PHARMACOLOGICAL ANALYSIS OF CANNABINOID RECEPTOR ACTIVITY IN ISOLATED NERVE-SMOOTH MUSCLE AND EPITHELIAL PREPARATIONS

RAJESH MAKWANA, B.Sc. (Hons.) Pharmacology

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Abstract

This study was directed at characterising the cannabinoid receptor activity modulating the electrical field stimulation (EFS) evoked contractions of the rat isolated ileum myenteric plexus longitudinal muscle (MPLM) preparation, and the capsaicin, nicotine and veratridine evoked secretory responses of the rat isolated colonic submucosal plexus-mucosal (SPM) preparation.

EFS of the MPLM preparation with single pulses at a repetition frequency of 0.05 Hz elicited a transient twitch contraction immediately in response to each electrical pulse. In contrast, stimulation of the MPLM preparation with 2 second trains of pulses every minute at a frequency of 30 Hz elicited a rapid transient rebound contraction on termination of each train of EFS. The non-selective cannabinoid receptor agonists AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 inhibited both EFS-evoked twitch and rebound contractions of the rat ileum MPLM elicited by 0.05 Hz and 30 Hz EFS respectively. The inhibition of the twitch contractions was competitively antagonised by the cannabinoid CB_1 receptor antagonist / inverse agonist SR 141716 with pK_B values of 8.60. In contrast, SR 141716 only antagonised the ability of AEA, Δ^9-THC and WIN 55,212-2 but not CP 55,940 to inhibit the rebound contractions with pA_2 values of 6.60. These observations extended to the inhibitory effect of WIN 55,212-2 on the twitch and rebound contractions of the guinea-pig ileum MPLM. The CB_2 antagonist / inverse agonist SR 144528 did not alter the effects of the agonists. Additionally, the inhibitory effect of AEA was refractory to the vanilloid $TRPV_1$ receptor antagonist capsazepine. WIN 55,212-3 a stereoisomer of WIN 55,212-2 was without effect on the rat MPLM. SR 141716 alone concentration-dependently increased the twitch contractions but inhibited the rebound contractions. Both types of the EFS-evoked contractions were abolished by the Na^+ channel blocker tetrodotoxin or the muscarinic acetylcholine (ACh) receptor antagonist atropine but not the nicotinic ACh receptor antagonist hexamethonium. None of the cannabinoids altered the contractions to exogenously applied ACh.

These data suggested that the cannabinoid agonists inhibited the twitch contractions through a stereospecific presynaptic CB_1 receptor-mediated reduction in the release of

ACh. Additionally, the inhibition of the rebound contractions occurred because of an inhibition of ACh release by a novel stereospecific presynaptic non-CB_1 -non CB_2 - non -TRPV1 site. The ability of SR 141716 to inhibit the rebound contractions and antagonise AEA, Δ^9-THC and WIN 55,212-2 may be though partial agonism at the non-CB_1-non CB_2-non-TRPV1 site.

The ability of SR 141716 to potentiate the twitch contractions by increasing the release of ACh suggested that the CB_1 receptor was constitutively active or was subjected to a tonic activation by endocannabinoid agonists. A comparison between the maximal enhancement of the twitch contractions of the rat and the guinea-pig ileum MPLM caused by three CB_1 receptor antagonists / inverse agonists AM 251, SR 141716 and O-2050 showed that each cannabinoid had a different maximum. This suggested inverse agonism. These data were supported with studies showing the lack of effect of three fatty acid amide hydrolase (FAAH) inhibitors AA-5HT, PMSF, URB–597 and VDM-11, an inhibitor of the AEA uptake transporter on EFS-evoked contractions. These studies showed that all three FAAH inhibitors increased the potency of exogenously applied AEA but not WIN 55,212-2, and that VDM-11 had no effect on the potency of exogenously applied AEA. This data suggested that a functional endocannabinoid tone and the uptake transporter were not present in the MPLM, but FAAH was present. These data provide supporting evidence that SR 141716 behaved as an inverse agonist in the MPLM to augment twitch contractions.

The interaction between CP 55,940 or WIN 55,212-2 with SR 141716 was investigated using the rat colonic SPM sheet. Both CP 55,940 and WIN 55,212-2 attenuated the secretory responses to capsaicin and nicotine in a SR 141716 sensitive manner. SR 140333, a neurokinin 1 receptor antagonist, abolished the capsaicin and nicotine. This suggested that CP 55,940 and WIN 55,212-2 inhibited the capsaicin and nicotine response through a CB_1 receptor-mediated inhibition of the release of substance P or neurokinin A. The sensitivity of the veratridine response to TTX and α-chymotrypsin and the failure of the cannabinoids to attenuate the response suggested the absence of the CB_1 receptor on the neurones releasing the undetermined neuropeptide.

Together, these data suggest that both the CB_1 receptor and non-CB1-non-CB_2 -non-TRPV1 receptor can mediate the inhibitory effects of cannabinoid agonists in the rat ileum MPLM depending on the frequency of EFS. These data also show that SR 141716 is an inverse agonist in the MPLM. In the SPM preparation, the CB_1 receptor appears to be involved in the modulation of some forms of peptidergic transmission.

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Abbreviations

Δ	Change
2-AG	2-Arachidonly glycerol
5-HT	Serotonin
95 % C.I	95 % Confidence intervals
AC	Adenylate cyclase
ACh	Acetylcholine
AEA	Arachidonyl-ethanolamide, Anandamide
ANOVA	Analysis of variance
ATP	Adenosine-triphosphate
^{O}C	Degrees Celsius
EFS	Electrical field stimulation
FAAH	Fatty acid amide hydrolase
n_H	Hill coefficient
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
cDNA	Complementary DNA
CNS	Central nervous system
DMSO	Dimethylsulphoxide
DNA	Deoxyribo nucleic acid
EC_{50}	Effective concentration for 50 % maximal response
E_{max}	Maximal response
ENS	Enteric nervous system
g	Grams
GTP	Guanosine 5-triphosphate
IPAN	Intrinsic primary afferent neurone
I_{SC}	Short circuit current
K_B	Equilibrium dissociation constant of an antagonist
K_I	Dissociation constant of a ligand
MAPK	Mitogen activated protein kinases
mACh	Muscarinic Acetylcholine receptor
MPLM	Myenteric plexus longitudinal muscle
nACh	Nicotinic acetylcholine receptor
NAdr	Noradrenaline
NO	Nitric oxide
pEC_{50}	-Log EC_{50}
pK_B	-Log K_B
pK_I	-Log K_I
R_t	Transmembrane tissue resistance
s.e.m	Standard error of the mean
SPM	Submucosal plexus-mucosal
Δ^9-THC	Δ^9-Tetrahydrocannabinol
$TRPV_1$	Transient receptor potential vanilloid type 1 receptor, VR_1
VIP	Vasoactive intestinal polypeptide

Publications

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CHAPTER 1:

GENERAL INTRODUCTION

1.1. *Cannabis sativa*

1.1.1. Botany

The Indian hemp represents one of mankind's oldest cultivated Old World plants originating from the plains of Central Asia, but through man's activities has been distributed widely around the world (Grinspoon & Bakalar, 1993; Peters & Nahas, 1999). The plant has been exploited through the process of selection for thousands of years for multiple desirable characteristics, some grown exclusively for their fibre and oil content, while others for their content of psychoactive chemicals (Grinspoon & Bakalar, 1993; Peters & Nahas, 1999). All of these varieties are generally classified under the Linnaeus system as a single species, *Cannabis sativa* L. (Schultes, 1970).

The cannabis plant is a fast growing annual with characteristic finely branched leaves, each of which are subdivided into at least five spear shaped leaflets with a saw tooth edge (Figure 1.0). The plant is either male or female, and under normal temperate growing conditions these are generated in roughly equal numbers (Iversen, 2000; Stearn, 1970).

A vast number of constituents representing almost all of the chemical classes, e.g. amino acids, flavonoids, nitrogenous compounds, hydrocarbons, steroids, sugars, are present in the plant. However, to date, at least seventy 21–carbon-containing terpenophenolic alkaloids which are unique to the plant have been identified (ElSohly & Slade, 2005; Lambert & Fowler, 2005). These compounds are commonly referred to as cannabinoids, or more specifically phytocannabinoids because of their plant origin.

Although a large variability exists in the amount and ratio of the chemical constituents of the plant, which is dependent on botanical and cultivation factors, Δ^9-tetrahydrocannabinol (Δ^9-THC; Figure 1.1) is the principal psychoactive phytocannabinoid (Gaoni & Mechoulam, 1964). Δ^9-THC is present in larger amounts, usually 1 - 5 % by weight, than other phytocannabinoids (Grinspoon & Bakalar, 1993). It is present in most parts of the plant including the leaves and flowers, but is most highly concentrated in fine droplets of resin produced by glands at the base of

the fine hairs that coat the leaves and the bracts of the female flower head (Stearn, 1970). Other major phytocannabinoids that are present in similar or lesser amounts than Δ^9-THC include cannabinol, cannabidiol, cannabingerol etc. The later phytocannabinoids are essentially non-psychoactive, but may possess pharmacological activity (ElSohly & Slade, 2005; Mechoulam, 1970).

Figure 1.0: *Cannabis sativa. L*. The (A) male and (B) female plants. Modified from Köhler FE (1887). *Cannabis sativa (Cannabaceae).* Available at http://caliban.mpiz-koeln.mpg.de/~stueber/koehler/CANNABIS.jpg

1.1.2. Historical uses and current legislation

Cannabis was possibly one of the most versatile plants cultivated not specifically for its herbal contents, but as a valuable source of natural fibre and oil. The strong structural fibres in its stalk and stems provided material for cloth and rope making, whilst the oil in its seeds was used as a lubricant, paint ingredient and for cooking (Grinspoon & Bakalar, 1993; Schultes, 1970).

Although the first descriptions of its medicinal properties were included by the Greek physician Dioscorides in his classic textbook of pharmacology, *De Materia Medica* ("The Materials of Medicine") in 60 AD, the curative properties of the plant were popularised into Western medicine by the British physician William O'Shaughnessy in 1830 (Iversen, 2000). O'Shaughnessy reported, observing the medicinal use of the drug in India for its effectiveness in the treatment of a variety of ailments such as asthma, constipation, convulsions, diarrhoea, epilepsy, nausea, rheumatic pain, tetanus and vomiting (O'Shaughnessy, 1842). Soon afterwards, both an extract and a tincture of cannabis were introduced into the British Pharmacopoeia in 1845, and were available for more than a hundred years (Iversen 2000). However, by the middle of the twentieth century, the availability of alternative treatments, increased recreational use of cannabis and difficulties in obtaining standardised supplies of the medicine led to the removal of both herbal preparations from the British Pharmacopoeia as the Misuse of Drugs Act (1971) declared that it was of no medical benefit and its use was illegal (Baker *et al*., 2003).

Today, despite the illegal possession, supply and use of cannabis for self–medication in the United Kingdom (U.K.), cannabis is a schedule C drug, as recognition that it does not carry the same risks as schedule B drugs such as amphetamine and barbiturates (Baker *et al*., 2003).

1.1.3. Modern therapeutic applications

The interest in the therapeutic properties of cannabis was renewed after the discovery and isolation of Δ^9-THC (Gaoni & Mechoulam, 1964). The chemical synthesis of the phytocannabinoid was followed by research into basic structure activity relationships (Razdan, 1986) and their metabolic routes (Agurell *et al*., 1986) with the aim of developing more potent and less lipophilic analogues, or compounds that separated the desirable medicinal properties from the psychoactive effects.

The first potent synthetic Δ^9-THC analogue to be used in man was nantradol, synthesised in the early 1980s by Pfizer Inc. in the United States of America (U.S.A), but this was subsequently replaced by levonantradol (Nantrodolum®) (Iversen, 2000). The synthetic cannabinoids had an advantage over Δ^9-THC as they were slightly water soluble and thus easier to formulate and deliver. In spite of proving more potent than morphine as analgesics and effective in blocking nausea and vomiting in patients undergoing cancer chemotherapy, the inability to separate the psychoactive property was a drawback to their use (Iversen, 2000). However, since levonantradol, a number of clinically useful cannabinoid based medicines have been developed and are currently available by prescription both in the U.K and U.S.A., whilst others are in clinical trials. Some Δ^9-THC based drugs that are already used clinically include dronabinol (Marinol®), which is available under prescription to counteract the AIDS wasting syndrome and nabilone (Cesamet®) another potent synthetic analogue of Δ^9-THC, with low psychotomimetic activity, which is marketed to curtail nausea and vomiting experienced by patients undergoing cancer chemotherapy (Pertwee, 2000). More recently, two other cannabinoid medicines have become available. These include rimonabant or Acomplia®, synthesised by Sanofi Recherchē in France, and Sativex®, developed by GW Pharmaceuticals in the U.K. Rimonabant®, an antagonist / inverse agonist of the central cannabinoid CB_1 receptor has been indicated as an anti-obesity drug and for the suppression of the reinforcing and rewarding properties of a number of drugs of abuse, such as alcohol, cocaine, heroin and nicotine (Carai *et al*., 2005), whereas Sativex®, a whole cannabis plant extract containing an equal proportion of Δ^9-THC and cannabidiol is being tested for clinical efficacy for the alleviation of symptoms of debilitating conditions such as multiple sclerosis, spinal cord injury and neurogenic pain (Smith, 2004).

1.2. Cannabinoid receptor subtypes

One of the earliest hypothesis concerning the mechanism of action of Δ^9-THC was attributed to its high lipophilicity, which could perturb the phospholipid bilayer of the cell plasma membrane and result in relatively non-specific disruption of cellular function (Hillard *et al*., 1985; Paton, 1975; Roth & Williams, 1979).

There is now convincing evidence for the existence of specific cannabinoid receptors, with two subtypes being described i.e. the CB_1 receptor (Section 1.2.1) and CB_2 receptor (Section 1.2.2), as defined by the IUPHAR Committee on Receptor Nomenclature (Howlett *et al*., 2002). Both receptors share common characteristics with other members of the G protein-coupled receptor family in their amino acid sequences and the seven transmembrane helical structures (Matsuda *et al*., 1990; Munro *et al*., 1993). Between them, both receptors mediate the majority of the pharmacological actions of Δ^9-THC.

Recent data have pointed to the existence of additional novel pharmacological targets for cannabinoids because of the observation of non-CB_1-non-CB_2-receptor mediated effects by number of established cannabinoid receptor agonists, either after blockade of the CB_1 and CB_2 receptors with selective antagonists or in transgenic $CB_1^{-/-}$, $CB_2^{-/-}$ and $CB_1^{-/-}$ / $CB_2^{-/-}$ receptor gene knockout animals and their tissues (for review see Pertwee, 2004).

1.2.1. Cannabinoid CB_1 receptor

The earliest evidence that a specific stereo-selective cannabinoid binding site mediated the pharmacological actions of Δ^9-THC and its analogues was provided by Howlett and co-workers using rat brain neuroblastoma N18TG2 cells (Howlett, 1984; Howlett *et al*., 1986). The authors of these studies proposed the existence of a stereo-specific $G_i/_o$ protein-coupled cannabinoid receptor on the observation that pairs of enantiomeric psychotropic cannabinoids inhibited the activity of the enzyme adenylate cyclase in a concentration-dependent, stereo-selective and pertussis-toxin sensitive manner. Soon afterwards, this hypothesis was validated by radioligand-binding assays performed on rat brain membranes with the demonstration of a

saturable, stereo-selective and high affinity membrane binding site for a range of psychotropic cannabinoids (Devane *et al*., 1988).

The first cannabinoid receptor, designated as the CB_1 receptor, was eventually cloned from the rat brain (Matsuda *et al*., 1990) and thereafter from the human brain and testis (Gerard *et al*., 1991) and subsequently from the mouse brain (Abood *et al*., 1997; Chakrabarti *et al*., 1995). This receptor has been noted to be highly conserved across the human, rat and mouse. Specifically, the nucleotide sequences of human and rat CB_1 receptor share 93 % homology, whereas those of human and mouse are 90 % similar and those of rat and mouse are 95 % identical (Gerard *et al*., 1991; Chakrabati *et al.,* 1995).

The anatomical distribution of this receptor extends primarily to neuronal tissues such as the brain, spinal cord and nerves of the autonomic nervous system, where it is localised on the soma and axon terminals to modulate neuronal excitability and neurotransmitter release respectively. In the central nervous system (CNS), the CB_1 receptor has been detected in high abundance in the hippocampus, cerebral and cerebellar cortices and basal ganglia (Herkenham *et al*., 1991; Glass *et al*., 1997). The heterogeneous receptor distribution in these discrete areas correlates well with the known effects of cannabinoids on memory, perception and motor function. The peripheral distribution pattern of the CB_1 receptor extends mainly to nerve terminals innervating visceral organs such as the adrenal glands, heart, lungs, gastrointestinal tract and genitourinary tract (Howlett *et al*., 2002; Pertwee, 1997).

The existence of subtypes of the CB_1 receptor has also been reported. For example, the minor splice variant CB_{1A} and CB_{1B} receptor have been detected in the human lung (Shire *et al*., 1995; Rinaldi-Carmona *et al*., 1996) and foetal human brain (Ryberg *et al*., 2005) respectively. Although these subtypes of the human CB_1 receptor appear to have a matching distribution pattern to that of the conserved CB_1 receptor in the human brain and peripheral tissues, their biological significance remains unknown (Shire *et al*., 1995). Other distinct CB_1 receptor mRNAs have also been detected in the C57BL/6 mouse brain, but again their significance remains unknown (Onaivi *et al*., 1996).

1.2.2. Cannabinoid CB_2 receptor

In 1993, the second cannabinoid receptor type, named as the CB_2 receptor, was isolated from the human promyelocytic leukaemia HL60 cells (Munro *et al*., 1993). In addition to it being a pertussis toxin-sensitive $G_i/_o$ protein-coupled receptor, this receptor was revealed to exhibit 68% identity to the CB_1 receptor within the transmembrane regions and 44% identity throughout the whole CB_1 protein (Munro *et al*., 1993). Since then, the receptor has been further isolated from the mouse and rat spleen and a myeloid leukaemia cell line (Brown *et al*., 2002; Griffin *et al*., 2000; Shire *et al*., 1996; Valk *et al*., 1997).

Unlike the CB_1 receptor nucleotide sequence, which appears to be highly conserved between the human, mouse and rat, the nucleotide sequence of the CB_2 receptor exhibits less homology between these mammalian species. For example, the human and mouse CB_2 receptor share 82% amino acid identity (Shire *et al*., 1996) whereas the mouse and rat 93% homologous (Brown *et al*., 2002; Griffin *et al*., 2000).

The distribution of the CB_2 receptor appears to be predominantly restricted to the periphery e.g. in cells of the immune system such as B and T lymphocytes, natural killer cells and monocytes (Galiegue *et al*., 1995). Given their location on these cells they are thought to be involved in chemotaxis and the modulation of the release of pro-inflammatory and anti-inflammatory mediators (Bouaboula *et al*., 1993). Visceral regions where CB_2 receptor mRNA expression has also been detected include the adrenal glands, small intestine, heart, lungs, pancreas, prostate, spleen, testis, tonsils and uterus (Munro *et al*., 1993; Galiegue *et al*., 1995; Walter *et al*., 2003). More recent studies have provided functional evidence for a presence of the CB_2 receptor on neurones of the brainstem, where its activation can inhibit emesis (Van Sickle *et al.,* 2005). The later results suggest that the CB_2 receptor could represent an alternative non-psychotropic therapeutic site of action of cannabinoid receptor agonists.

1.3. Cannabinoid receptor ligands

Since the isolation and elucidation of the chemical structure of Δ^9-THC in 1964, many potent, stereo-isomeric, high affinity, selective and non-selective cannabinoid receptor ligands have been both synthesised and assayed *in vivo* and *in vitro*. This sub-section briefly introduces the classes of cannabinoid receptor ligands with regard to their chemical class and pharmacological activity.

1.3.1. Agonists

These have been classified according to their chemical structures into at least four main groups: classical (Section 1.3.1.1), non-classical (Section 1.3.1.2), aminoalkylindole (Section 1.3.1.3) and eicosanoid (Section 1.3.1.4).

1.3.1.1. Classical agonists

Members of this group are characterised by a dibenzopyran ring system in their structure (Howlett *et al*., 2002). This class include the phytocannabinoids e.g. Δ^9-THC (Figure 1.1) and its synthetic analogues such as HU-210 (Mechoulam *et al*., 1988) and the clinically tested dronabinol, nantradol, levonantradol, nabilone and the two components of Sativex® i.e. Δ^9-THC and cannabidiol (Section 1.1.3). Other unique cannabinoids belonging to this class are the water-soluble compounds O-1057 and O-2545 from Organix (Pertwee *et al*., 2000, Litchman *et al*., 2000; Martin *et al*., 2006).

Like Δ^9-THC, most classical cannabinoids that bind to the CB_1 receptor also have affinity for the CB_2 receptor. However, there are significant differences in the magnitude of potencies and efficacies between the various compounds, which are suggested to be related to the intrinsic efficacy of the ligand, stimulus-response coupling capacity of the bioassay and the density of the cannabinoid receptor (Howlett *et al*., 2002). The affinity values (equilibrium dissociation constant, pK_I) of Δ^9-THC for the *in vitro* displacement of [^{3}H]-CP 55,940 from CB_1 and CB_2 receptor binding sites range from 7.80 to 8.41 respectively (Bayewitch *et al.,* 1996; Felder *et al.,* 1995; Showalter *et al.,* 1996; Rhee *et al.,* 1997; Rinaldi-Carmona *et al.,* 1994).

Structure-activity relationship studies have revealed that the phenolic hydroxyl group and the aliphatic side chain are crucial for potency and affinity for this class of cannabinoids (Goutopoulous & Makriyannis, 2002). The presence of chiral centres in classical cannabinoids such as Δ^9-THC and HU210 has made it possible to develop enantiomers of this class of cannabinoids. These stereo-chemical changes have shown that the pharmacological activity of the (-) enantiomers is greater than that of the (+) enantiomers in terms of receptor affinity and potency (Howlett *et al*., 2002) at both cannabinoid receptor subtypes.

1.3.1.2. Non-classical agonists

This class of synthetic cannabinoids consists of bicyclic and tricyclic analogues of Δ^9-THC that lack the dibenzopyran ring found in the classical cannabinoids. The prototypical compound of this class is the Pfizer Inc. compound CP 55,940 (Johnson & Melvin, 1986; Melvin *et al*., 1993; Figure 1.1), of which the tritiated radioactive form was used to demonstrate the existence of cannabinoid binding sites in the rat brain (Devane *et al*., 1988).

In many bioassays, CP 55,940 behaves as a full agonist and displays high affinity and potency at both cannabinoid receptor subtypes with pK_I and potency (pEC_{50}) values between 8.30 and 9.30 respectively (Felder *et al*., 1995; Rinaldi-Carmona *et al.,* 1994; Ross *et al*., 1999; Showalter *et al.,* 1996). The presence of chiral centres and the development of the (+)-enantiomer of CP 55,940 i.e. CP 56,667 has been exploited to demonstrate the stereo-selective property of the cannabinoid receptor (Pertwee *et al*., 1993). Like classical cannabinoids, the phenolic hydroxyl group and the aliphatic side chain of the non-classical cannabinoids are considered to be essential for cannabinoid receptor activity (Goutopoulous & Makriyannis, 2002).

1.3.1.3. Aminoalkyindole agonists

Although structurally unrelated to the other classes of cannabinoid receptor agonists, members of this class typified by the Sterling Winthrop Inc. compound *R*-(+)-WIN 55,212-2 (WIN 55,212-2; Figure 1.1) display the full spectrum of cannabinoid effects both *in vivo* and *in vitro* (Pacheco *et al*., 1991; Compton *et al*., 1992).

Site-directed mutagenesis studies on the cannabinoid receptor genes and molecular modelling studies of the receptor proteins have revealed that WIN 55,212-2 binds differently to receptor than the other three classes of cannabinoids. However, the receptor binding has been suggested to occur in a manner that allows mutual displacement of WIN 55,212-2 by other classes of cannabinoid receptor ligands (Houston & Howlett, 1998).

WIN 55,212-2 behaves as a full agonist and displays high affinity and potency at both cannabinoid receptor types (Howlett *et al*., 2002), whereas its *S*-(-)-enantiomer i.e. WIN 55,212-3 is inactive. Typical pK_I and pEC_{50} values for WIN 55,212-2 at both receptor subtypes range between 7.80 and 9.55 respectively (Felder *et al.,* 1995; Rinaldi-Carmona *et al.,* 1994; Showalter *et al.,* 1996).

The tritiated form of WIN 55,212-2 has been used to characterise and map the distribution of the cannabinoid receptor in the rat brain (Jansen *et al*., 1992; Kuster *et al*., 1993). A structural requirement for this class of cannabinoids to exhibit cannabimimetic activity is the presence of the 3-aroyl moiety (Goutopoulous & Makriyannis, 2002).

1.3.1.4. Eicosanoid agonists

These are a series of arachidonic acid derivatives that often referred to as the endogenous cannabinoids or endocannabinoids. Of the endocannabinoids, arachidonyl-ethanolamide (anandamide, AEA; Figure 1.1), isolated from the pig brain (Devane *et al*., 1992), and 2-arachidonoly glycerol (2-AG), discovered in the canine gut (Mechoulam *et al*., 1995) and rat brain (Sugiura *et al*., 1995), are the most widely investigated.

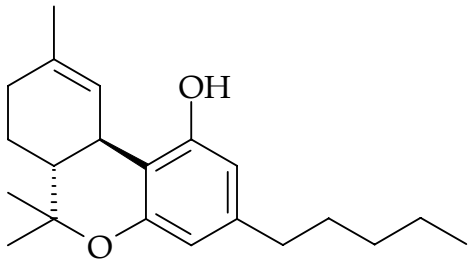
Other major endocannabinoids include nolandin-ether isolated from the pig brain (Hanus *et al*., 2001), virodhamine, discovered in the rat brain (Porter *et al*., 2002) and *N*-arachidonoyl-dopamine, detected in both rat and bovine brain (Huang *et al*., 2002).

Studies with AEA have shown that this cannabinoid binds marginally more readily to the CB_1 receptor than to the CB_2 receptor with pK_I values of around 7.05 and 5.71 respectively (Felder *et al.,* 1995; Showalter *et al.,* 1996).

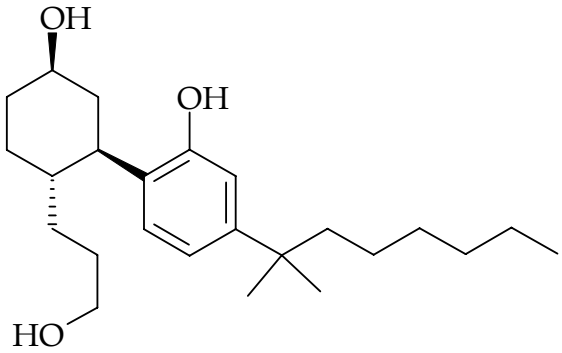
In some CB_1 receptor-expressing systems, AEA and 2-AG resemble Δ^9-THC in demonstrating different degrees of efficacy. For example, Mackie *et al.,* (1993) showed that AEA at maximally active concentrations produced a sub-maximal inhibition of N-type Ca^{2+} currents in N18 mouse neuroblastoma cells relative to that obtained with WIN55212-2. Furthermore, AEA was able to antagonise the response to WIN55212-2, indicating that the both agonists were likely to be acting at the same receptor and that AEA was behaving as a partial agonist. By contrast, in cultured rat hippocampal neurones, Twitchell *et al.,* (1997) have shown that the maximal inhibition of Ca^{2+} currents carried by N- and P/Q-type channels in response to AEA was similar to that of WIN55212-2 and CP 55,940. The differences in efficacy of AEA between these experimental cell types could be attributed to differences in the density of the CB_1 receptor, the stimulus-response coupling capacity of the cells or the susceptibility of AEA to metabolic degradation in these cells.

Far fewer structure-activity relationship studies have been performed with 2-AG compared to AEA. For a molecule such as AEA that lacks chiral centres, structural modifications have yielded potent chiral centre containing CB_1 receptor selective analogues such as the fatty acid amide hydrolase (FAAH) enzyme resistant *R*-(+)-methanandamide (Abadji *et al*., 1994). Other notable selective CB_1 receptor agonists derived from AEA include the commercially available arachidonoyl-2'-chloroethanolamide and arachidonoyl-cyclopropylamide (Hillard *et al*., 1999). It has been proposed that the hexyl saturated carbon chain and the amide moiety are essential for activity (Howlett *et al*., 2002).

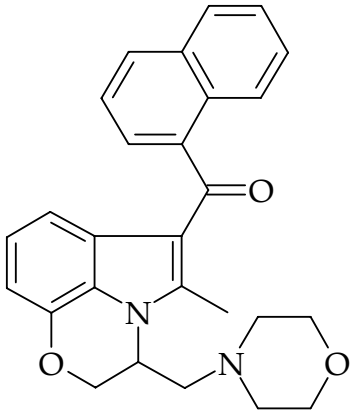
Although AEA has been commonly referred to as a cannabinoid, a large body of evidence has emerged since its discovery that it is also capable of eliciting a number of non-CB_1-non-CB_2 receptor-mediated effects, for example, through activation of the transient receptor potential vanilloid type 1 receptor (TRPV1) (Zygmunt *et al*., 1999) (Section 1.5)



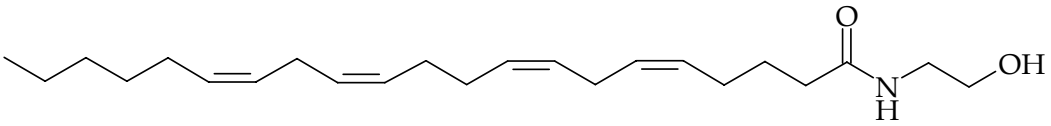
Δ^9-THC (Classical)



CP 55,940 (Non-classical)



WIN 55,212-2 (Aminoalkylindole)



AEA (Eicosanoid)

Figure 1.1: Chemical structures of the cannabinoid receptor agonists used in the present study.

1.3.2. Antagonists

Since the most widely used cannabinoid receptor agonists i.e. Δ^9-THC, CP 55,940, WIN 55,212-2 and AEA are capable of activating both subtypes of the cannabinoid receptor, selective, high affinity, potent and reversible competitive antagonists have been developed to pharmacologically block the effect of the agonists and to distinguish between the receptor subtypes.

However, in addition to their antagonist activity, a consistent observation with the majority of these antagonists is their ability to independently elicit effects that are opposite in direction to those produced by the agonists in a number of *in vitro* and *in vivo* bioassays. These antagonist-mediated effects have been interpreted as evidence for either inverse agonism via an agonist-independent reduction in the constitutive activity of the receptor, or an antagonism of the endogenous agonist-mediated tonic activation of the receptor (Pertwee, 2005b).

1.3.2.1. CB_1 receptor antagonists / inverse agonists

Several classes of structurally unrelated, potent and surmountable antagonists/inverse agonists have been developed since the cloning of the CB_1 receptor protein. These include the diarylpyrazoles such as the widely used Sanofi Recherché compound SR 141716, which is known as rimonabant and Acomplia® (Section 1.1.3, Rinaldi-Carmona *et al*., 1994), its analogue AM251 (Gatley *et al*., 1997), the benzofuran LY 320135 from Eli Lilly (Felder *et al*., 1998) and the aminoalkylindole WIN 56,098 (Pacheco *et al*., 1991).

Tritium-labelled SR 141716 and AM 251 have also been synthesised for use in radioligand binding studies to test novel cannabimimetic ligands for CB_1 receptor binding (Pertwee, 1999). Structure-activity relationship studies with SR 141716 have indicated that the 2,4-dichlorophenyl and amide moieties are essential for CB_1 receptor affinity whereas the 5-position of the pyrazole ring is important for receptor potency.

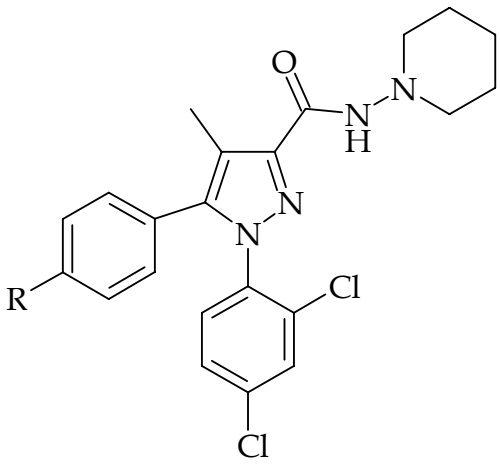
Several radioligand binding and functional assays have shown that pK_I value of SR 141716 ranges between 7.93 and 8.74 at the CB_1 receptor (Felder *et al.,* 1995;

Rinaldi-Carmona *et al.,* 1994; Showalter *et al.,* 1996) and around 4.88 at the CB_2 receptor (Felder *et al.,* 1998).

Recently, a number of CB_1 receptor antagonists have been developed and claimed to behave as neutral antagonists i.e. ligands devoid of intrinsic efficacy. But, because very few studies have been performed with these compounds, the full spectrum of their pharmacological effects remains to be characterised. Some examples of putative neutral antagonists are: O-1184, a derivative of Δ^8-THC (Ross *et al*., 1999), O-2050 a sulphonamide analogue of Δ^9-THC (Martin *et al*., 2002) and O-2654 derived from cannabidiol (Thomas *et al*., 2004)

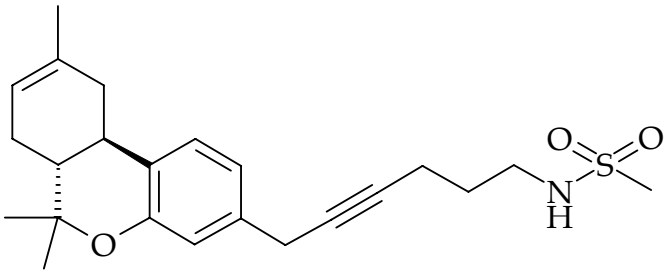
1.3.2.2. CB_2 receptor antagonists / inverse agonists

Ligands in this group are structurally dissimilar from the CB_1 antagonists / inverse agonists (Section 1.3.2.1). Some widely used CB_2 antagonists / inverse agonists include the diarylpyrazoles such as SR 144528 from Sanofi Recherchē (Rinaldi-Carmona *et al*., 1998) and the aminoalkylindole, AM630 (Ross *et al*., 1999). These compounds demonstrate affinities of around 9.50 and 7.50 at the CB_2 respectively and around 5.00 and 5.29 at the CB_1 receptor respectively (Ross *et al*., 1999; Rinaldi-Carmona *et al*., 1998).

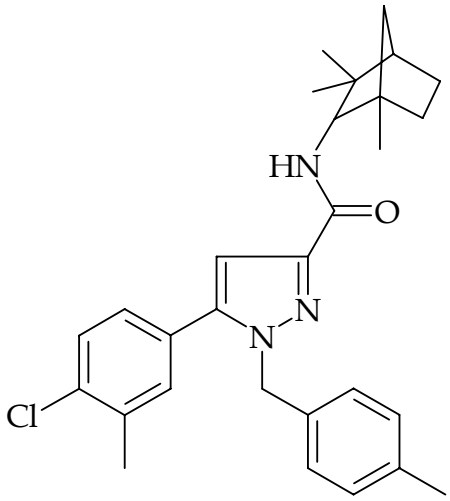


SR 141716 (CB_1): R= Cl

AM 251 (CB_1): R = I



O-2050 (CB_1)



SR 14458 (CB_2)

Figure 1.2: Chemical structures of the cannabinoid receptor antagonists used in the present study. The selectivity of the receptor subtype is indicated in brackets.

1.3.3. Allosteric modulators

In addition to the presence of the primary (orthosteric) binding site on the cannabinoid receptor with which agonists / antagonists / inverse agonists interact, two recent studies have provided evidence for the existence of an allosteric binding site on the CB_1 receptor through which certain compounds interact and cause a conformational change in the receptor structure, and hence alter the affinity and/or intrinsic efficacy of cannabinoids acting at the primary binding site (Price *et al.,* 2005; Horswill *et al.,* 2007).

Org 27569, Org 27759 and Org 29647, developed by Organon Research U.K., and PSNCBAM-1 developed by Prosidion U.K., have been demonstrated to separately modulate the affinity and stimulus response coupling capacity of the CB_1 receptor in an allosteric manner. For example, these compounds have been reported to augment the binding of [^{3}H]-CP 55,940 and [^{3}H]-SR 141716 to the CB_1 receptor in mouse brain membranes and human CB_1 receptor-transfected human embryonic kidney HEK-293 cells, but non-competitively reduce the tissue maxima of the CP 55,940 in functional assays such as the [^{35}S]-guanosine-5'-triphosphate-γS ([^{35}S]-GTPγS) assay on mouse brain membranes and attenuation of the electrically evoked contractions of the mouse isolated vas deferens.

Because these compounds do not compete with cannabinoids for the primary binding site, nor do they display an inherent effect of their own, these observations have led to the suggestion that the Organon and Prosidion compounds behave as allosteric enhancers of cannabinoid binding but inhibitors of efficacy. *In vivo* studies with PSNCBAM-1 in rat model of acute food intake have shown that this compound significantly reduced food intake and body weight in a manner similar to SR 141716 (Horswill *et al.,* 2007). These early studies have pointed to a possible usefulness of targeting the allosteric site as a novel target for therapeutic exploitation.

1.4. Cannabinoid receptor signal mechanisms

Depending on the bioassay system, the cannabinoid receptor can couple to either $G_{i/o}$ or G_s family of G proteins to transduce intracellular signals (Abaji *et al*., 1994; Calandra *et al*., 1999). The G protein isoform utilised can be distinguished pharmacologically with pertussis toxin to which the former is sensitive.

The [^{35}S]GTPγS assay is one of the most common *in vitro* functional bioassays used for demonstrating the activation of the cannabinoid receptor in whole cells or cell membranes either naturally expressing, or transfected with, one or both cannabinoid receptor subtypes (Howlett *et al.,* 2002). The assay measures the ability of cannabinoid receptor agonists to stimulate the coupling of the α-subunit of the G protein to the agonist-occupied receptor, whilst the membrane bound guanosine-diphosphate is exchanged with the metabolically stable analogue of GTP ([^{35}S]GTPγS). The α-[^{35}S]GTPγS complex regulates the activity of ion channels and intracellular enzymes e.g. adenylate cyclase and mitogen activated protein kinases that regulate important cell functions (Howlett & Mukhopadhyay, 2000). Figure 1.3 shows a diagrammatic representation of the signal transduction pathways of the cannabinoid receptor.

1.4.1. Modulation of adenylate cyclase activity

Both cannabinoid receptor subtypes can alter cellular function by modulating cellular levels of the intracellular signalling molecule cyclic adenosine monophosphate (cAMP) with which they do by coupling to adenylate cyclase through $G_{i/o}$ and G_s proteins.

The negative coupling of the receptor to adenylate cyclase through $G_{i/o}$ proteins is associated with an inhibition of the synthesis of cAMP (Howlett *et al*., 1984; Howlett *et al*., 1986) whereas positive coupling with adenylate cyclase through G_s proteins results in the stimulation of the synthesis of cAMP (Felder *et al*., 1998). The ability of cannabinoids to modulate cellular levels of cAMP has been demonstrated to regulate many aspects of cellular function, such as the contractile activity of smooth muscle, the gating properties of ion channels on neurones etc. (Childers & Deadwyler, 1996).

In vitro functional evidence that the CB_1 receptor-induced inhibition of electrically evoked contractions of myenteric plexus longitudinal muscle (MPLM) preparation of guinea-pig small intestine, and the mouse vas deferens can be modulated by manipulating the cellular levels of cAMP has been demonstrated by Coutts and Pertwee, (1998) and Pertwee *et al*., (1996b). In the guinea-pig ileum MPLM, pre-incubation of the tissue with drugs that mimicked the action of cAMP, e.g. 8-bromo-cyclic-AMP, or increased intracellular levels of cAMP by stimulating its production, e.g. with forskolin, or inhibiting its catabolism, e.g. with 3-isobutyl-1-methylxanthine, significantly attenuated the maximal inhibitory response to WIN 55,212-2 (Coutts & Pertwee, 1998). In the mouse isolated vas deferens, in the presence of forskolin, concentrations of CP 55,940 that inhibited electrically-evoked contractions produced a concentration-related inhibitory effect on cAMP production via a SR 141716-sensitive manner presumably due to an action at the CB_1 receptors (Pertwee *et al*., 1996b).

1.4.2. Modulation of mitogen activated protein kinases

The activation of both cannabinoid receptor subtypes is known to cause the phosphorylation and activation of p42/44 and p38 mitogen-activated protein kinases (MAPK) through an interaction with $G_i/_o$ proteins. These enzyme-signalling pathways regulate gene transcription and expression of factors involved in important cellular functions associated with synaptic plasticity, cell division, growth, differentiation and apoptosis (Bouaboula *et al*., 1995; Rueda *et al*., 2000).

1.4.3. Modulation of ion channel function

Data from electrophysiological studies have shown that the CB_1 receptor, but not the CB_2 receptor, couples predominantly through $G_{i/o}$ proteins to certain subtypes of voltage-gated calcium (Ca^{2+}) and potassium (K^+) channels. The CB_1 receptor has been reported to couple negatively to the N, P, Q and L-types of the Ca^{2+} channel (Caufield & Brown, 1992; Gebremedhin *et al*., 1999; Mackie *et al*., 1993; 1995), but positively to A-subtype and inward-rectifying K^+ channels (Mackie *et al.,* 1995; McAllister *et al.,* 1999).

