for a further 18 h. Cell-free supernatants were harvested for interleukin-2 assay by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments. *Denotes significant difference from WIN55212-2-treated cells ($P<0.05$, two-way ANOVA followed by Bonferroni's post hoc test, $n=5$).

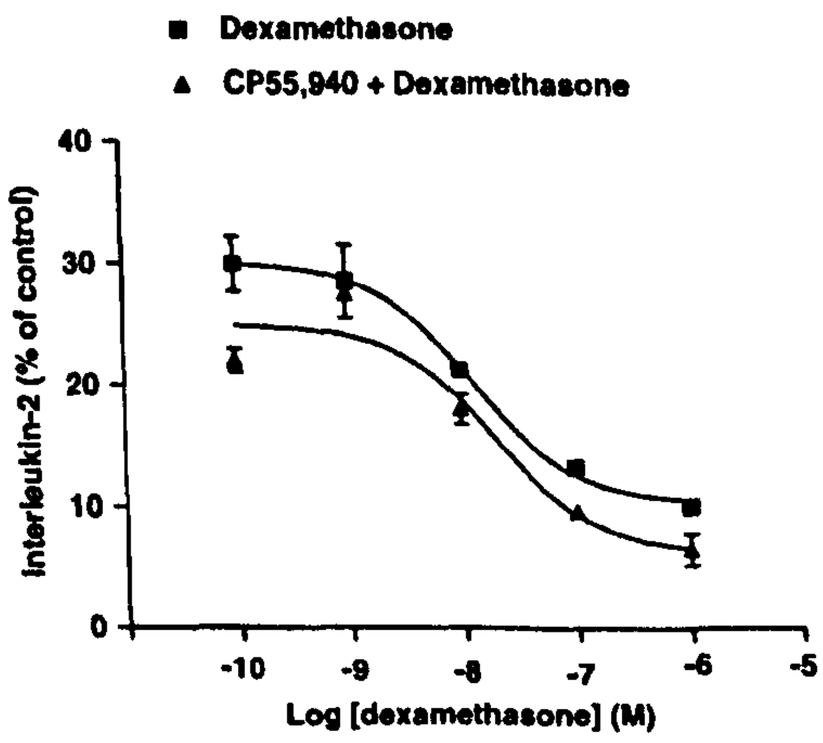

Fig. 7. Effect of CP55,940 on dexamethasone-induced inhibition of interleukin-2 release from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were preincubated with CP55,940 (10^{-6} M) for 30 min before addition of dexamethasone (10^{-10}–10^{-6} M) for 2 h. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg/ml) for a further 18 h. Cell-free supernatants were harvested for interleukin-2 assay by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments.

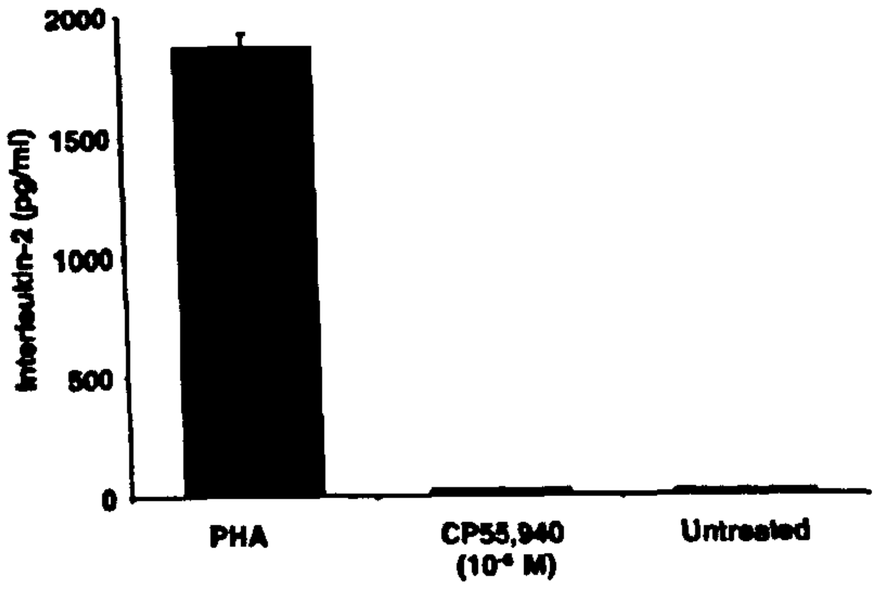

Fig. 8. Effect of CP55,940 on the secretion of interleukin-2 from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were stimulated with phytohaemagglutinin (10 μg/ml) or CP55,940 (10^{-5} M) for 18 h. Cell-free supernatants were harvested for interleukin-2 assay by ELISA as described in Materials and methods. Data are means and S.E.M. of five separate experiments.