For example, in neuronal cells such as rat cortical and cerebellar brain slices and N18 and NG108-15 brain neuroblastoma-glioma hybrid cells, the negative coupling of the

CB_1 receptor with N, P and Q-types of the Ca^{2+} channel has been implicated in the inhibition of neurotransmitter release as a consequence of a reduction in the influx of Ca^{2+} ions into nerve terminals. The inhibition of the L-subtype Ca^{2+} currents in cat brain arterial smooth muscle cells has suggested as a mechanism by which cannabinoids produce vasodilatation (Gebremedhin *et al*., 1999).

The positive coupling of the CB_1 receptor to the A- and inward-rectifying types of the K^+ channel in neuronal cells has also been suggested to be a mechanism by which cannabinoids inhibit neuronal excitability. A possible mechanism by which the activation of the CB_1 receptor would inhibit neurotransmitter release would through an activation of these K^+ channels to increase the efflux of K^+ ions from the neurones thereby raising the threshold potential for the generation and propagation of an action potential.

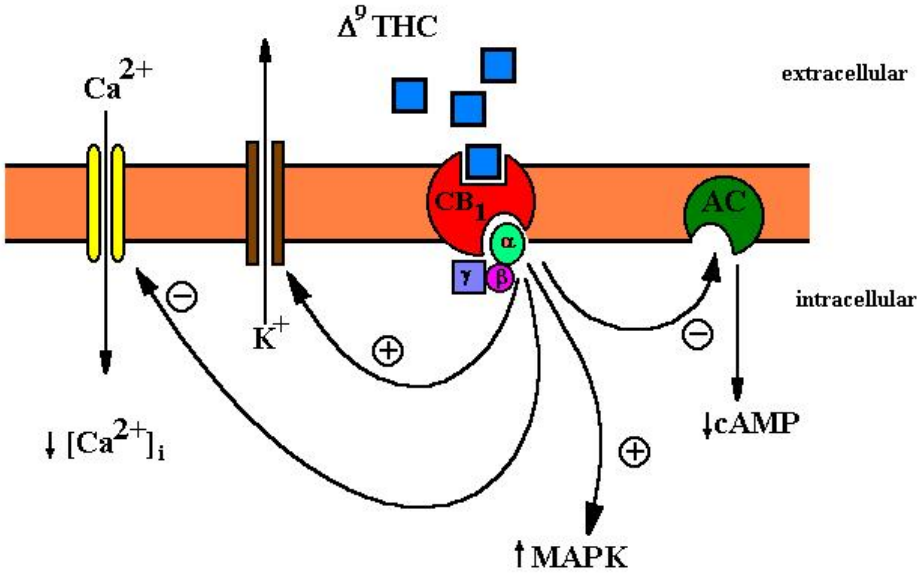


Figure 1.3: Signal transduction pathways of the CB_1 receptor. The presence of Δ^9-THC causes the CB_1 receptor to couple through the $G_{i/o}$ proteins negatively to adenylate cyclase (AC) to reduce the formation of cyclic adenosine monophosphate (cAMP), decrease Ca^{2+} conductance by inhibition of the voltage-gated Ca^{2+} channels (N, P, Q and L types and couple positively to stimulate the K^+ channel (A and inward rectifying types) to hyperpolarise the cell membrane and stimulate mitogen-activated protein kinases (MAPK). With the exception of coupling to ion channels, the CB_2 receptor utilises similar signalling pathways as the CB_1 receptor.

1.5. Other novel pharmacological targets

The availability of both selective antagonists of the CB_1 and CB_2 receptor and transgenic $CB_1^{-/-}$, $CB_2^{-/-}$, and $CB_1^{-/-}$/ $CB_2^{-/-}$ receptor gene knock out mice (Ledent *et al.,* 1999; Zimmer *et al.,* 1999) has provided an approach to characterise the biological role of these two receptors and to investigate whether additional cannabinoid receptor types exist.

As discussed in detail elsewhere (Begg *et al*., 2005; Pertwee, 2004; 2005), since the discovery of the CB_1 and CB_2 receptors, a large body of evidence has emerged to suggest that additional cannabinoid receptor subtypes may exist. Some evidence also exists to suggest that a number of non-cannabinoid receptors possess binding sites for some established CB_1 and CB_2 receptor cannabinoids (Begg *et al*., 2005; Pertwee, 2004).

1.5.1. Transient receptor potential vanilloid type 1 receptor

Originally cloned by Caterina *et al*. (1997) from a rat dorsal root ganglion sensory neurone cDNA library, the transient receptor potential vanilloid type 1 (TRPV1) receptor is non-selective ligand-gated ion channel which has been shown to be expressed along the entire length of sensory neurones in several peripheral tissues including the cardiovascular, respiratory, gastrointestinal and genitourinary systems (Szallasi & Blumberg, 1999). The receptor can be activated by capsaicin, protons and heat, and hence it has been suggested to transduce noxious chemical and physical stimuli (Szallasi & Blumberg, 1999).

The existence of a possible cannabinoid-vanilloid receptor system cross-talk was proposed by Di Marzo *et al*. (1998) on observing close structural similarities between the AEA and the capsaicin analogue olvanil. This hypothesis was confirmed with the demonstration that AEA induced calcitonin gene-related peptide release from perivascular nerves and relaxation of rat and guinea-pig isolated arterial strips was blocked by the TRPV1 receptor antagonist capsazepine but not SR 141716 (Zygmunt *et al*., 1999). Furthermore, radio-ligand binding assays have confirmed that AEA displays affinity for the rat and human TRPV1 receptor (Zygmunt *et al*., 1999; Smart *et al*., 2000).

Further evidence has also been obtained from other *in vitro* functional assays for a role of the TRPV1 receptor in mediating a number of the pharmacological actions of AEA that are not blocked by antagonists of the CB_1 and CB_2 receptor. For example, the AEA-induced inhibition of electrically-evoked contractions of the mouse vas deferens has been shown to occur through both CB_1 and TRPV1 receptor over the same concentration range (Ross *et al*., 2001). In the guinea-pig ileum MPLM preparation, Mang *et al*. (2001) have shown that AEA increased both basal ACh release from the myenteric plexus and resting tone of the longitudinal muscle through a stimulation of the TRPV1 receptor. In the same preparation AEA acting via the TRPV1 receptor potentiated the inhibitory effect of endogenously released GABA (Begg *et al*., 2002b). In the guinea-pig bronchus AEA was found to induce smooth muscle contraction via a capsazepine-sensitive, SR 141716 insensitive manner (Craib *et al*., 2001; Tucker *et al*., 2001).

1.5.2. Putative CB_2-like receptor

Palmitoylelthanolamide, an endogenous structural analogue of AEA, has been reported to display cannabimimetic activity in several functional bioassays inspite of being known to lack affinity for both known cannabinoid receptor types (Felder *et al*., 1993, Showalter *et al*., 1996, Lambert *et al*., 1999). Although palmitoylethanolamide has been reported to decrease locomotor activity (Adams *et al*., 1995) and intestinal transit (Capasso *et al*., 2001) in mice and relax rat isolated mesenteric arteries (White & Hiley, 1998) in similar manner to AEA, the antinociceptive and anti-inflammatory activity of the former fatty acid amide, unlike of the later, are attenuated by SR 144528, albeit weakly, but not by SR 141716 or capsazepine (Calignano *et al*., 2001; Berdyshev *et al*., 1998).

Whilst this putative receptor awaits to be cloned, some evidence for its existence has been obtained from studies on the mouse vas deferens. Griffin *et al*. (1997) have shown that the mouse vas deferens contains CB_2-like mRNA, and that the putative receptor was capable of mediating the inhibitory action of palmitolyethanolamide on the electrically evoked contractions of this tissue *in vitro*.

1.5.3. Putative SR 141716 sensitive, non-CB_1, non-CB_2 receptor

Studies have shown that AEA, its metabolically stable analogue methanandamide, and cannabidiol and its analogues such as abnormal cannabidiol and O-1602, can produce a concentration related relaxation of rat or mouse pre-contracted isolated mesenteric arteries from both wild type and $CB_1^{-/-}$ or $CB_1^{-/-}$ / $CB_2^{-/-}$ knock out mice (Jarai *et al*., 1999; Wagner *et al*., 1999). This effect has been reported not to be reproduced by the other established cannabinoid receptor agonists (Wagner *et al*., 1999), nor is it antagonized by concentrations of SR 141716 which are sufficient to block the CB_1 receptor.

Recent reports suggest that the recently identified and cloned GPR 55 orphan receptor (Sawdargo *et al*., 1999) displays similar properties to the putative SR 141716 sensitive, non-CB_1, non-CB_2 cannabinoid receptor (Brown *et al*., 2005; Sjögren *et al*., 2005). The GPR 55 receptor, originally cloned from the human brain, has therefore been proposed to be the third G protein coupled cannabinoid receptor.

1.5.4. Putative AEA and WIN 55,212-2 sensitive receptor

The existence of a novel G protein-coupled receptor in the mouse brain which can be activated by both AEA and WIN 55,212-2, but not by members of the other classes of the cannabinoid receptor agonist has been proposed by both Di Marzo *et al*. (2000) and Breivogel *et al*. (2001). Both AEA and WIN 55,212-2 have been reported to induce [^{35}S]GTPγS binding in brain membranes of $CB_1^{-/-}$ receptor knock out mice in an SR 141716-SR 144528-and capsazepine-insensitive manner. Because maximal concentrations of AEA produced a submaximal level of stimulation of [^{35}S]GTPγS binding relative to that induced by WIN 55,212-2, and that AEA was also capable of reducing the maximal stimulation produced by the later cannabinoid to its own maxima, these data suggested that both cannabinoids acted through a common mechanism.

1.5.5. Allosteric sites on non-cannabinoid receptors

Other than the established cannabinoid receptor subtypes, i.e. CB_1 and CB_2 receptors, and putative cannabinoid receptors (Section 1.5.1 to 1.5.4), a few recent studies have provided evidence for the presence of binding sites for cannabinoids on some

established non-cannabinoid receptors. Through these binding sites, cannabinoids have been demonstrated act in an allosteric manner to either enhance or reduce the affinity and/or stimulus-response coupling capacity of the non-cannabinoid receptors. Some examples of non-cannabinoid receptors that have been suggested to possess allosteric binding sites for cannabinoids include the serotonin 5-HT_3 receptor, muscarinic acetylcholine (mACh) m_1 and m_4 and nicotinic acetylcholine (nACh) receptors and glutamate NMDA receptor.

In both rat nodose ganglion cells naturally expressing the 5-HT_{3A} receptor and 5-HT_{3A} receptor-transfected human endothelial HEK-293 cells, AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 have been shown to non-competitively inhibit 5-HT-induced inward currents in a non-CB_1-non-CB_2 receptor-mediated manner without altering the affinity of the 5-HT_{3A} receptor for 5-HT (Fan, 1995; Barann *et al*., 2002). These data have suggested that these cannabinoids receptor agonists can behave as allosteric antagonists of 5-HT_{3A} receptor efficacy but not affinity. Because of the physiological role played by the 5-HT_{3A} receptor in emesis, and since cannabinoids are useful clinically for alleviating emesis induced by chemotherapy drugs, a direct allosteric inhibitory effect of cannabinoids on the 5-HT_{3A} receptor may account for the anti-emetic effect of cannabinoids.

Convincing evidence for a direct role of cannabinoids in modulating mACh receptor affinity through a mechanism that is unrelated to their actions on the cannabinoid receptor has been provided by Christopoulous & Wilson, (2001) using radioligand binding assays on Chinese hamster ovary cells expressing the m_1 and m_4 mACh receptors, but not the cannabinoid receptor. These authors showed that AEA, methanandamide and SR 141716 displaced the binding of the selective m_1 and m_4 mACh subtype receptor ligands [^{3}H]-*N*-methylscopolamine and [^{3}H]-quinuclidinyl benzilate in a non-competitive manner. Although the biological significance of these data remain unknown, given the abundant expression of the m_1 and m_4 ACh receptor in the brain and their role in cognition, these findings may explain the well known inhibitory effects that cannabinoids have on learning and memory.

Studies by Oz *et al*. (2003; 2004) have revealed the presence of an allosteric site for cannabinoids on the nACh receptor. In these studies, both AEA and CP 55,940 have

been found to inhibit nicotine-induced inward currents in *Xenopus* oocytes transfected with the nACh receptor in a non-competitive manner through a non-CB_1-non-CB_2 receptor-mediated action.

In contrast to the inhibitory effect of cannabinoids on the binding or stimulus response coupling capacity the receptors described above, AEA and methanandamide have been demonstrated to act allosterically on the NMDA receptor to potentiate NMDA induced currents in *Xenopus* oocytes transfected with NMDA receptors (Hampson *et al*., 1998). These data suggest that these cannabinoids can allosterically enhance the efficacy of the NMDA receptor.

1.6. Cannabinoid bioassays

Because of the many diverse pharmacological actions of cannabinoids in man, several *in vivo* animal, and cell and tissue based *in vitro*, bioassays have been developed to elucidate the mechanism of action of the cannabinoids and for the study of their structure-activity relationships.

1.6.1. In vivo bioassays

1.6.1.1. Mouse tetrad model

This model exploits the ability of cannabinoid agonists to simultaneously induce anti-nociception, catalepsy, hypothermia and sedation in mice within the same dose range and time frame, such that all four effects can be determined in the same animal (Martin *et al.,* 1995).

Although individually these four behaviours are not unique to cannabinoids, their observation together has been regarded to be unique to cannabinoids. The rank order of potencies of cannabinoid receptor agonists determined using this model shows good correlation with the rank order of potencies for cannabinoids acting through the CB_1 receptor. Because the tetrad effects are readily antagonised by CB_1 receptor antagonists and are absent in $CB_1^{-/-}$ transgenic mice, this model is a useful CB_1 receptor assay (Howlett *et al.,* 2002).

However, whilst cannabinoid agonist activity can be readily assessed with this assay, compounds with inverse agonist or partial agonist activity often provide inconsistent results due to their dual pharmacological activity (Howlett *et al*., 2002; Martin *et al.,* 1995).

1.6.1.2. Drug discrimination model

In this test, rats or monkeys are trained to press one of two levers to obtain a food reward and then after receiving injections of a cannabinoid such as Δ^9-THC or its vehicle. After the discrimination has been learned, the animal is monitored for its ability to press a specific lever for the food reward under the influence of a test drug or Δ^9-THC (Howlett *et al.,* 2002; Pertwee, 1997). On the test day, the lever chosen

tells the experimenter whether the test drug produces similar effects to those of Δ^9-THC.

Although drug discrimination studies are a reliable way of determining, with a high degree of accuracy, whether test substances are as like or unlike the drug used for training, they are very time consuming because of the extensive animal training required. Furthermore, this model is also prone to provide false positives when compounds exhibiting dual pharmacological activity are assayed.

Structure-activity relationship studies have revealed that this model serves as a reliable CB_1 receptor assay because of the excellent correlation between the rank orders of potencies of cannabinoid determined with other CB_1 assays and also that SR 141716 antagonises discriminative properties of the animals (Pertwee, 1997).

1.6.1.3. Learning and memory models

These models usually involve the use of the eight-arm radial or water maze to evaluate the ability of cannabinoids to impair working memory and spatial navigation. Rats or mice are trained to navigate to a particular hidden location or platform first before, and then under, the influence of cannabinoids.

Although these models are not specific for cannabinoids, their blockade or reversal by CB_1 receptor antagonists demonstrates their viability as a CB_1 receptor assay (Howlett *et al.*, 2002).

1.6.2. In vitro bioassays

1.6.2.1. Radio-ligand binding assay

This assay allows a quantitative measurement of the affinity of a compound of interest for the cannabinoid receptor by determining the concentration of the test compound that is required to displace a radio-labelled cannabinoid ligand of known concentration from the cannabinoid receptor.

A major disadvantage of this assay is that it cannot be used to distinguish between agonists and antagonists because it does not provide information about the efficacy of the compound (Howlett *et al.*, 2002).

Commonly used radio-labelled cannabinoids include [^{3}H]-SR 141716, [^{3}H]-CP 55,940 and [^{3}H]-WIN 55,212-2. In order to yield useful information about the affinity of the unlabelled test compound, the binding assay must be performed with tissues or cells that contain either the CB_1 receptor or CB_2 receptor but not both.

1.6.2.2. cyclic-AMP assay

This enzyme-based functional assay exploits the ability of cannabinoids to either increase or decrease the basal or drug-induced synthesis of cAMP by stimulating or inhibiting the activity of adenylate cyclase respectively. This assay is based on the principle of competition between cAMP present in a biological sample with a fixed amount of alkaline phosphatase or peroxidase labelled cAMP for sites on a cAMP specific polyclonal antibody. The colour development of a substrate solution added to the reaction medium is used to determine the activity of the bound enzyme. The intensity of the colour is inversely proportional to the concentration of cAMP in the sample. This assay can be performed on whole tissues or cultured cells expressing the cannabinoid receptor naturally or cultured cell lines transfected with the cannabinoid receptor subtypes (Howlett *et al.,* 2002; Pertwee, 1997) to provide information about the affinity, potency and efficacy of the compounds under study.

1.6.2.3. Isolated nerve-smooth muscle preparations

The mouse isolated vas deferens and guinea-pig ileum MPLM are two simple and reliable functional cannabinoid bioassays. These tissues exploit the ability of cannabinoid receptor agonists to inhibit the electrical field stimulation-(EFS-)-evoked contractions of the longitudinal muscle of these tissues in a concentration-dependent and stereo-specific manner. In both tissues, the observed effects are mediated predominantly through the activation of the CB_1 receptor located on nerve terminals involved in neurotransmitter release. Although these bioassays are sensitive, they lack specificity as other classes of drugs also inhibit the EFS-evoked contractions. However, the lack of specificity can be overcome by the use of selective receptor antagonists. Cannabinoids with agonist, antagonists / inverse agonist or partial agonist activity can be easily studied with these assays (Howlett *et al.,* 2002).

1.7. Endocannabinoid system

To date, at least five endocannabinoids have been discovered; AEA (Devane *et al*., 1992), 2-AG (Mechoulam *et al*., 1995; Sugiura *et al*., 1995), noladin ether (Hanus *et al*., 2001), *N*-arachidonoyl-dopamine, (Huang *et al*., 2002), palmitoylethanolamide (Kuehl *et al*., 1957) and virodhamine (Porter *et al*., 2002). Since their discovery, the major proteins and the molecular mechanisms for their biosynthesis, action and inactivation, as well as other pharmacological targets have been characterised (De Petrocellis *et al*., 2004). Collectively, this signalling system has been called the endocannabinoid system.

Of all the endocannabinoids, the biosynthetic and degradative pathway of AEA is the best characterised. AEA is synthesised from the phospholipase D-catalysed hydrolysis of *N*-arachidonyl-phosphatidylethanolamides, which are in turn synthesised from membrane-derived phosphoglycerides and phosphatidylethanolamides by a Ca^{2+}-dependent *N*- acyltransferase (Di Marzo *et al*., 1994; Cadas *et al*., 1997) (Figure 1.4). An alternative pathway by which AEA is synthesised involves the condensation of ethanolamine and free fatty acid (Di Marzo *et al*., 1994).

The termination of AEA signalling appears to be regulated by a two-step process via a putative membrane transporter followed by enzymatic hydrolysis by FAAH (Deutsch & Chin, 1993; De Petrocellis *et al*., 2004; Di Marzo *et al*., 1994; Freund *al*., 2003). Although the transporter has been characterised, it has not been cloned. There is evidence both for and against the existence of this protein, which remains an unresolved controversial subject. FAAH is a membrane bound hydrolase enzyme which shows broad specificity for a number of endocannabinoids (Freund *al*., 2003; Piomelli *et al*., 2005). $FAAH^{-/-}$ mice have also been developed and have confirmed the importance of FAAH for the inactivation of AEA in the brain (Piomelli *et al*., 2005).

A large number of studies have shown that AEA and 2-AG are not stored in vesicles, but are released on demand from various cells in response to a rise in intracellular Ca^{2+} and/or activation of cell surface receptors (see Freund *al*., 2003 for review). In the brain, AEA and 2-AG release have been demonstrated in several regions such as

the hippocampus, hypothalamus and cerebellum, where the somatodendritic region of the post-synaptic cells releases these transmitters retrogradely towards the pre-synaptic neurones in response to nerve stimuli to modulate neuronal excitability (Alger, 2002; Freund *al*., 2003). AEA and 2-AG release has also been demonstrated to occur from non-neuronal sites such as endothelial cells of blood vessels (Deutsch *et al.,* 1997; Sugiura *et al.,* 1998).

Several studies have also demonstrated that levels of a number of endocannabinoids are enhanced during pathophysiological conditions (Schmid, 2002). Because of their diverse actions, endocannabinoids can have both protective and toxic effects on cellular function.

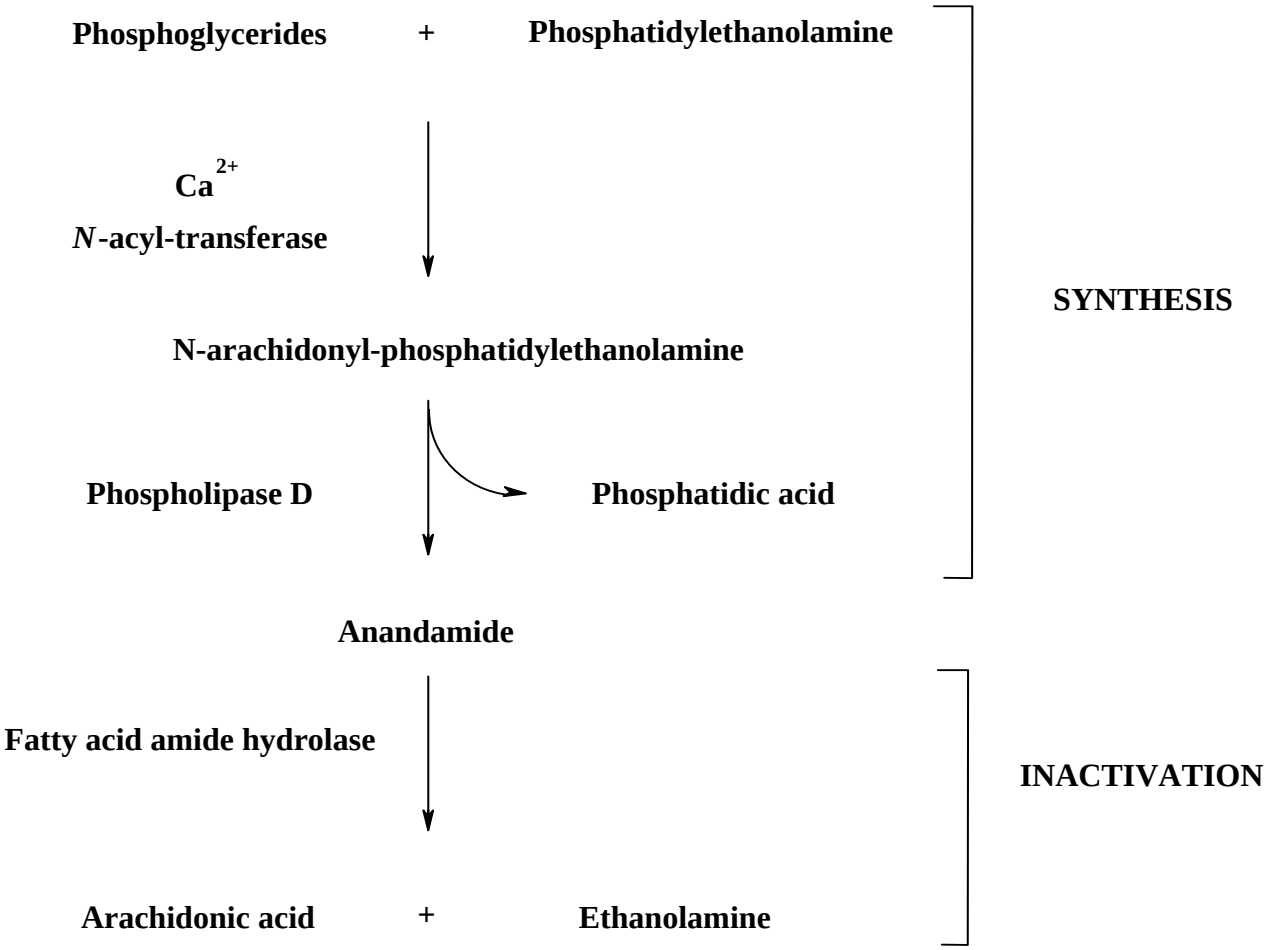


Figure 1.4: Biosynthesis and metabolic pathway for the endocannabinoid anandamide. A rise in intracellular Ca^{2+} triggers *N*-acyl-transferase to transfer arachidonic acid from the phosphoglyceride to the phosphatidylethanolamine. Phospholipase D then cleaves N-arachidonoyl-phosphatidylethanolamine to synthesise AEA. The microsomal enzyme FAAH inactivates AEA into free arachidonic acid and ethanolamine.

1.8. Cannabinoids and the intestinal tract

For centuries, various botanical preparations of *Cannabis sativa* have been used as herbal remedies for a variety of intestinal disorders such as diarrhoea, intestinal pain, ulcerative colitis etc. (Coutts & Izzo, 2004; Hornby & Prouty, 2004; Izzo & Coutts, 2005). However, it has only been in the last two decades years that the mechanisms by which cannabinoids produce their therapeutic effect in the intestinal tract have begun to be elucidated, primarily because of the identification of a functional endocannabinoid system in the tract (for review see Coutts & Izzo, 2004; Duncan *et al*., 2005b; Hornby & Prouty, 2004; Izzo & Coutts, 2005; Izzo *et al*., 2001b, Massa *et al*., 2005; Pertwee, 2001; Pinto *et al*., 2002).

1.8.1. Endocannabinoid system in the enteric nervous system

The enteric nervous system (ENS) is the major site of action of cannabinoids in the intestinal tract. This system consists of nerve cell bodies and their processes which are capable of functioning independently but also under the extrinsic control of the CNS via the vagal, pelvic and splanchnic nerves (Furness *et al*., 2003).

The ENS consists of two ganglionated nerve plexuses, the myenteric plexus and the submucosal plexus, which contain a variety of neurones to modulate the major physiological processes that occur in the intestinal tract i.e. motility, secretion, digestion, absorption and elimination. The myenteric plexus lies between the longitudinal and circular muscle layers to control the movements of the intestinal tract, whereas the submucosal plexus lies near the lumen between the circular muscle layer and submucosa to control water and electrolyte transport (Furness *et al*., 2003) (Figure 1.5).

The neurones of the ENS have been classified into many groups based on their morphology, electrophysiology, key neurotransmitters and projections (Kunze & Furness, 1999). However, for an understanding of their role in modulating intestinal function, they have been divided into four classes as defined by their function. These are the motor neurones, secretomotor/vasomotor neurones, interneurones and intrinsic primary afferent neurones (IPANs) (Kunze & Furness, 1999).

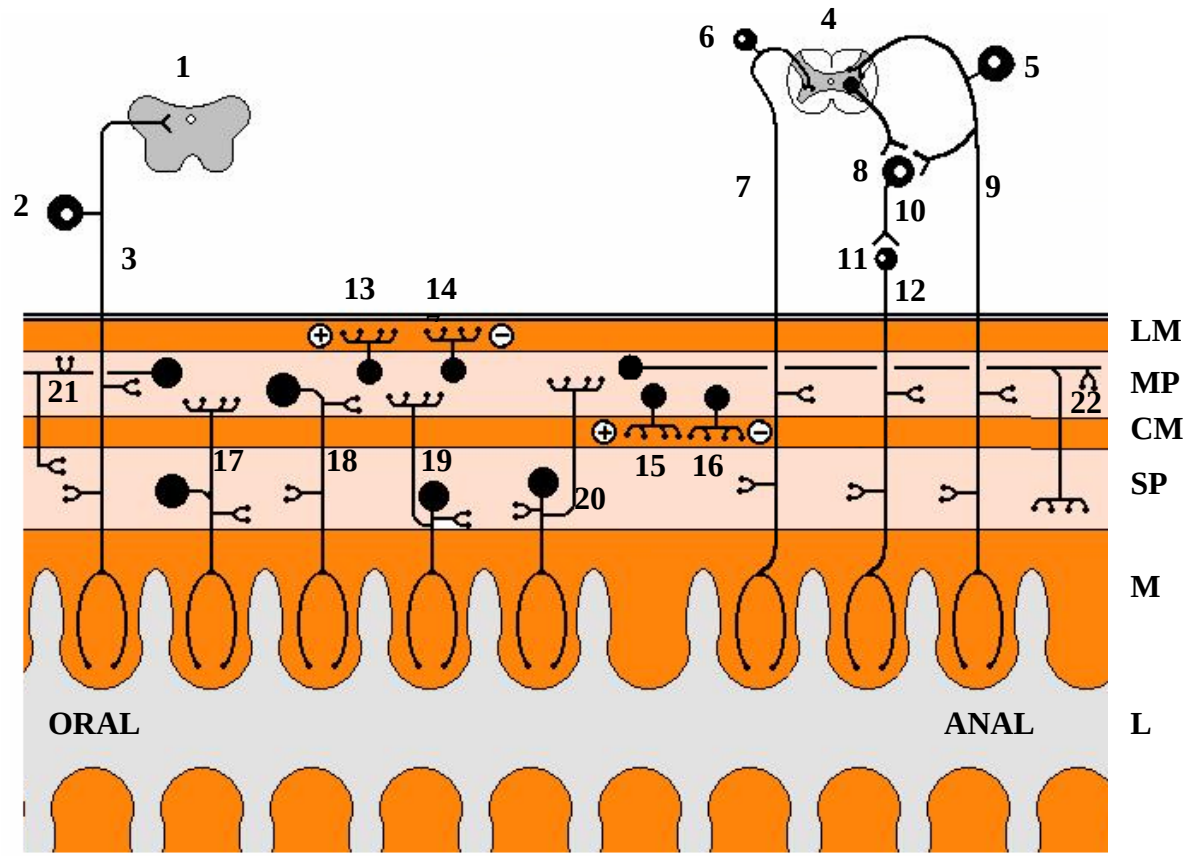


Figure 1.5: The structure of the intestinal tract and type of enteric neurones as defined by their function and projections (Modified from Furness *et al*., 2004). ⊕, excitatory; Θ, inhibitory; LM, longitudinal muscle; MP, myenteric plexus; CM, circular muscle; SP, submucosal plexus; M, mucosa; L, lumen.

1. Brain stem
2. Nodose ganglion
3. Vagus nerve
4. Spinal cord
5. Dorsal root ganglion
6. Pelvic ganglion
7. Pelvic nerve
8. Sympathetic ganglion
9. Spinal primary afferent neurone
10. Splanchnic and Lumbar nerves
11. Celiac and Mesenteric ganglions
12. Celiac and Mesenteric nerves
13. Excitatory longitudinal muscle motor neurone
14. Inhibitory longitudinal muscle motor neurone
15. Excitatory circular muscle motor neurone
16. Inhibitory circular muscle motor neurone
17. Submucosal intrinsic primary afferent neurone
18. Myenteric intrinsic primary afferent neurone
19. Cholinergic secretomotor neurone
20. Non-cholinergic secretomotor neurone
21. Ascending interneurone
22. Descending interneurone

1.8.1.1. Motor neurones

The enteric motor neurones transmit both excitatory and inhibitory stimuli from adjacent neurones to contract and relax the muscle layers respectively (Brookes *et al*., 2001; Furness *et al*., 2003). They have been classified as either excitatory or inhibitory, as defined by the effect they have in the contractility of the smooth muscle layers (Figure 1.5). The primary transmitter of the excitatory neurones is ACh, which acts through the m_3ACh receptor to induce muscle contraction. Other transmitters which can be released from the excitatory motor terminals include 5-HT and substance P which act predominantly through the $5HT_2$ and neurokinin NK_1 receptors respectively (Brookes *et al*., 2001; Furness *et al*., 2003).

In the human, guinea-pig, pig and rat ileal and colonic myenteric plexus, the CB_1 receptor has been shown to be completely co-localised with choline acetyltransferase (ChAT), a marker of ACh-containing neurones (Coutts *et al.,* 2002; Hinds *et al.,* 2006; Kulkarni-Narla & Brown, 2000). A high degree of co-localisation of the CB_1 receptor with immunoreactivity for substance P-containing neurones has also been reported in the pig intestinal tract (Kulkarni-Narla & Brown, 2000). These immunohistochemical data strongly support functional evidence from *in vitro* organ bath studies that activation of the CB_1 receptor inhibits the peristaltic reflex (Heinemann *et al.,* 1999; Izzo *et al*, 2000) and EFS-evoked contractions of the guinea-pig ileum by reducing the release of ACh and substance P from the myenteric plexus (Coutts & Pertwee, 1997; Izzo *et al*, 1998; Mang *et al.,* 2001). Similar data have also been obtained from organ bath studies with the human ileum and colon (Croci *et al.,* 1998; Hinds *et al.,* 2006; Manara *et al.,* 2002) and mouse colon (Mancinelli *et al.*, 2001). These data are also consistent with results obtained from a plethora of *in vivo* studies demonstrating an enteric CB_1 receptor-, but not CB_2 receptor-, mediated delay in the transit of intra-gastrically or intra-duodenally administered non-absorbable markers along the small intestine in rats and mice (Calignano *et al*., 1997; Colombo *et al*., 1998; Izzo *et al.,* 1999a, 2000, 2001a; Landi *et al*., 2002).

However, it is noteworthy that despite the convincing immunohistochemical evidence that the CB_1 receptor is completely co-localised with cholinergic neurones, data from two electrophysiological studies performed on the guinea-pig ileal MPLM have shown that not all neurally-(EFS-and nicotine-) evoked cholinergic responses of this

tissue were sensitive to the inhibitory actions of WIN 55,212-2 and CP 55,940, and those whose activity was attenuated by these cannabinoid agonists were not always mediated through the CB_1 receptor (Lopez-Redondo *et al.,* 1997; Sones *et al.,* 2004). These data suggest that there may be certain subclasses of excitatory cholinergic motor neurones which do not contain the CB_1 receptor or that novel subtypes of the cannabinoid receptor are present in the ENS of the guinea-pig.

A recent study has provided immunohistochemical evidence for the co-localisation of the CB_2 receptor with nerves expressing calretinin, a marker for excitatory motor neurones in the rat ileal myenteric plexus (Duncan *et al.,* 2005a). However, the co-localisation of this receptor with specific markers of excitatory neurotransmitters was not reported. Thus, it clear that further studies are now required to investigate whether the CB_2 receptor has a functional role in modulating intestinal transit.

The inhibitory motor neurones involved in the relaxation of the smooth muscle layers are known to release a combination of transmitters such as nitric oxide (NO), noradrenaline (NAdr), adenosine triphosphate (ATP), vasoactive intestinal polypeptide (VIP) (Brookes *et al*., 2001; Furness *et al*., 2003; Kunze & Furness, 1999). Immunohistochemical studies performed on the human, mouse and pig colonic myenteric plexus have shown that the immunoreactivity of the CB_1 receptor does not overlap with markers of inhibitory neurones and transmitters (Hinds *et al.,* 2006; Kulkarni-Narla & Brown, 2000; Pinto *et al.,* 2002). Furthermore, cannabinoid receptor agonists have no effect on inhibitory junctional potentials of the mouse colon or relaxations of the human colon evoked by EFS of the inhibitory nerves (Hinds *et al.,* 2006; Storr *et al.,* 2004). These data suggest that activation of the CB_1 receptor decreases intestinal transit by attenuating the release of contractile transmitters from excitatory motor neurones and not by facilitating of the release of inhibitory transmitters.

1.8.1.2. Secretomotor/vasomotor neurones

The secretomotor/vasomotor neurones reside in the submucosal plexus but project to the mucosa and submucosal blood vessels to regulate water and electrolyte levels and blood flow through local reflex circuits respectively (Brookes *et al*., 2001; Costa *et al.,* 2000). These neurones are also classified as either excitatory or inhibitory. ACh, substance P and VIP acting via the m_3ACh, NK_1 and $VPAC_1$ receptors respectively, are the predominant excitatory transmitters which cause an increase in water and electrolyte secretion (Costa *et al.,* 2000; Furness *et al*., 2003). NAdr is the principle neurotransmitter released upon activation of the inhibitory secretomotor neurones and acts through the α_1 and α_2 adrenoceptors to decrease submucosal blood flow and secretion of electrolytes and water respectively (Costa *et al.,* 2000; Furness *et al*., 2003)

In the guinea-pig ileal submucosal plexus, the CB_1 receptor has been associated with all VIP-containing neurones (MacNaughton *et al.,* 2004). However, a similar immunoreactivity is absent in the pig submucosal plexus (Kulkarni-Narla & Brown, 2000). These data suggest a species specific difference in the distribution of the CB_1 receptor. *In vitro* functional studies using the Ussing chamber technique to measure electrogenic ion transport, have shown that activation of the CB_1 receptor in the guinea-pig and rat ileal submucosal plexus-mucosal (SPM) preparations with WIN 55,212-2 caused an inhibition of the capsaicin- and EFS-evoked increases in electrolyte secretion respectively. These inhibitory effects were not on the epithelial cells because the secretory responses to forskolin and carbachol, which act directly on the epithelium to elicit secretion, were not affected by WIN 55,212-2 treatment (MacNaughton *et al.,* 2004; Tyler *et al.,* 2000). Moreover, in extrinsically denervated segments of the guinea-pig ileum, the inhibitory effect of WIN 55,212-2 on the response to EFS was completely lost, suggesting that cannabinoids appear to act first through extrinsic primary afferent neurones to modulate submucosal secretomotor neurone function (MacNaughton *et al.,* 2004). Far fewer studies have examined the role of the endocannabinoid system in the regulation of intestinal secretion *in vivo*. Oral administration of croton oil (Izzo *et al.,* 2001b) or cholera toxin (Izzo *et al.,* 2003) to mice has been reported to cause diarrhoea and fluid accumulation in the small intestine through a disruption of the submucosa, activation of enteric neurones

and release of endogenous secretagogues. Treatment with CP 55,940 was shown to concentration-dependently inhibit the diarrhoea and fluid accumulation in a SR 141716-sensitive and SR 144528-insensitive manner, suggesting the involvement of the CB_1 receptor and not the CB_2 receptor.

1.8.1.3. Interneurones

Interneurones behave as relay neurones to convey information between and within the myenteric and submucosal plexus. They are classified as either ascending (orally projecting) or descending (anally projecting), and are suggested to be involved in local neural circuits which control the propulsive function of the intestine (Brookes *et al*., 2001; Kunze & Furness, 1999) (Figure 1.5). Neurotransmission in the ascending pathways is cholinergic via the nACh receptor but non-cholinergic in the descending pathways, probably involving ATP, 5-HT, NO and VIP (Furness *et al* 2003). The ascending neurones which release ACh are excitatory and may contain the CB_1 receptor to modulate local motility.

Some functional evidence for the presence of the CB_1 receptor on ascending interneurones has been provided from studies demonstrating the CB_1 receptor-mediated inhibitory effect of WIN 55,212-2, CP 55,940 and methanandamide on the distension evoked peristaltic reflex of the guinea-pig isolated ileum. In this assay, all three cannabinoids inhibited the peristaltic reflex by increasing the threshold pressure and volume required for eliciting the reflex, whilst concomitantly decreasing the ejection pressure for the emptying phase (Heinemann *et al*., 1999; Izzo *et al*., 2000). This suggested that cannabinoid agonists inhibited the peristaltic activity by blockade of ascending excitatory motor pathways. These data have been shown to extend to the peristaltic reflex of the mouse isolated colon (Mancinelli *et al*., 2001).

1.8.1.4. Intrinsic primary afferent neurones

IPANs are sensory nerves that respond to chemical and mechanical stimuli (Furness *et al*., 2004). They connect to motor neurones, interneurones, secretomotor neurones and other IPANs to initiate reflex pathways involved in the control of gut movement, water and electrolyte secretion and blood flow (Brookes *et al*., 2001). IPANs also have efferent functions in that they release transmitters onto the mucosal epithelium

and the smooth muscle layers (Figure 1.5). Key transmitters released from these nerves are ACh, VIP, 5-HT and substance P (Furness *et al*., 2004).

Co-localisation between the immunoreactivity of the CB_1 receptor with ChAT, and calbindin, a marker for IPANS has been demonstrated in the rat and guinea-pig ileal myenteric plexus (Coutts *et al.,* 2002). Additionally, IPANS containing immunoreactivity for ChAT, substance P and the CB_1 receptor have been demonstrated in the pig ileal and colonic submucosal plexus (Kulkarni-Narla & Brown, 2000). These data indicate that cannabinoids can modulate the activity of motor neurones, interneurones and secretomotor neurones indirectly by acting on the IPANS.

1.8.2. Other components of the endocannabinoid system in the intestinal tract

In addition to the presence of the cannabinoid receptor in the ENS, levels of both AEA and 2-AG high enough to cause receptor activation have been reported in homogenates of the intestine of guinea-pig (Guagnini *et al.,* 2006), human (Wright *et al.,* 2005; Valenti *et al.,* 2005), mouse (Izzo *et al.,* 2001a), rat (Katayama *et al.,* 1997; Valenti *et al.,* 2005) and dog (Mechoulam *et al.,* 1995). However, in addition to species-related differences in the levels of AEA and 2-AG in the intestinal tract, significant regional differences in the levels of these endocannabinoids have been noted, which may reflect a difference in the activity of these endocannabinoids.

Unlike in the brain, neither the sources nor mechanisms by which endocannabinoids are released in the intestinal tract have been elucidated. However, the mechanisms for the inactivation of the endocannabinoids have been suggested to be present in the mouse small intestine because *in vivo* administration of VDM-11, an inhibitor of the putative endocannabinoid uptake transporter, into mice treated with cholera toxin caused a significant decrease in the diarrhoea in an SR 141716-sensitive manner (Izzo *et al.,* 2001a). Additionally, VDM-11 has also been shown to worsen ileus in mice caused by an abdominal injection of acetic acid in an SR 141716-sensitive manner (Mascolo *et al.,* 2002). These data indicate the presence of both the endocannabinoid uptake transporter and an active endocannabinoid agonist tone in the mouse small intestine.

To date, FAAH has only been isolated from the ileum of the rat and not of other species (Katayama *et al*., 1997). In the rat ileum, FAAH immunoreactivity has been detected on cell bodies and nerve fibres in both the myenteric and submucosal plexus (Duncan *et al.,* 2005b). However, the classes of enteric neurones that express FAAH have not been identified. *In vivo* administration of two FAAH inhibitors, AA-5-HT or palmitoylisopropylamide, into mice has been shown to delay small intestinal motility in an SR 141716-sensitive manner (Capasso *et al.,* 2005). Moreover, the inhibitory effect of the FAAH inhibitors was absent in both $CB_1^{-/-}$ mice and $FAAH^{-/-}$ mice suggesting that the decrease in intestinal transit caused by FAAH inhibitors was through a potentiation of the inhibitory effect of tonically released endocannabinoid agonists at the CB_1 receptor.

The presence of an active endocannabinoid tone in several *in vivo* and *in vitro* bioassays has been suggested because CB_1 receptor antagonists alone produce effects which are opposite in direction to those produced cannabinoid receptor agonists. For example, while *in vivo* administration of cannabinoid receptor agonists into rodents delays intestinal transit, SR 141716 alone increases intestinal transit (Colombo *et al.,* 1998; Izzo *et al.,* 1999a, b; 2001a). Similarly, while cannabinoid receptor agonists cause an inhibition of the EFS-evoked contractions of the guinea-pig ileum MPLM, the presence of SR 141716 alone augments the amplitude of the contractions. Whilst these effects of SR 141716 are suggestive of an ongoing endocannabinoid agonist activity, these data have to be interpreted with caution because SR 141716 is known to have inverse agonist activity (Pertwee, 2005).

1.9. Aim and objectives

1.9.1. Aim

The rat has long served as a common *in vivo* model for characterising the pharmacological actions of cannabinoids on intestinal motility and secretion. Given the lack of *in vitro* functional bioassays for evaluating cannabinoid effects on these intestinal functions of this species, the aim of this study was to investigate the viability of the contractile function of the isolated ileum myenteric plexus longitudinal muscle (MPLM) preparation and the secretory function of the isolated colonic submucosal plexus-mucosal (SPM) preparation as novel rat *in vitro* intestinal cannabinoid bioassays, and to pharmacologically characterise the cannabinoid receptor subtypes modulating the neurally evoked responses of these tissues.

1.9.2. Objectives

1.9.2.1. Contraction studies

- To investigate whether representatives of the four main classes of structurally different cannabinoid receptor agonists i.e. AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 inhibited the EFS evoked contractions of rat ileum MPLM.
- To identify the cannabinoid receptor subtypes mediating the effect of the four cannabinoid receptor agonists under the EFS conditions using cannabinoid receptor subtype selective antagonists / inverse agonists in the rat isolated ileum MPLM.
- To determine whether any non-cannabinoid receptor-mediated effects of the cannabinoid receptor agonists and antagonists / inverse agonists observed the under the EFS conditions in the rat ileal MPLM extended to the guinea-pig ileal MPLM.
- To seek evidence for the presence of a functional endogenous cannabinoid tone in the rat MPLM and to determine whether the evidence extended to the guinea-pig ileum MPLM and rat vas deferens.

1.9.2.2. Secretion studies

- To investigate the role of the CB_1 receptor in modulating the pharmacologically evoked secretory function of the rat colonic SPM preparation.

CHAPTER 2:

GENERAL MATERIALS and METHODS

2.1. Animals

Tissues were obtained from male Wistar rats (400 to 550 g) and Dunkin-Hartley or Heston-2 guinea-pigs of either sex (500 to 800 g). All animals were bred at the Biological Services Unit of the University of Hertfordshire, U.K. from stock originating at Charles River Laboratories, U.K. and Harlan, U.K. respectively.

The animals were housed in rooms with a controlled temperature (22 ± 1 ^{O}C), relative humidity (55 ± 10 %) and 12 hour light-dark cycle with food and water available *ad libitum*. Rats were killed by carbon dioxide (CO_2) asphyxiation, whilst guinea-pigs were killed by a blow to the head followed by exsanguination. All animal care and killing was conducted in accordance with requirements of the Animals (Scientific Procedures) Act 1986 and the University of Hertfordshire ethical review committee.

2.2. Isolated nerve-smooth muscle preparation contraction studies

2.2.1. Tissue dissection: Myenteric plexus longitudinal muscle preparation

A 15 cm segment of the ileum was excised from the small intestine both rats and guinea-pigs and immersed in Krebs-Henseleit solution A of the composition (in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5, and gassed with 95 % O_2 and 5 % CO_2 at room temperature (21 ± 4 ^{O}C).

A maximum of four 2 cm length MPLM strips were dissected from the ileum segment by the method of Paton & Zar (1968) after discarding the first 5 cm length closest to the ileo-caecal junction. Briefly, a 2 cm segment of the ileum was flushed of its contents with Krebs-Henseleit solution A and slipped over a glass rod of 5 mm diameter. After trimming the mesentery, the longitudinal muscle with the adhered myenteric plexus was separated from the rest of the ileum by gentle stroking with a cotton bud soaked with the Krebs-Henseleit solution A, starting at the mesenteric border and working along and around the length and circumference of whole ileum (Figure 2.0). Cotton sutures were tied at both ends of the strip, and the tissue was suspended between a pair of platinum wire electrodes, approximately 1 cm in length and 5 mm apart, and a Dynamometer UF1 isometric force transducer (Pioden

Controls, U.K.). The electrode with the tissue was then immersed in either 5, 10 or 30 ml jacketed organ baths containing Krebs-Henseleit solution A at 33 or 37 ^{O}C and bubbled with a mixture of 95 % O_2 and 5 % CO_2 (Figure 2.1). The majority of the experiments were carried out at 33 ^{O}C in order to dampen the irregular spontaneous activity of the tissues, particularly of the rat ileum MPLM, to enable the evoked responses to be quantified easily. Pilot studies showed that running the experiments at 33 ^{O} C had no significant effect on the ability of the tissues to respond to EFS or any drug. Therefore, data from experiments carried out at the different bath temperatures have been pooled.

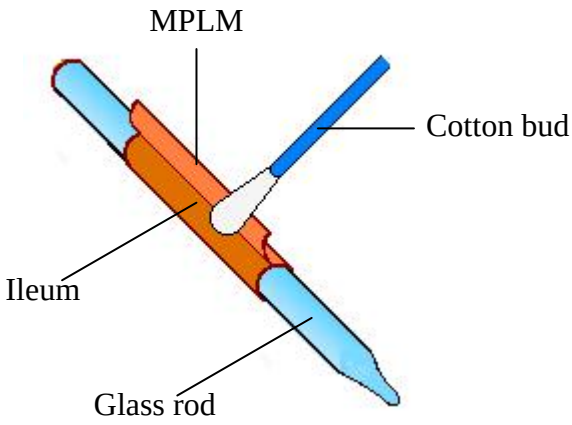


Figure 2.0: Separation of the myenteric plexus-longitudinal muscle from an ileum segment.

2.2.2. Tissue dissection: Vas deferens

Both vasa deferentia were dissected from the rats and placed in Krebs-Henseleit solution B of the composition (in mM): NaCl 118.3, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5, and gassed with 95 % O_2 and 5 % CO_2 at room temperature (21 ± 4 ^{O}C). Krebs-Henseleit solution B differed from Krebs-Henseleit solution A (Section 2.2.1) in that Mg^{2+} was excluded. After expelling the contents from both vas deferentia by gentle squeezing, cotton sutures were tied at both ends and the tissue was suspended between a pair of platinum wire electrodes, approximately 1 cm in length and 5 mm apart, and a Dynamometer UF1 isometric force transducer (Pioden Controls, U.K). The electrodes with the tissues were then immersed in 30 ml organ baths containing Krebs-Henseleit solution B and bubbled with 95 % O_2 and 5 % CO_2 and maintained at 37 ^{O}C.

2.2.3. Apparatus set-up and maintenance

The organ baths were connected by silicone tubing to reservoirs containing the Krebs-Henseleit solutions via jacketed heating coils. The temperature of the organ baths and heating coils were controlled thermostatically with water circulated by thermocirculators (Harvard Apparatus, U.K.).

Changes in tension of the tissues was recorded isometrically in units of grams (g) using the isometric force transducers connected to a MacLab Chart Version 3.5 data-acquisition system on an Apple Macintosh computer (Apple Macintosh, U.K.), a PowerLab Chart Version 5.2 data-acquisition system (AD Instruments, U.K.) on a Personal computer (Dell, U.K.) or MX216 or MultiTrace-4 chart recorders (Lectromed, U.K.).

For experiments involving EFS, the electrodes were connected to Grass S11 or S88 stimulators (Grass Instruments, U.S.A) or a Multistim D330 (Digitimer, U.K.) stimulator via a variable cycle timer (Harvard Apparatus, U.K).

Cannabinoids have been shown to stick to glassware due to their high lipophilicity. At the end of every experiment involving the use of cannabinoids, the organ baths and stimulation electrodes were washed thoroughly with 70 % ethanol followed by 2 M hydrochloric acid and copious amounts of distilled water.

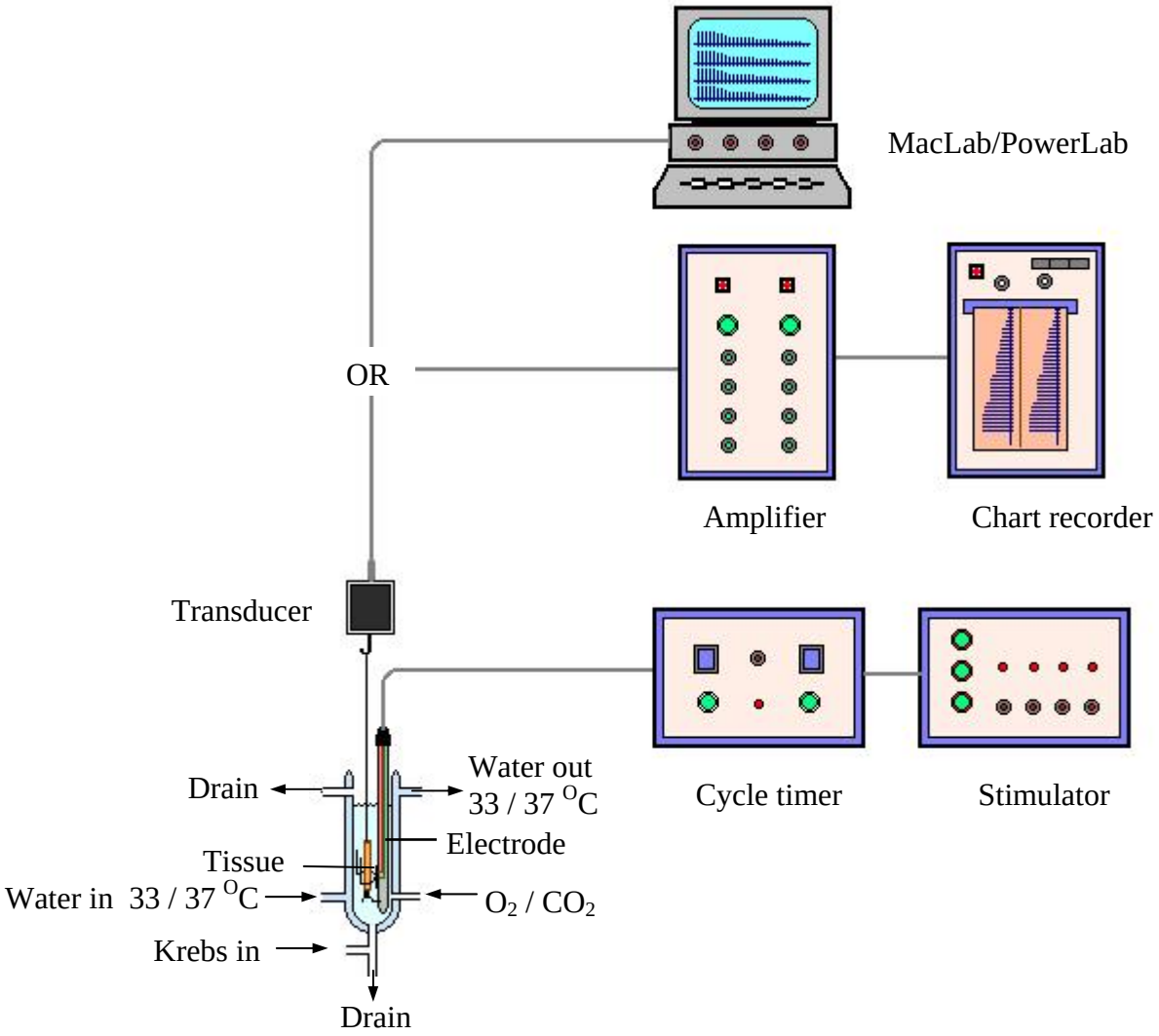


Figure 2.1: Schematic diagram of the apparatus for recording the motility of the isolated nerve-smooth muscle preparations (not to scale).

2.2.4. Electrical field stimulation parameters

The EFS parameters employed for evoking contractions of rat and guinea-pig ileum MPLM and the rat vas deferens are summarised in Table 2.0. The parameters have been chosen from previous studies where some of the cannabinoid ligands used in the present study have been reported to produce an effect. Where these are not available, EFS parameters from studies on equivalent tissues were used.

Table 2.0: Electrical field stimulation parameters and the contractile transmitters released by the isolated nerve-smooth muscle preparations

Tissue	Frequency	Pulse width	Voltage	Contractile transmitter	Reference
Rat ileum MPLM	0.05 Hz	0.5 ms	110 % supramaximal	ACh	-
	30 Hz for 2 sec every min	0.5 ms	110 % supramaximal	ACh	Borelli *et al.*, 2004
Guinea-pig ileum MPLM	0.1 Hz	0.5 ms	110 % supramaximal	ACh	Gill *et al.*, 1970
	30 Hz for 2 sec every min	0.5 ms	110 % supramaximal	ACh	-
Rat vas deferens	0.1 Hz	0.5 ms	110 % supramaximal	ATP	Pertwee *et al.*, 1993

2.2.5. Experimental design

2.2.5.1. Myenteric plexus-longitudinal muscle preparation

2.2.5.1.1. Electrical field stimulation studies

After 10 minutes since placing the tissues in the organ baths, each tissue was stretched by 0.5 g and allowed to equilibrate for a further 50 minutes until EFS was commenced. As summarised in Table 2.0, the rat and guinea-pig ileum MPLM strips were subjected to EFS over the entire duration of the experiment, either with single pulses repeated at a frequency of 0.05 Hz or 0.1 Hz respectively, or with trains of pulses for 2 seconds every minute at a frequency of 30 Hz.

Each pulse at any given frequency was of 0.5 ms duration and at a voltage that was 10 % greater than that required to elicit maximal contractions. 110 % supramaximal voltage was employed to ensure that the neurones sensitive to the respective frequency within the field of stimulus were stimulated and that the EFS conditions were comparable with previous studies (Coutts & Pertwee 1997; Mang *et al*., 2001; Pertwee *et al*., 1996).

No drugs were added until the amplitude of EFS-evoked contractions had become consistent for a minimum of a 30 minute period. The time taken for the amplitude of the contractions to become uniform was about 3 to 4 hours. It was also noted that the amplitude of the EFS-evoked responses could remain stable for a further 4 hours without the need to renew the bathing Krebs-Henseleit solution. On each day, the drug treatments were randomised between the organ baths. Only one concentration-response curve was constructed per tissue, as previous studies have shown that due to their lipophilicity, cannabinoids cannot be washed from the tissue by replenishing the organ bath with drug free Krebs-Henseleit solution (Pertwee *et al.*, 1992).

For tissues subjected to EFS with single pulses (0.05 Hz for rat ileum MPLM and 0.1 Hz for guinea-pig ileum MPLM), concentration-response curves to the cannabinoid receptor agonists were constructed with a 20 minute dosing interval. When competition studies were performed, the cannabinoid receptor antagonists / inverse agonists were administered 20 minutes prior to the addition of the cannabinoid receptor agonists.

When both the rat and guinea-pig ileum MPLM were subjected to EFS with trains of pulses at 30 Hz, concentration-response curves to the cannabinoid receptor agonists were constructed with a 30 minute dosing interval. When competition studies were performed, the cannabinoid receptor antagonists / inverse agonists were administered 30 minutes prior to the addition of the cannabinoid receptor agonists.

For all other competition studies, such as with the FAAH inhibitors (see Chapter 4), the FAAH inhibitors or their vehicles were administered 20 minutes prior to the addition of a cannabinoid receptor agonists. The concentration-response curves to the cannabinoid receptor agonists were constructed with a 20 minute dosing interval in the presence of the FAAH inhibitors.

All experiments were performed in parallel with relevant vehicle treated and time controls.

2.2.5.1.2. Muscarinic acetylcholine receptor stimulation studies

If a cannabinoid was found to alter the amplitude of EFS-evoked contractions, its ability to produce a similar effect on contractions elicited by exogenously applied ACh was investigated. Two experimental designs were used for carrying out this objective. The first involved administering the highest concentration of the cannabinoid, which maximally increased or decreased the amplitude of EFS-evoked contractions, between two semi-logarithmic ACh (10^{-10} to 10^{-5} M) cumulative concentration-response curves constructed 30 minutes apart. The second experimental design involved administering a single concentration of a cannabinoid in half-logarithmic increments between single administrations of ACh (10^{-6} M) over 30 minute intervals on a single piece of tissue. For both experimental designs, each tissue acted as its own control. All experiments were performed in parallel with relevant vehicle treated and time controls.

2.2.5.2. Vas deferens

After 10 min since placing the tissues in the organ baths, each tissue was stretched by 0.5 g and allowed to equilibrate for a further 50 minutes until EFS was commenced. No drugs were added until the amplitude of EFS-evoked contractions had become consistent for a minimum of a 30 minute period. The time taken for the amplitude of

the contractions to become uniform was about 1 hour. It was also noted that the amplitude of the EFS-evoked responses could remain stable for a further 4 hours without the need to renew the bathing Krebs-Henseleit solution. On each day, the drug treatments were randomised between the organ baths.

Cumulative concentration-response curves to the cannabinoids were constructed cumulatively with a dosing interval of 20 minutes. Only one concentration-response curve was constructed per tissue. Competition studies were only performed with WIN 55,212-2 and SR 141716 for the purpose of comparison to the EFS experiments on the rat ileum MPLM. SR 141716 was added 20 minutes prior to the addition of WIN 55,212-2. All experiments were performed in parallel with relevant vehicle-treated and time controls

2.2.5.3. Data analysis

This section describes the methods used for quantifying and analysing the effects of the cannabinoid receptor agonists in the presence and absence of the competing ligands on tissues stimulated with EFS (Section 2.2.5.3.1) and ACh (Section 2.2.5.3.2).

2.2.5.3.1. EFS studies

2.2.5.3.1.1. Quantification of the agonist data in the absence and presence of a competing ligand

The inhibition of the EFS-evoked contractions by a cannabinoid receptor agonist in the presence of a competing ligand or its vehicle was calculated in percentage terms using Equation 2.0:

$$\% \text{ Inhibition of contraction} = 100\left(\frac{y-x}{y}\right) \qquad \text{(Equation 2.0)}$$

where x denotes the amplitude of the evoked contractions after each addition of the agonist and y the amplitude immediately before the first addition of the competing ligand or its vehicle.

2.2.5.3.1.2. Quantification of the inherent effect of the competing ligand

During some competition experiments, depending on the tissue and/or frequency of EFS employed, the presence of certain competing ligands alone either enhanced or reduced the amplitude of the EFS-evoked contractions (see Results in Chapter 3). Therefore, the enhancement and (or) reduction of the amplitude of the EFS-evoked contractions was calculated using the methods describe in Section 2.2.5.3.1.2.1 and 2.2.5.3.1.2.2 respectively.

2.2.5.3.1.2.1. Quantification of the enhancement of the contractions

The increase in the amplitude of the EFS-evoked contractions by a ligand was calculated in percentage terms using Equation 2.1:

$$\% \text{ Potentiation of contraction} = 100\left(\frac{x-y}{y}\right) \qquad \text{(Equation 2.1)}$$

where x denotes the amplitude of the evoked contractions after each addition of the ligand and y the amplitude immediately before the first addition of the same ligand.

2.2.5.3.1.2.2. Quantification of the reduction of the contractions

The reduction in the amplitude of the EFS-evoked contractions by a ligand was calculated in percentage terms using Equation 2.2:

$$\% \text{ Inhibition of contraction} = 100 - \left(100\frac{x}{y}\right) \qquad \text{(Equation 2.2)}$$

where x denotes the amplitude of the evoked contractions after each addition of the ligand and y the amplitude immediately before the first addition of the same ligand.

2.2.5.3.1.3. Graphical presentation of the data

Individual agonist concentration-response curves in the absence and presence of a competing ligand were fitted via non-linear regression to the four-parameter Hill equation (Equation 2.3), using GraphPad PRISM 4.0 for Windows (GraphPad Software, CA., U.S.A);

$$E = \text{Basal} + \frac{E_{max} - \text{Basal}}{1 + 10^{(\text{LogEC}_{50} - \text{Log[A]})^{n_H}}} \qquad \text{(Equation 2.3)}$$

where E denotes response, Log [A] the logarithm of the concentration of an agonist A, n_H the midpoint slope of the curve, Log EC_{50} the logarithm of the midpoint location parameter along the concentration axis, and E_{max} and Basal the upper and lower asymptotes respectively.

The concentration-response data were plotted as the mean ± standard error of the mean (mean ± s.e.m). Shifts in the agonist concentration concentration-response curve by the presence of a competing ligand was compared by a one-way ANOVA, followed by a Dunnett's post hoc test. The probability $P < 0.05$ was taken to be statistically significant.

2.2.5.3.1.4. Quantification of the augmentation and antagonism of the agonist action by the presence of a competing ligand

The cannabinoid receptor agonist data quantified in the absence and presence of a competing ligand devoid of inherent activity yielded concentration-response curves with similar upper and lower curve asymptotes. Therefore, the EC_{50} for each agonist concentration-response curve in the absence and presence of a competing ligand was chosen for the measurement of the shift as a concentration ratio.

In contrast, the cannabinoid receptor agonist data quantified in the presence of a competing ligand with inherent activity yielded concentration-response curves with similar upper asymptotes but different lower asymptotes. Because under these circumstances the EC_{50} value of each agonist concentration-response curve in the absence and presence of the competing ligand did not result in an equivalent level of response, the concentration ratios were measured from the agonist concentrations

corresponding to the 50 % equieffective response level in the absence and presence of the competing ligand.

Equations 2.4 and 2.5 show the formulae used for measuring the concentration ratio for the leftward and rightward shift of an agonist concentration-response curve in the presence of a competing ligand respectively.

$$\text{Concentration ratio} = \frac{A}{A'} \qquad \text{(Equation 2.4)}$$

$$\text{Concentration ratio} = \frac{A'}{A} \qquad \text{(Equation 2.5)}$$

In both Equation 2.4 and 2.5, *A* denotes the EC_{50} value of the cannabinoid receptor agonist in the absence of the competing ligand whereas *A'* represents either the EC_{50} value or the concentration corresponding to the 50 % effect of the agonist in the presence of a given concentration of a competing ligand respectively. Thus, for every concentration of the competing ligand, a corresponding value of the concentration ratio was calculated.

2.2.5.3.1.4.1. Measurement of the antagonist affinity and /or potency

When a series of concentration ratio values were available from experiments in which multiple concentrations of an antagonist were used, a Schild plot was constructed to estimate the pK_B of the antagonist using the method of Arunlakshana & Schild, 1959. The Schild plot represented a linear regression of Log (Concentration ratio - 1) upon Log [B], for determining both the gradient and the 95% Confidence Intervals (C.I.) of the slope, and the pK_B of the antagonist. Each plot was fitted via the linear regression function (Equation 2.6) using GraphPad PRISM 4.0.

$$y = m\,x + pK_B \qquad \text{(Equation 2.6)}$$

where *y* denotes the logarithm of the concentration ratio minus one i.e. Log (Concentration ratio - 1), *x* the logarithm of a given concentration of an antagonist B i.e. (Log [B]), *m* the gradient of the slope i.e. the ratio of the change in Log

(Concentration ratio - 1) for each unit change in Log [B], and pK_B the negative logarithm of Log [B] when Log (Concentration ratio - 1) = 0.

If the gradient of the Schild plot was not significantly different from unity, the antagonism was taken to be simple competitive. However, if the slope was significantly different from unity, but the 95 % C.I of the slope included unity, the plot was re-fitted by constraining the slope to unity. The intercept of the Log [B] axis where Log (Concentration ratio – 1) = 0 was read as the pK_B of the antagonist.

By definition, the pK_B represented the equilibrium dissociation constant of the agonist-receptor complex, and the negative logarithm of the antagonist concentration that occupied 50 % of the receptors at equilibrium. Hence the pK_B was a measure of the affinity of the antagonist for a specific receptor (Arunlakshana & Schild, 1959).

When only a single value for the concentration ratio was available from experiments in which a single concentration of an antagonist B was used, the antagonist potency (pA_2) was calculated using the Gaddum-Schild equation (Equation 2.7; Schild, 1947);

$$pA_2 = \text{Log (Concentration ratio} - 1) - \text{Log [B]} \qquad \text{(Equation 2.7)}$$

The pA_2 represented the negative logarithm of the concentration of the antagonist B that produced a rightward shift of the agonist concentration-response curve by two logarithmic units. Because the pA_2 was derived from a single measurement, it represented an empirical measure of the potency of the antagonist and an estimate of the pK_B. In contrast, the pK_B value was taken to represent both the affinity and potency the antagonist.

As the pK_B and pA_2 values of an antagonist were agonist-and system-independent measures of antagonism, they were used to classify the cannabinoid receptor subtypes mediating the inhibitory effect of the cannabinoid receptor agonists on the EFS-evoked contractions of the various isolated nerve-smooth muscle preparations.

2.2.5.3.2. Muscarinic acetylcholine receptor stimulation studies

The amplitude of the contractions to cumulative additions of ACh in the presence of the cannabinoid receptor ligands or their vehicle were expressed as a percentage mean ± s.e.m of the amplitude of the maximal contraction to ACh (10^{-5} M). This concentration of ACh was determined from the initial ACh concentration-response curve constructed on each tissue in the absence of the cannabinoids or their vehicle.

Shifts in the ACh concentration-response curves were compared by a one-way ANOVA, followed by a Dunnett's post hoc test. The probability $P < 0.05$ was taken to be statistically significant.

2.3. Isolated nerve-epithelial preparation secretion studies

2.3.1. Tissue dissection

A 3 cm length segment of the proximal colon was excised from rats and immersed in Krebs-Henseleit solution A of the composition (in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5, and gassed with 95 % O_2 and 5 % CO_2 at room temperature (21 ± 4 ^{O}C).

The segment was opened longitudinally along the mesenteric border after trimming the mesentery and rinsing the faecal contents away with Krebs-Henseleit solution A. The tissue was then pinned as flat sheet with the basolateral (serosal) side up on a cork board lined with a piece of paper towel soaked with Krebs-Henseleit solution A. The smooth muscle layers with the attached myenteric plexus were then gently stripped away by blunt dissection leaving a submucosal plexus-mucosal (SPM) sheet (Figure 2.2). A maximum of two SPM sheets was prepared from the 3 cm segment.

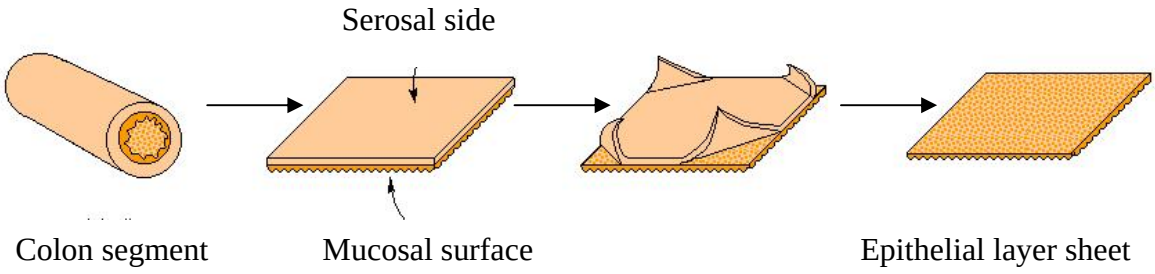


Figure 2.2: Separation of the submucosal plexus-mucosal sheet from a segment of the colon.

2.3.2. Apparatus set-up and maintenance

Each SPM sheet was lightly clamped between the halves of the Ussing chambers (WP Instruments, U.S.A.) with a circular window surface area of 0.63 cm^2. Both apical and basolateral surfaces of the SPM sheet were bathed with 5 ml of Krebs-Henseleit solution A maintained at 37 ^{O}C and circulated with a stream of 95 % O_2 and 5 % CO_2 (Figure 2.3).

The chambers were connected through 3M KCl agar electrodes to a voltage clamp apparatus (DVC 1000, WP Instruments, U.S.A.) and the preparations automatically short-circuited by voltage clamping the tissue at a holding potential of 0 mV. The short circuit current (I_{sc}, Section 2.3.4) was continuously recorded in units of $\mu A.cm^{-2}$ on a MacLab data-acquisition recording system running the Chart version 3.5 software (AD Instruments, U.K.) on an Apple Macintosh computer (Apple Macintosh, U.K.).

At the end of every experiment involving cannabinoids, the chambers were washed thoroughly with 70 % ethanol followed by 2 M hydrochloric acid and copious amounts of distilled water to ensure the complete removal of residual cannabinoids. The 3M KCl-agar electrolyte in the electrodes was also renewed regularly.

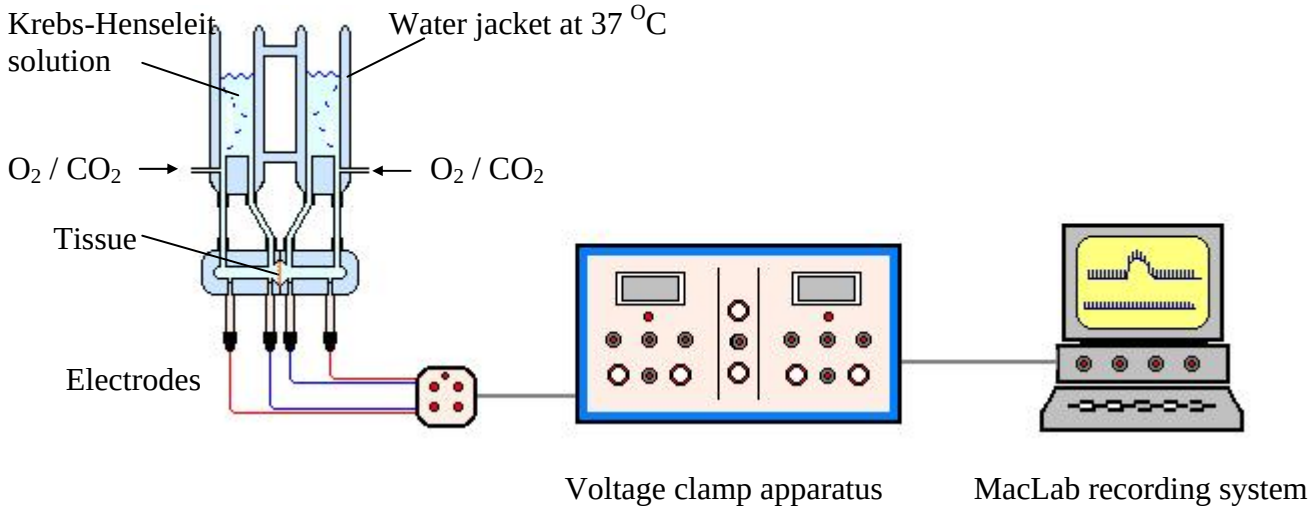


Figure 2.3: Schematic diagram of the Ussing chamber apparatus for recording ion transport in the isolated nerve-epithelial preparation (not to scale).

2.3.3. Experimental design

A minimal 60 minute equilibration period was established before addition of any drug to the tissues. During this time the basal I_{sc} and R_t had stabilised. Only one concentration of a nerve stimulant or a cannabinoid was used per tissue because the tissues developed tachyphylaxis to nerve stimulants and that the drugs could not be washed out because of the design of the chambers.

Drugs were only added to the basolateral side of the stripped tissue, as Tyler *et al*, (2000) have demonstrated that cannabinoids do not produce an effect when added to the apical surface. Cannabinoid receptor agonists were added 30 minutes prior to the addition of a nerve stimulant. In competition studies, the CB_1 receptor antagonist SR 141716 was administered 30 minutes prior to the addition of a cannabinoid receptor agonist. All experiments were performed in parallel with relevant vehicle treated and time controls.

2.3.4. Data analysis

By definition, the I_{sc} was taken as a measure of active ion transport. A positive change in the I_{sc} in response to stimulation of ion transport by a neurotransmitter or exogenous agonist indicated a net basolateral to apical anion flux or a cation flux in the opposite direction, and this increase was recorded as an upward deflection on the computer. The amplitude of the response was taken a measure of the intensity of the secretory response.

Changes in the transmembrane tissue resistance (R_t) *as* an indicator of the integrity/leakiness of the tissue were monitored over the entire duration of the experiment by intermittently changing the membrane potential to from 0 to 2 mV for 2 seconds every 30 seconds. The R_t was calculated using Ohm's Law (Equation 2.8):

$$R_t = 2 \text{ mV} \ / \Delta I_{sc}. \qquad \text{(Equation 2.8)}$$

The change in the I_{sc} and R_t were measured as units of $\mu A.cm^{-2}$ and Ω respectively as the mean ± s.e.m. Comparisons between the data were performed using a Student's unpaired *t*-test and the probability $P < 0.05$ was taken to be statistically significant.

Table 2.1: Drugs used in the study

NAME	CELLULAR ACTION	SOLVENT	SUPPLIER
Acetylcholine (ACh)	Endogenous nACh / mACh receptor agonist	Distilled water	Sigma-Aldrich (Poole, U.K.)
AM 251	Cannabinoid CB_1 receptor antagonist / inverse agonist	Ethanol	Tocris-Cookson (Bristol, U.K.)
Anandamide (AEA)	Endogenous cannabinoid receptor and Vanilloid TRPV1 receptor agonist	Ethanol	Tocris-Cookson (Bristol, U.K.)
Arachidonyl serotonin (AA 5-HT)	FAAH inhibitor	Ethanol	Axxora Life Sciences (Nottingham, U.K.)
Atropine	mACh receptor antagonist	Distilled water	Sigma-Aldrich (Poole, U.K.)
(*E*)-Capsaicin	Vanilloid TRPV1 receptor agonist	Ethanol	Tocris-Cookson (Bristol, U.K.)
Capsazepine	Vanilloid TRPV1 receptor antagonist	Ethanol	Tocris-Cookson (Bristol, U.K.)
α-Chymotrypsin (Type I from bovine pancreas)	Endopeptidase	Krebs-Henseleit solution	Sigma-Aldrich (Poole, U.K.)

CP 55,940	Cannabinoid receptor agonist	Ethanol	Pfizer (Kent, U.K.)
Hexamethonium	nACh receptor antagonist	Distilled water	Sigma-Aldrich (Poole, U.K.)
L-NAME	Nitric oxide synthase inhibitor	Distilled water	Sigma-Aldrich (Poole, U.K.)
(-) Nicotine	nACh receptor agonist	Distilled water	Sigma-Aldrich (Poole, U.K.)
O-2050	Cannabinoid CB_1 receptor antagonist	Ethanol	Tocris-Cookson (Bristol, U.K.)
Phenylmethyl sulphonylfluoride (PMSF)	FAAH inhibitor	Ethanol	Sigma-Aldrich (Poole, U.K.)
PPADS	P_2X purinoceptor antagonist	Distilled water	Tocris-Cookson (Bristol, U.K.)
Prazosin	α_1 Adrenoceptor antagonist	Dimethyl sulphoxide	Sigma-Aldrich (Poole, U.K.)
SR 140333	Neurokinin NK_1 receptor antagonist	Ethanol	Sanofi-Recherche (Montpellier, France)

SR 141716	Cannabinoid CB_1 receptor antagonist / inverse agonist	Ethanol	Sanofi-Recherche (Montpellier, France)
SR 144528	Cannabinoid CB_2 receptor antagonist / inverse agonist	Ethanol	Sanofi-Recherche (Montpellier, France)
Tetrodotoxin (TTX)	Voltage dependent Na^+ channel inhibitor	Distilled water	Tocris-Cookson (Bristol, U.K.)
URB 597	FAAH inhibitor	Ethanol	Axxora Life Sciences (Nottingham, U.K.)
Vasoactive Intestinal Polypeptide (VIP)	VIP receptor agonist	Distilled water	Sigma-Aldrich (Poole, U.K.)
[D-p-Cl-Phe^6,$Leu1^{17}$]-VIP (VIP 6-28)	VPAC receptor antagonist	Distilled water	Sigma-Aldrich (Poole, U.K.)
Veratridine	Voltage dependent Na^+ channel activator	Ethanol	Sigma-Aldrich (Poole, U.K.)
WIN 55,212-2	Cannabinoid receptor agonist	Ethanol	Tocris-Cookson (Bristol, U.K.)
WIN 55,212-3	Optical isomer of WIN 55,212-2	Dimethyl sulphoxide	Sigma-Aldrich (Poole, U.K.)

All chemicals used for preparing the Krebs-Henseleit solutions and the solvents for dissolving the drugs (Table 2.1), with the exception of distilled water, were purchased from Fisher Scientific (Loughborough, U.K.). The total volume of the drugs or solvent added to the organ baths or Ussing chambers did not exceed 1 % of the total bath volume.

CHAPTER 3:
ISOLATED NERVE-SMOOTH MUSCLE PREPARATIONS

Pharmacological Analysis of Cannabinoid Receptor Activity in the Rat Isolated Ileum Myenteric Plexus Longitudinal Muscle Preparation

3.1. Introduction

A large body of evidence has emerged over the last two decades from a plethora of *in vivo* studies, which have been performed almost exclusively on the mouse and rat (Pertwee, 2001), that the predominant action of psychotropic cannabinoids on the intestinal tract is a reduction in the propulsive (Carai *et al.,* 2006; Calignano *et al*., 1997; Chesher *et al*., 1973; Colombo *et al*., 1998; Izzo *et al*., 1999a, 1999b, 2000, 2001a; Landi *et al.,* 2002; Mathison *et al.,* 2004) and secretory (Izzo *et al*., 1999a, 1999b, 2000, 2003; Shook & Burks, 1989) function of the small intestine. The former action has been invariably studied by evaluating either the distance travelled or the delay in the time taken for the transit of an orally or intra-duodenally administered non-absorbable marker from the pylorus to the caecum of the animal.

Whilst *in vivo* bioassays have long played an important role in characterising the pharmacology of cannabinoids on intestinal motility under both physiological and pathophysiological states, the mechanism of the depressant action has been inferred from *in vitro* studies performed on segments or strips of the isolated ileum of the guinea-pig (Coutts & Pertwee, 1997; Gill *et al.,* 1970; Mang *et al*., 2001; Pertwee *et al*., 1996; Izzo *et al.,* 1998) and to a lesser extent of human (Croci *et al*., 1998; Guagnini *et al*., 2006; Manara *et al*., 2002).

Therefore, to date the guinea-pig ileum has been the standard *in vitro* bioassay for screening cannabinoids for activity on the intestinal tract. The *in vitro* studies on this tissue have focussed on the ability of cannabinoids to inhibit either the peristaltic reflex in segments of whole ileum (Heinemann *et al*., 1999; Izzo *et al*., 2000), synaptic transmission (Lopez-Redondo *et al*., 1997), or the low frequency EFS-evoked release of a variety of neurotransmitters (Begg *et al*., 2002a, 2002b; Coutts & Pertwee 1997; Mang *et al*., 2001) or subsequent contraction of the longitudinal (Coutts & Pertwee 1997; Mang *et al*., 2001; Pertwee *et al*., 1996) or circular (Izzo *et al*., 1998) smooth muscle layers.