(21.8 ± 6.3 pg/ml, $n=5$), which was not significantly ($P>0.05$) different from the basal release (Fig. 8).

4. Discussion

Cannabinoid receptor ligands have potential utility as anti-inflammatory drugs for the treatment of many disease conditions primarily because of their immunosuppressive actions, but their psychoactive effects limit their therapeutic benefits. Emerging evidence suggests that cannabinoids produce many of their immunosuppressive effects by inhibiting T cell responses (for reviews, see Klein et al., 1998a,b; Parolaro, 1999). A significant proportion of these studies have been conducted on cell lines and transfected cells derived from rats or mice (Kaminski et al., 1992; Condie et al., 1996; Massi et al., 2000). While these systems provide useful information for the understanding of the functional properties of cannabinoid receptors, extrapolating these data to man may be hindered by problems of species differences and the artificial nature of the cell lines and transfected cells in which receptors are overexpressed (Kenakin et al., 1995). Consequently, we have investigated the effects of a range of cannabinoid receptor ligands on phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells, a human immune cell.

In the present study, we have shown that a nonselective cannabinoid receptor agonist WIN55212-2 (Felder et al., 1995) and a selective cannabinoid CB_2 receptor agonist JWH 015 (Huffman et al., 1996) evoked a significant concentration-related inhibition of phytohaemagglutinin-induced interleukin-2 release from human peripheral blood mononuclear cells. The nonselective and synthetic cannabinoid CP55,940 (Felder et al., 1995), produced a small, nonsignificant inhibition of interleukin-2 release from human peripheral blood mononuclear cells whereas the plant cannabinoid, Δ^9-tetrahydrocannabinol and the selective cannabinoid CB_1 receptor agonist, ACEA (Hillard et al., 1999), were ineffective in inhibiting phytohaemagglutinin-induced release of interleukin-2. The inhibition of phytohaemagglutinin-induced release of interleukin-2 evoked by WIN55212-2 was not antagonised by pretreatment of the cells with SR141716A, a cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona et al., 1994). However, SR144528, a cannabinoid CB_2 receptor antagonist (Rinaldi-Carmona et al., 1998), significantly attenuated the inhibitory effects of WIN55212-2. Taken together, these data suggest that the observed effects were mediated by a cannabinoid CB_2-like receptor.

Peripheral blood mononuclear cells, used in the present study, comprised 95% lymphocytes and 5% monocytes. In adult blood, lymphocytes comprise approximately 83% of the mononuclear cells (Dien and Lentner, 1970), suggesting that the buffy coat cells used by us contained fewer monocytes than expected, or that the isolation process results in a selective loss of monocytes. The buffy coat cells used by us are a by-product of the preparation of plasma for human use, and it is possible that the more adherent monocytes are lost in the handling of blood to produce plasma and then in the preparation of mononuclear cells by us resulting in a preparation enriched with nonadherent lymphocytes.

In the present study, we cultured human peripheral blood mononuclear cells in foetal calf serum-free medium. While it is conventional to include foetal calf serum in cell culture medium (for example, Corrigan et al., 1995), we chose not to include it because plasma proteins have been shown to bind cannabinoids and reduce their potency (Dewey, 1986), that is, this process acts as an agonist uptake/removal process. Furthermore, if this binding were saturable, over the concentration range studied, then this could influence the data obtained particularly when attempting to characterise antagonist activity (Kenakin and Beek, 1981). Thus, we elected to negate the influence of protein binding in our experiments by omitting foetal calf serum from the medium. When unstimulated peripheral blood mononuclear cells were incubated for 18 h in serum-free medium, no significant change in cell numbers nor a change in cell viability was observed. This may be unexpected since serum contains the growth factors necessary for cell survival and proliferation. However, in our experiments, unstimulated lymphocytes released a small, nonsignificant amount of interleukin-2. This basal release of interleukin-2 may have been sufficient to maintain lymphocytes in a viable, functional state but be insufficient to promote cell replication.