Results from these studies have suggested that psychotropic cannabinoids decrease intestinal motility by reducing the release of contractile neurotransmitters such as acetylcholine (ACh) from the myenteric plexus (Coutts & Pertwee 1997; Mang *et al*.,

2001) through an activation of the CB_1 receptor located on somatodendritic and terminal regions of the neurones (Coutts *et al*., 2002).

Although the guinea-pig ileum has served as a useful bioassay for describing the inhibitory action of cannabinoids on intestinal transit observed in the mouse or rat *in vivo*, it is important that isolated ileal tissues from the latter species are used for studying cannabinoid effects. Firstly, because a large number of models of disturbed intestinal motility of man have been created using the rat and mouse (Izzo *et al*., 1999a, 1999b, 2000, 2001a, 2003; Mascolo *et al*., 2002; Mathison *et al.,* 2004) and secondly, to date, the guinea-pig has not been employed for investigating cannabinoid effects on ileal transit.

The availability of an *in vitro* ileal bioassay from the rat and mouse would not only make it possible for a better comparison to be made between the data obtained from both types of studies, but may offer the opportunity to understand more about the mode of action of existing and future cannabinoid drugs on ileal motility under both physiological and pathophysiological conditions. Additionally, the existence of possible novel cannabinoid receptor subtypes or targets may be revealed.

Previous studies have reported that the conservation between the rat and human mRNAs of the cannabinoid receptors is slightly higher compared to that between the mouse and human (Chakrabarti *et al*., 1995; Shire *et al*., 1996). Moreover, the mRNAs for both the cannabinoid receptors and their expression have been identified and mapped in the myenteric plexus of the rat ileum (Coutts *et al*., 2002; Duncan *et al*., 2005; Valenti *et al*., 2005). Furthermore, a number the endogenous cannabinoid ligands, exemplified by AEA and 2-AG, (Fegley *et al*., 2005; Gomez *et al*., 2002; Mascolo *et al*., 2002; Izzo *et al*., 2003; Valenti *et al*., 2005) along with the mechanisms for their enzymatic inactivation (Katayama *et al*., 1997) have been shown to be present in the rat ileum. Therefore, the purpose of the present study was to investigate whether the rat ileum MPLM served as a suitable and robust *in vitro* ileal cannabinoid receptor bioassay by assessing the interaction between representatives of the four main classes of cannabinoid receptor agonists i.e. AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 and the CB_1 and CB_2 receptor selective

antagonists / inverse agonists SR 141716 and SR 144528 respectively. To aid in the interpretation of data obtained on the rat ileum MPLM, some experiments were performed on the guinea-pig ileum MPLM preparation.

3.2. Methods

MPLM strips were dissected from the ileum of both rats and guinea-pigs (Section 2.1) and set up in organ baths as described in Section 2.2.1.

The experiments under EFS and muscarinic stimulation conditions were performed by the methods described in Section 2.2.5.1.1 and 2.2.5.1.2 respectively.

The data obtained from the experiments with EFS and ACh stimulation were quantified and analysed by the methods described in Section 2.2.5.3.1 and 2.2.5.3.2 respectively.

The compounds used in this study were: ACh, AEA, atropine, capsazepine, CP 55,940, hexamethonium, L-NAME, SR 141716, SR 144528, TTX, Δ^9-THC, WIN 55,212-2 and WIN 55,212-3. See Table 2.1 for the solvents and suppliers of the compounds used.

3.3. Results

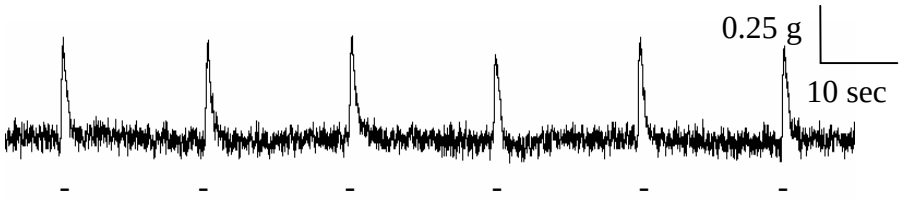
3.3.1. The EFS-evoked responses of the rat ileum MPLM

Stimulation of the rat ileum MPLM strip with single pulses at a repetition frequency of 0.05 Hz elicited a transient twitch contraction every 20 seconds. Each twitch contraction occurred immediately in response to the electrical pulse (Figure 3.0a). In contrast, stimulation of the MPLM strip with 2 second trains of pulses at a frequency of 30 Hz every minute elicited a biphasic response. This response consisted of a transient relaxation during the train of pulses followed by a rapid transient rebound contraction on termination of the EFS (Figure 3.0b).

The amplitude of the relaxations to EFS at 30 Hz were noted to diminish over the first 20 minutes of stimulation as the basal tension of the tissues decreased from 0.5 g to about 0.2 g. By contrast, the amplitude of the rebound contractions gradually increased during this period and continued to increase for up to 3 to 4 hours without altering the basal tension of the tissue. The amplitudes of the twitch and rebound contractions after 3 to 4 hours of EFS were 0.45 ± 0.13 g (n = 100) and 1.42 ± 0.34 g (n = 100) respectively. As shown in Figure 3.1, the contractions evoked by both frequencies of EFS were significantly ($P < 0.05$, unpaired t test) attenuated by treatment with either atropine (10^{-6} M) or TTX (10^{-6} M) but not hexamethonium (10^{-4} M).

Preliminary experiments showed that the relaxations elicited by EFS at 30 Hz were abolished by treatment with TTX (10^{-6} M, data not shown) and L-NAME (10^{-4} M, data not shown). Additionally, the presence of L-NAME had no effect on the amplitude or timing of the rebound contractions.

(a)



(b)

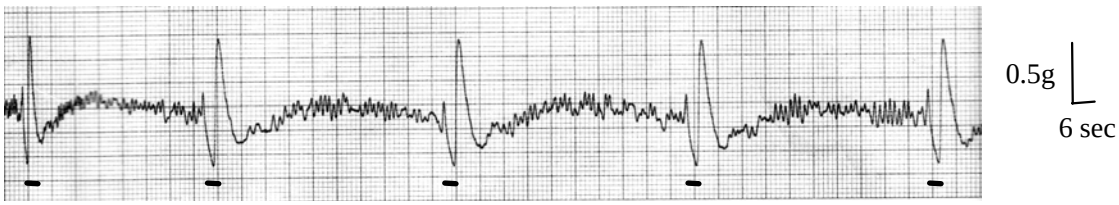


Figure 3.0: Representative traces of the EFS-evoked responses of the rat ileum MPLM. (a) Twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage. Each upward deflection represents a contraction. The dots represent the time at which the EFS pulses were delivered. Upward deflections represent the contractions. (b) Biphasic responses to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage. Note the transient relaxations during the 2 second stimulation periods and the subsequent the rebound contractions. The downward deflections denote relaxations whilst upward deflections represent contractions. The dots represent the time at which the EFS pulses were delivered

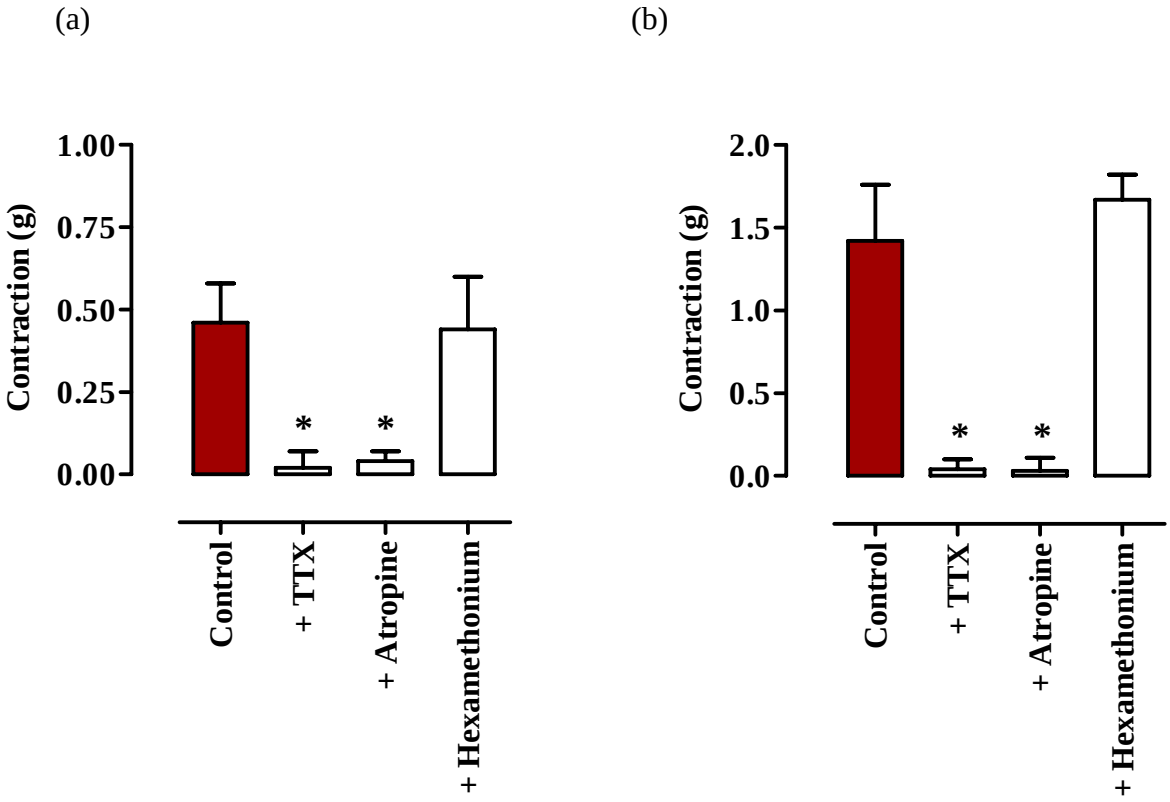


Figure 3.1: Bar graphs representing the amplitude of the EFS-evoked contractions of the rat ileum MPLM in the absence (control column, each n = 18) and presence of TTX (10^{-6} M, each n = 6), atropine (10^{-6} M, each n = 6) or hexamethonium (10^{-4} M, each n = 6). (a) Data for the twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage (b) Data for the rebound contractions to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage. * represents a statistical difference ($P < 0.05$, unpaired *t* test) compared to the control column of each data set. Values are represented as the mean ± s.e.m.

3.3.2. Effect of the cannabinoid receptor agonists on the EFS-evoked contractions of the rat ileum MPLM

AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 all caused a concentration-related inhibition of the contractions of the rat ileum MPLM elicited by EFS with single pulses at 0.05 Hz and trains of pulses at 30 Hz (Figures 3.3 and 3.5). Figure 3.2 shows representative traces of the contractions to both frequencies of EFS in the absence and presence of CP 55,940.

All four agonists caused a similar maximal inhibition of the contractions evoked by both frequencies of EFS (Figures 3.3 and 3.5, Table 3.0). However, the twitch contractions evoked by EFS at 0.05 Hz were attenuated more readily by each agonist, at concentrations less than 10^{-6} M, than the rebound contractions elicited by EFS at 30 Hz. A comparison between the pEC_{50} values of each agonist under the two EFS conditions indicated that all four agonists were significantly ($P < 0.05$, unpaired *t* test) more potent inhibitors of the twitch contractions than of the rebound contractions (Table 3.0).

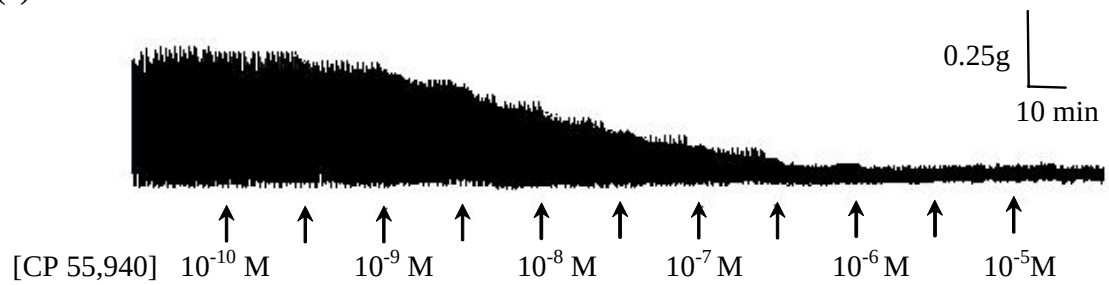
A comparison between the rank orders of the pEC_{50} values of the four agonists on tissues subjected to the two EFS conditions showed no correlation. The rank order of the pEC_{50} values of the agonists on tissues subjected to EFS at 0.05 Hz was CP 55,940 = WIN 55,212-2 > AEA > Δ^9-THC, whereas that for tissues subjected to EFS at 30 Hz was AEA > CP 55,940 = Δ^9-THC = WIN 55,212-2. The lack of correlation between the rank orders of the pEC_{50} values of the agonists was attributed to differences in the degree of reduction in the pEC_{50} value of each agonist with the increase in the frequency of EFS. These differences are revealed by a comparison between ratios of the EC_{50} value of each agonist under the two EFS conditions. Table 3.0 shows that the pEC_{50} value of CP 55,940 and WIN 55,212-2 was reduced by around one logarithmic unit greater than the pEC_{50} value of AEA and Δ^9-THC as a result of the increase in the frequency of EFS.

The rate of onset of the inhibition of the contractions by each agonist was both slow and dependent on the frequency of EFS employed. The maximal inhibition of the contractions at each concentration of an agonist was achieved within 20 and 30 minutes of administration on tissues stimulated at 0.05 Hz and 30 Hz respectively. Figure 3.6 shows the time courses for the maximal inhibition of the twitch and rebound contractions by all four agonists at 10^{-6} M and 10^{-4} M respectively.

Despite the differences in the rank orders of the pEC_{50} values of the four agonists under the two EFS conditions, the rank orders of rate of onset of the inhibition of the contractions were similar under both EFS conditions i.e. WIN 55,212-2 > CP 55,940 > Δ^9-THC > AEA. The time taken for WIN 55,212-2, CP 55,940, Δ^9-THC and AEA to produce a 50 % inhibition of the contractions evoked by EFS at 0.05 Hz was 4, 5, 7 and 9 minutes respectively, whereas that at 30 Hz was 7, 9, 11 and 12 minutes respectively.

Unlike WIN 55,212-2, its stereoisomer WIN 55,212-3 was a significantly ($P < 0.05$ unpaired *t* test) less efficacious inhibitor of the EFS-evoked contractions over the same concentration range under both EFS conditions (Table 3.0).

(a)



(b)

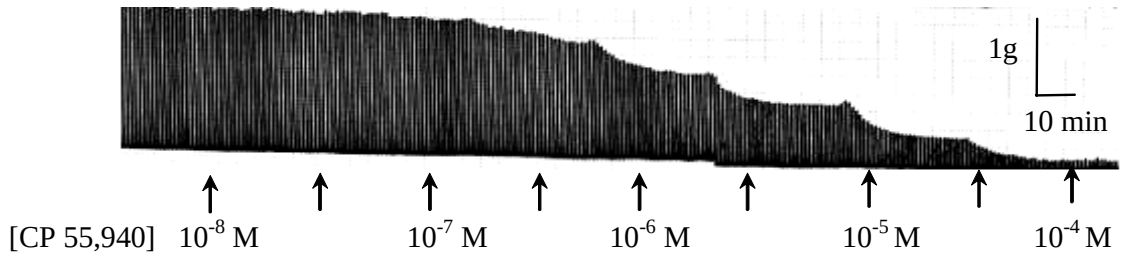
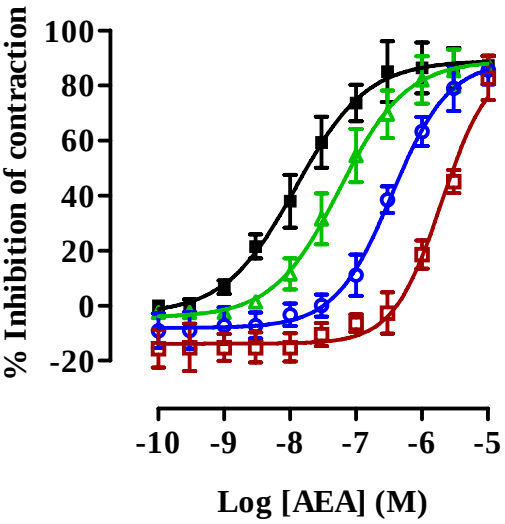


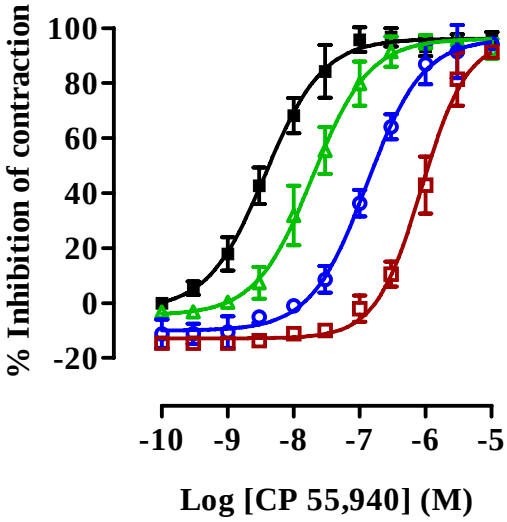
Figure 3.2: Representative traces of the EFS-evoked contractions of the rat ileum MPLM in the absence and presence of CP 55,940 added in half-logarithmic unit increments. Unlabelled arrows represent intermediate concentrations. (a) Trace illustrating the twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage in the absence and presence CP 55,940 (10^{-10} to 10^{-5} M) (b) Trace illustrating the rebound contractions to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage in the absence and presence of CP 55,940 (10^{-8} to 10^{-4} M).

Figure 3.3: Concentration-response curves for the inhibition of the EFS (0.05 Hz frequency, 0.5 ms duration, 110 % supramaximal voltage) evoked twitch contractions of the rat ileum MPLM by (a) AEA, (b) CP 55,940, (c) Δ^9-THC and (d) WIN 55,212-2 constructed in the presence of ethanol (■) or SR 141716 (10^{-8} M (Δ), 10^{-7} M (○) or 10^{-6} M (□)). Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the twitch response measured immediately before the addition of any drug to the organ bath. Vertical lines indicate s.e.m, n = 6 for each curve. SR 141716 or ethanol was added 20 minutes before the first addition of an agonist.

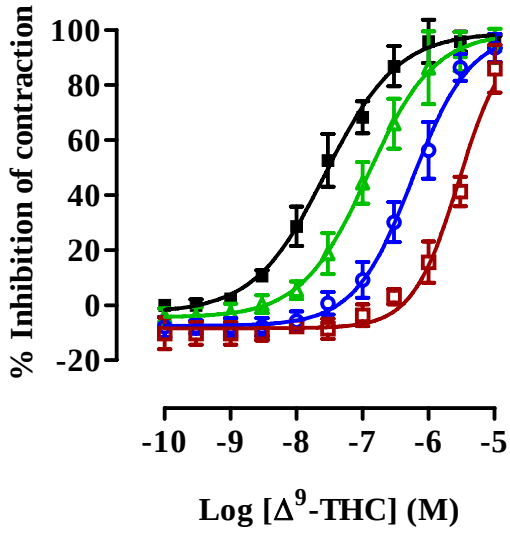
(a)


% Inhibition of contraction
100
80
60
40
20
0
-20
-10
-9
-8
-7
-6
-5
Log [AEA] (M)

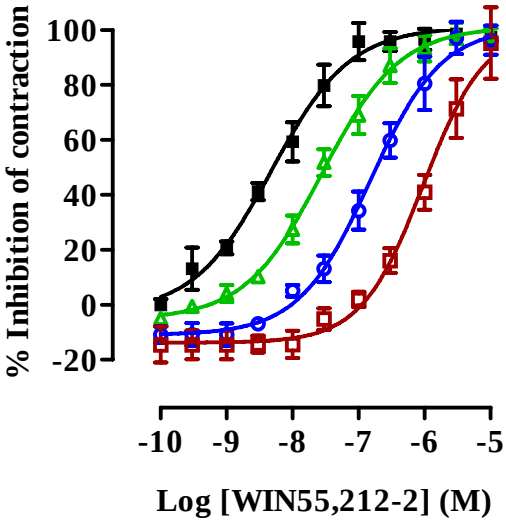
(b)


% Inhibition of contraction
100
80
60
40
20
0
-20
-10
-9
-8
-7
-6
-5
Log [CP 55,940] (M)

(c)


% Inhibition of contraction
100
80
60
40
20
0
-20
-10
-9
-8
-7
-6
-5
Log [Δ9-THC] (M)

(d)


% Inhibition of contraction
100
80
60
40
20
0
-20
-10
-9
-8
-7
-6
-5
Log [WIN55,212-2] (M)

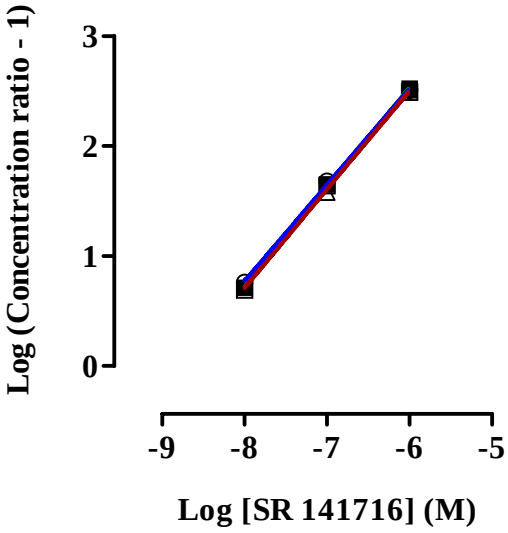
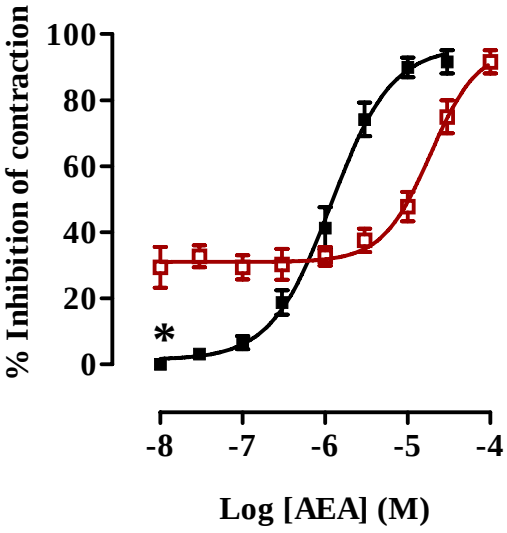


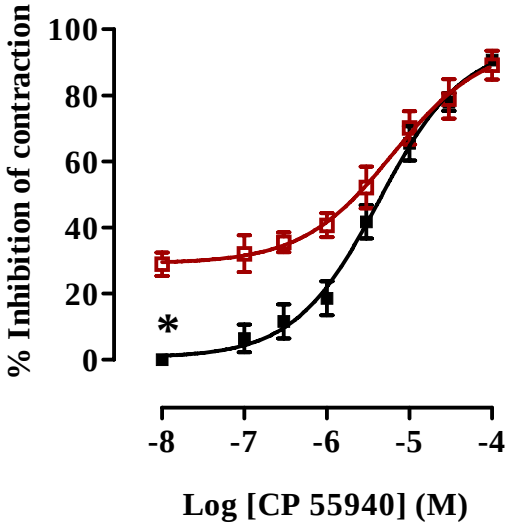
Figure 3.4: Schild plot for SR 141716 for the antagonism of the inhibition of the EFS-(0.05 Hz frequency, 0.5 ms duration, 110 % supramaximal voltage) evoked twitch contractions of the rat ileum MPLM by AEA (○), CP 55,940 (■), Δ^9-THC (□) and WIN 55,212-2 (Δ). The concentration ratios were calculated using the agonist concentrations corresponding to the 50 % equieffective inhibition level in the absence and presence of SR 141716 (10^{-8} to 10^{-6} M). The intersection of the slope with the abscissa where Log (Concentration ratio – 1) = 0 represents the pK_B value of SR 141716 against each of the four agonists.

Figure 3.5: Concentration-response curves for the inhibition of the EFS- (30 Hz frequency, 2 seconds every minute, 0.5 ms duration, 110 % supramaximal voltage) evoked rebound contractions of the rat ileum MPLM by (a) AEA, (b) CP 55,940, (c) Δ^9-THC and (d) WIN 55,212-2 constructed in the presence of ethanol (■) or SR 141716 (10^{-6} M (□)). Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the twitch response measured immediately before the addition of any drug to the organ bath. Vertical lines indicate s.e.m, $n \geq 6$ for each curve. SR 141716 or ethanol was added 30 minutes before the first addition of an agonist.

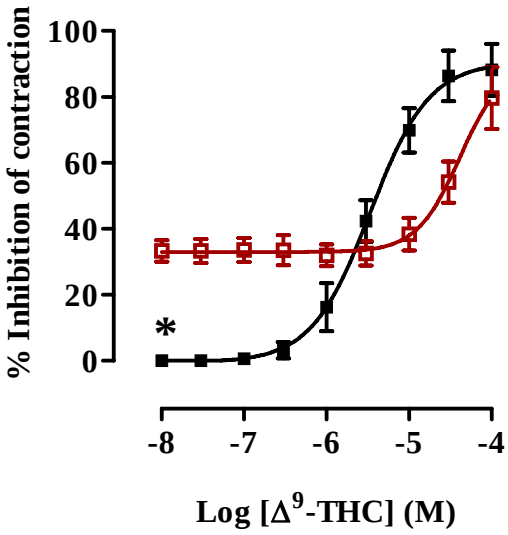
(a)


% Inhibition of contraction
100
80
60
40
20
0
*
-8
-7
-6
-5
-4
Log [AEA] (M)

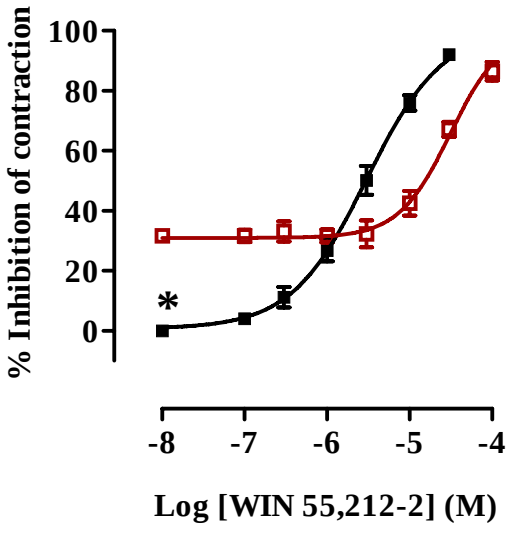
(b)


% Inhibition of contraction
100
80
60
40
20
0
*
-8
-7
-6
-5
-4
Log [CP 55940] (M)

(c)


% Inhibition of contraction
100
80
60
40
20
0
*
-8
-7
-6
-5
-4
Log [Δ9-THC] (M)

(d)


% Inhibition of contraction
100
80
60
40
20
0
*
-8
-7
-6
-5
-4
Log [WIN 55,212-2] (M)

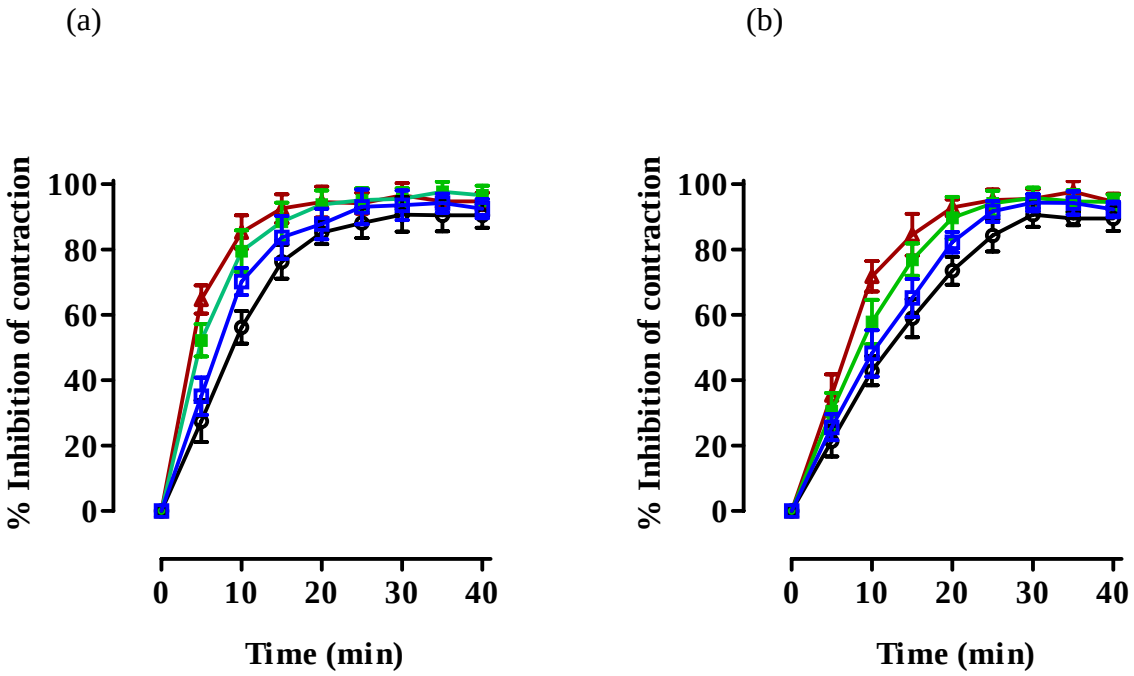


Figure 3.6: Comparison of the time-response curves for the inhibition of the EFS-evoked contractions of the rat ileum MPLM by AEA (○), CP 55,940 (■), Δ^9-THC (□) and WIN 55,212-2 (Δ). (a) Data for the twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage in the presence of 10^{-6} M of each agonist. (b) Data for the rebound contractions to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage in the presence of 10^{-4} M of each agonist. Each symbol represents the mean value of the inhibition of the contractions expressed as a percentage reduction of the amplitude of the contractile response measured immediately before the first addition of the agonist. Vertical lines indicate s.e.m, n = 4 for each curve.

Table 3.0: A comparison of the potency (pEC_{50}), tissue maximal response (E_{max}) and potency ratios of the various cannabinoid receptor agonists for the inhibition of the EFS-evoked contractions of the rat ileum MPLM.

Cannabinoid agonist	$pEC_{50\ (0.05\ Hz)}$	$E_{max\ (0.05\ Hz)}$ (%)	$pEC_{50\ (30\ Hz)}$	$E_{max\ (30\ Hz)}$ (%)	$\text{Log}\left[\frac{EC_{50\ (30\ Hz)}}{EC_{50\ (0.05\ Hz)}}\right]$
AEA	7.91 ± 0.02	87.28 ± 3.45	5.90 ± 0.03 *	91.59 ± 3.48	2.0
CP 55,940	8.42 ± 0.02	96.64 ± 3.32	5.37 ± 0.04 *	90.77 ± 1.63	3.0
WIN 55,212-2	8.31 ± 0.04	97.25 ± 1.12	5.49 ± 0.01 *	92.10 ± 1.36	2.8
Δ^9-THC	7.56 ± 0.02	95.78 ± 7.88	5.47 ± 0.01 *	88.24 ± 7.84	2.1
WIN 55,212-3	7.45 ± 0.04	4.02 ± 2.12 #	6.03 ± 0.12	8.65 ± 3.53 #	1.4

Agonist pEC_{50}, E_{max} values were derived by non-linear regression analysis for tissues stimulated electrically with either single pulses of 0.5 ms duration, 110 % supramaximal voltage at 0.05 Hz frequency or 2 second trains of pulses of 0.5 ms duration and 110 % supramaximal voltage at a frequency of 30 Hz every minute. * represents a significantly ($P < 0.05$, unpaired *t* test) lower pEC_{50} compared to pEC_{50} of the same agonist in tissues stimulated at 0.05 Hz. # represents a significantly ($P < 0.05$, unpaired *t* test) lower E_{max} compared to the E_{max} of WIN 55,212-2 on tissues under similar EFS conditions. Values are represented as mean ± s.e.m, $n \geq 6$.

3.3.3. Effect of the cannabinoid receptor agonists on the EFS-evoked contractions of the rat ileum MPLM in the presence of SR 141716

In tissues stimulated with single pulses at 0.05 Hz, the presence of increasing concentrations of SR 141716 (10^{-8} to 10^{-6} M) caused significant ($P < 0.05$, ANOVA and Dunnett's test) progressive degrees of dextral shifts of the AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 concentration-response curves with no significant ($P < 0.05$, paired *t* test) effect on the E_{max} of each agonist (Figure 3.3).

An sum-of-squares F test on the Hill slope parameters of each family of agonist concentration-response curves in the absence and presence of SR 141716 indicated that the slope of each curve was not significantly ($P > 0.05$) different from unity. Hence, the dextral shifts of the agonist concentration-response curves caused by SR 141716 were considered parallel.

In contrast, a sum-of-squares F test on the E_{max} parameter of the agonist concentration-response curves in the absence and presence of SR 141716 revealed that a significant ($P < 0.05$) improvement in the sum-of-squares could be achieved by constraining the E_{max} to be shared between each data set. Therefore, each family of curves was re-fitted to Equation 2.3 (Chapter 2) with the E_{max} shared between each data set. Figure 3.3 represents the agonist concentration-response curves in the absence and presence of SR 141716 after constraining the E_{max} to be shared between each family of curves for all four agonists.

Schild analysis (Figure 3.4) of the rightward displacements of the concentration-response curves of each agonist by SR 141716 (Figure 3.3) yielded the estimates of the pK_B value and slope shown in Table 3.1. In all instances, the slope of the Schild plots were significantly ($P < 0.05$, unpaired *t* test) less than unity. But, the estimates of the pK_B value obtained from these slopes were similar irrespective of the agonist that was used.

Because the experimentally determined dose ratios represented a sample from the complete population of the infinite dose ratios that could be determined using infinite concentrations of SR 141716, the 95 % C.I. of the slope of each Schild analyses was calculated to investigate whether random sample variation may have produced the non-unit slopes, and if the sample data came from the population describing simple competitive antagonism i.e. unit slope.

As shown in Table 3.1, the 95 % a C.I. value of the slopes of each Schild analysis contained unity. Therefore, each Schild plot was re-plotted by constraining the slope to unity to estimate the pK_B value of SR 141716, with the assumption that the interaction between SR 141716 and each of the agonists was simple competitive and that random variation caused the deviation from unit slope.

The estimates of the pK_B value of SR 141716 obtained before and after constraining the slopes of the Schild plots to unity are listed in Table 3.1. It can be seen that the estimates of the pK_B value of SR 141716 yielded from the Schild plots with the unit slopes were slightly higher than those obtained from the non-unit slopes. Nonetheless, the pK_B values of SR 141716 obtained were similar irrespective of the agonist that was used.

As mentioned above, the concentrations of each agonist required to inhibit the rebound contractions elicited by EFS at 30 Hz were significantly higher than those necessary for inhibiting the twitch contractions to EFS at 0.05 Hz. Additionally, it was impossible to prepare very highly concentrated stock solutions of each cannabinoid agonist. Therefore, the pA_2 values of SR 141716 were calculated using the Gaddum Schild equation following incubation of a single concentration of 10^{-6} M.

SR 141716 (10^{-6} M) caused small but significant ($P < 0.05$, ANOVA and Dunnett's test) rightward displacements of the concentration-response curves of AEA, Δ^9-THC and WIN 55,212-2 but not CP 55,940 (Figure 3.5). As shown in Table 3.1, the estimates of the pA_2 values of SR 141716 for the antagonism of WIN 55,212-2, AEA and Δ^9-THC were similar against these three agonists, but significantly lower than the pK_B value obtained in the twitch contraction experiments.

In addition to its antagonist activity, the presence of SR 141716 alone caused a significant ($P < 0.05$, paired *t* test) concentration dependent augmentation of the amplitude of the twitch contractions to EFS at 0.05 Hz. The augmentation of the twitch contractions can be easily seen in Figure 3.3 as the progressive concentration dependent downward shift of the bottom asymptote of each agonist concentration-response curve in the presence of SR 141716. Figure 3.7 shows a concentration response curve for the augmentation of the twitch contractions by SR 141716 (10^{-8} to 10^{-5} M). The amplitude of the contractions in the presence of SR 141716 at 10^{-6} and 10^{-5} M were significantly ($P < 0.05$, paired *t* test) larger than those in the presence of ethanol. The maximum potentiation of the contractions at 10^{-5} M corresponded to an increase of 12.45 ± 4.12 % (n = 6). The pEC_{50} value of SR 141716 from this data was 7.52 ± 0.02.

In contrast to the augmentation of the twitch contractions, SR 141716 alone significantly ($P < 0.05$, paired t test) inhibited the rebound contractions elicited by EFS at 30 Hz. The inhibition of the rebound contractions can be easily seen in Figure 3.5 as the elevation of the bottom asymptote of the agonist concentration-response curves in the presence of SR 141716 (10^{-6} M). Higher concentrations of SR 141716 were not used for antagonism studies because this cannabinoid produced a further concentration related inhibition of the rebound contractions (Figure 3.7). The inhibition of the contractions caused by SR 141716 at 10^{-6} M was 28.3 ± 4.4 % (n = 9) whereas that at the maximal concentration used i.e. 10^{-5} M was 76.1 ± 3.7 % (n = 9). The pEC_{50} value of SR 141716 from Figure 3.7 was 5.70 ± 0.01.

The rate of onset of the action of SR 141716 was slower on tissues subjected to EFS at 30 Hz than those stimulated with 0.05 Hz. The maximal effect at each concentration of SR 141716 was achieved within 20 and 30 minutes of administration on tissues stimulated at 0.05 Hz and 30 Hz respectively. Figure 3.8 shows the time courses for the maximal inhibition of the twitch and rebound contractions by SR 141716 at 10^{-5} M. The time taken for SR 141716 to produce a 50 % enhancement and inhibition of the twitch and rebound contractions to EFS at 0.05 Hz and 30 Hz was 4 and 6 minutes respectively.

Table 3.1: A comparison of the estimates of the pK_B and pA_2 values of SR 141716 against AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 for the inhibition of the EFS-evoked contractions of the rat ileum MPLM.

Cannabinoid receptor agonist	Experimentally fitted slope	Constrained slope	pK_B from the experimentally fitted slope	pK_B from the constrained slope	pA_2
AEA	0.88 ± 0.03* (0.55 to 1.21)	1.00	7.78 ± 0.18 (5.45 to 10.1)	8.65 ± 0.07 (8.33 to 8.97)	6.91
CP 55,940	0.91 ± 0.02* (0.65 to 1.16)	1.00	7.96 ± 0.14 (6.15 to 9.77)	8.63 ± 0.06 (8.39 to 8.87)	–
Δ^9-THC	0.90 ± 0.03* (0.53 to 1.27)	1.00	7.91 ± 0.20 (5.32 to 10.5)	8.60 ± 0.06 (8.35 to 8.87)	6.83
WIN 55,212-2	0.88 ± 0.03* (0.60 to 1.18)	1.00	7.78 ± 0.16 (5.77 to 9.90)	8.61 ± 0.06 (8.33 to 8.89)	6.62

The experimentally fitted slope column represents the slopes of the Schild plots of SR 141716 obtained from a line of best fit, for the antagonism of the four cannabinoid receptor agonists in tissues subjected to EFS at 0.05 Hz frequency, 0.5 ms duration, 110 % supramaximal voltage. The constrained slope column represents the unit slopes of the Schild plots of SR 141716 for the same experiments. Where appropriate, the 95 % C.I. values are shown in parenthesis. * represents significantly ($P < 0.05$, unpaired *t* test) lower than unity. The pK_B values of SR 141716 represent the negative logarithmic concentrations of SR 141716 on the Schild plot where the experimentally fitted and constrained slopes of the Schild plot intersected with the abscissa. The pA_2 values represent the negative logarithmic concentrations of SR 141716 calculated using the Gaddum-Schild equation for the antagonism of the cannabinoid receptor agonists by SR 141716 (10^{-6} M) on tissues stimulated with 30 Hz frequency, 2 seconds every minute, 0.5 ms duration, 110 % supramaximal voltage. – represents no antagonism. Where appropriate values represent mean ± s.e.m.