In the present study, inhibition of phytohaemagglutinin-induced release of interleukin-2 by WIN55212-2 and JWH 015 was observed at concentrations greater than those required to displace a radiolabelled cannabinoid receptor ligand in receptor binding studies (>1 μM) (Felder et al., 1995; Showalter et al., 1996). However, the potency of WIN55212-2 in the present study is similar to that reported by others in studies on a murine macrophage cell line (RAW264.7) (Ross et al., 2000). It is noteworthy that the

K_d values reported from cannabinoid binding studies are usually higher in experiments where purified receptors or transfected cells have been used (Howlett, 1995; Slipetz et al., 1995). This difference has been ascribed to loss of activity of lipophilic cannabinoids due to nonspecific interactions with cells and serum (Howlett, 1995; Slipetz et al., 1995). Furthermore, the pA_2 value for the cannabinoid CB_2 receptor antagonist SR144528 reported in this study is significantly lower than the pK_i value reported for this compound on Chinese hamster ovary cells transfected with CB_2 receptors (Iwamura et al., 2001). It is lower than that previously obtained by us in studies on epithelial cells (Ihenetu et al., 2003), although the potency of SR144528 in the present study is similar to that reported by others in experiments on a murine macrophage cell line (Ross et al., 2000). One explanation for this difference may be due to the level of cannabinoid CB_2 receptor expression in mononuclear cells compared to that in other tissues, coupled with the lipophilic nature of these compounds reducing the actual concentration of antagonist available at the receptor. Clearly, further experiments are required to determine why SR144528 is apparently less potent as a cannabinoid CB_2 receptor antagonist on monocytes compared with other tissues.

In line with the present study, it is noteworthy that few studies to date have reported functional effects of cannabinoids via cannabinoid CB_2 receptors at concentrations less than 1 μM (Ross et al., 2000). Furthermore, in transfected cell lines, the stoichiometry of key regulatory proteins may be altered resulting in responses distinct from those found in primary cells (Kenakin et al., 1995). Thus, it seems possible that our finding that cannabinoid agonists were less potent in human peripheral blood mononuclear cells when compared to data published by others may reflect a low level of cannabinoid receptor expression in these cells.

Other published work suggests that cannabinoids can stimulate cytokine release. In contrast to our findings, Derocq et al. (1995) were able to show that low concentrations of CP55,940 significantly ($P<0.05$) increased DNA synthesis in human tonsilar B cells, a primary cell system that expresses high levels of cannabinoid CB_2 receptors (Galiegue et al., 1995). Other studies showing effects of cannabinoids at low concentrations include experiments in which the cannabinoid receptor agonists CP55,940 or WIN55212-2 caused increased expression of IL-8 in HL-60 cells transfected with cannabinoid CB_2 receptors (Jbilo et al., 1999; Derocq et al., 2000). However, these cannabinoid CB_2 receptor agonists still increased IL-8 expression when wild type HL-60 cells were used (Derocq et al., 2000; Jbilo et al., 1999). These findings suggest that HL-60 cells have a higher level of endogenous cannabinoid CB_2 receptor expression than human peripheral blood mononuclear cell since, in the present study, the cannabinoid receptor agonist CP55,940 did not induce the release of interleukin-2 from peripheral blood mononuclear cells, even after incubation for 18 h.

Other published work has also shown that cannabinoids may either increase or decrease interleukin-2 release from immune cells depending on the experimental conditions and the cells studied (Pross et al., 1992; Watzl et al., 1991). In the murine lymphocyte cell line, EL4.IL-2, Δ^9-tetrahydrocannabinol and cannabidiol inhibited phorbol myristyl acetate/ionophore-induced interleukin-2 mRNA expression and interleukin-2 release in a concentration-dependent manner (Condie et al., 1996; Jan et al., 2002). In contrast, in phytohaemagglutinin-activated human peripheral blood mononuclear cells, Δ^9-tetrahydrocannabinol and cannabidiol did not inhibit interleukin-2 release, although these cannabinoid receptor ligands did inhibit the release of other cytokines (Watzl et al., 1991), findings that are consistent with those reported in the present study. Thus, it appears that the choice of cell and the stimulus used to provoke cytokine release may influence the inhibitory activity of cannabinoid receptor agonists. Such an effect is not unique to cannabinoid receptor agonists and has been noted in studies with other classes of agonists (e.g. Kenakin, 1982; Kenakin et al., 1995). The exact reason for the differences between the findings of the present study and those described above is still unclear and additional experiments are necessary to resolve these discrepancies.