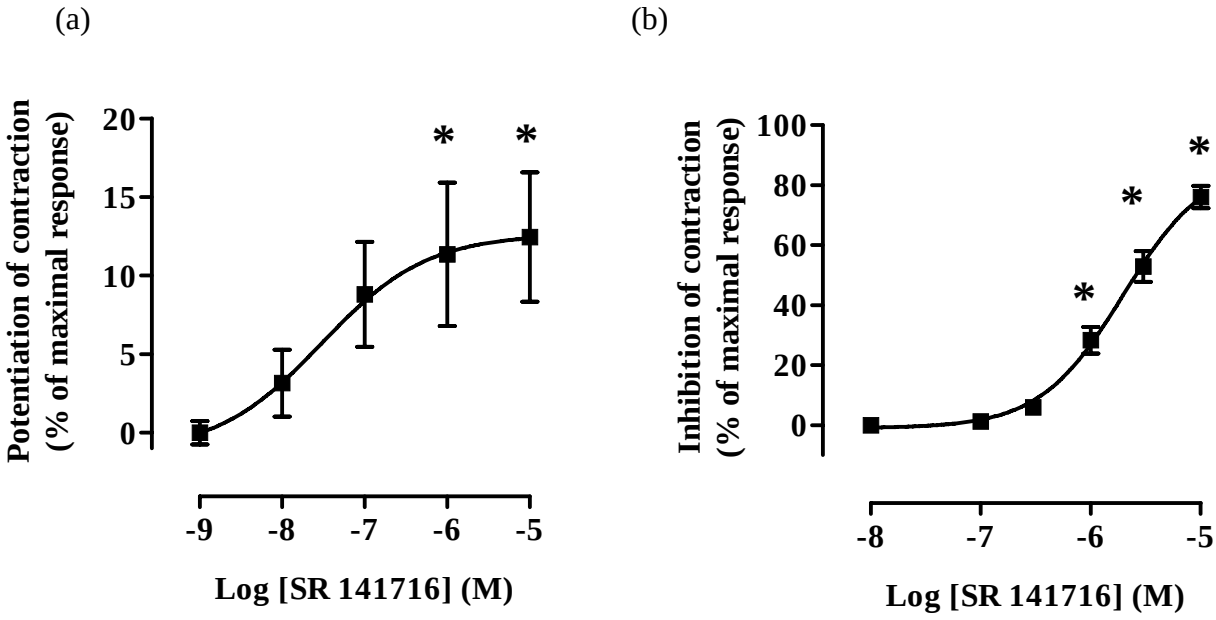


Figure 3.7: Concentration-response curves for the effect of SR 141716 on the EFS-evoked contractions of the rat ileum MPLM (a) Data for the enhancement of the twitch contractions evoked by EFS at 0.05 Hz frequency, 0.5 ms duration, 110 % supramaximal voltage. Each symbol represents the mean value of increase in the contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the addition of SR 141716 to the organ bath (n = 6). Increasing concentrations of SR 141716 were added every 20 minutes. (b) Data for the inhibition of the rebound contractions evoked by EFS at 30 Hz frequency, 2 seconds every minute, 0.5 ms duration, 110 % supramaximal voltage. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage of the amplitude of the contraction measured immediately before the addition of SR 141716 to the organ bath (n = 9). Increasing concentrations of SR 141716 were added every 30 minutes. Vertical lines indicate s.e.m. . Both curves were fitted by non-linear regression analysis. * represents a statistical difference ($P < 0.05$, paired *t* test) compared to amplitude in the absence of SR 141716 i.e. 0 %

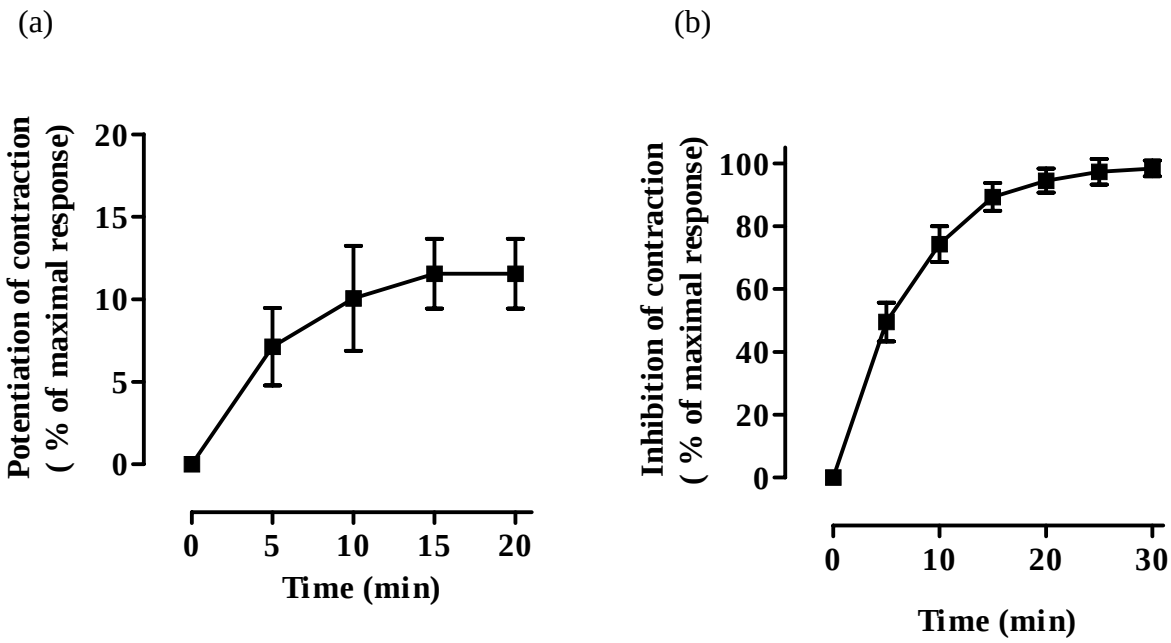


Figure 3.8: Time-response curves for the change in the amplitude of the EFS-evoked contractions of the rat ileum MPLM by SR 141716 (10^{-5} M). (a) Data for the enhancement of the twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage. Each symbol represents the mean value of increase of the contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the addition of SR 141716 to the organ bath (n = 6). (b) Data for the inhibition of the rebound contractions to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage of the amplitude of the contraction measured immediately before the addition of SR 141716 to the organ bath (n = 9). Vertical lines indicate s.e.m.

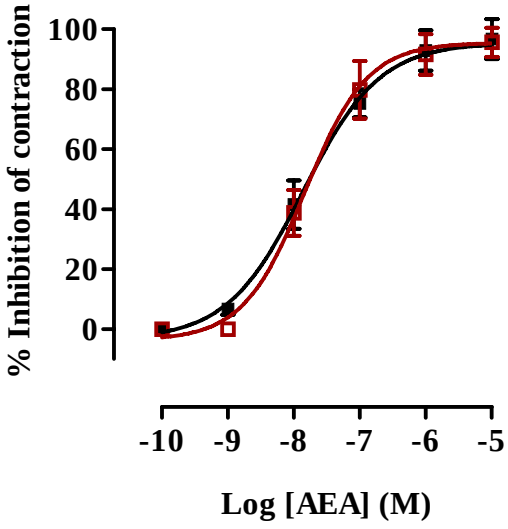
3.3.4. Effect of the cannabinoid receptor agonists on the EFS-evoked contractions of the rat ileum MPLM in the presence of SR 144528

Pre-treatment of the rat ileum MPLM with SR 144528 (10^{-6} M) did not cause a displacement of the AEA, CP 55,940 Δ^9-THC or WIN 55,212-2 concentration-response curves under either of the EFS conditions (Figures 3.9 and 3.10).

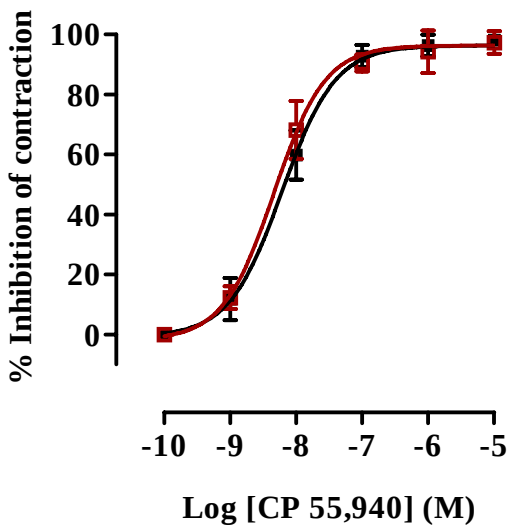
SR 144528 alone had no effect on the amplitude of the EFS-evoked contractions evoked by either frequency of EFS.

Figure 3.9: Concentration-response curves for the inhibition of the EFS- (0.05 Hz frequency, 0.5 ms duration, 110 % supramaximal voltage) evoked twitch contractions of the rat ileum MPLM by (a) AEA, (b) CP 55,940, (c) Δ^9-THC and (d) WIN 55,212-2 constructed in the presence of ethanol (■) or SR 144528 (10^{-6} M (□)). Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the twitch response measured immediately before the addition of any drug to the organ bath. Vertical lines indicate s.e.m. n = 6 for each curve. SR 144528 or ethanol was added 30 minutes before the first addition of an agonist.

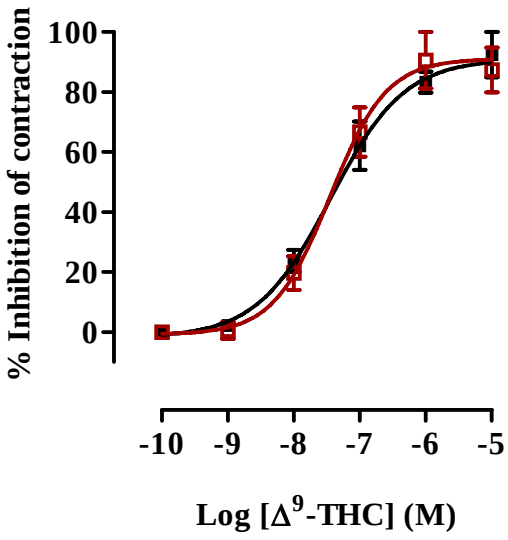
(a)


% Inhibition of contraction
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
Log [AEA] (M)

(b)


% Inhibition of contraction
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
Log [CP 55,940] (M)

(c)


% Inhibition of contraction
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
Log [Δ9-THC] (M)

(d)

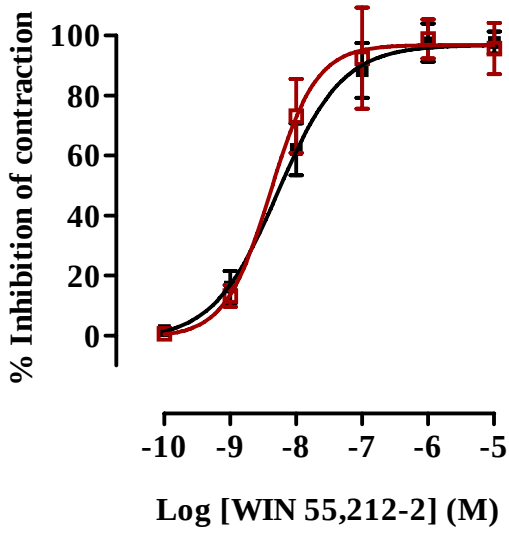
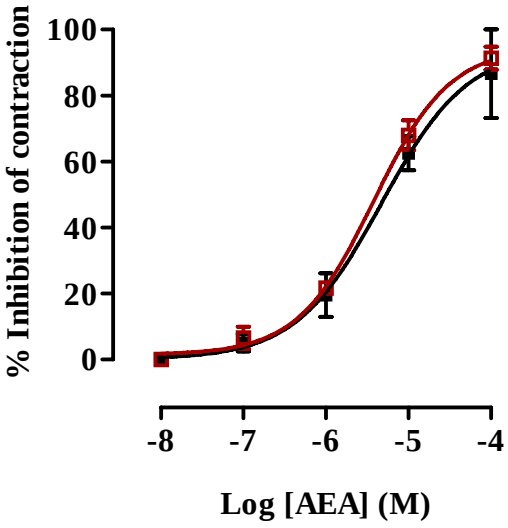
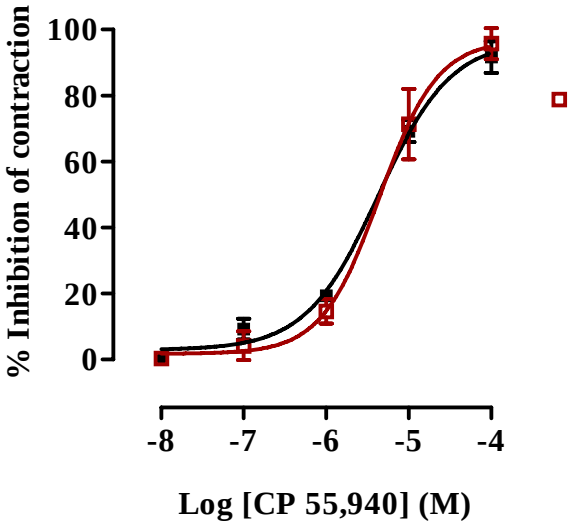

% Inhibition of contraction
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
Log [WIN 55,212-2] (M)

Figure 3.10: Concentration-response curves for the inhibition of the EFS (30 Hz frequency, 2 seconds every minute, 0.5 ms duration, 110 % supramaximal voltage) evoked rebound contractions of the rat ileum MPLM by (a) AEA, (b) CP 55,940, (c) Δ^9-THC and (d) WIN 55,212-2 constructed in the presence of ethanol (■) or SR 144528 (10^{-6} M (□)). Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the twitch response measured immediately before the addition of any drug to the organ bath. Vertical lines indicate s.e.m, n = 6 for each curve. SR 144528 or ethanol was added 30 minutes before the first addition of an agonist.

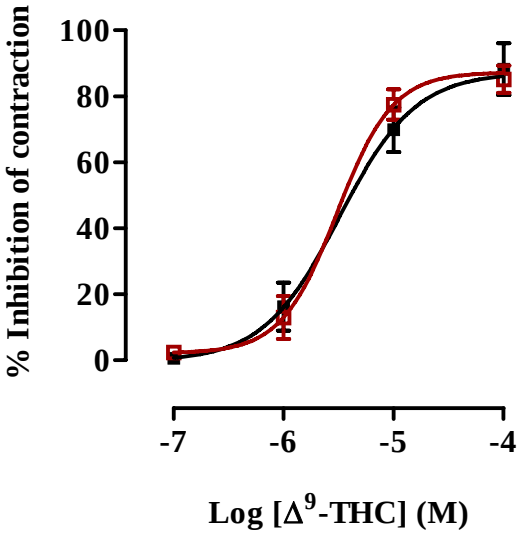
(a)


% Inhibition of contraction
100
80
60
40
20
0
-8
-7
-6
-5
-4
Log [AEA] (M)

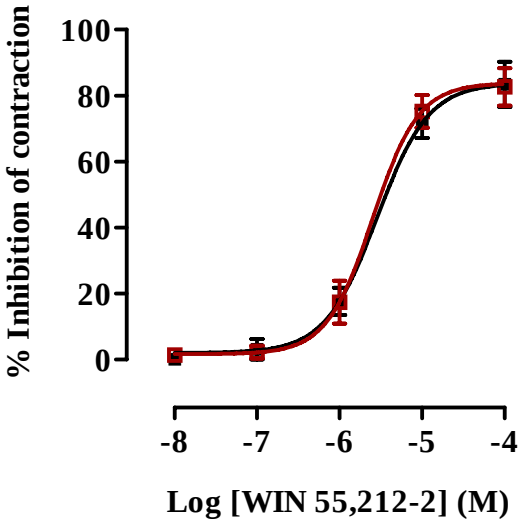
(b)


% Inhibition of contraction
100
80
60
40
20
0
-8
-7
-6
-5
-4
Log [CP 55,940] (M)

(c)


% Inhibition of contraction
100
80
60
40
20
0
-7
-6
-5
-4
Log [Δ9-THC] (M)

(d)


% Inhibition of contraction
100
80
60
40
20
0
-8
-7
-6
-5
-4
Log [WIN 55,212-2] (M)

3.3.5. Effect of the AEA on the EFS-evoked contractions of the rat ileum MPLM in the presence of capsazepine

Figure 3.11 shows that the presence of capsazepine (10^{-5} M) did not alter the ability of AEA to attenuate the twitch and rebound contractions of the rat ileum MPLM evoked by EFS at 0.05 Hz and 30 Hz respectively.

Capsazepine alone at this concentration significantly ($P < 0.05$, paired *t* test) inhibited both types of the EFS-evoked contractions to a similar degree by 22.3 ± 5.5 % (n = 6) and 15.35 ± 6.14 % (n = 6) respectively (Figure 3.11).

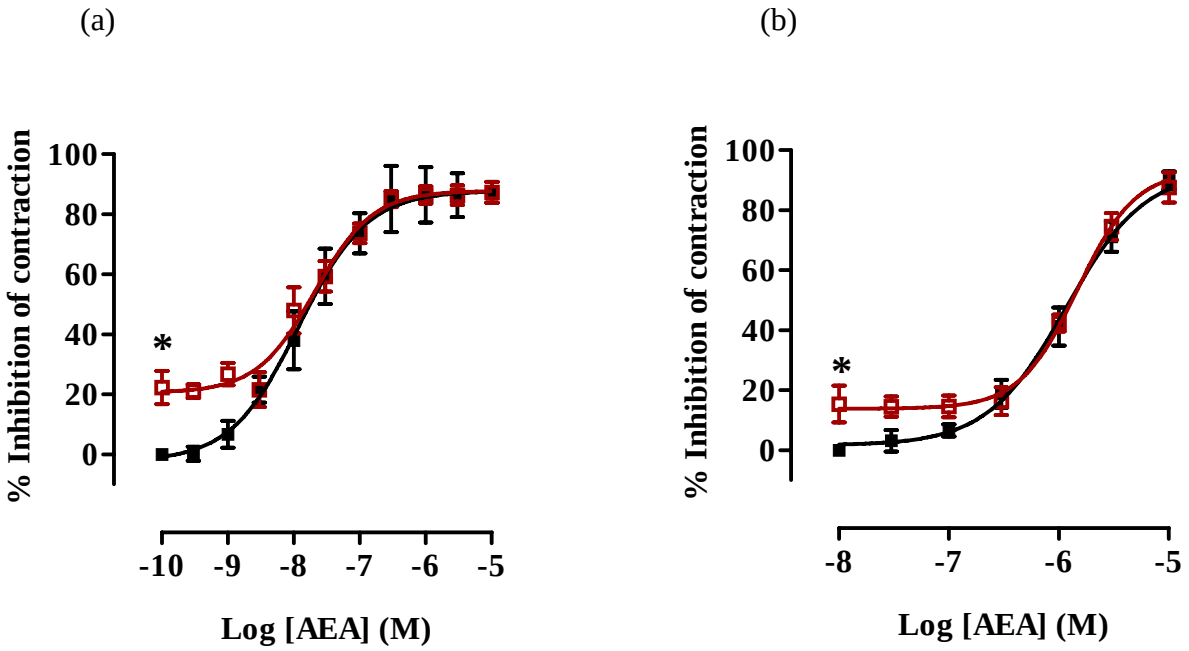


Figure 3.11: Concentration-response curves for the inhibition of the EFS-evoked contractions of the rat ileum MPLM by AEA constructed in the presence of ethanol (■) or capsazepine (10^{-5} M (□)). (a) Data for the twitch contractions to EFS at 0.05 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage (b) Data for the inhibition of the rebound contractions to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of the contractions expressed as a percentage reduction of the amplitude of the contractions measured immediately before the addition of capsazepine to the organ bath (n = 6). Vertical lines indicate s.e.m. Capsazepine or ethanol was added 30 minutes before the first addition of AEA.* represents a statistical difference ($P < 0.05$, paired *t* test) in the inhibition of contractions caused by the presence of capsazepine.

3.3.6. The EFS-evoked responses of the guinea-pig ileum MPLM

Stimulation of the guinea-pig ileum MPLM strip with single pulses at a repetition frequency of 0.1 Hz elicited a transient twitch contraction of 1.62 ± 0.75 g (n = 60) during the duration of the electrical pulse. In contrast, stimulation with 2 second trains of pulses every minute at a frequency of 30 Hz elicited a rebound contraction of 3.31 ± 0.48 g (n = 60) at the end of the 2 second train of pulses. Figure 3.12 shows that both types of contractions were abolished by treatment with either TTX (10^{-6} M) or atropine (10^{-6} M) but not hexamethonium (10^{-4} M).

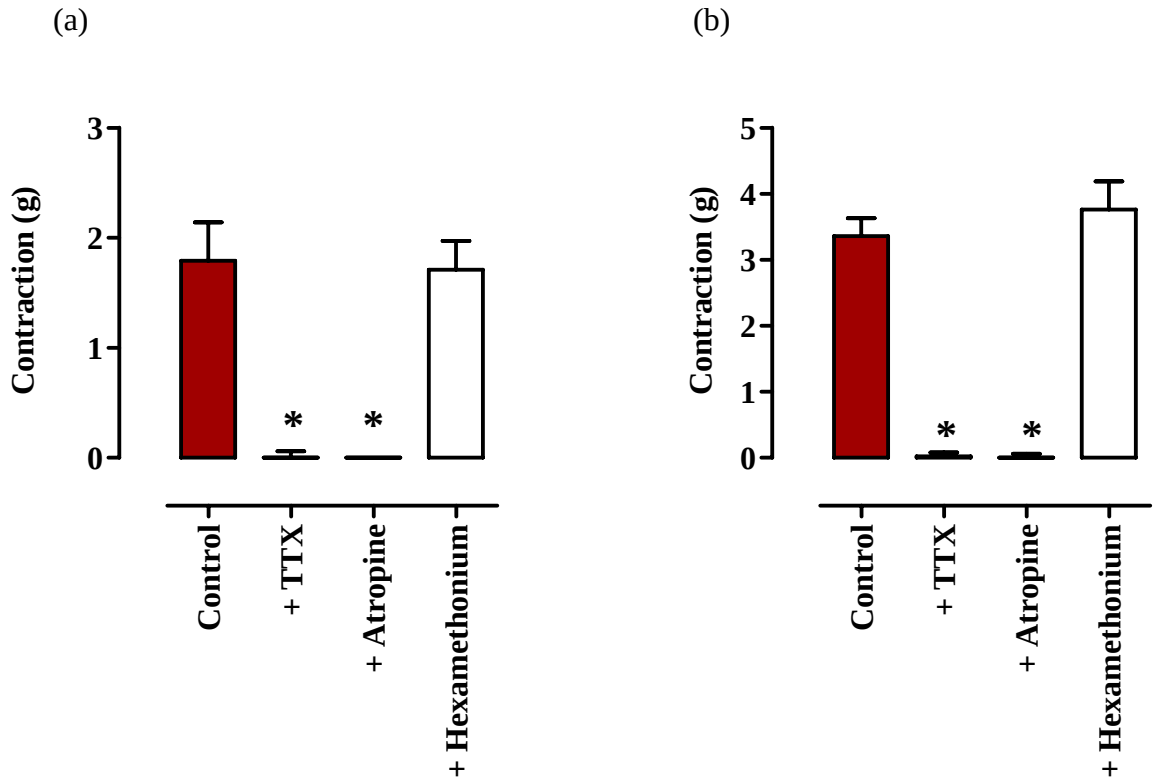


Figure 3.12: Bar graphs representing the amplitude of the EFS-evoked contractions of the guinea-pig ileum MPLM in the absence (control column, each n = 18) and presence of TTX (10^{-6} M, each n = 6), atropine (10^{-6} M, each n = 6) or hexamethonium (10^{-4} M, each n = 6). (a) Data for the twitch contractions to EFS at 0.1 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage (b) Data for the rebound contractions to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage. * represents a statistical difference ($P < 0.05$, unpaired *t* test) compared to the control column of each data set. Values are represented as the mean ± s.e.m.

3.3.7. Effect of WIN 55,212-2 on the EFS-evoked contractions of the guinea-pig ileum MPLM in the absence and presence of SR 141716

WIN 55,212-2 caused a concentration-dependent inhibition of the contractions of the guinea-pig ileum MPLM elicited by EFS with single pulses at 0.1 Hz and trains of pulses at 30 Hz with a similar E_{max} (Figure 3.13). The pEC_{50} values for the inhibition of twitch and rebound contractions were 8.62 ± 0.04 (n = 6) and 6.25 ± 0.03 (n = 6) respectively. This cannabinoid receptor agonist was chosen for this experiment because previous studies (Pertwee *et al.*, 1996; Coutts & Pertwee, 1997) have demonstrated its stereospecific effects in this tissue.

In similar manner to the rat ileum MPLM (Section 3.3.2), the twitch contractions of the guinea-pig ileum MPLM evoked by EFS at 0.1 Hz were attenuated by significantly ($P < 0.05$, unpaired *t* test) lower concentrations of WIN 55,212-2 than the rebound contractions elicited by EFS at 30 Hz . The pEC_{50} values of WIN 55,212-2 for the inhibition of the twitch contractions of the guinea-pig ileum MPLM and rat ileum MPLM were very similar. In contrast, WIN 55,212-2 was a 0.75 logarithmic unit more potent in the guinea-pig ileum MPLM than in the rat ileum MPLM for the inhibition of the rebound contractions elicited by EFS at 30 Hz.

The onset and duration of action of WIN 55,212-2 at each concentration was similar to that on rat ileum MPLM under both EFS conditions (data not shown). Pre-exposure to SR 141716 (10^{-6} M) resulted in a significant ($P < 0.05$, ANOVA and Dunnett's test) parallel dextral shift of the mean WIN 55,212-2 concentration-response curve without a reduction in the mean E_{max} under either EFS conditions (Figure 3.13). The pA_2 values of SR 141716 for the rightward displacement of the WIN 55,212-2 concentration-response curve on tissues subjected to EFS at 0.1 Hz and 30 Hz were 7.97 and 6.71 respectively.

SR 141716 (10^{-6} M) alone caused a significant ($P < 0.05$, paired *t* test) 55.34 ± 5.53 % (n = 6) increase in the amplitude of the twitch contractions. By contrast, SR 141716 alone at this concentration had no effect on the amplitude of the rebound contractions of this tissue.

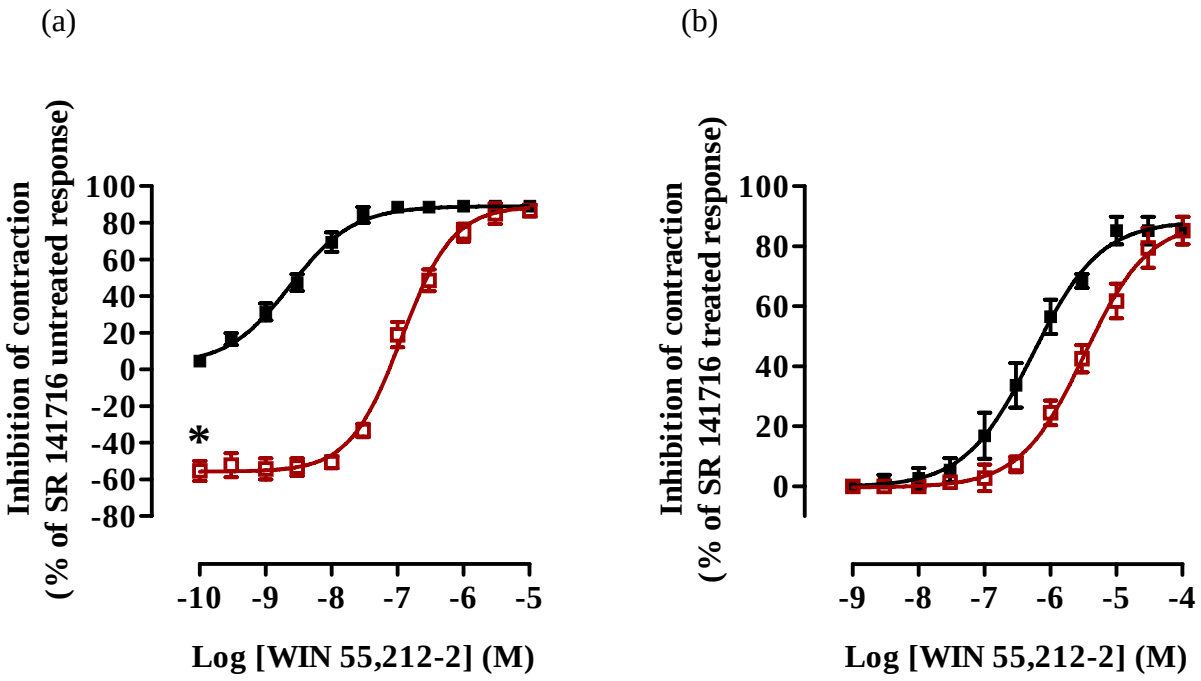


Figure 3.13: Concentration-response curves for the inhibition of the EFS-evoked contractions of the guinea-pig ileum MPLM by WIN 55,212-2 in the absence and presence of ethanol (■) or SR 141716 (10^{-6} M, □). (a) Data for the twitch contractions to EFS at 0.1 Hz frequency, 0.5 ms duration and 110 % supramaximal voltage (b) Data for the rebound contractions to EFS at 30 Hz for 2 seconds every minute, 0.5 ms duration and 110 % supramaximal voltage. Each curve was fitted by non-linear regression analysis. Each symbol represents the mean value of inhibition of EFS-evoked contractions expressed as a percentage reduction of the amplitude of contractions measured immediately before the addition of any drug to the organ bath (n = 6). SR 141716 or ethanol was added 20 and 30 minutes before the first addition of WIN 55,212-2 on tissues subjected to EFS at 0.1 Hz and 30 Hz respectively. * represents a statistical difference ($P < 0.05$, paired *t* test) compared to amplitude in the absence of SR 141716 i.e. 0 %.

3.3.8. Effect of the cannabinoids on the ACh-evoked contractions of the rat ileum MPLM

AEA (10^{-4} M), CP 55,940 (10^{-4} M), Δ^9-THC (10^{-4} M) and WIN 55,212-2 (10^{-4} M) which represented the concentrations that produced a marked inhibition of the EFS-evoked contractions of the rat ileum MPLM (Sections 3.3.2 and 3.3.3), had no effect on basal tension of this tissue nor did their presence alter the amplitude of the contractions elicited by single (Figure 3.14, for CP 55,940) or cumulative (Figure 3.15) additions of exogenously applied ACh (10^{-6} M).

Similarly, SR 141716 (10-5 M) also had no effect on the basal tension of this tissue or the contractions elicited by exogenously applied ACh (Figure 3.15).

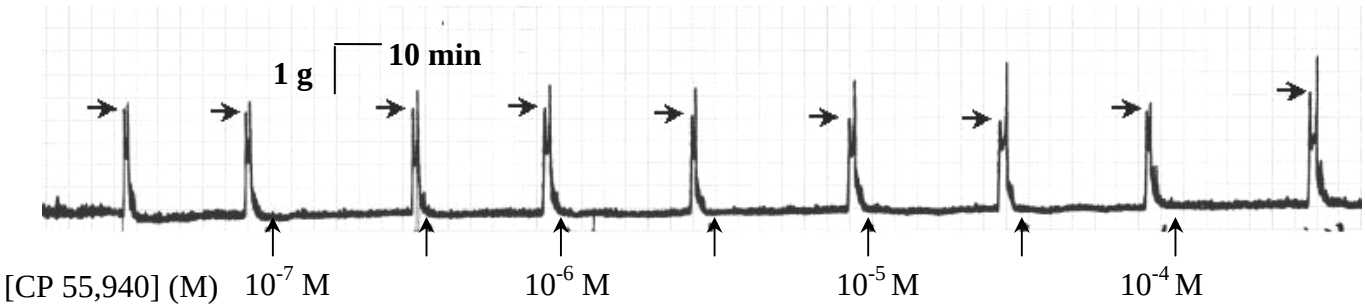


Figure 3.14: Trace illustrating the contraction of the rat ileum MPLM to single administrations of ACh (10^{-6} M) in the presence of CP 55,940 added in half-logarithmic unit increments. Unlabelled arrows represent intermediate concentrations of CP 55,940. → represents the peak of the ACh-evoked contraction.

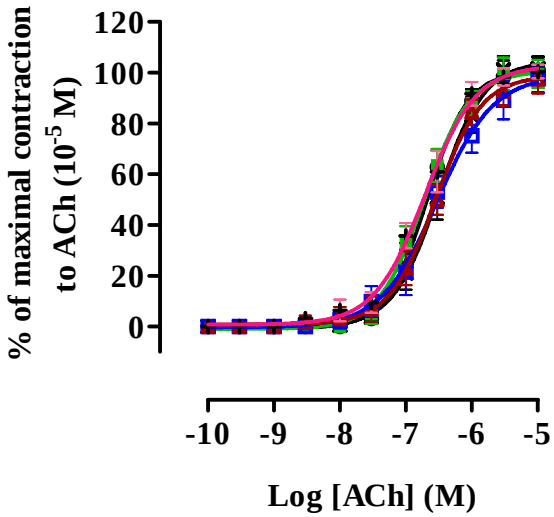


Figure 3.15: Concentration-response curves to exogenously applied ACh constructed in the presence of ethanol (**X**), AEA (○, 10^{-4} M), CP 55,940 (■, 10^{-4} M), Δ^9-THC (□, 10^{-4} M), WIN 55,212-2 (Δ, 10^{-4} M) or SR 141716 (+, 10^{-5} M) on the rat ileum MPLM. Each curve was fitted by non-linear regression analysis. The amplitude of the contractions to cumulative additions of ACh in the presence of ethanol or a cannabinoid were expressed as a percentage of the maximal contraction to ACh obtained at 10^{-5} M from an initial ACh concentration-response curve constructed on each tissue. Ethanol or a cannabinoid was added 30 minutes before constructing the second ACh concentration-response curve. n = 6 for all curves. All values represent mean ± s.e.m.

3.3.9. Effect of the cannabinoids on the ACh evoked contractions of the guinea-pig ileum MPLM

The presence of WIN 55,212-2 (10^{-4} M) or SR 141716 (10^{-4} M) which represented the concentrations that markedly altered the amplitude of the EFS-evoked contractions of the guinea-pig ileum MPLM (Section 3.3.7) had no effect on basal tension of this tissue nor did their presence alter the amplitude of the contractions elicited by cumulative additions of exogenously applied ACh (Figure 3.16).

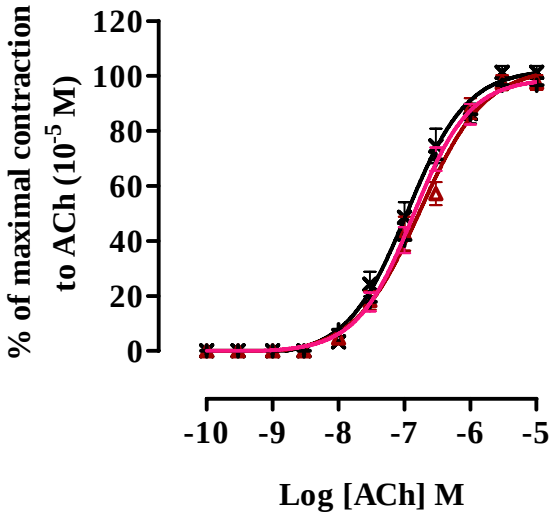


Figure 3.16: Concentration-response curves to exogenously applied ACh constructed in the presence of ethanol (**X**), WIN 55,212-2 (Δ, 10^{-4} M) or SR 141716 (+, 10^{-5} M) on the guinea-pig ileum MPLM. The amplitude of the contractions to cumulative additions of ACh in the presence of ethanol or a cannabinoid were expressed as a percentage of the maximal contraction to ACh obtained at 10^{-5} M from an initial ACh concentration-response curve constructed on each tissue. Ethanol or a cannabinoid was added 30 minutes before constructing the second ACh concentration-response curve. n = 6 for all curves. All values represent mean ± s.e.m.

3.4. Discussion

The results obtained show that the ability of representatives of the four established classes of structurally different cannabinoid receptor agonists to inhibit the EFS-evoked contractions of the guinea-pig and human ileal MPLM extends to the rat ileal MPLM. More importantly, they have revealed that the ability of each of the four agonists to act selectively through the CB_1 receptor was dependent on the frequency of EFS employed because clear differences in their rank order of potencies and the estimates of the pK_B and pA_2 values of SR 141716 were identified against the agonists between the two EFS conditions. Moreover, as demonstrated using WIN 55,212-2 and SR 141716, these observations appeared to extend to the guinea-pig ileum MPLM under similar EFS conditions.

In the rat MPLM, the potencies estimated for all four agonists to inhibit of the EFS-evoked twitch contractions of this tissue and their rank order of potencies i.e. CP 55,940 = WIN 55,212-2 > AEA > Δ^9-THC were consistent with those previously reported at the guinea-pig, human, mouse and rat CB_1 receptor (Martin *et al*., 2000; Pertwee & Fernando, 1996; Pertwee *et al*., 1996; Rinaldi-Carmona *et al*., 1998; Showalter *et al*., 1996). These findings together with the observation that the presence of increasing concentrations of SR 141716 caused similar agonist independent, progressive parallel and surmountable dextral shifts of the concentration response curves of each agonist suggested that the inhibitory effect each agonist was mediated by the activation of the CB_1 receptor.

Schild analysis of the interaction between each of the four agonists and SR 141716 yielded Schild plots with slopes of less than unity, but pK_B values of SR 141716 which were in line with its pK_B value previously reported at the CB_1 receptor (Rinaldi-Carmona *et al*., 1994) and irrespective of the agonist that was used. This result suggested that the interaction between SR 141716 and each of the four agonists was not strictly simple competition at the CB_1 receptor. However, because the 95 % C.I. values of the experimentally fitted slopes of each Schild analysis included unity, it was possible that the experimentally fitted slopes were not equal to unity because of random variation in the sample of the dose ratios. A re-fit of each Schild plot with a

unit slope yielded estimates of the pK_B value of SR 141716 which were again both consistent with its pK_B value reported at the CB_1 receptor (7.93-8.74; Rinaldi-Carmona *et al.*, 1994) and independent of the agonist that was used. These data suggested that the inhibition of the EFS-evoked twitch contractions of the rat ileum MPLM caused by all four agonists was through full agonism at the CB_1 receptor and that the interaction between each agonist and SR 141716 at this receptor was simple competitive. These results supported previous immunohistochemical (Coutts *et al.*, 2002) and *in vivo* (Izzo *et al.*, 1999a, 199b) data demonstrating the presence of the CB_1 receptor in this tissue and its functional role in delaying small intestinal transit *in vivo* respectively.

There are two reasons for believing that the antagonism produced by SR 141716 was not through functional antagonism, i.e. the agonists and SR 141716 were not counteracting each other's effect by producing opposing responses through separate receptors. Firstly, the maximal enhancement of twitch contractions produced by the highest concentration of SR 141716 was not large enough to completely offset the maximal inhibitory effect of each agonist. Secondly, the estimates of the pK_B values of SR 141716 obtained were similar irrespective of the agonist that was used.

In contrast to the CB_1 receptor pharmacology of all four agonists for the inhibition of the EFS-evoked twitch contractions of the rat ileum MPLM, the agonist potencies and their corresponding rank of order for the inhibition of the EFS-evoked rebound contractions of this tissue, i.e. AEA > CP 55,940 = Δ^9-THC = WIN 55,212-2, were not consistent with activity at either the CB_1 or CB_2 receptor (Rinaldi-Carmona *et al.*, 1998; Showalter *et al.*, 1996). This observation suggested that the inhibition of the rebound contractions of the rat ileum MPLM was not mediated through the activation of either the CB_1 or CB_2 receptor.

An unexpected but consistent finding was that the SR 141716 only antagonised the ability of AEA, Δ^9-THC and WIN 55,212-2, but not CP 55,940 to inhibit the EFS-evoked rebound contractions of the rat MPLM. Moreover, the pA_2 values of SR 141716 calculated against the former three agonists, albeit being similar, were around 2 logarithmic units lower than its pK_B value reported at the CB_1 receptor. This finding

suggested that AEA, Δ^9-THC and WIN 55,212-2 interacted with SR 141716 through a receptor distinct from the CB_1 receptor. The failure of SR 141716 to antagonise the inhibition of the rebound contractions caused by CP 55,940 indicated that this agonist did not interact with either the CB_1 receptor or the receptor mediating the inhibitory effect of AEA, Δ^9-THC and WIN 55,212-2.

The inhibition of the rebound contractions caused by AEA, Δ^9-THC and WIN 55,212-2 was unlikely to be mediated through a part activation of CB_1 receptor because, if the inhibition occurred through this receptor, the inhibitory effect of CP 55,940 should have also been susceptible to antagonism by SR 141716. These observations suggested that the CB_1 receptor did not mediate the inhibition of the rebound contractions of the rat ileum MPLM caused by all four agonists.

Relative to the inhibitory effects of WIN 55,212-2, its enantiomer WIN 55,212-3 was a significantly poor inhibitor of both types of the EFS-evoked contractions of the rat ileal MPLM. This observation suggested that the inhibitory effects of at least WIN 55,212-2 on this tissue was stereo-specific and supportive of a receptor-mediated action of this agonist under both EFS conditions. Indeed, at least at the cannabinoid receptor, WIN 55,212-2 demonstrates far greater affinity and intrinsic efficacy than its enantiomer in several *in vivo* and *in vitro* assays (Compton *et al.*, 1992; Izzo *et al.*, 1999; Pacheco *et al.*, 1993).

It is well established that all four agonists used in the present study do not demonstrate marked selectivity for either the CB_1 or CB_2 receptor. Moreover, AEA is also known to activate the TRPV1 receptor (Zygmunt *et al.*, 1999; Smart *et al.*, 2000). The failure of SR 144528 to antagonise the inhibitory effects of each of the four agonists under both EFS conditions suggested that none of the four agonists acted through the CB_2 receptor to attenuate the EFS-evoked contractions. This finding was consistent with previous data from similar *in vitro* contraction studies on the guinea-pig and human ileal MPLM (Croci *et al.*, 1998; Manara *et al.*, 2005; Mang *et al.*, 2001) and *in vivo* studies evaluating the inhibitory effect of these cannabinoids on small intestinal transit in the rat and mouse (Izzo *et al.*, 1999a, 1999b, 2000a, 2003).

Recent reports have shown the presence of the mRNA of the CB_2 receptor and the receptor protein in rat ileal myenteric plexus (Duncan *et al*., 2005a, 2006; Storr *et al.*, 2002). However, the ability of cannabinoid receptor agonists to delay small intestinal transit of the rat *in vivo* through acting at this receptor has been reported to occur only after inflammation of the small intestine, specifically that induced with the bacterial endotoxin lipopolysaccharide (Mathison *et al.*, 2004) and not other inflammatory agents (Izzo *et al.*, 1999a; 1999b). Because the MPLM strips used in the present study were prepared from healthy rats, it is unlikely that the CB_2 receptor mediated the inhibitory effects of all four agonists under both EFS conditions. These data are also supported by the failure of SR 144528 to antagonise the inhibitory of the agonists

The failure of capsazepine to antagonise the inhibitory effects of AEA under both EFS conditions suggested that the TRPV1 receptor was either absent or not activated by this agonist in this tissue. It was unlikely that the TRPV1 receptor was absent in tissue because the mRNA and immunoreactivity for this receptor has been identified in the rat ileum MPLM (Anavi-Goffer *et al.*, 2002). Moreover, in the present study, capsazepine alone caused an inhibition of the both types of EFS-evoked contractions of this tissue, possibly through a TRPV1 receptor mediated direct relaxation of the longitudinal muscle as reported previously by Nocerino *et al.*, (2002).

This study also demonstrated an inhibitory effect of WIN 55,212-2 on both the twitch and rebound contractions of the guinea-pig ileum MPLM elicited by EFS. The potency of this agonist for inhibiting the twitch contractions was similar to that reported previously at the CB_1 receptor in this tissue (Coutts & Pertwee, 1997; Pertwee *et al*., 1996) and to that obtained in the rat ileum MPLM when subjected to similar EFS conditions. This inhibitory effect was likely to be mediated through the activation of the CB_1 receptor because it was antagonised by SR 141716 with a pA_2 value in agreement with its pK_B value at the CB_1 receptor. However, as was observed in the rat ileum MPLM, the potency of WIN 55,212-2 to inhibit the rebound contractions of the guinea-pig ileum MPLM was also significantly lower than its potency measured for the inhibition of the twitch contractions. Moreover, the potency obtained was inconsistent with that reported previously at both the CB_1 and CB_2 receptor (Rinaldi-Carmona *et al*., 1998; Showalter *et al*., 1996). Because the pA_2 value of SR 141716 calculated for this antagonism was over a logarithmic unit less

than its pK_B value at the CB_1 receptor, the inhibition of the rebound contraction caused by WIN 55,212-2 was unlikely to be mediated through the CB_1 receptor.

The similarity between the pA_2 value of SR 141716 obtained against WIN 55,212-2 in the guinea-pig ileum MPLM and those obtained against the same agonist, AEA and Δ^9-THC in the rat MPLM, for the inhibition of the rebound contractions of both tissues suggested that the MPLM of both species contained the same putative non-CB_1-non-CB_2 receptor, in addition to the CB_1 receptor. Moreover, in addition to the presence of these two receptors in the rat ileum MPLM, this tissue also appeared to contain an additional non-CB_1-non-CB_2 receptor that mediated the inhibitory effect of CP 55,940.

The twitch and rebound contractions of both MPLM tissues to EFS were abolished by TTX or atropine but not hexamethonium. This suggested that the electrical stimulus indirectly provoked contractions of the longitudinal smooth muscle layer, through the mACh receptor, by stimulating the release of ACh from post-ganglionic nerve terminals of the myenteric plexus. Because the relaxations of the rat ileum MPLM observed during the first 20 minutes of EFS at 30 Hz were abolished by TTX and L-NAME, it was likely that these responses were mediated by the release of NO from the myenteric plexus. The failure of EFS to induce relaxations of the guinea-pig ileum MPLM preceding the rebound contractions suggests that either an inhibitory transmitter was not released from this tissue or the amount released was not sufficient to produce a relaxation of the muscle.

None of the four agonists, nor SR 141716 at the highest concentrations used had, any effect on the resting tone or contractions of the rat ileum MPLM evoked by exogenously applied ACh. Similar observations were on the made on guinea-pig ileum MPLM in the presence of WIN 55,212-2 and SR 141716. Therefore, in agreement with previous studies (Coutts & Pertwee, 1997; Mang *et al.,* 2001), the effects of the cannabinoids on the EFS-evoked contractions of both tissues may be ascribed to a pre-synaptic modulation of the release of ACh from the myenteric plexus and not a post-junctional interaction with the mACh receptor on the smooth muscle or an interference with the hydrolysis of ACh by ACh esterase.

All cannabinoids assayed displayed a slow onset of action on the EFS-evoked contractions of both MPLM tissues. The slow kinetics may be attributed to the relative low aqueous solubility and high lipophilicity of the compounds, which would cause them to be sequestered by the solubilising agent and delay the equilibration between the cannabinoids molecules in free solution and those present in the tissue. The low solubility of these drugs in water would be expected to delay their onset of action by limiting their rate of diffusion across the aqueous phase from the solubilising agent to the tissue. In the rat ileum MPLM, the good correlation between the rank order of the onset of action of the four agonists i.e. WIN 55,212-2 > CP 55,940 > Δ^9-THC > AEA and their rank order of logarithmic octanol-water partition coefficients i.e. 3.4, 4.6, 5.5 and 6.3 respectively (Valvetti *et al.,* 2004) suggests that the rate of onset of action of each agonist was slow because of their low aqueous solubility, high lipophilicity and slow receptor kinetics. Because the rank orders of onset of the inhibition of contractions by all four agonists in the rat ileum MPLM correlated well between the two EFS conditions, it is conceivable that the differences in the rates of onset of inhibition between the two EFS conditions were not attributed to differences in the diffusion of the agonists through the tissue to the receptor. The differences between the rates of onset of action between the two EFS conditions supports the notion that the inhibitory effects under the two EFS conditions were not mediated through a common receptor.

In the absence of other drugs, SR 141716 alone produced a small but significant concentration dependent increase in the amplitude of the EFS-evoked twitch contractions of the rat ileum MPLM. Consistent with results from previous studies, SR 141716 at the single concentration used caused a marked increase in the amplitude of the EFS-evoked twitch contractions of the guinea-pig ileum MPLM (Coutts & Pertwee, 1997; Pertwee *et al.,* 1996). This augmentation of the twitch contractions may be through an SR 141716-induced antagonism of the tonic activation of the CB_1 receptor by endogenously released CB_1 receptor agonists. Indeed, recent reports have shown that levels of both AEA and 2-AG high enough to cause the activation CB_1 receptor have been detected in the rat and guinea-pig ileum MPLM (Katayama *et al.,* 1997; Valenti *et al.,* 2005; Guagnini *et al.,* 2006). Alternatively, the augmentation of

the twitch contractions may be through an unmasking of the constitutive activity of the CB_1 receptor via inverse agonism.

Whilst SR 141716 increased the amplitude of the EFS-evoked twitch contractions of both ileal MPLM tissues, it did not do the same to the EFS-evoked rebound contractions. This might be because there are different subtypes of the cannabinoid receptor in these tissues as suggested by the difference between the pK_B and pA_2 values of SR 141716 between the two EFS conditions.

Taken together, the data in this study have provided evidence that the rat ileum MPLM served as a viable *in vitro* ileal cannabinoid receptor bioassay as the guinea-pig ileum MPLM. Representatives of the four main classes of structurally different cannabinoid receptor agonists were shown to attenuate the EFS-evoked contractions of this tissue in a concentration dependent, presynaptic and stereospecific manner. Evidence was provided for a functional role of the CB_1 receptor and a putative non-CB_1-non-CB_2 but SR 141716-sensitive and agonist dependent receptor in selectively mediating the inhibitory effects of the cannabinoid receptor agonists on the twitch and rebound contractions of the rat ileum MPLM respectively. These observations were shown to extend to the guinea-pig ileum subjected to similar EFS conditions. Neither the CB_2 receptor nor the TRPV1 receptor were involved in mediated the inhibitory effects of the cannabinoid receptor agonists or AEA under either EFS conditions respectively. The ability of SR 141716 to cause an augmentation of the EFS-evoked twitch contractions suggests either the presence of an endocannabinoid tone or a constitutively active CB_1 receptor.

CHAPTER 4:

ISOLATED NERVE-SMOOTH MUSCLE PREPARATIONS

Studies into the Mechanism of the SR 141716-Mediated Increase in the Amplitude of the Twitch Contractions of the Rat Myenteric Plexus Longitudinal Muscle Preparation

4.1. Introduction

In the previous chapter, it was shown that in addition to its antagonist activity at the CB_1 receptor, SR 141716 alone caused an augmentation of the EFS-evoked twitch contractions of both the rat and guinea-pig ileal MPLM. Two common explanations that have been offered to describe such antagonist mediated changes in responses are the unmasking of a constitutive activity of the receptor via inverse agonism, or the antagonism of tonically released endogenous agonists from the receptor. To date, SR 141716 has only been conclusively shown to behave as an inverse agonist in recombinant cell-based assays monitoring cAMP accumulation or [^{35}S]-GTPγS incorporation (Bouaboula *et al*., 1997; Landsman *et al*., 1997; MacLennnan *et al*., 1997) in which the CB_1 receptor was over-expressed. Whilst inverse agonism and constitutive activity of the CB_1 receptor have been easily demonstrated and characterised in receptor over-expression systems, it has appeared unlikely that such activity could occur in intact tissues in which receptor expression was likely to be at a natural physiological level.

Over the last decade, a large body of evidence has emerged to indicate that a functional endocannabinoid tone may be present in the intestinal tract, primarily because of the identification of AEA and 2-AG (Fegley *et al*., 2005; Gomez *et al*., 2002; Guagnini *et al.,* 2006; Mechoulam *et al.,* 1995; Mascolo *et al*., 2002; Izzo *et al*., 2003) and the mechanisms for their inactivation, via facilitated uptake by a putative transporter protein (Mascolo *et al*., 2002) followed by enzymatic hydrolysis such as by FAAH (Katayama *et al*., 1997). Furthermore, the demonstration that *in vivo* administration of inhibitors of FAAH or the putative uptake transporter into rodents mimics the inhibitory effects of exogenously administered cannabinoid receptor agonists has added weight to a possible physiological inhibitory role of an endocannabinoid agonist tone on intestinal motility (Capasso *et al*., 2005; Pinto *et al*., 2002). However, the stimulatory effect of SR 141716 on the intestinal tract may not be attributed unequivocally to the presence of ongoing local enteric endocannabinoid agonist activity because the existence of a possible constitutive activity of the CB_1 receptor in the small intestine has not been investigated.

The purpose of this study to make an attempt to elucidate the mechanism by which SR 141716 alone augmented the EFS-evoked twitch contractions of the rat ileum MPLM. Parallel experiments were also performed on the guinea-pig ileum MPLM because of the common use of this tissue as a cannabinoid bioassay. Additionally, some experiments were also performed on the rat vas deferens, because despite it possessing a functional pre-synaptic CB_1 receptor, in this tissue SR 141716 does not alter the amplitude of the EFS-evoked contractions (Lay *et al.*, 2000).

Recently, a number of ligands have been developed and suggested to behave as potent "silent" CB_1 receptor antagonists (Pertwee, 2005b). Among these is O-2050, a commercially available sulphonamide analogue of Δ^9-tetrahydrocannabinol, which has been reported to be devoid of agonist or inverse agonist activity on the EFS-evoked contractions of the mouse vas deferens (Martin *et al*., 2002). Therefore, the availability of O-2050 offered the opportunity to elucidate the mechanism by which SR 141716 alone produced its inherent effects. For example, if O-2050 was found not to augment the EFS-evoked contractions of the rat and guinea-pig ileum MPLM, evidence would be obtained to suggest that SR 141716 behaved as an inverse agonist and that an endocannabinoid tone was absent.

In this study, the effect of three inhibitors of FAAH; AA-5HT (arachidonoyl-serotonin), PMSF (phenylmethylsulphonyl fluoride) and URB-597 and of VDM-11, an inhibitor of the putative endocannabinoid uptake transporter were also evaluated on the MPLM tissues, as an approach to unmask an inhibitory endocannabinoid tone by limiting the inactivation of tonically released endocannabinoid agonists.

Additionally, the effects of AA-5HT, PMSF, URB-597 and VDM-11 were examined on the potency of exogenously applied AEA and WIN 55212-2 as inhibitors of the EFS-evoked contractions of the MPLM tissues. The latter cannabinoid receptor agonist was used because it is not a substrate of FAAH as lacks an amide linkage, and in addition to its common use as an agonist, it has been shown to have stereospecific activity at the CB_1 receptor in these tissues (Coutts & Pertwee, 1997; see Chapter 2).

4.2. Methods

MPLM strips were dissected from the ileum of both rats and guinea-pigs (Section 2.1) and set up in organ baths as described in Section 2.2.1. The experiments under EFS and muscarinic stimulation conditions were performed by the methods described in Section 2.2.5.1.1 and 2.2.5.1.2 respectively.

The data obtained from the experiments with EFS and ACh stimulation were quantified and analysed by the methods described in Section 2.2.5.3.1 and 2.2.5.3.2 respectively.

The vas deferens was dissected from the rats (Section 2.1) by the method described in Section 2.2.2. The experiments on the rat vas deferens under EFS stimulation conditions were performed by the methods described in Section 2.2.5.2. The data obtained from the experiments with EFS were quantified and analysed by the methods described in Section 2.2.5.3.1.

The compounds used in this study were: AA-5HT, ACh, AEA, AM 251, atropine, O-2050, PPADS, Prazosin, PMSF, SR 141716, TTX, WIN 55,212-2, URB-597 See Table 2.1 for the solvents and suppliers of the compounds used.

4.3. Results

4.3.1. Effect of SR 141716, AM 251 and O-2050 on EFS-evoked twitch contractions of the rat and guinea-pig ileum MPLM

SR 141716, AM 251 and O-2050 all caused a concentration-dependent enhancement of the EFS-evoked twitch contractions of both the rat and guinea-pig ileum MPLM (Figure 4.1). The twitch contractions in the presence of these cannabinoids were abolished by application of either TTX (10^{-6} M) or atropine (10^{-6} M). Figure 4.0 shows representative traces of the augmentation of the twitch contractions of both tissues by SR 141716.

All three cannabinoid antagonists produced different degrees of maximal enhancement of the twitch contractions in both tissues. The E_{max} values of all three antagonists were over 20 times ($P < 0.05$, unpaired *t* test) greater in the guinea-pig ileum MPLM than in the rat ileum MPLM (Table 4.0). Nevertheless, the rank order of the E_{max} values were similar in both tissues i.e. AM 251 > SR 141716 > O-2050.

Because each set of curves in Figure 4.1 had different maxima and that the slopes were not parallel, a direct comparison between the pEC_{50} values of the three cannabinoids was not possible. A comparison between the concentrations corresponding to the 5 % and 150 % level of enhancement of the contractions of the rat and guinea-pig ileum MPLM curves, respectively, indicated that the rank order of potency of the three cannabinoids on both tissues was similar i.e. AM 251 > SR 141716 > O-2050.

The rate of onset of the potentiation of the contractions by each cannabinoid was slow on both tissues, with the maximal enhancement at each concentration being achieved within 20 minutes of administration. Figure 4.2 shows the time courses for the maximal enhancement of the twitch contractions of the rat and guinea-pig ileum MPLM by all three cannabinoids at a concentration of 10^{-6} M.

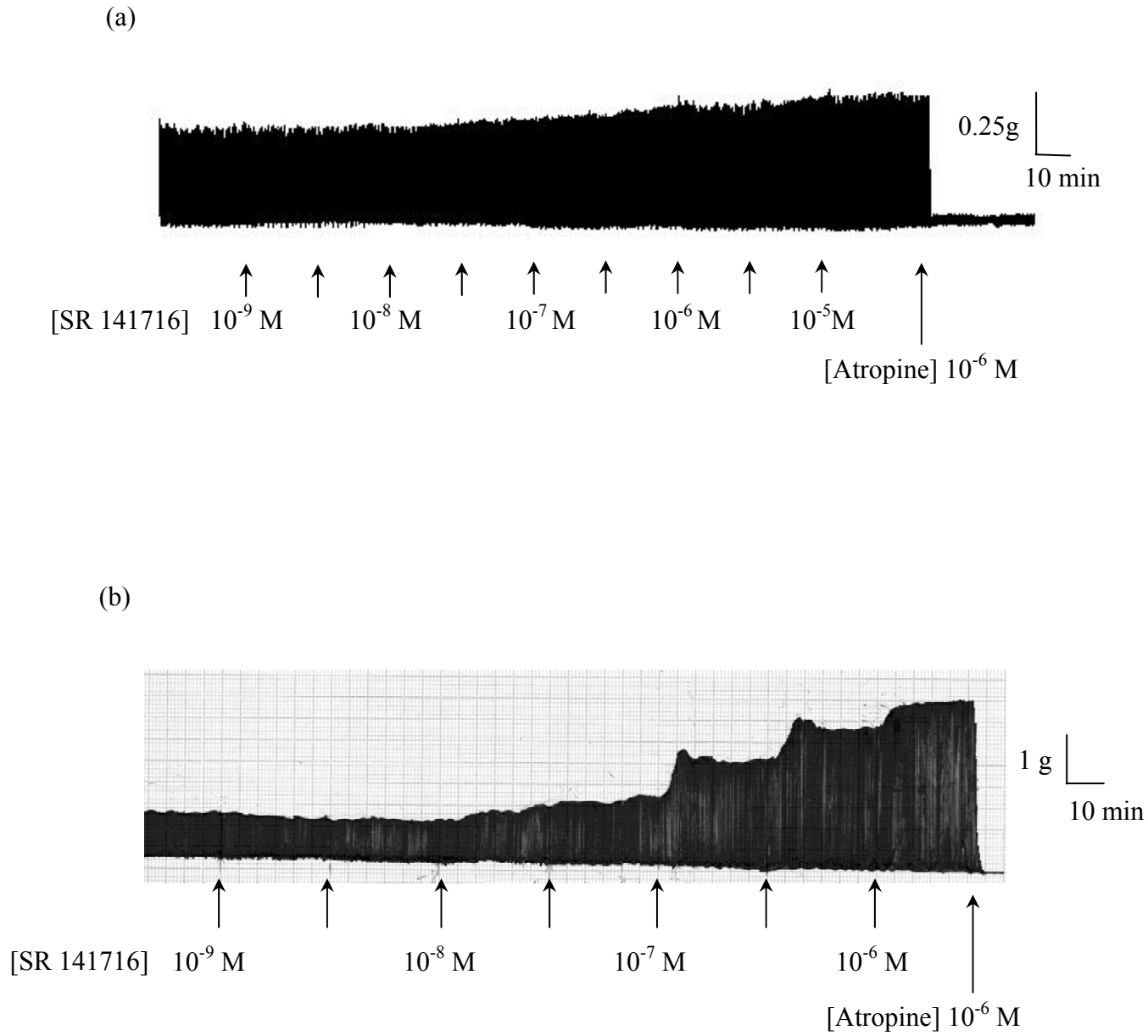


Figure 4.0: Representative traces of the EFS-evoked twitch contractions of the rat and guinea-pig ileum MPLM in the absence and presence of SR 141716 added in half-logarithmic unit increments. Unlabelled arrows represent intermediate concentrations. (a) Trace illustrating the twitch contractions of the rat ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.05 Hz in the absence and presence SR 141716 (b) Trace illustrating the twitch contractions of the guinea-pig ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz in the absence and presence SR 141716.

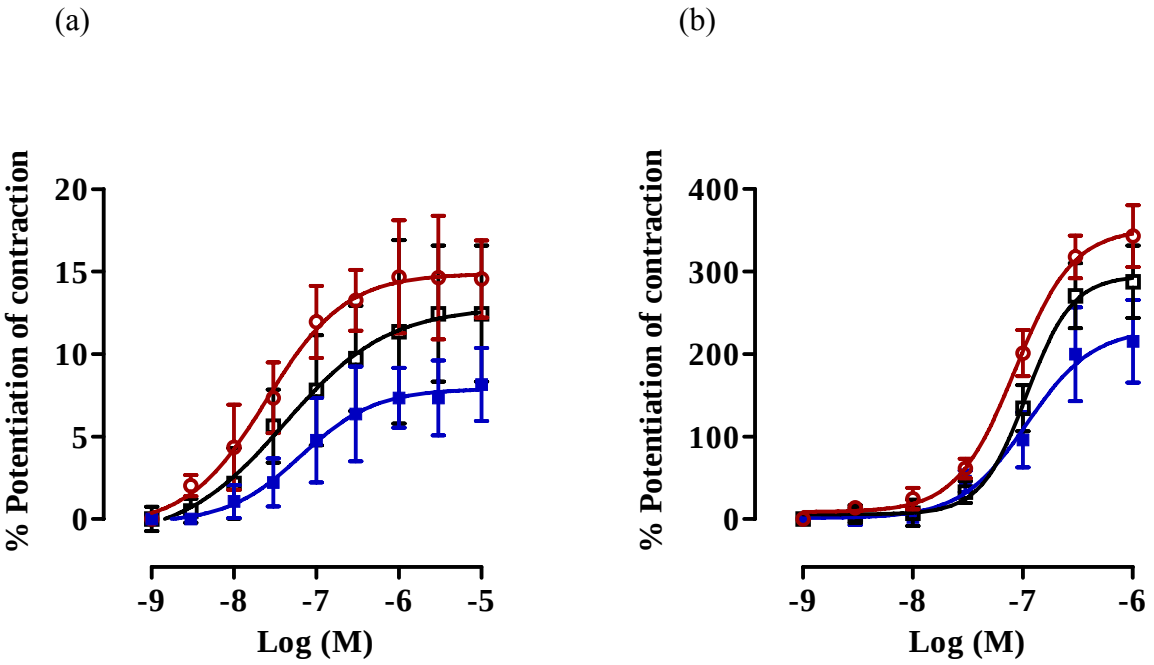


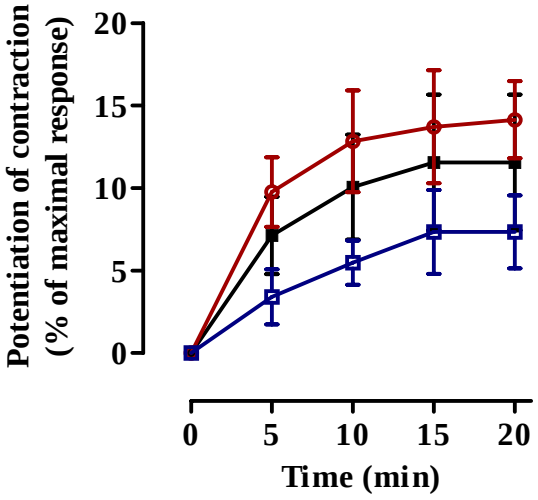
Figure 4.1: Concentration-response curves for the enhancement of the EFS-evoked twitch contractions of the rat and guinea-pig ileum MPLM by SR 141716 (□), AM 251 (○) and O-2050 (■). Each curve was fitted by non-linear regression analysis. (a) Data for the twitch contractions of the rat ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.05 Hz (b) Data for the twitch contractions of the guinea-pig ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz. Each symbol represents the mean value of potentiation of the twitch contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the addition of the same cannabinoid to the organ bath (n = 6). Vertical lines indicate the s.e.m.

Table 4.0: A comparison of the potency (pEC_{50}) and tissue maximal response (E_{max}) and the ratio of the E_{max} values of the enhancement of the EFS-evoked contractions of the rat and guinea-pig ileum MPLM by the CB_1 receptor antagonist / inverse agonists SR 141716, AM 251 and O-2050.

	Rat ileum MPLM		Guinea-pig ileum MPLM		
Cannabinoid	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	$\frac{E_{max\,(guinea-pig)}(\%)}{E_{max\,(rat)}(\%)}$
SR 141716	7.46 ± 0.04	12.45 ± 4.12	6.97 ± 0.01	287.8 ± 43.9 *	23.1
AM 251	7.60 ± 0.02	14.55 ± 2.34	7.07 ± 0.01	343.0 ±37.5 *	23.5
O-2050	7.19 ± 0.01	8.15 ± 2.21	6.96 ± 0.03	215.5 ± 50.2 *	26.4

pEC_{50} and E_{max} values were derived by non-linear regression analysis for the rat and guinea-pig ileum MPLM strips stimulated electrically with single pulses of 0.5 ms duration, 110 % supramaximal voltage at 0.05 Hz and 0.1 Hz frequency respectively. * represents a significantly ($P < 0.05$, unpaired t test) greater E_{max} value than the E_{max} value for the same cannabinoid obtained on the rat ileum MPLM. Where appropriate, values are represented as mean ± s.e.m of $n \geq 6$ experiments.

(a)



(b)

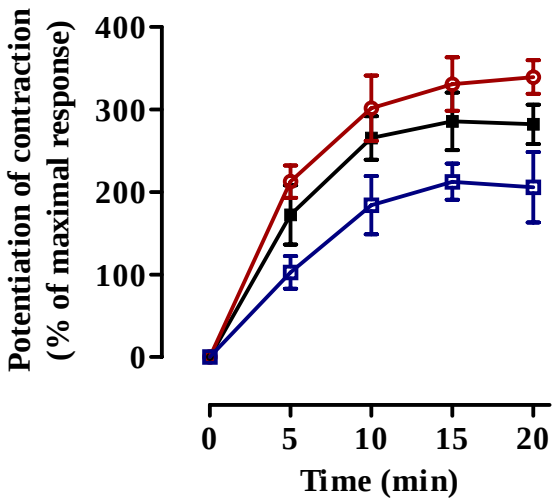


Figure 4.2: Comparison of the time-response curves for the enhancement of the amplitude of the EFS-evoked twitch contractions of the rat and guinea-pig ileum MPLM by SR 141716 (□), AM 251 (○) and O-2050 (■) at 10^{-6} M. (a) Data for the twitch contractions of the rat ileum MPLM elicited by single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.05 Hz. (b) Data for the twitch contractions of the guinea-pig ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz. Each symbol represents the mean value of potentiation of the twitch contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the addition of the same cannabinoid to the organ bath (n = 4). Vertical lines indicate the s.e.m.

4.3.2. Effect of SR 141716, AM 251 and O-2050 on EFS-evoked twitch contractions of the rat vas deferens

Stimulation of the rat vas deferens with single pulses at a repetition frequency of 0.1 Hz elicited a transient twitch contraction of 1.27 ± 0.23 g (n = 60) every 10 seconds during the duration of the electrical pulse (Figure 4.3). Figure 4.4 shows that the twitch contractions were abolished by treatment with either TTX (10^{-6} M) or PPADS (10^{-6} M) but not prazosin (10^{-6} M).

Cumulative additions of SR 141716, AM 251 and O-2050 from 10^{-9} M to 10^{-5} M every 20 minutes had no effect on the basal tension or the EFS-evoked twitch contractions of this tissue (data not shown).

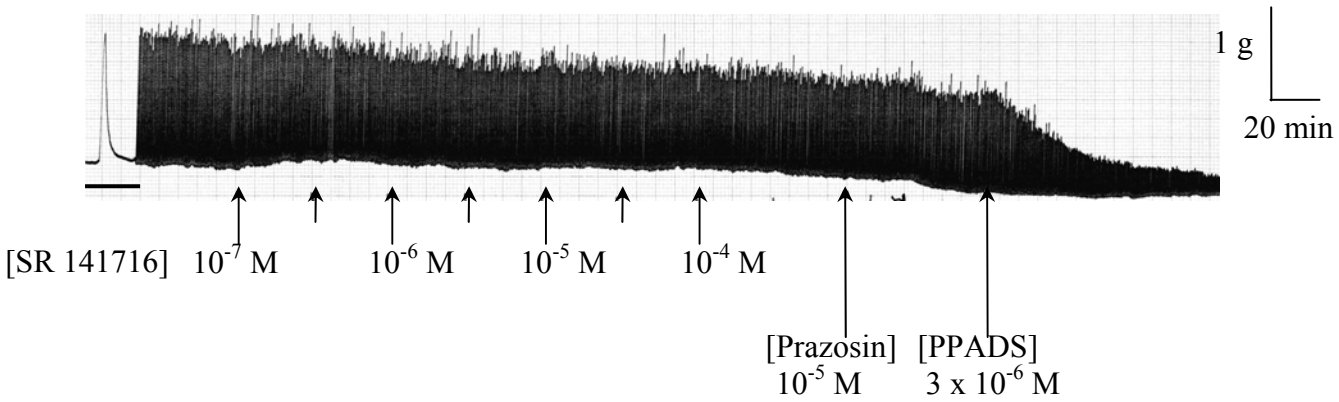


Figure 4.3: Representative trace of the EFS-evoked twitch contraction of the rat vas deferens to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz in the absence and presence of prazosin (10^{-6} M) and PPADS (10^{-6} M). SR 141716 was added in half-logarithmic unit increments. Unlabelled arrows represent intermediate concentrations. Solid line indicates the monophasic contraction of the rat vas deferens to EFS on a fast time base. Subsequent contractions are recorded on a slow time base.

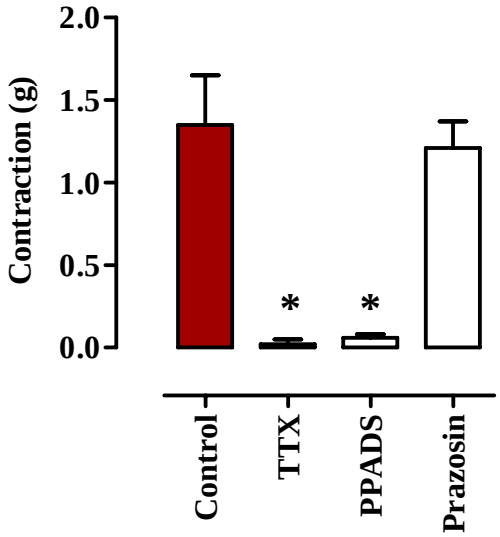


Figure 4.4: Bar graphs representing the amplitude of the EFS-evoked twitch contractions of the rat vas deferens evoked by single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz in the absence (control column, n = 18) and presence of TTX (10^{-6} M, n = 6), PPADS (10^{-6} M, n = 6) or prazosin (10^{-6} M, n = 6). * represents a statistical difference ($P < 0.05$, unpaired *t* test) compared to the control column. Values are represented as the mean ± s.e.m.

4.3.3. Effect of AA-5HT, PMSF and URB-597 alone and on the potency of AEA and WIN 55,212-2 as inhibitors of the EFS-evoked twitch contractions of the rat and guinea-pig ileum MPLM

Cumulative additions of AA-5HT (10^{-9} to 10^{-5} M), PMSF (10^{-6} to 10^{-4} M) or URB-597 (10^{-9} to 10^{-5} M) every 15 minutes had no effect on basal tension or the EFS-evoked twitch contractions of both the rat and guinea-pig ileum MPLM (data not shown).

The effect of AA-5HT and URB-597 at concentrations higher than 10^{-5} M could not be investigated because of difficulties in preparing sufficiently concentrated stock solutions of these compounds.

Pre-incubation of the tissues for 20 minutes with AA-5HT (10^{-6} M), PMSF (10^{-4} M) or URB-597 (3×10^{-8} M) resulted in a significant ($P < 0.05$, ANOVA and Dunnett's test) parallel leftward displacement of the AEA concentration-response curve on both the rat and guinea-pig ileum MPLM without a change in E_{max} (Figure 4.5) or slope. The presence of these concentrations of AA-5HT, PMSF and URB-597 increased the potency of AEA by 2.0, 22.1 and 20.2 times on the rat ileum MPLM respectively, and by 3.2, 32.4 and 48.8 times on the guinea-pig ileum MPLM respectively.

By contrast, pre-treatment of the rat and guinea-pig ileum MPLM for 20 minutes with AA-5HT (10^{-6} M), PMSF (10^{-4} M) or URB-597 (3×10^{-8} M) had no effect on the location and E_{max} of the WIN 55,212-2 concentration-response curve (Figure 4.6).

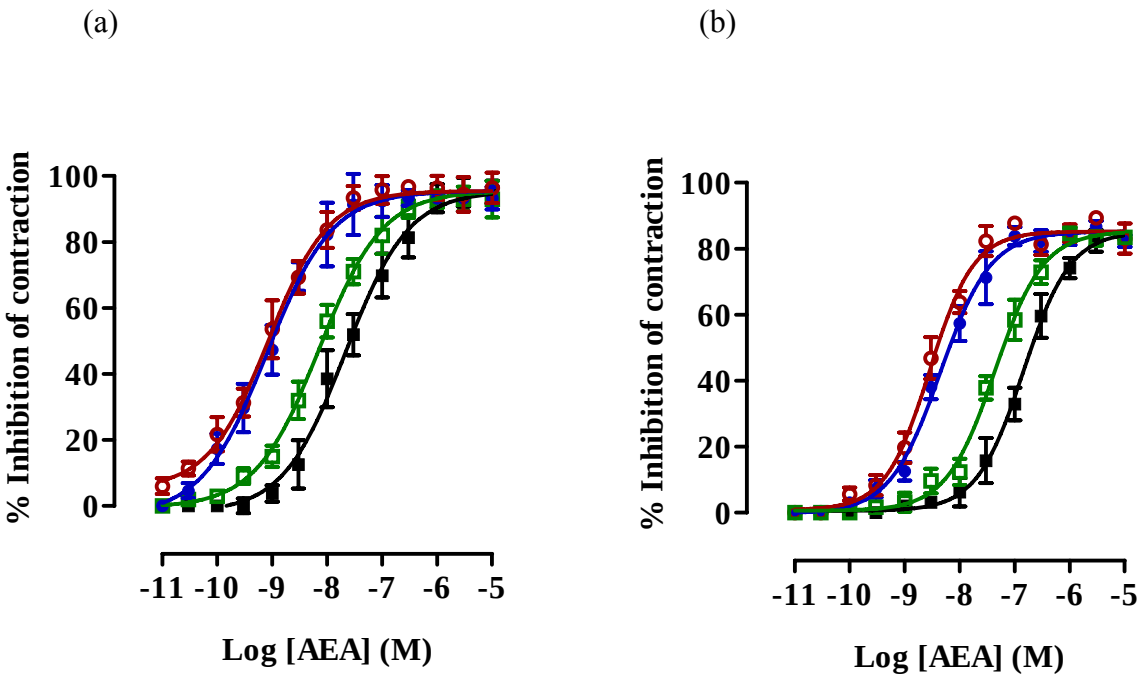


Figure 4.5: Concentration-response curves for the inhibition of the EFS-evoked contractions of the rat and guinea-pig ileum MPLM by AEA in the presence of ethanol (■), AA-5HT (□, 10^{-6} M), PMSF (●, 10^{-4} M) or URB-597 (○, 3 x 10^{-8} M). Each curve was fitted by non-linear regression analysis with the upper asymptote constrained to a common value. (a) Data for the twitch contractions of the rat ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.05 Hz (b) Data for the twitch contractions of the guinea-pig ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz. Each symbol represents the mean value of inhibition of twitch contractions expressed as a percentage of the amplitude of the contraction measured immediately before the addition of any drug to the organ bath (n = 6). Vertical lines indicate the s.e.m. AA-5HT, PMSF or URB-597 or ethanol was added 20 minutes before the first addition of AEA.

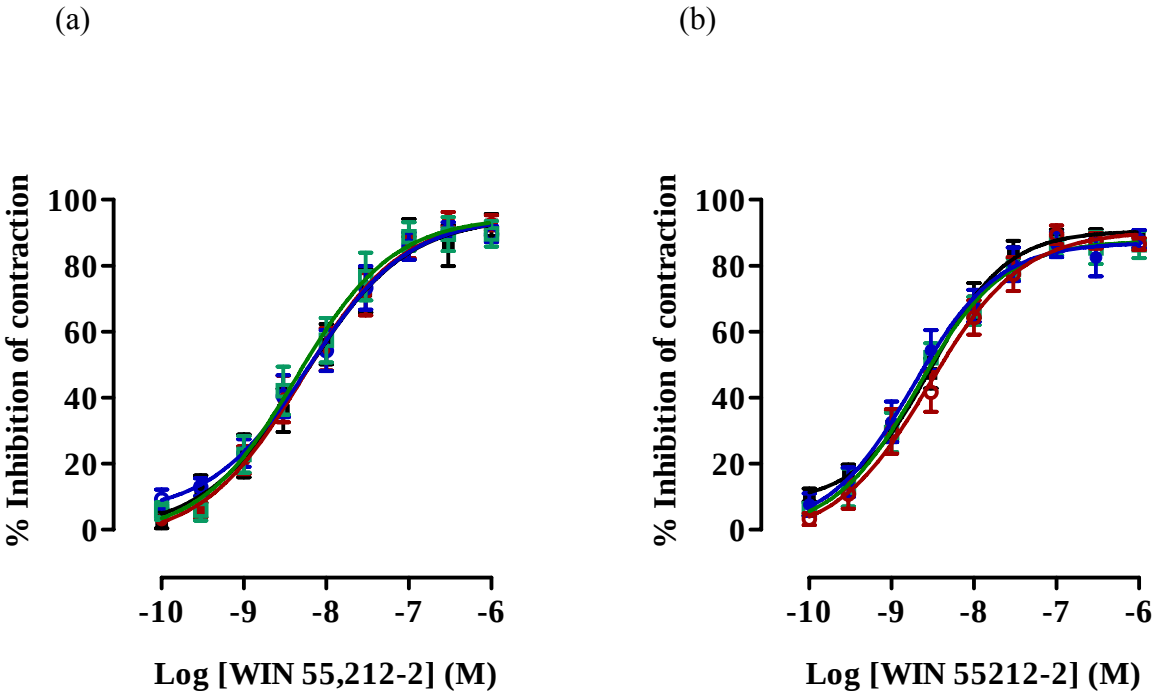


Figure 4.6: Concentration-response curves for the inhibition of the EFS-evoked contractions of the rat and guinea-pig ileum MPLM by WIN 55,212-2 in the presence of ethanol (■), AA-5HT (□, 10^{-6} M), PMSF (●, 10^{-4} M) or URB-597 (○, 3 x 10^{-8} M). Each curve was fitted by non-linear regression analysis with the upper asymptote constrained to a common value. (a) Data for the twitch contractions of the rat ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.05 Hz (b) Data for the twitch contractions of the guinea-pig ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz. Each symbol represents the mean value of inhibition of twitch contractions expressed as a percentage of the amplitude of the contraction measured immediately before the addition of any drug to the organ bath (n = 6). Vertical lines indicate the s.e.m. AA-5HT, PMSF or URB-597 or ethanol was added 20 minutes before the first addition of WIN 55,212-2.

4.3.4. Effect of VDM-11 alone and on the potency of AEA as an inhibitor of the EFS-evoked twitch contractions of the rat and guinea-pig ileum MPLM

Cumulative additions of VDM-11 (10^{-9} to 10^{-5} M) every 15 minutes had no effect on basal tension or EFS-induced contractions of rat and guinea-pig ileum MPLM (data not shown). Concentrations of VDM-11 higher than 10^{-5} M were not investigated. Pre-incubation of the tissues for 20 minutes with VDM-11 (10^{-5} M) did not shift the AEA concentration-response curve on either tissues (Figure 4.7).

(a) (b)

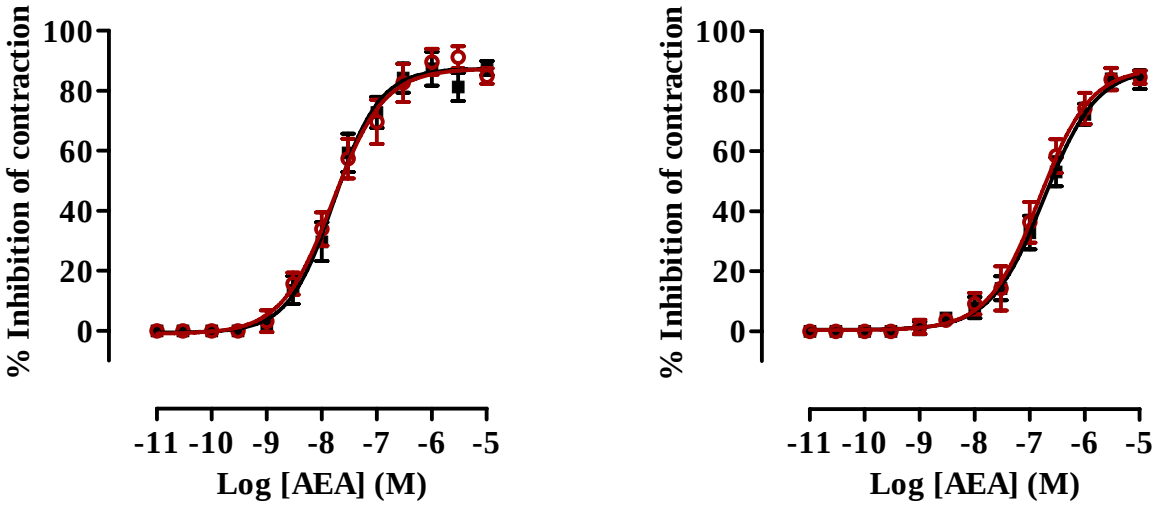


Figure 4.7: Concentration-response curves for the inhibition of the EFS-evoked contractions of the rat and guinea-pig ileum MPLM by AEA in the presence of ethanol (■) or VDM-11 (○, 10^{-5} M). Each curve was fitted by non-linear regression analysis with the upper asymptote constrained to a common value. (a) Data for the twitch contractions of the rat ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.05 Hz (b) Data for the twitch contractions of the guinea-pig ileum MPLM to single pulses of 0.5 ms duration and 110 % supramaximal voltage repeated at a frequency of 0.1 Hz. Each symbol represents the mean value of inhibition of twitch contractions expressed as a percentage of the amplitude of the contraction measured immediately before the addition of any drug to the organ bath (n = 6). Vertical lines indicate the s.e.m. VDM-11 or ethanol was added 20 minutes before the first addition of AEA.