We and others have shown that a range of cannabinoid ligands including WIN55212-2, CP55,940 and Δ^9-tetrahydrocannabinol act as agonists at the peripheral cannabinoid CB_2 receptor to cause inhibition of tumour necrosis factor-α-induced release of interleukin-8 in HT-29 cells (Ihenetu et al., 2001) and to inhibit adenylate cyclase activity in Chinese hamster ovary cells transfected with cannabinoid CB_2 receptors (Bayewitch et al., 1995), respectively. However, in the present study, CP55,940 only marginally and nonsignificantly inhibited phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells while Δ^9-tetrahydrocannabinol had no effect in inhibiting this release. Receptor binding studies have demonstrated that these two agonists have affinity for cannabinoid CB_2 receptors on immune cells (Bouaboula et al., 1993; Galiegue et al., 1995; Kaminski et al., 1992). Thus, one explanation for this lack of activity could be due to a low level of efficacy combined with a relatively low level of cannabinoid CB_2 receptor expression. Similar effects have been reported in experiments with partial agonists in other receptor systems (Kenakin and Beek, 1982). This hypothesis is supported by the ability of CP55,940 and Δ^9-tetrahydrocannabinol to inhibit the effects of WIN55,212-2. In the present study, both compounds shifted concentration–effect curves for WIN55,212-2-induced inhibition of interleukin-2 release, to the right. In the case of CP55,940, the small inhibitory effect on interleukin-2 release adds further weight to the hypothesis that it is acting as a weak partial agonist at cannabinoid CB_2 receptors relative to the effect observed with WIN55212-2.

Given the apparent potency of CP55,940 at cannabinoid CB_2 receptors, reported by others (Showalter et al., 1996), it

is possible that the lack of inhibitory effect on phytohaemagglutinin-induced interleukin-2 release is because the inhibitory effect is negated by additional release of interleukin-2 induced by CP55,940. Such an effect has been reported by others (Jbilo et al., 1999) and could also explain the apparent antagonism of the inhibitory action of WIN55212-2 by CP55,940. However, this is clearly not the case since when human peripheral blood mononuclear cells were incubated with CP55,940 for 18 h, no release of interleukin-2 was seen, adding support to the hypothesis that, in our experiments, CP55,940 acts at cannabinoid CB_2 receptors on human peripheral blood mononuclear cells to antagonise the effects of WIN55212-2.

To test the specificity of CP55,940 in antagonising the effect of WIN55,212-2, we studied the effect of CP55,940 in antagonising dexamethasone-evoked inhibition of phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells. CP55,940 did not antagonise dexamethasone-evoked inhibition of phytohaemagglutinin-induced release of interleukin-2 but marginally potentiated its effect. In order to investigate whether high concentration of CP55,940 evoked the release of interleukin-2 on its own, a point which could account for its poor activity in inhibiting phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells, we studied the effect of CP55,940 (10^{-5} M) on the release of interleukin-2 from human peripheral blood mononuclear cells in the absence of phytohaemagglutinin. In these experiments, CP55,940 alone did not stimulate the release of interleukin-2 from phytohaemagglutinin. Taken together, these results show that CP55,940 appears to be specific in antagonising WIN55212-2-mediated inhibition of phytohaemagglutinin-induced interleukin-2 release from human peripheral blood mononuclear cells and does not, on its own, evoke the release of interleukin-2. Δ^9-Tetrahydrocannabinol exhibited similar profiles (data not shown). Previously, other laboratories have demonstrated that Δ^9-tetrahydrocannabinol antagonised HU293a and HU210 (nonselective cannabinoid receptor agonists) induced inhibition of forskolin-stimulated adenylyl cyclase in Chinese hamster ovary cells transfected with CB_2 receptors (Bayewitch et al., 1996). To our knowledge, the present study is the first report of CP55,940 acting as a partial agonist/antagonist at a cannabinoid CB_2 receptor-mediated event in a native system.

In summary, we have demonstrated that WIN55212-2 and JWH 015 evoke inhibition of interleukin-2 release from human peripheral blood mononuclear cells. The selective cannabinoid CB_2 receptor antagonist SR144528 antagonised WIN55212-2 inhibition of phytohaemagglutinin-induced release of interleukin-2 from human peripheral blood mononuclear cells whereas the cannabinoid CB_1 receptor antagonist SR141716A had no effect. Furthermore, CP55,940 and Δ^9-tetrahydrocannabinol behaved as partial agonists/antagonists under our experimental conditions, indicating that they possess affinity for, but low efficacy at, cannabinoid CB_2 receptors. Thus, this study adds to and extends the body of knowledge suggesting that cannabinoids modulate immune cell function and suggests that some ligands have partial agonist activity at cannabinoid CB_2 receptors. The structures of the cannabinoid receptor ligands utilised in the above study could therefore serve as models for the synthesis of novel and more selective cannabinoid compounds for therapeutic use.

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