4.3.5. Effect of AEA on the EFS-evoked contractions of the guinea-pig ileum MPLM in the absence and presence of SR 141716

The ability of both AEA and WIN 55,212-2 to attenuate the twitch contractions of the rat ileum MPLM, and of the latter agonist to inhibit the twitch contractions of the guinea-pig ileum MPLM were shown to be mediated through the activation of the CB_1 receptor (Chapter 3, Sections 3.3.3 and 3.3.7). Because of the use of AEA in Section 4.3.3 and 4.3.4, the effect of this agonist was investigated on the twitch contractions of the guinea-pig ileum MPLM in the presence and absence of SR 141716 to characterise the receptor mediating the inhibitory effect of this agonist.

AEA caused a concentration-dependent inhibition of the twitch contractions of the guinea-pig ileum MPLM with a pEC_{50} value of 6.81 ± 0.02 (n = 6, Figure 4.8). This value was significantly ($P < 0.05$, unpaired *t* test) lower than the pEC_{50} value obtained on the rat ileum MPLM (Section 3.3.3), but similar to that obtained previously on this tissue (Mang *et al*., 2001).

The onset and duration of action of AEA at each concentration was similar to that observed on the rat ileum MPLM (data not shown). Pre-treatment of the tissue with SR 141716 (10^{-7} M) caused a significant ($P < 0.05$, ANOVA and Dunnett's test) parallel dextral shift of the AEA concentration-response curve without a reduction in the E_{max} value (Figure 4.8). The pA_2 value of SR 141716 for this antagonism was 7.90.

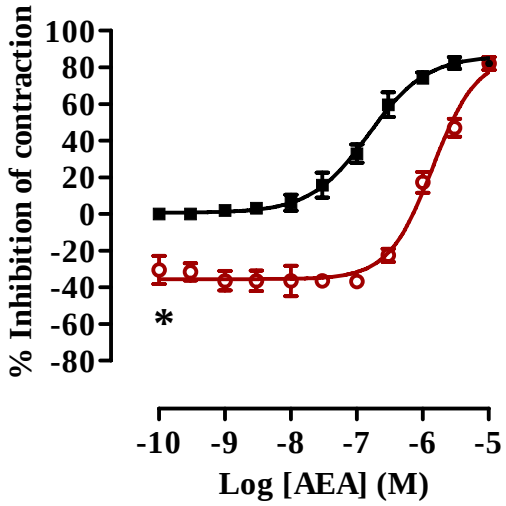


Figure 4.8: Concentration-response curves for the inhibition of the EFS (0.1 Hz, 0.5 ms duration, 110 % supramaximal voltage) evoked twitch contractions of the guinea-pig ileum MPLM by AEA in the presence of ethanol (■) or SR 141716 (10^{-7} M, ○). Each curve was fitted by non-linear regression analysis with the upper asymptote constrained to a common value. Each symbol represents the mean value of inhibition of EFS-evoked contractions expressed as a percentage reduction of the amplitude of contractions measured immediately before the addition of any drug to the organ bath (n = 6). Vertical lines represent the s.e.m. SR 141716 or ethanol was added 20 minutes before the first addition of AEA. * represents a statistical difference ($P < 0.05$, paired *t* test) compared to amplitude in the absence of SR 141716 i.e. 0 %.

4.3.6. Effect of WIN 55,212-2 on the EFS-evoked contractions of the rat vas deferens in the absence and presence of SR 141716

To rule out the possibility that the failure of SR 141716, AM 251 and O-2050 to augment the EFS-evoked contractions of the this tissue (Section 4.3.2) may have been a consequence of the absence of the CB_1 receptor, the effect of WIN 55,212-2 was investigated in the absence and presence of SR 141716.

WIN 55,212-2 caused a concentration related inhibition of the twitch contractions of this tissue with a pEC_{50} value of 7.76 ± 0.02 (n = 5). The onset of the inhibition of the contractions by WIN 55,212-2 was slow, with the maximal inhibition at each concentration being achieved within 30 minutes of administration (data not shown).

The presence of SR 141716 (10^{-6} M) caused a significant ($P < 0.05$, ANOVA and Dunnett's test) parallel dextral shift of the WIN 55,212-2 concentration-response curve without a reduction in the E_{max} value (Figure 4.9). The pA_2 value of SR 141716 for the antagonism of WIN 55,212-2 was 7.81

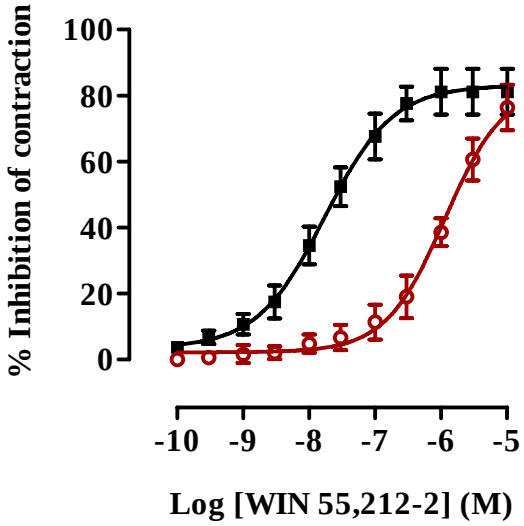


Figure 4.9: Concentration-response curves for the inhibition of the EFS-evoked twitch contractions (0.1 Hz, 0.5 ms duration, 110 % supramaximal voltage) of the rat vas deferens by WIN 55,212-2 in the presence of ethanol (■) or SR 141716 (10^{-6} M, ○). Each curve was fitted by non-linear regression analysis with the upper asymptote constrained to a common value. Each symbol represents the mean value of inhibition of EFS-evoked contractions expressed as a percentage reduction of the amplitude of contractions measured immediately before the addition of any drug to the organ bath (n = 5). Vertical lines indicate the s.e.m. SR 141716 or ethanol was added 20 minutes before the first addition of WIN 55,212-2.

4.3.7. Effect of SR 141716, AM 251 and O-2050 on ACh-evoked contractions of the rat and guinea-pig ileum MPLM

AM251 (10^{-5} M), SR 141716 (10^{-5} M) and O-2050 (10^{-5} M) used at the concentrations that produced a marked augmentation of the EFS-evoked twitch contractions of the rat and guinea-pig ileum MPLM (Section 4.3.1), had no effect on basal tension of this tissue nor did their presence alter the amplitude of the contractions elicited by cumulative additions of exogenously applied ACh (Figure 4.10).

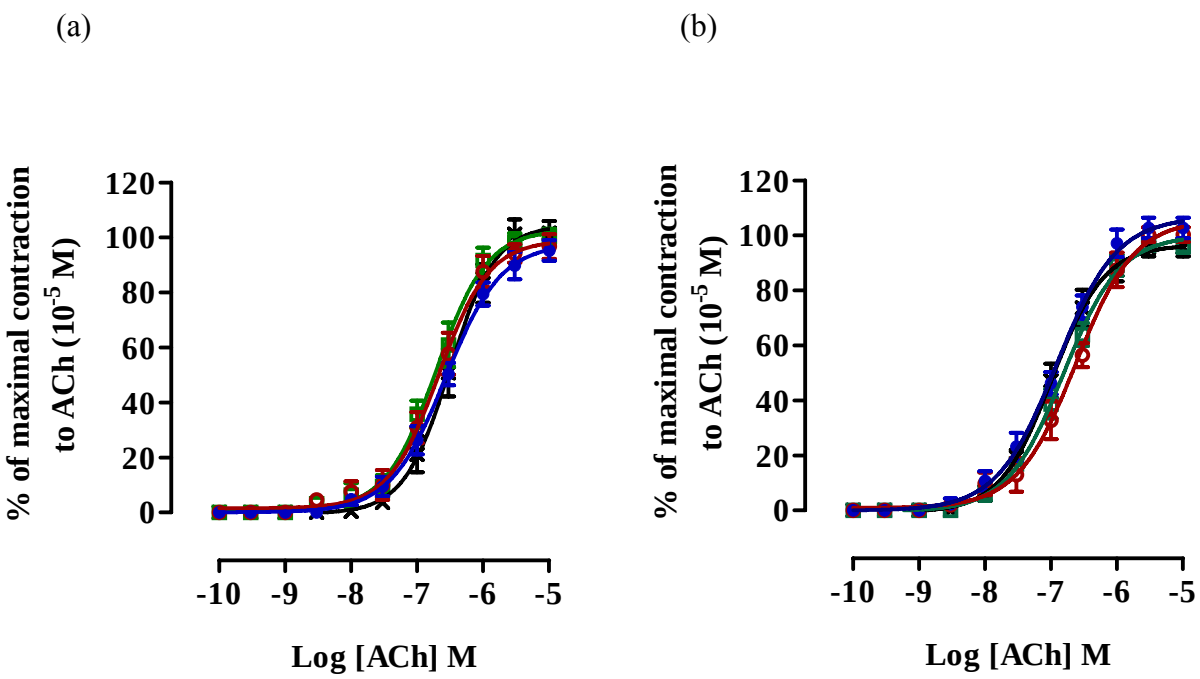


Figure 4.10: Concentration-response curves of exogenously applied ACh constructed in the presence of ethanol (**X**), AM251 (○, 10^{-5} M), SR 141716 (□, 10^{-5} M) or O-2050 (●, 10^{-5} M). Each curve was fitted by non-linear regression analysis. (a) Data for the rat ileum MPLM. (b) Data for the guinea-pig ileum MPLM. The amplitude of the contractions to cumulative additions of ACh in the presence of ethanol or a cannabinoid were expressed as a percentage of the maximal contraction to ACh obtained at 10^{-5} M from an initial ACh concentration-response curve constructed on each tissue. Ethanol or the cannabinoid was added 30 minutes before constructing the second ACh concentration-response curve. n = 6 for all curves. All values represent mean ± s.e.m.

4.3.8. Effect of AEA and WIN 55,212-2 on ACh-evoked contractions of the guinea-pig ileum MPLM

Both AEA (10^{-5} M; Section 4.3.5) and WIN 55,212-2 (10^{-4} M; Section 3.3.7) at the concentrations that produced a marked inhibition of the EFS-evoked twitch and rebound contractions of the guinea-pig ileum MPLM respectively, had no effect on basal tension of this tissue nor did their presence alter the amplitude of the contractions elicited by cumulative additions of exogenously applied ACh (Figure 4.11).

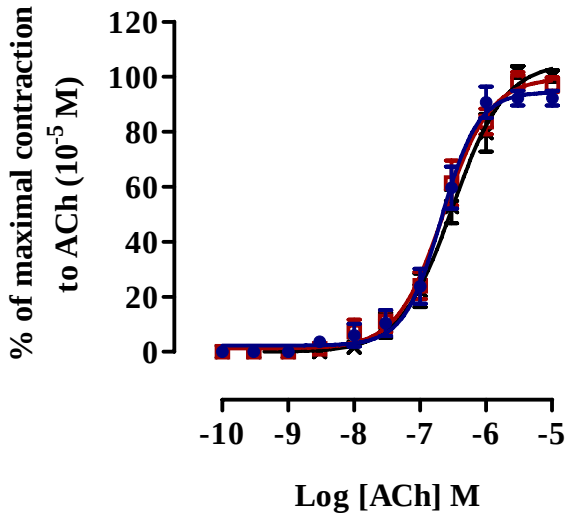


Figure 4.11: Concentration-response curves of exogenously applied ACh constructed in the presence of ethanol (**X**), AEA (□, 10^{-5} M) or WIN 55,212-2 (●, 10^{-4} M) on the guinea-pig ileum MPLM. Each curve was fitted by non-linear regression analysis. The amplitude of the contractions to cumulative additions of ACh in the presence of ethanol or a cannabinoid were expressed as a percentage of the maximal contraction to ACh obtained at 10^{-5} M from an initial ACh concentration-response curve constructed on each tissue. Ethanol or the cannabinoid was added 30 minutes before constructing the second ACh concentration-response curve. n = 6 for all curves. All values represent mean ± s.e.m.

4.4. Discussion

The purpose of this study was to investigate whether the mechanism by which SR 141716 increased the EFS-evoked twitch contractions of the rat and guinea-pig MPLM was through inverse agonism or the antagonism of an inhibitory effect of tonically released endocannabinoid agonists at the CB_1 receptor.

There are two main reasons for believing that the enhancement of the twitch contractions caused by SR 141716 was mediated through the CB_1 receptor and not another pharmacological target. Firstly, as was shown in the experiments with the rat MPLM in Chapter 3, the estimates of the pK_B value of SR 141716 obtained for the antagonism of the inhibitory effect of AEA, CP 55,940, Δ^9-THC and WIN 55,212-2 were both independent of the agonist that was used and similar to its pK_B value previously reported at the CB_1 receptor. Secondly, as was shown in the present study, the ability of SR 141716 to cause a concentration-dependent augmentation of the twitch contractions of both the rat and guinea-pig MPLM was shared by AM251 and O-2050 at concentrations which are known to act selectively through the CB_1 receptor.

The sensitivity of the EFS-evoked twitch contractions of both tissues to TTX and atropine but not hexamethonium, both before and after the augmentation of the twitch contractions by SR 141716, AM 251 or O-2050, together with the lack of effect of these cannabinoid receptor blockers on contractions elicited by exogenously applied ACh suggested that the enhancement of the twitch contractions was because of an increase in the release of ACh from the myenteric post-ganglionic nerve terminals and not a sensitisation of the post-junctional mACh receptor on the smooth muscle or an elevation of neurally released ACh through an inhibition of ACh esterase.

The observation that all three cannabinoid antagonists were more efficacious on the guinea-pig MPLM than on the rat MPLM could be ascribed to differences in the stimulus-response coupling capacity of the tissues, or the lower quanta of ACh output from the rat MPLM.

The data also demonstrated that the log concentration-response curves for all three antagonists had different E_{max} values with respect to each other, with AM 251 being the most efficacious followed by SR 141716 and O-2050. This observation provided strong evidence for a constitutive activity of the CB_1 receptor and possibly that these cannabinoid antagonists were behaving as inverse agonists in these tissues, because if the potentiation of contraction produced by each ligand was due to a displacement of endocannabinoid agonists from the CB_1 receptor, the maxima of all three concentration-response curves should have been equal, assuming that the amount of such displacement was equal.

Given that the concentrations of all three antagonists used were about 3 logarithmic units higher than their respective pK_B values at the CB_1 receptor, but within the selectivity range for the CB_1 receptor, it was unlikely that tonically released endocannabinoid agonists may have been capable of binding to a receptor that was already saturated with the antagonist.

It is noteworthy that O-2050 also potentiated the twitch contractions of both MPLM tissues at concentrations previously shown to be devoid of activity on the EFS-evoked contractions of the mouse vas deferens (Martin *et al*., 2002). Although the reason for this discrepancy is not clear, this finding could be ascribed to differences in the tissue, animal species, neurotransmitter released or the stimulus-response coupling mechanisms.

Previous studies have shown that differences in the pharmacological actions of a number cannabinoids have been known to occur in different bioassays. For example, AM 630, an analogue of WIN 55,212-2, has been shown to behave as a competitive antagonist in the mouse vas deferens (Pertwee *et al.,* 1995), an agonist in the guinea-pig MPLM (Pertwee *et al.,* 1996), but possess no cannabinoid activity in the mouse urinary bladder (Pertwee & Fernando, 1996). Moreover, in Chinese hamster ovarian cells transfected with the human CB_1 receptor, the same compound has been shown to behave as an inverse agonist (Landsman *et al.,* 1998).

The observation that the twitch contractions of the rat vas deferens were not altered by SR 141716, AM 251 or O-2050 at concentrations which would be expected to

completely occupy the CB_1 receptor, suggested that this the tissue either did not contain the CB_1 receptor, or that the receptor was not constitutively activite or was not subjected to tonic activation by endogenously released cannabinoid agonists. However, the presence of the CB_1 receptor was shown by the demonstration that the inhibitory effect of WIN 55,212-2 was antagonised by SR 141716 with a pA_2 value in line with its pK_B value at the CB_1 receptor.

It is known that the EFS of the rat vas deferens elicits a twitch contraction to single pulses and a biphasic contraction to trains of pulses. The twitch contraction and the first contraction of the biphasic response have been attributed to the release of ATP whilst the second contraction of the biphasic response is because of the release of NAdr (Ventura, 1998). In the present study, EFS-evoked twitch contractions of the rat vas deferens were attenuated by PPADS and TTX but not prazosin. This result suggested that the contractions of the vas deferens were caused an action of neurally released ATP on the P_2X purinoceptors. The failure of SR 141716, AM 251 and O-2050 to augment the EFS-evoked contractions of the rat vas deferens suggests that CB_1 receptor in this tissue was neither subjected to a tonic activation by endocannabinoid agonists or constitutively active. In comparison with the data from MPLM tissues, these data suggest that the expression of inverse agonist activity or the presence of an endocannabinoid tone was dependent on the tissue used and / or neurotransmitter released.

Data from studies on cell-based assays have indicated that the termination of endocannabinoid signalling is regulated by a two-step process, involving a translocation of released endocannabinoids from the extracellular space into cells via a putative uptake transporter, followed by enzymatic hydrolysis, predominantly by FAAH to form arachidonic acid and ethanolamine from AEA, or glycerol from 2-AG (Deutsch & Chin, 1993; Cravatt *et al*., 1996).

Given that high levels of a large number of endocannabinoids including AEA and 2-AG have been detected in both the rat and guinea-pig ileum (see references in the Introduction) and that their protection from inactivation by cellular uptake or enzymatic degradation by FAAH inhibits intestinal motility *in vivo* in mice (Capasso *et al*., 2005; Pinto *et al*., 2002), an attempt was made to unmask the presence of a

possible functional inhibitory endocannabinoid agonist tone in the MPLM by evaluating the effects of VDM-11, AA-5HT, PMSF and URB-597.

It was shown that cumulative additions of VDM-11 up to 10^{-5} M, a concentration which was almost equal to its EC_{50} determined in the rat RBL-2H3 basophilic leukaemia and C6 glioma cells (De Petrocellis *et al.,* 2000) did not attenuate the EFS-evoked contractions. Similarly, application of all three FAAH inhibitors by themselves up to concentrations which have been previously shown to completely inactivate FAAH and lack affinity for the CB_1 receptor *in vitro* (Bisogno *et al.,* 1998; Deutsch *et al.,* 1997; Kathuria *et al.,* 2003) also failed to attenuate the EFS-evoked contractions of either MPLM strips.

Whilst these results confirmed that VDM-11, AA-5HT, PMSF and URB-597 did not possess cannabinoid activity, they suggested that pharmacologically active endocannabinoids or substrates of the putative uptake transporter and FAAH were not released from the MPLM in response to EFS. These data supported the notion that the CB_1 receptor was likely to be constitutively active and that the three CB_1 antagonists behaved as inverse agonists.

To investigate whether the failure of VDM-11 and the three FAAH inhibitors to inhibit the EFS-evoked contractions may have been attributable to an absence of a functional endocannabinoid uptake transporter or FAAH in these tissues, or that the tissues were not incubated with the inhibitors for a sufficient period of time, the inhibitory effect of exogenously applied AEA in the absence and presence of these inhibitors was compared. The data from these experiments showed that the presence of any one of the three FAAH inhibitors, but not VDM-11, resulted in a significant increase in the potency of exogenously applied AEA to inhibit the EFS-evoked contractions of the MPLM from both species. These findings suggested that unlike the putative endocannabinoid uptake transporter, FAAH was likely to be both present and functional in the MPLM. Furthermore, because the concentration ratio for the potentiation of the inhibitory effect of AEA was reasonably similar in both the rat and guinea-pig ileum MPLM, it was likely that both tissues contained the same isoform of FAAH.

The lack of an effect of all three FAAH inhibitors on the potency of WIN 55,212-2 as an inhibitor of the EFS-evoked contractions of both tissues not only confirmed that this agonist was not a substrate of FAAH, but it added weight to the evidence that FAAH was present in the MPLM of both animal species and that the potentiation of the inhibitory effect of AEA by all three FAAH inhibitors was specific. Moreover, this observation also indicated that the FAAH inhibitors did not simply augment the sensitivity of the CB_1 receptor to AEA.

It has been recognised that unlike classical neurotransmitters such as ACh, endocannabinoid agonists including AEA and 2-AG are not stored in synaptic vesicles in nerves following synthesis, instead they are synthesised and released on demand in response to nerve stimuli (Di Marzo *et al*., 2004). Considering that the MPLM tissues in the present study were being stimulated, and therefore, that the release of these endocannabinoids was probably being induced, the levels of these endocannabinoids would be anticipated to be high. However, given that an inhibitory effect of the FAAH inhibitors alone was not observed, it appears that a functional endocannabinoid tone was absent in the MPLM, as suggested by the data with the three CB_1 receptor antagonists. Alternatively, it could be possible that the levels of the endocannabinoid agonists were not sufficient to maintain a tonic activation of the CB_1 receptor.

Previous investigators have shown that *in vivo* administration of PMSF (McVey *et al.,* 2003) or AA-5HT (Capasso *et al.,* 2005) produced a concentration related inhibition of small intestinal transit in rats and mice respectively. This effect was ascribed to a potentiation of the inhibitory action of tonically released endocannabinoid agonists at the CB_1 receptor, because high levels of AEA and 2-AG were detected in homogenates of biopsies of the small intestine. Although the effects of FAAH inhibitors have not been evaluated on intestinal transit of the guinea-pig *in vivo*, similar levels of AEA and 2-AG have been measured in the MPLM of the ileum of this species (Guagnini *et al.,* 2006). However, as suggested above, it is unlikely that these levels would be sufficient to maintain a tonic activation of the CB_1 receptor, and explain the potentiation of the EFS-evoked contractions by the CB_1 antagonists.

Whilst the effect of URB-597 has not been investigated on small intestinal transit of rodents *in vivo*, this FAAH inhibitor has been shown to selectively elevate the levels of AEA in the brain without affecting the levels of this endocannabinoid in the rat duodenum at concentrations that abolished the activity of FAAH (Fegley *et al.,* 2005). This finding indicates that the hydrolysis of AEA in the rat may occur via at least two enzymes, and that in this species other endocannabinoids may modulate small intestinal transit. Apart from FAAH, several enzymes including monoacylglycerol lipase and *N*-palmitoylethanolamine hydrolase have been implicated to be involved in inactivating endocannabinoids in the small intestine (van der Stelt & Di Marzo, 2004; Ueda *et al.,* 2000). However, given that FAAH is capable of hydrolysing a broad spectrum of established and putative endocannabinoids including 2-AG and palmitolyethanolamide, which are substrates of monoacylglycerol lipase and *N*-palmitoylethanolamine hydrolase respectively, it appears unlikely that these ligands were released in the rat and guinea-pig MPLM to inhibit the EFS-evoked contractions of these tissues.

In the present study, it was unlikely that the removal of the mucosa-submucosal plexus during the preparation of the MPLM resulted in the loss of the source of the endocannabinoid agonists, hence was responsible for the lack of an inhibitory effect of the FAAH inhibitors on the EFS-evoked contractions of the MPLM, because all three CB_1 receptor antagonists still were capable of augmenting the EFS-evoked contractions of the MPLM

While the data obtained suggests that SR 141716 augments intestinal transit via inverse agonism and not because of an antagonism of an endocannabinoid agonist tone, it may be possible that, by using an isolated preparation, the endocannabinoid agonist activity was not detected because the endocannabinoids may behave as hormones *in vivo*, in addition to their role as neurotransmitters in the brain. For instance, it has been demonstrated that the AEA can be synthesised by endothelial cells of the mesenteric vasculature (Randall *et al.,* 1996). Considering that these vessels are known to supply blood to the small intestine, it could be possible that *in vivo* endocannabinoid agonists are synthesised in the mesenteric vasculature but produce an inhibitory effect on the enteric transmission via a endocrine hormonal effect. However, while these speculations may be valid *in vivo*, they do not appear to

apply to the MPLM *in vitro*. Assuming that SR 141716 was an inverse agonist, because the CB_1 receptor was constitutively active in the MPLM, its ability to augment the EFS-evoked contractions may be explained using the theoretical "two state" receptor model (Leff, 1995). According to this model, the CB_1 receptor would exist in at least two interchangeable states, an active state, in which the receptor was coupled to the effector pathways to suppress the release of ACh, and an inactive sate, in which the receptor was not coupled to the effector pathways and thereby would increase the release of ACh. In the absence of any ligands, both conformations would exist at equilibrium; therefore, the CB_1 receptor would be inhibiting the release of ACh. However, as an inverse agonist, SR 141716 having preferential affinity for the inactive state would bind to the inactive state of the CB_1 receptor and shift the equilibrium to reduce the number of receptors in the active conformation, thereby reversing the suppression of the release of ACh.

In conclusion, this study showed that under the experimental conditions employed, the ability of SR 141716 to potentiate the EFS-evoked contraction of both the rat and guinea-pig ileum was attributed to an inverse agonist action at the CB_1 receptor and not because of an antagonism of tonically released endocannabinoid agonists.

CHAPTER 5:

ISOLATED NERVE-EPITHELIAL PREPARATION

Investigation into the Role of the Cannabinoid CB_1 Receptor In Modulating Pharmacologically Evoked Secretory Function of the Rat Colonic Submucosal Plexus-Epithelial Preparation.

5.1. Introduction

A number of studies (Izzo *et al*., 1999a, 1999b, 2000, 2003; Shook & Burks, 1989) have reported that *in vivo* administration of cannabinoid receptor agonists into rats and mice causes a dose-dependent suppression of basal and evoked small intestinal motility and secretion over the same range of doses (Izzo *et al*., 1999a, 1999b, 2000, 2003). Because cannabinoids are known to potently inhibit the peristaltic reflex (Heinemann *et al*., 2000; Mancinelli *et al.,* 2001; Izzo *et al.,* 2000) and the EFS-evoked contractions of both the longitudinal (Croci *et al.,* 1999; Guagnini *et al.,* 2006; Manara *et al.,* 1998; Pertwee *et al.,* 1996;) and circular (Hinds et al., 2006; Manara *et al.,* 1998) smooth muscle layer of segments of the both the small and large intestine of a number of mammals *in vitro*, it is possible that the anti-diarrhoeal action of cannabinoids observed *in vivo* is through the inhibition of intestinal propulsion, which results in a slower elimination of intestinal contents, and indirectly, to more efficient absorption of water and electrolytes due to the longer contact time with the mucosa. Alternatively, the possibility also exists that the cannabinoids themselves stimulate absorption of the luminal water and electrolytes.

To date, only two studies have investigated the effect of cannabinoids on intestinal secretion *in vitro* (MacNaughton *et al.,* 2004; Tyler *et al.,* 2000). These studies, performed on submucosal plexus-mucosal (SPM) preparations of the guinea-pig and rat ileum have shown that WIN 55,212-2 attenuated the EFS- and capsaicin- evoked secretory responses of these tissues through a CB_1 receptor-mediated reduction in the neuronal release of ACh and VIP respectively (MacNaughton *et al.,* 2004; Tyler *et al.,* 2000). Although these bioassays have provided good evidence for a specific anti-diarrhoeal action of cannabinoids, it is surprising that the effect of cannabinoids has not been investigated on secretory responses of the colonic tissues, considering that the colon is the region of the gut which plays an important role maintaining the homeostatic balance of water and electrolytes in the body (Rao, 2004).

Therefore, the aim of this study was to explore the viability of the rat colonic SPM preparation as an *in vitro* cannabinoid bioassay for colonic ion transport. A previous *in vivo* study (Izzo *et al.,* 1999a) has shown that while WIN 55,212-2 and CP 55,940 both equipotently decreased upper gastrointestinal transit, the later unlike the former,

did not modify castor-oil-induced diarrhoea. Therefore, this study examined the effects of both CP 55,940 and WIN 55,212-2 on the secretory responses of the rat isolated colonic epithelial sheet evoked by pharmacological stimulation of the nerves with capsaicin, nicotine and veratridine. This method of neural stimulation was chosen because the Ussing set-up employed was not customised for EFS studies.

5.2. Methods

SPM sheets were prepared from the colon dissected from rats (Section 2.1) by the method described in Section 2.3.1 and mounted in Ussing chambers as described in Section 2.3.2.

The experiments on the SPM preparation were carried out as described in Section 2.3.3.

The compounds used in this study were: α-chymotrypsin, atropine, capsaicin, CP 55,940, nicotine, SR 141716, SR 144528, SR 140333, TTX, WIN 55,212-2, veratridine, VIP 6-28. See Table 2.1 for the solvents and suppliers of the compounds used.

The data obtained from the experiments were quantified by the methods described in Section 2.3.4.

5.3. Results

5.3.1. The basal I_{SC} and R_t of the rat colonic SPM preparation

The SPM sheets exhibited a basal I_{SC} of 21.7 ± 2.4 μA.cm^{-2} (n = 45) and a R_t of 96.2 ± 15.4 Ω (n = 45) when voltage clamped at 0 mV. These parameters were not altered by the presence of atropine (10^{-6} M, 15 minutes, n = 6), α-chymotrypsin (4 units ml^{-1}, 30 minutes, n = 4), SR 140333 (10^{-6} M, 15 minutes, n = 6), TTX (10^{-6} M, 10 minutes, n = 6), VIP 6-28 (10^{-6} M, 15 minutes, n = 5) or the solvents of these drugs.

CP 55,940 (10^{-6} M, 30 minutes, n = 6), WIN 55,212-2 (10^{-6} M, 30 min, n = 6), SR 141716 (10^{-6} M, 30 min, n = 6) were also without effect. Figures 5.0 and 5.1 show the lack of effect of these drugs on the basal I_{SC} and R_t respectively.

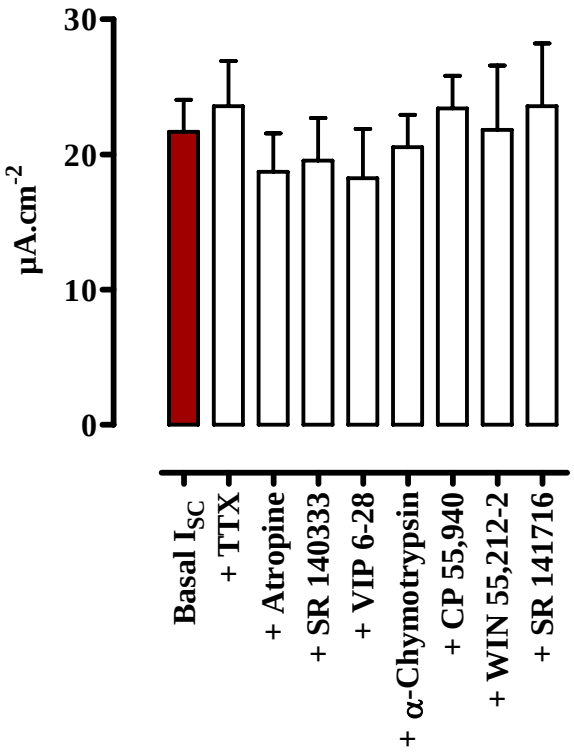


Figure 5.0: Bar graphs showing the lack of effect of a range of drugs on the basal I_{SC} of the rat colonic SPM preparation voltage clamped at 0 mV in Ussing chambers. Each bar represents the mean ± s.e.m. value of the basal I_{SC} in the absence (n = 45, closed column) and presence (open columns) of TTX (10^{-6} M, n = 6), atropine (10^{-6} M, n = 6), SR 140333 (10^{-6} M, n = 6), VIP 6-28 (10^{-6} M, n = 5), α-Chymotrypsin (4 units ml^{-1}, n = 4), CP 55,940 (10^{-6} M, n = 6), WIN 55,212-2 (10^{-6} M, n = 6) or SR 141716 (10^{-6} M, n = 6).

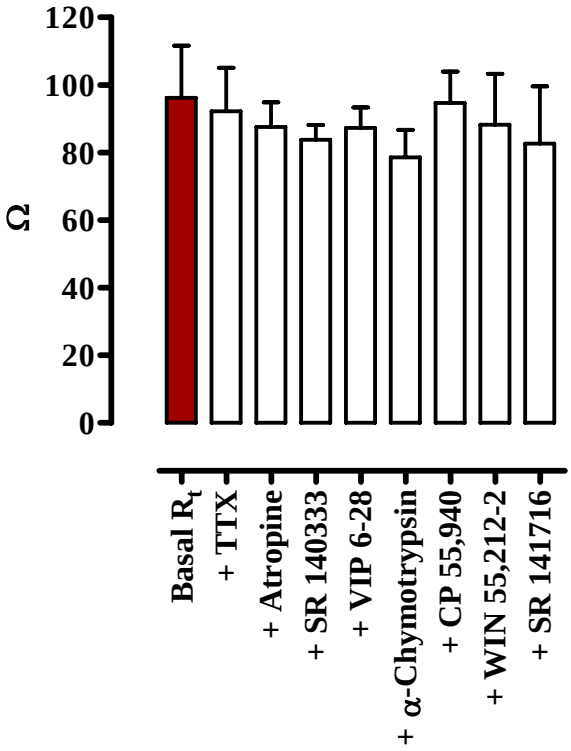


Figure 5.1: Bar graphs showing the lack of effect of a range of drugs on the basal R_t of the rat SPM preparation calculated using Ohm's law from the change in basal I_{SC} in response to an intermittent change in voltage clamped from 0 mV to 2 mV. Each bar represents the mean ± s.e.m. value of the basal R_t in the absence (n = 45, closed column) and presence (open columns) of TTX (10^{-6} M, n = 6), atropine (10^{-6} M, n = 6), SR 140333 (10^{-6} M, n = 6), VIP 6-28 (10^{-6} M, n = 5), α-Chymotrypsin (4 units ml^{-1}, n = 4), CP 55,940 (10^{-6} M, n = 6), WIN 55,212-2 (10^{-6} M, n = 6) or SR 141716 (10^{-6} M, n = 6).

5.3.2. Effect of CP 55,940 and WIN 55,212-2 on the capsaicin-evoked change in I_{SC} and R_t of the rat colonic SPM preparation in the absence and presence of SR 141716.

Capsaicin (10^{-6} M) evoked a significant ($P < 0.05$, unpaired *t*-test) transient increase in I_{SC} of 19.6 ± 2.7 μA.cm^{-2} (n = 15, Figures 5.2 and 5.3). The evoked I_{SC} peaked within a minute of application of capsaicin and returned to the original basal value within 3 minutes. As shown in Figure 5.2, the presence of capsaicin had no effect on the R_t of the preparation.

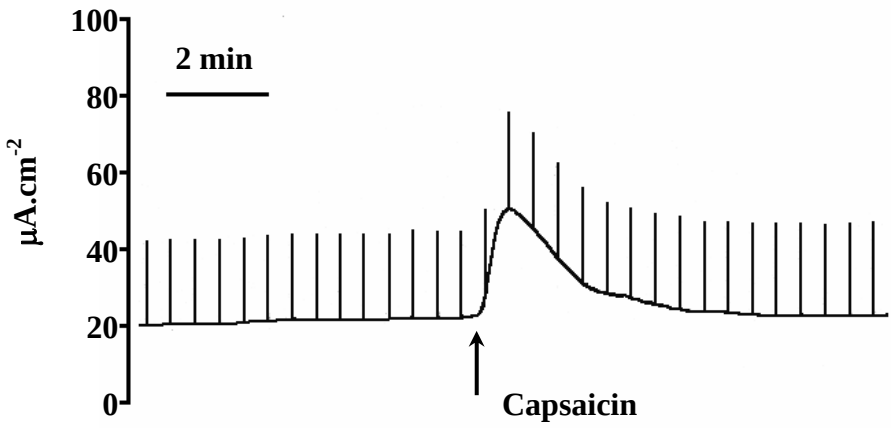


Figure 5.2: Trace illustrating the capsaicin- (10^{-6} M) evoked increase in the I_{SC} of the rat SPM preparation observed as a transient upward deflection from a basal I_{SC} of 20 μA.cm^{-2}. The small spikes represent the change in I_{SC} in response to the automatic change in the holding potential from 0 mV to 2 mV by the voltage clamp apparatus, intermittently for 2 seconds every 30 seconds. The R_t was calculated using Ohm's Law i.e. R_t = 2 mV / Δ I_{sc}.

As shown in Figure 5.3, the capsaicin-evoked I_{SC} was abolished ($P < 0.05$, unpaired *t*-test) by the presence of TTX (10^{-6} M, 10 minutes, n = 3) or SR 140333 (10^{-6} M, 15 minutes, n = 6) but not atropine (10^{-6} M, 15 minutes, n = 6).

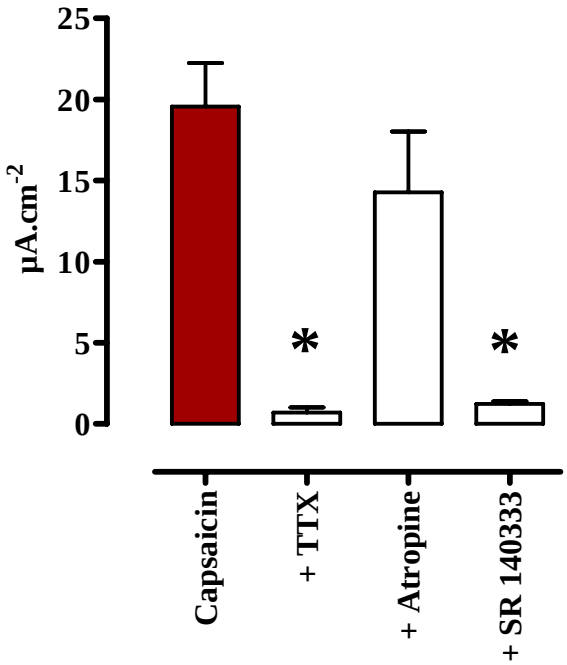


Figure 5.3: Bar graph representing the amplitude of the capsaicin- (10^{-6} M) evoked I_{SC} by the rat SPM preparation in the absence (closed column, n = 15) and presence (open column) of TTX (10^{-6} M, n = 3), SR 140333 (10^{-6} M, n = 6) or atropine (10^{-6} M, n = 6). * indicates a statistical difference ($P < 0.05$, unpaired *t*-test) compared to the capsaicin column.

Pre-treatment for 30 minutes with either CP 55,940 (10^{-6} M, n = 12, Figure 5.4) or WIN 55,212-2 (10^{-6} M, n = 6, Figure 5.5) caused a significant ($P < 0.05$, unpaired *t*-test) 72.18 % and 71.17 % inhibition of the capsaicin evoked I_{SC} respectively.

The inhibitory effects of both CP 55,940 and WIN 55,212-2 were completely ($P < 0.05$, unpaired *t*-test) antagonised by a 30 minute pre-exposure to SR 141716 (10^{-6} M, n = 6 against each agonist). At this concentration, SR 141716 alone (n = 6 for both Figure 5.4 and 5.5, 6 minutes) had no effect on the capsaicin response.

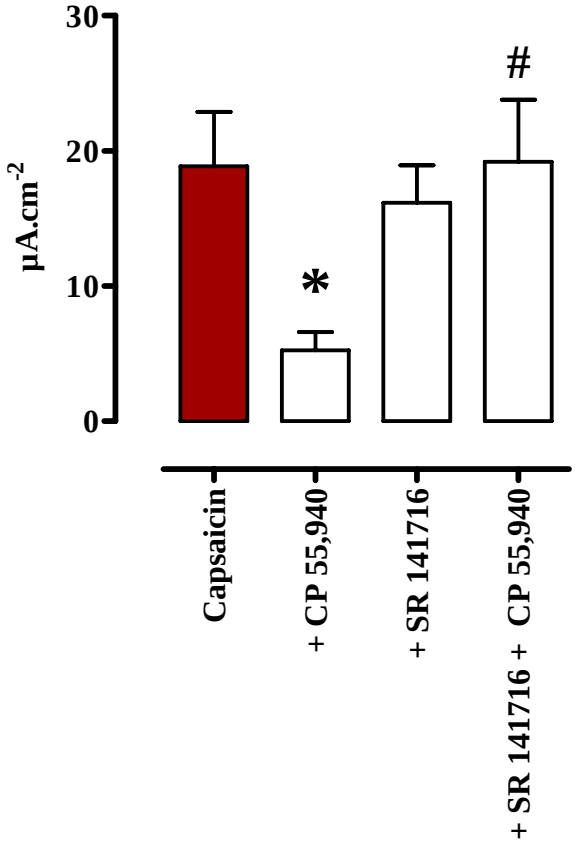


Figure 5.4: Bar graph representing the amplitude of the capsaicin- (10^{-6} M) evoked I_{SC} by the rat SPM preparation in the presence of ethanol (closed column, n = 26), CP 55,940 (10^{-6} M, n = 12) or SR 141716 (10^{-6} M, n = 6), or both CP 55,940 (10^{-6} M) and SR 141716 (10^{-6} M, n = 6). * indicates a significant difference (P < 0.05) compared to the capsaicin control response. # indicates a significant ($P < 0.05$, unpaired *t*-test) difference compared to the capsaicin response in the presence of CP 55,940.

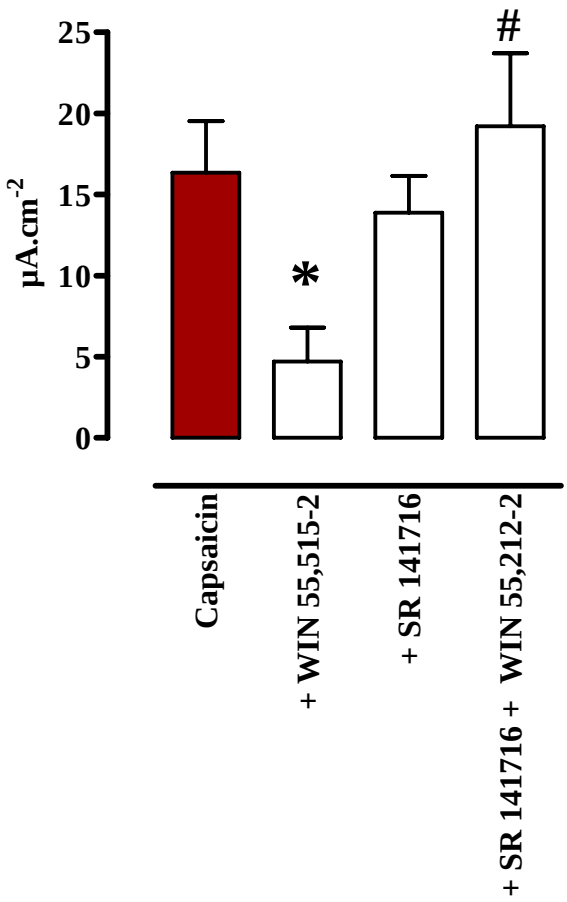


Figure 5.5: Bar graph representing the amplitude of the capsaicin- (10^{-6} M) evoked I_{SC} by the rat SPM preparation in the presence of ethanol (closed column, n = 20), WIN 55,212-2 (10^{-6} M, n = 6) or SR 141716 (10^{-6} M, n = 6), or both WIN 55,212-2 (10^{-6} M) and SR 141716 (10^{-6} M, n = 8). * indicates a significant difference ($P < 0.05$, unpaired *t*-test) compared to the capsaicin control response. # indicates a significant difference ($P < 0.05$, unpaired *t*-test) compared to the capsaicin response in the presence of WIN 55,212-2.

5.3.3. Effect of CP 55,940 and WIN 55,212-2 on the nicotine-evoked I_{SC} and R_t of the rat colonic SPM preparation in the absence and presence of SR 141716.

Application of nicotine (10^{-4} M) to the SPM preparation resulted in a significant ($P < 0.05$, unpaired *t*-test) transient increase in I_{SC} of 56.6 ± 6.4 μA.cm^{-2} (n = 13, Figure 5.6 and 5.7). The evoked I_{SC} peaked within a minute of application of nicotine and returned to the original basal value within 5 minutes. Figure 5.5 also shows that nicotine had no effect on the basal R_t of the tissues.

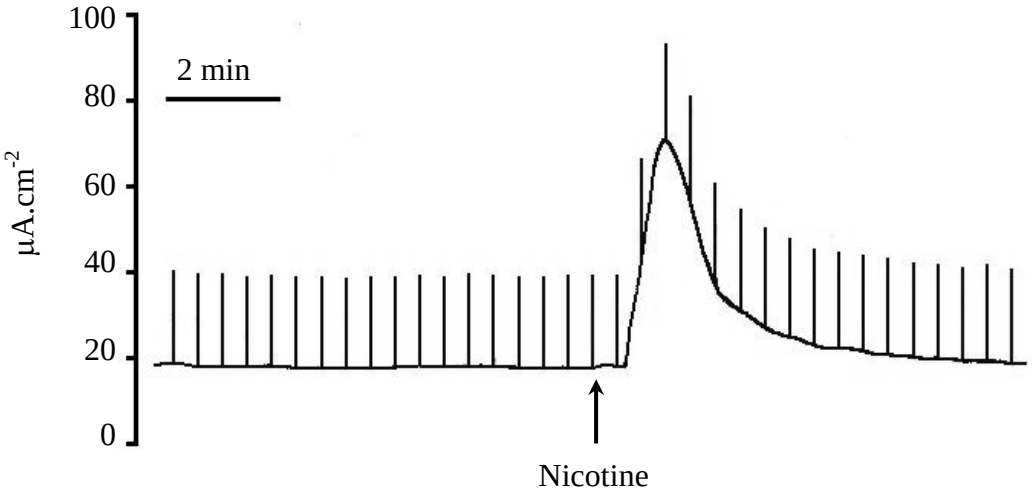


Figure 5.6: Trace illustrating the nicotine (10^{-4} M) evoked increase in the I_{SC} of the rat SPM preparation observed as a transient upward deflection from a basal I_{SC} of 18.8 μA.cm^{-2}.

As shown in Figure 5.7, the nicotine evoked I_{SC} was abolished ($P < 0.05$, unpaired *t*-test) by the presence of TTX (10^{-6} M, 10 minutes, n = 3) or SR 140333 (10^{-6} M, 15 minutes, n = 6) but not atropine (10^{-6} M, 15 minutes, n = 6).

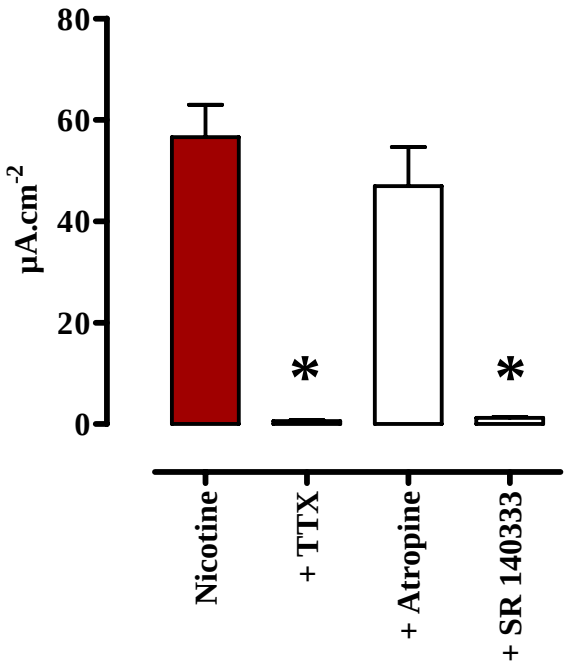


Figure 5.7: Bar graph representing the amplitude of the nicotine- (10^{-4} M) evoked change in I_{SC} by the rat SPM preparation in the absence (closed column, n = 15) and presence (open column) of TTX (10^{-6} M, n = 3), SR 140333 (10^{-6} M, n = 6) or atropine (10^{-6} M, n = 6). * indicates a statistical difference ($P < 0.05$, unpaired *t*-test) compared to the nicotine column.

Pre-treatment for 30 minutes with either CP 55,940 (10^{-6} M, n = 12, Figure 5.8) or WIN 55,212-2 (10^{-6} M, n = 6, Figure 5.9) caused a significant ($P < 0.05$, unpaired *t*-test) 72.2 % and 71.2 % inhibition of the capsaicin evoked I_{SC} respectively.

The inhibitory effect of both CP 55,940 and WIN 55,212-2 was completely ($P < 0.05$, unpaired *t*-test) antagonised by a 30 minute pre-exposure to SR 141716 (10^{-6} M, n = 6 against each agonist). At this concentration, SR 141716 alone (n = 6 for both Figure 5.8 and 5.9, 6 minutes) had no effect on the nicotine response.

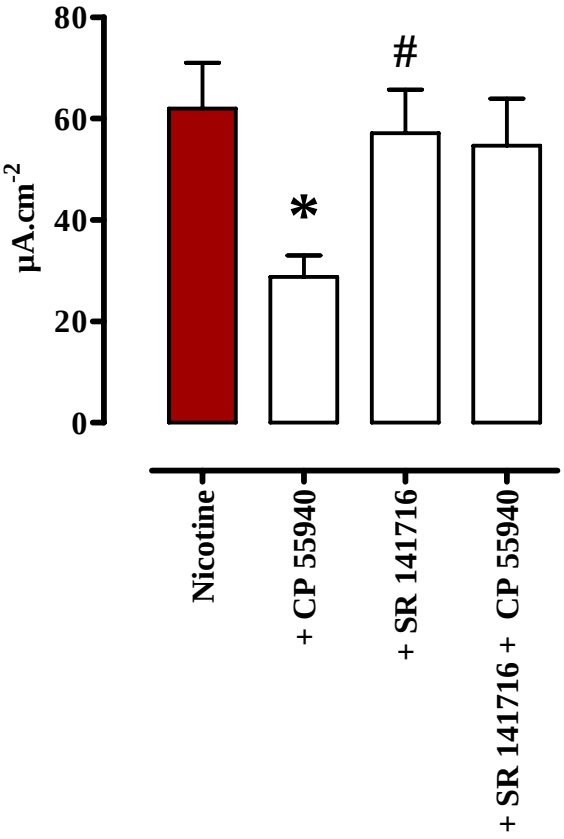


Figure 5.8: Bar graph representing the amplitude of the nicotine- (10^{-4} M) evoked I_{SC} by the rat SPM preparation in the presence of ethanol (closed column, n = 21), CP 55,940 (10^{-6} M, n = 7) or SR 141716 (10^{-6} M, n = 7), or both CP 55,940 (10^{-6} M) and SR 141716 (10^{-6} M, n = 7). * indicates a significant difference ($P < 0.05$, unpaired *t*-test) compared to the nicotine control response. # indicates a significant difference ($P < 0.05$, unpaired *t*-test) compared to the nicotine response in the presence of CP 55,940.

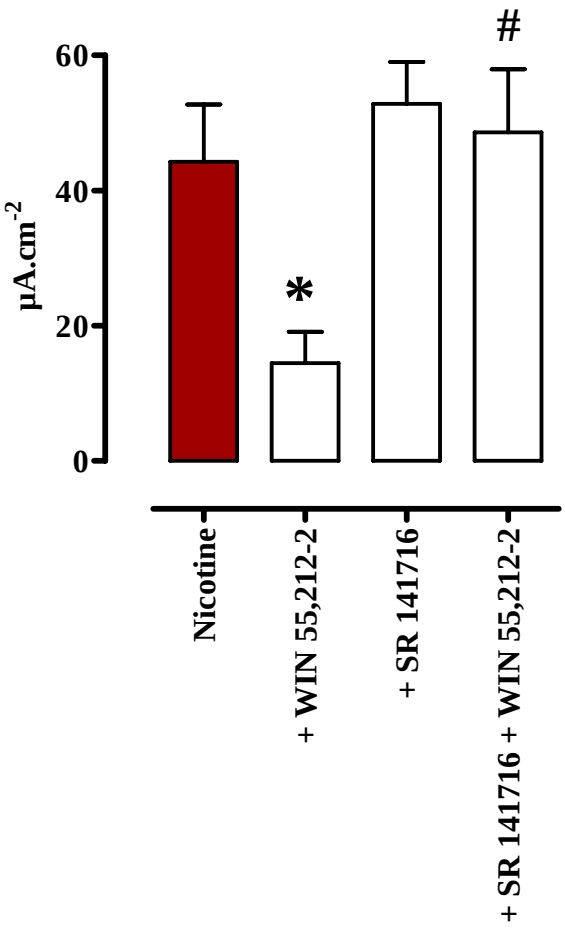


Figure 5.9: Bar graph representing the amplitude of the nicotine- (10^{-4} M) evoked change in I_{SC} by the rat SPM preparation in the presence of ethanol (closed column, n = 19), WIN 55,212-2 (10^{-6} M, n = 6) or SR 141716 (10^{-6} M, n = 6), or both WIN 55,212-2 (10^{-6} M) and SR 141716 (10^{-6} M, n = 7). * indicates a significant difference ($P < 0.05$, unpaired *t*-test) compared to the nicotine control response. # indicates a significant difference ($P < 0.05$, unpaired *t*-test) compared to the nicotine response in the presence of WIN 55,212-2.

5.3.4. Effect of CP 55,940 and WIN 55,212-2 on the veratridine-evoked change in I_{SC} and R_t of the rat colonic SPM preparation in the absence and presence of SR 141716.

Veratridine (3 x 10^{-5} M) evoked a sustained increase in I_{SC} of 52.83 ± 6.54 μA.cm^{-2} (n = 26) above the basal I_{SC}, without altering the R_t (Figure 5.10 and 5.11). The evoked I_{SC} peaked within 3 minutes of application of this stimulant and the response could remain sustained for up to 1 hour before gradually returning to the basal value within another hour.

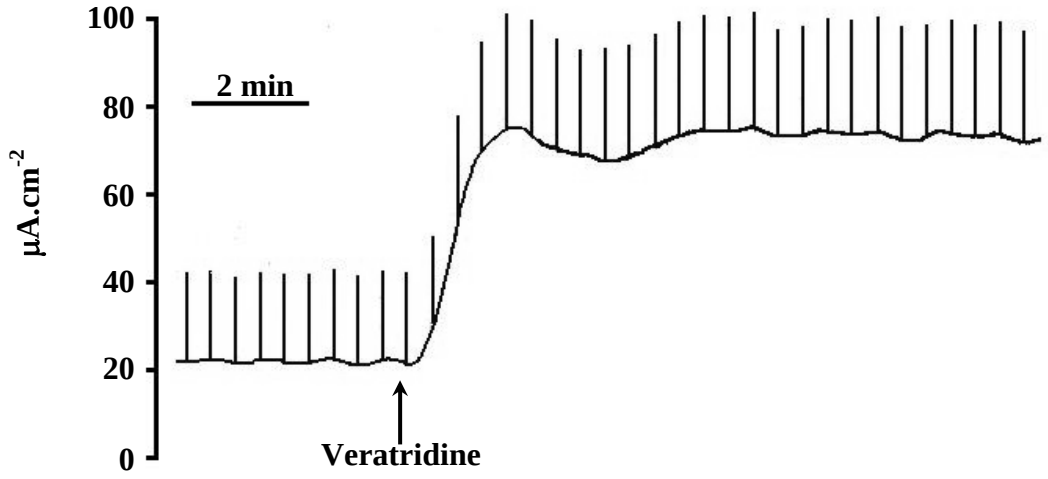


Figure 5.10: Trace illustrating the veratridine- (3 x 10^{-5} M) evoked increase in the I_{SC} of the rat SPM preparation.

As shown in Figure 5.11, the veratridine-evoked response was abolished by treatment with either TTX (10^{-6} M, 10 minutes, n = 3) or α-chymotrypsin (4 units ml^{-1}, 20 minutes, n = 6) but not atropine (10^{-6} M, 15 minutes, n = 6), SR 140333 (10^{-6} M, 15 minutes, n = 6) or VIP 6-28 (10^{-6} M, 15 minutes, n = 5).

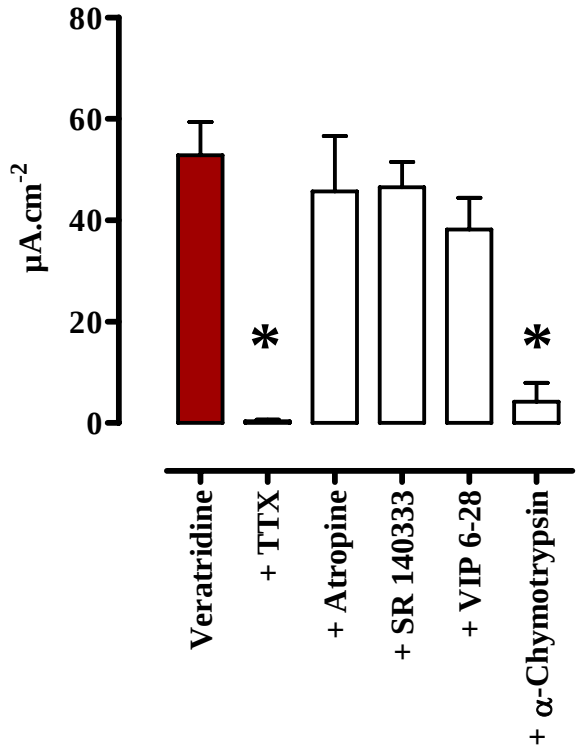


Figure 5.11: Bar graph representing the amplitude of the veratridine- (3 x 10^{-5} M) evoked increase in I_{SC} by the rat SPM preparation in the absence (closed column, n = 26) and presence (open column) of TTX (10^{-6} M, n = 3), atropine (10^{-6} M, n = 6), SR 140333 (10^{-6} M, n = 6), VIP 6-28 (10^{-6} M, n = 5) or α-chymotrypsin (4 units ml^{-1}, n = 6). * indicates a statistical difference ($P < 0.05$, unpaired *t*-test) compared to the veratridine column.

Pre-treatment of the tissues for 30 minutes with either CP 55,940 (10^{-6} M, n = 8) or WIN 55,212-2 (10^{-6} M, n = 6) had no significant ($P > 0.05$, unpaired *t*-test) effect on the veratridine- (3 x 10-5 M, n = 12) evoked response (Figure 5.12).

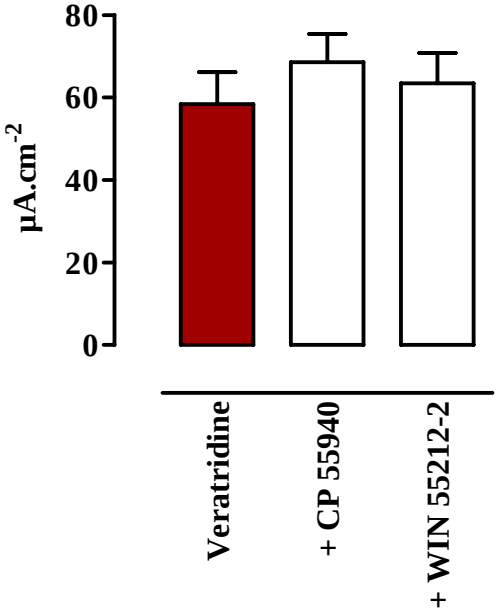


Figure 5.12: Bar graph representing the amplitude of the veratridine (3 x 10^{-5} M) evoked increase in I_{SC} by the rat SPM preparation in the presence of ethanol (closed column, n = 12), CP 55,940 (10^{-6} M, n = 8) or WIN 55,212-2 (10^{-6} M, n = 6).

5.4. Discussion

In the present study, it was demonstrated that basolateral application of capsaicin or nicotine evoked a transient secretory response in the rat colonic SPM preparation as measured as an increase in the I_{SC}. Because the secretory responses to both stimulants were abolished by TTX or SR 140333 but not atropine, it was likely that both capsaicin and nicotine stimulated the same population of secretomotor neurones of the submucosal plexus to release substance P or neurokinin A, but not ACh. Indeed, activation of the TRPV1 or nACh receptor by capsaicin or nicotine, respectively, has been shown to elicit similar transient increases in I_{SC} in other intestinal tissues such as rat distal colon (Cox *et al.*, 1993; Yarrow *et al.*, 1988) guinea-pig ileum (MacNaughton *et al.*, 2003) and mouse jejunum (Sheldon *et al.*, 1989).

The finding that the veratridine-evoked sustained secretory response was only blocked by TTX and α-chymotrypsin, but not atropine, SR 140333 or VIP 6-28 indicated that this stimulant activated the submucosal plexus to induce the release of a neuropeptide that was distinct from substance P and VIP. To date, the secretory neurotransmitter released by veratridine in intestinal tissues has proven difficult to identify primarily because this stimulant is known to cause a sustained stimulation of neurones through persistent activation of the voltage gate Na^+ channels which are involved in the generation and propagation of action potentials.

It was shown that the presence of either CP 55,940 or WIN 55,212-2 at concentrations which act selectively through the CB_1 receptor in intestinal tissues caused a significant attenuation of the I_{SC} evoked by capsaicin and nicotine. These results support and extend earlier findings for the inhibitory effect of WIN 55,212-2 on the EFS-evoked secretory response of the rat ileum (Tyler *et al.*, 2000) and the capsaicin-stimulated secretory response of the guinea-pig ileum (MacNaughton *et al.*, 2003). The presence of SR 141716, at a concentration which would be predicted to occupy all of the CB_1 receptor population, blocked the inhibitory effects of both CP 55,940 and WIN 55,212-2 on the capsaicin- or nicotine- evoked secretory responses. This result suggested that both cannabinoid receptor agonists inhibited the secretory response to capsaicin and nicotine through a CB_1 receptor-mediated inhibition of the

release of substance P and/or neurokinin A. Indeed previous studies have shown that the CB_1 receptor and TRPV1 receptor are colocalised in the submucosal plexus of at least the guinea-pig ileum (MacNaughton *et al.,* 2003). Furthermore, similar presynaptic CB_1 receptor activation has been shown to inhibit the release of substance P from the airways (Nemeth *et al.,* 2003; de Vries *et al.,* 2001), dorsal root ganglion neurons (Tognetto *et al.,* 2001) and spinal cord (Lever & Malcangio, 2002).

Although CP 55,940 and WIN 55,212-2 have been shown to demonstrate affinity for the CB_2 receptor, it was unlikely that this receptor was involved in modulating the secretory responses to capsaicin and nicotine in the rat SPM sheet because, SR 141716 completely antagonised the inhibitory effect of the cannabinoid agonists, and data from *in vivo* studies have shown that the administration of the CB_2 receptor antagonist SR 144528 does not attenuate the inhibitory effect of these agonists (Izzo *et al.,* 1999a, 1999b, 2000, 2003).

It is well known that the CB_1 receptor is coupled through $G_{i/o}$ proteins, positively to A-type and inwardly rectifying K^+ channels and negatively to Na^+ and N- and P/Q-type Ca^{2+} channels (Caufield and Brown, 1992; Mackie *et al.,* 1993; Howlett *et al.,* 2002). Thus, in the present study, the inhibition of the capsaicin- and nicotine- evoked secretory responses of the colonic SPM preparations via of a reduction in the release of substance P and/or neurokinin A could be attributed to the suppression of the excitability of the neurones of the submucosal plexus via the modulation of these ion channels upon activation of the CB_1 receptor.

The lack of effect of CP 55,940 and WIN 55,212-2 on the veratridine- evoked secretory response suggested that the CB_1 receptor was absent on the neurones activated by veratridine. These data suggest that the cannabinoid agonist were not inhibiting the Na^+ channel directly. It was demonstrated that the SPM preparation displayed a resting basal I_{SC} in the absence of exogenously applied drugs. It is well known that the basal I_{SC} is due to the leakage of ions from the tight junctions between the epithelial cells (Baird, 1994). Thus, the finding that the treatment with TTX, atropine, SR 140333, VIP-628, CP 55,940, WIN 55,212-2 or SR 141716 failed to alter the basal I_{SC} of the SPM preparation suggested that these compounds were not directly acting post-synaptically on the epithelial cells. The observation that both CP

55,940 and WIN 55,212-2 failed to attenuate the secretory response to veratridine supported the lack of a non-specific effects of these cannabinoids. Furthermore, the lack of effect of these cannabinoids on the R_t added weight to the notion that the effects of the cannabinoids were not non-specific.

In addition to its antagonist activity, SR 141716 alone has been shown to increase intestinal fluid secretion when administered into rodents (Izzo, *et al.*, 1999a, 199b, 2000, 2003). Furthermore, diarrhoea is among the adverse effects associated with the intake of SR 141716 for the management of body weight by obese patients (Van Gaal *et al.*, 2005). Two common explanations that have been offered to explain such antagonist-mediated changes in basal or evoked responses have been a possible: inverse agonist activity of SR 141716, or a confounding presence of an endocannabinoid tone. In the present study it was demonstrated that SR 141716 alone failed to alter the basal I_{SC} and the I_{SC} evoked by capsaicin and nicotine. Thus, it was likely that in the rat colonic SPM preparation either endocannabinoid agonists were not released tonically or that SR 141716 appeared to behave as a neutral antagonist because the CB_1 receptor was not constitutively active. Although this finding was in agreement with studies on SPM sheets of the rat (Tyler *et al.*, 2000) and guinea-pig (MacNaughton *et al.*, 2003) ileum, endocannabinoid agonists such as AEA and 2-AG have been detected in the rat colonic submucosa. Moreover, the levels measured have been suggested to be sufficient to cause the activation of the CB_1 receptor (D'Argenio *et al.*, 2006). The failure of SR 141716 to augment the secretory response to capsaicin and nicotine suggest that the endocannabinoid agonists levels were either insufficient to activate the CB_1 receptor because of their lower rate of synthesis or rapid hydrolysis.

In conclusion, the results from the present study extend the role of the CB_1 receptor in selectively modulating neurally-evoked secretory responses of the rat colon. Additionally, the results indicate that the anti-diarrhoeal action of cannabinoids observed *in vivo* may not be attributed exclusively to the blockade of intestinal propulsion, which may result in slower elimination of the intestinal contents and, indirectly, to a more efficient absorption of water and electrolytes due to the longer contact time with the mucosa.

CHAPTER 6:
GENERAL DISCUSSION

6.1. General discussion

The experimental work described in this thesis was directed at addressing two primary aims. Firstly, to explore the viability of the electrically-evoked contractions of the rat isolated ileum MPLM preparation and of the pharmacologically evoked secretory responses of the isolated colonic SPM preparation as novel *in vitro* intestinal nerve smooth muscle and epithelial cannabinoid bioassays respectively. Secondly, to characterise the cannabinoid receptors mediating the effects produced in these preparations.

Data obtained in Chapter 3 provided evidence for the suitability of the rat isolated ileum MPLM preparation as a cannabinoid bioassay. In this tissue, representatives of the four established classes of cannabinoid receptor agonists caused a concentration-dependent inhibition of both the EFS-evoked twitch and rebound contractions in a pre-synaptic and stereo-specific manner. The inhibition of both types of contractions was attributed to an inhibition of the excitation of the smooth muscle indirectly by a reduction in the release of ACh from post-ganglionic neurones of the myenteric plexus.

That the cannabinoid agonists inhibited the twitch and rebound contractions in the nanomolar and micromolar concentration ranges, respectively, and that the rank of order of the pEC_{50} values of the agonists did not correlate between the two EFS conditions suggested that the inhibition of both types of the contractions was not mediated through a common receptor. This interpretation was supported by the demonstration that SR 141716 antagonised the inhibition of the twitch contractions caused by each agonist with pK_B values consistent with its antagonist activity at the CB_1 receptor, whereas it only blocked the inhibition of the rebound contractions produced by AEA, Δ^9-THC and WIN 55,212-2, but not CP 55,940, with pA_2 values lower than its reported antagonistic activity at both the CB_1 and CB_2 receptors. Using similar EFS conditions, and WIN 55,212-2 as the cannabinoid receptor agonist, these findings were shown to extend to the guinea-pig ileum MPLM preparation. In this tissue, the pEC_{50} values of WIN 55,212-2 and the pA_2 values of SR 141716 were in line with those obtained in the rat ileum MPLM under comparable EFS conditions.

Because the inhibition of both types of contractions of the rat ileum MPLM by each agonist was not antagonised by SR 144528 and neither was the inhibitory effect of AEA blocked by capsazepine, strong evidence was obtained for the lack of a functional role of the CB_2 and $TRPV_1$ receptors in modulating the EFS-evoked contractions of this tissue. Taken together, these data suggested that the inhibition of the twitch contractions of both the rat and guinea-pig ileum MPLM were mediated by activation of the pre-synaptically located CB_1 receptor whereas the inhibition of the rebound contractions, of at least the former tissue, was mediated through the activation of two pre-synaptically located putative non-CB_1-non-CB_2-non-$TRPV_1$ receptors.

Data obtained in Chapter 3 also showed that in addition to its antagonist activity SR 141716 alone caused an augmentation of the twitch contractions of both the rat and guinea-pig ileum MPLM, but an attenuation of the rebound contractions of only the former tissue. The ability of SR 141716 to enhance the amplitude of the twitch contractions was suggestive of an increase in the release of ACh by either an antagonism of the inhibitory effect of tonically released endocannabinoid agonists or inverse agonism at the CB_1 receptor. The inhibition of the rebound contractions and antagonist activity SR 141716 was attributed to an uncharacterised partial agonist activity at the putative SR 141716 sensitive non-CB_1-non-CB_2-non-$TRPV_1$ receptor.

The presence of a functional CB_1 receptor in the rat ileum MPLM was consistent with previous immunohistochemical and *in vivo* data showing the presence of the CB_1 receptor protein in this tissue and its functional role in delaying small intestinal transit in the rat *in vivo* respectively. In contrast, from the data available, neither of the putative non-CB_1-non-CB_2-non-$TRPV_1$ receptors appeared to fit the pharmacological profile of the novel pharmacological targets for cannabinoids summarised in Chapter 1 (Section 1.5). For instance, as mentioned above, the refractoriness of the AEA induced inhibition of the rebound contractions to antagonism by capsazepine demonstrated the lack of involvement of the $TRPV_1$ receptor. The inability of AEA to stimulate the $TRPV_1$ receptor could be ascribed to the poor affinity and lack of efficacy of AEA at this receptor in the MPLM.

The inhibition of the rebound contractions caused by each of the four cannabinoids was unlikely to be mediated by the activation of the putative CB_2-like receptor for two reasons. Firstly, because this putative receptor has been characterised to be only stimulated by palmitoylelthanolamide, an eicosanoid structurally related to AEA, and not by any of the four cannabinoids used in the present study (Pertwee, 2004; 2005). Secondly, although the putative CB_2-like receptor has been suggested to be weakly antagonised by SR 144528 (Calignano *et al*., 2001; Berdyshev *et al*., 1998; Pertwee, 2005), the inability of this antagonist to block the inhibitory effect each of the four cannabinoid agonists on the MPLM suggested the absence of this receptor in this tissue. An investigation into the effect of palmitoylelthanolamide on EFS-evoked contractions of the MPLM could be examined to determine whether the putative CB_2-like receptor is present in the MPLM and if it can modulate EFS-evoked contractions of this tissue.

The putative SR 141716-sensitive, non-CB_1-non-CB_2 receptor which has been reported to mediate the vasodilator actions of AEA, methanandamide, cannabidiol and its analogues is known not to be activated by CP 55,940, WIN 55,212-2 and Δ^9-THC. Therefore, it is unlikely that the putative cannabinoid receptors identified in the MPLM are identical to the putative cannabinoid receptor found in vascular tissues. O-1918, a cannabidiol analogue, has been reported to behave as a selective antagonist of the putative vascular cannabinoid receptor (Begg *et al.,* 2005). An investigation into the inhibitory effects of the four cannabinoid receptor agonists in the presence O-1918 could be examined to determine whether the putative cannabinoid receptors in the MPLM are similar to or different from the putative cannabinoid receptor in the vasculature.

The putative cannabinoid receptors in the MPLM do not appear to be similar to the putative AEA and WIN 55,212-2 receptor described in the brain by both Di Marzo *et al*. (2000) and Breivogel *et al*. (2001). This is because the putative cannabinoid receptors in the MPLM were activated not only by AEA and WIN 55,212-2 but also by Δ^9-THC and CP 55,940.

Taken together, the identification of the two putative non-CB_1-non-CB_2-non-$TRPV_1$ receptors in the MPLM adds to the list of novel pharmacological targets for cannabinoids. Whether the putative non-CB_1-non-CB_2-non-$TRPV_1$ receptors identified in the MPLM serves a physiological role in modulating intestinal motility *in vivo* remains to be established.

Experiments described in Chapter 4 were an attempt to elucidate whether the mechanism by which SR 141716 augmented the twitch contractions of the rat and guinea-pig ileum MPLM was caused by an antagonism of an endocannabinoid agonist tone or inverse agonism at the CB_1 receptor. The evidence collected showed that an endocannabinoid tone was absent in these tissues and that SR 141716 behaved as an inverse agonist. The absence of a tonic inhibitory effect of endocannabinoid agonists was demonstrated by the lack of an attenuation of the twitch contractions in response to administration of inhibitors of FAAH, i.e. AA-5HT, PMSF and URB-597, and VDM-11, an inhibitor of the putative endocannabinoid uptake transporter protein. If endocannabinoid agonists were tonically released by EFS, the inhibitors of FAAH or the putative uptake transporter should have inhibited the twitch contractions by limiting the hydrolysis, the putative uptake of tonically released endocannabinoid agonists, or both. Because the presence of AA-5HT, PMSF or URB-597 selectively enhanced the inhibitory effect of exogenously applied AEA but not WIN 55,212-2 by a similar degree in both tissues, it was likely that both tissues contained FAAH. The lack of effect of VDM-11 on the potency of exogenously applied AEA suggested that the putative uptake transporter was absent in both tissues, that the uptake of AEA by the myenteric plexus was most likely by passive diffusion and that SR 141716 was an inverse agonist and not a simple competitive antagonist at the CB_1 receptor.

Convincing evidence for the inverse agonist activity of SR 141716 was obtained from a comparison between the augmentation of the twitch contractions produced by this cannabinoid and that produced by two other CB_1 receptor antagonists i.e. AM 251 and O-2050. These experiments showed that the maximal enhancement produced by SR 141716 was lower than that produced by AM 251 but greater than that by O-2050. These data suggested that SR 141716 enhanced the EFS-evoked twitch contractions of both tissues by inverse agonism, because, if the potentiation of contraction produced by each ligand was due to a displacement of endocannabinoid agonists from

the CB_1 receptor, the maxima of SR 141716, AM 251 and O-2050 should have been equal.

Experiments performed on the rat isolated vas deferens in Chapter 4 showed that neither SR 141716, AM 251 nor O-2050 increased the EFS-evoked ATP-mediated twitch contractions of this tissue. Additionally, the pA_2 value of SR 141716 obtained against WIN 55,212-2 was in agreement with its pK_B and pA_2 values estimated in the twitch contraction experiments of the rat and guinea-pig ileum MPLM. These data suggested that SR 141716 behaved as a neutral CB_1 antagonist in the rat vas deferens and that the constitutive activity of the CB_1 receptor was dependent on the tissue employed, neurotransmitter released or stimulus response coupling capacity of the drug-receptor complex.

Experiments described in Chapter 5 aimed to investigate the role of the CB_1 receptor in modulating the basal and capsaicin-, nicotine- and veratridine-evoked electrogenic secretory function of the rat isolated colonic SPM preparation. These experiments showed that the basal I_{SC} and R_t of the SPM preparation were not maintained by neuronal cholinergic or peptidergic tone, but most likely by the epithelial cells themselves. The lack of effect of CP 55,940, WIN 55,212-2 and SR 141716 on the basal I_{SC} and R_t was most likely because of an absence of the cannabinoid receptor on the epithelial cells. The presence of either CP 55,940 or WIN 55,212-2 selectively attenuated the transient increase in the I_{SC} evoked by capsaicin and nicotine but not the sustained increase in the I_{SC} elicited by veratridine. Moreover, the inhibition of the capsaicin and nicotine responses by CP 55,940 and WIN 55,212-2 was antagonised by the presence of SR 141716. Because the transient increase in the I_{SC} elicited by both capsaicin and nicotine was both neurogenic and sensitive to the blockade of the NK_1 receptor, it was likely that both CP 55,940 and WIN 55,212-2 inhibited the neuronal release of substance P and neurokinin A by activation of the CB_1 receptors. The sustained increase in the I_{SC} elicited by veratridine was shown to be mediated by the release of a neuropeptide distinct from substance P, neurokinin A and VIP. The lack of an effect of CP 55,940 and WIN 55,212-2 on the veratridine response suggested that the CB_1 receptor was not involved in the release of all neuropeptides. The lack of effect of SR 141716 to alter the capsaicin- and nicotine- evoked increase in the I_{SC} suggested that the CB_1 receptors modulating the release of substance P and neurokinin

A from the submucosal plexus was not constitutively active and that this cannabinoid behaved as a neutral CB_1 receptor antagonist. These data added weight to the notion that the constitutive activity of the CB_1 receptor was dependent on the tissue employed, neurotransmitter released or stimulus response coupling capacity of the drug-receptor complex.

In conclusion, this project has shown that the rat ileum MPLM and colonic SPM preparations serve as suitable *in vitro* intestinal nerve smooth muscle and epithelial cannabinoid bioassays respectively.

6.2 Future work

Experiments described in Chapter 3 were restricted to investigating the effect of the cannabinoids on contractions of the rat ileum MPLM to two frequencies of EFS. An examination of the effect of the cannabinoids on contractions to a range of frequencies of EFS would help characterise the EFS frequency range over which the cannabinoids act separately on one or both of the CB_1 and non-CB_1-non-CB_2-non-$TRPV_1$ receptors. These experiments may also uncover the presence of additional cannabinoid targets that can modulate the EFS-evoked contractions of the MPLM.

An investigation into the effects of the cannabinoids on the EFS-evoked release of ACh from the MPLM in response to the EFS parameters used for eliciting the twitch and rebound contractions would confirm the ability of the cannabinoids to modulate the EFS-evoked contractions by a modulation of ACh release. The effects of a cannabinoid on [^{3}H]-ACh release could be studied from [^{3}H]-choline pre-incubated MPLM strips as described by Mang *et al.*, (2001). In this method, samples of the Krebs-Henseleit solution bathing the electrically stimulated MPLM strips can be collected at intervals in the absence and presence of cannabinoids. The radioactivity content of the samples can be quantified by liquid scintillation spectrometry to give a measure of the change in the outflow of the evoked [^{3}H]-ACh in the presence of the cannabinoid. Alternatively, both ACh release and muscle contraction can be studied simultaneously using the cascade super-fusion technique (Finkleman, 1930; Gaddum, 1953). In this technique, two MPLM strips can be suspended one below the other between isometric transducers and superfused with Krebs-Henseleit solution in the

absence and presence of cannabinoids. EFS of only the top i.e. donor MPLM strip would cause a contraction and the release of ACh of this strip. The ACh released from the donor strip would then contract the second i.e. recipient MPLM strip being superfused by the superfusate. The release of ACh can be easily verified by the inclusion of atropine or TTX in the superfusing Krebs-Henseleit solution. The inclusion of a cannabinoid agonist in the superfusing Krebs-Henseleit solution would inhibit the contractions of both MPLM strips. The inhibition EFS-evoked contractions of the donor strip would be explained with the same reasons as mentioned above for the organ baths studies. The attenuation of the contractions of the recipient MPLM strip would be indicative of the inhibition of ACh release from the donor MPLM strip because, as shown in the organ baths studies, cannabinoids do not interact with the muscle mACh receptors.

To date, *in vivo* intestinal transit studies have not analysed the motility patterns of the small intestine following administration of cannabinoids. The collective *in vivo* data suggests that activation of the CB_1 receptor causes an inhibition of the propulsive patterns of contractile activity such as peristalsis as has been observed *in vitro. In vitro* functional studies with the rat ileum MPLM may be useful in characterising the mechanism of the action of the cannabinoids on different EFS-evoked cholinergic and non-cholinergic-non-adrenergic motility patterns. These studies could be extended to the effects of cannabinoids on the MPLM preparation from rats with an inflamed ileum.

An experiment investigating the effect of cannabinoid agonists and SR 141716 on ACh-mediated contractions of the rat ileum MPLM elicited by pharmacological stimulation of the myenteric plexus, such as with nicotine and veratridine would provide comparative data for the EFS experiments. These studies may also provide insights into the role of the different receptors in relating the difference in the receptors modulating the twitch and rebound contractions of the MPLM.

The ability of SR 141716, AM 251 and O-2050 to increase the amplitude of the twitch contractions of the rat and guinea-pig ileum MPLM preparations was suggestive of inverse agonism at the CB_1 receptor. This conclusion was supported by the failure of the inhibitors of FAAH and the endocannabinoid uptake transporter to inhibit the

twitch contractions. Compelling evidence for the absence of an endocannabinoid tone may be obtained by measuring the levels of AEA and 2-AG from samples of the Krebs-Henseleit solution bathing the MPLM strips in the absence and presence of EFS using liquid chromatography and mass spectroscopy techniques. These experiments could be performed in the absence and presence of inhibitors of FAAH or the endocannabinoid uptake transporter to complement the data from the organ bath studies.

In experiments described in Chapter 4, the ability of AM 251 and O-2050 to augment the twitch contractions of the MPLM tissues was taken to be mediated by the CB_1 receptor. An estimation of the pK_B values of these cannabinoids would be necessary to confirm their action at the CB_1 receptor.

A major experimental limitation of the studies on the colonic SPM preparation was the inability to wash the capsaicin and nicotine after each response. A modification of the perspex Ussing chambers by including ports for draining the Krebs-Henseleit solution from the chambers may allow repeated secretory responses to be evoked in a single preparation. An examination of the interaction between a range of concentrations of the cannabinoid agonists and SR 141716 would allow precise characterisation of the CB_1 receptor. These studies could be extended to inflamed colonic SPM preparations.

The cannabinoids used in the present study represent a small fraction of cannabinoids that have been assayed *in vivo.* Little is known about the pharmacology of the many other endocannabinoids and phytocannabinoids and their interaction with other receptor systems that modulate intestinal motility and secretion. The rat ileum MPLM and colonic SPM preparations may also prove useful in characterisation of the pharmacology these cannabinoids and their interactions with other receptor systems.

CHAPTER 7:
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